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MICROBIOLOGY (C GENCO, SECTION EDITOR)

The Microbiome of Oral Squamous Cell Carcinomas: a Functional Perspective

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Abstract

Purpose of Review This decade has witnessed increasing interest in the potential role of the oral microbiome in head and neck cancers, particularly oral squamous cell carcinoma (OSCC). Most studies have focused on the bacterial component of the microbiome (bacteriome), but the fungal component (mycobiome) is also receiving attention. In this review, we provide an overview of mechanisms by which the microbiome can contribute to oral carcinogenesis, and summarize results from clinical studies, especially focusing on those reporting functional microbiome analysis. Synthesizing and illustrating the evidence, we also suggest a new "passenger-turning-driver" functional model for the role of the microbiome in oral cancer.

Recent Findings In vitro studies provide convincing evidence for the carcinogenicity of the periodontal bacteria *Fusobacterium nucleatum* and *Porphyromonas gingivalis.* However, results from clinical studies are inconsistent, with significant variations in composition of the microbiome associated with oral cancer. Methodological differences may partially explain the differing conclusion. However, variations observed may also reflect functional redundancy: the phenomenon that different species may be enriched in different samples, but still serve the same functions. Indeed, functional analyses of the bacteriome associated with oral cancer have revealed more consistent results, namely enrichment of a virulent, inflammatory bacteriome in the tumors.

Summary Apart from oncoviruses associated with a special entity of oral cancer, no consistent evidence implicates specific microbial species in OSCC etiology. Instead, the disturbed function of an initially "passenger" microbiome within the tumor microenvironment likely contributes to tumor progression by sustaining chronic inflammation.

Keywords Carcinoma · High-throughput nucleotide sequencing · Mouth neoplasms · Microbiota · Mycobiome · Squamous cell

Introduction

Oral cancer (predominantly squamous cell carcinoma) is a subset of head and neck cancers (HNCs) affecting the oral cavity proper, i.e., mouth anterior to the palatine tonsils, also referred to as intra-oral. In the international databases, oral cancer is classified as "lip and oral cavity": ICD-10 sites C00-C06. These constitute the 16th most prevalent malignancy worldwide, accounting for an estimated 247,563 new cases and 177,384 deaths annually [1]. There is, however, marked geographical and cultural variation. In much of South Asia, for example, oral cancer is the most common cancer among males, perhaps sixth among women, and

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second overall [2]. In the USA, the corresponding numbers are ~ 35,130 and 7410, respectively, although the anatomical sub-sites do not precisely match those used in the worldwide data [3]. There is a male predilection and the tongue is the most commonly affected sub-site [3]. In the West, nearly 74% of oral squamous cell carcinoma (OSCC) cases are attributed to tobacco smoking and heavy alcohol consumption [4], while in South Asia and the Pacific, smokeless tobacco and areca (betel) nut chewing are the major risk factors [5]. Unlike cancers of the oropharynx, only a small fraction of OSCC cases (2–6%) are attributed to human papilloma virus (HPV) infection [6, 7]—although greater proportions are reported from the Asia-Pacific Region—but there are questions about definitions of head and neck sub-sites and laboratory methods [8]. Around 15% of all OSCC cases remain unexplained by any of the known risk factors. In addition, and despite advances in cancer treatment modalities, OSCC continues to have poor prognosis with 5-year survival rates less than 50% in much of the world [9, 10]. These challenges have triggered scientists to search for novel risk factors and prognosis modifiers that eventually could present targets for preventive or therapeutic interventions. Particularly, there has been increasing interest in the role of the oral microbiome in oral carcinogenesis [11].

Apart from the debate on its origin [12], the term "microbiome" is currently used to refer to "all microorganisms in a particular habitat and their collective genomes" [13]. The microbiomes associated with the human body, including the oral microbiome, and their role in health and disease have been studied extensively [14]. Due to differences in key ecological factors, such as redox potential, attachment legends, moisture level, acidity, etc., there are significant variations in the composition of the microbiome from one body site to another, and even between sub-sites in close proximity, e.g., sub- and supra-gingival plaque [15]. The human microbiomes form complex, but balanced (homeostatic), communities that are compatible with health, a status increasingly referred to as normobiosis. Under certain circumstances, however, this balance may be disturbed, leading to perturbations in the composition and function of the microbiome (dysbiosis) that are associated with transition from health to disease [16]. In the oral cavity, dental caries and periodontitis are typical examples of diseases associated with microbial dysbiosis. Bacteria are the predominant microorganisms in the oral cavity and therefore most research so far has focused on the bacterial component of the oral microbiome—the oral bacteriome. However, there is increasing interest in less abundant microbial communities, such as those involving fungi, the oral mycobiome [17, 18], and viruses, the oral virome [19], respectively, and their potential role in oral diseases. The advent of high-throughput molecular technologies, especially next-generation sequencing (NGS), has revolutionized the study of microbial communities. An overview of nucleic acid sequencing-based methods currently used to study the oral microbiome is provided in Fig. 1.

Evidence is emerging for the oral microbiome playing a role in oral cancer, a topic we reviewed comprehensively in 2016 [11]. Nonetheless, there has been a virtual explosion in research in this area (at least 18 additional studies since that report), with evolving concepts regarding the nature of contributions by the oral microbiome to oral carcinogenesis, which consequently warrants revisiting the topic. In this concise review, we summarize results from clinical studies on the bacteriome and mycobiome associated with oral cancer, and provide an overview of carcinogenic properties of some oral microbes. We also propose a new functional model for the role of the microbiome in oral carcinogenesis.

Carcinogenicity of Specific Oral Microbes

The Classical Microbial Suspects: Viruses

The vast majority of microbes designated as Class 1 carcinogens by the International Agency for Research on Cancer (IARC) are viruses. Therefore, viruses come to mind first when raising a question regarding the role of microorganisms in cancer. As far as HNCs are concerned, two families of oncogenic viruses are particularly important: Human Papillomaviruses (HPVs) and Human Herpesviruses (HHVs). There is currently incontrovertible evidence that a small number of so-called high-risk (hr) HPVs are responsible for a global epidemic of oropharyngeal cancer. By now, cancer of the oropharynx has replaced cancer of the uterine cervix as the most common HPV-related cancers in the USA [20]. A smaller proportion of intra-oral cancers are also caused by hrHPVs. Although these HPVs are epitheliotropic, cancerous lesions mostly arise in the mucosa associated with lymphoid tissue, due to interactions between lymphocytes and keratinocytes, namely in the posterior third of the tongue (such lesions located in the palatine tonsils and elsewhere in Waldeyer's tonsillar ring are not classified as intra-oral). The underlying cellular mechanisms are well understood: HPV E6 and HPV E7 oncogenes interfere with p53 and retinoblastoma gene proteins, respectively, and thereby block their tumor suppressor actions [21]. Consequently, there are encouraging possibilities to block the oncogenic pathways with interfering ribonucleic acid (RNA) or by gene editing [22].

Among the HHVs, the Epstein-Barr virus (HHV-4) is the major cause of nasopharyngeal cancers, a distinct biological entity [23]. However, there is limited evidence that HHV-4 may play a role in oral cancer [21, 24]. HHV-8 is the etiologic agent for Kaposi's sarcoma located in the mouth and elsewhere that is prevalent in immunosuppressed individuals, particularly in patients with acquired immune deficiency syndrome (AIDS) [21, 25]. The virus is carried in the oropharynx and can be recovered from saliva/oral fluid samples [21, 26]. There is circumstantial evidence that Herpes Simplex Viruses (HSV), both HSV-1 and HSV-2, are associated with oral (and

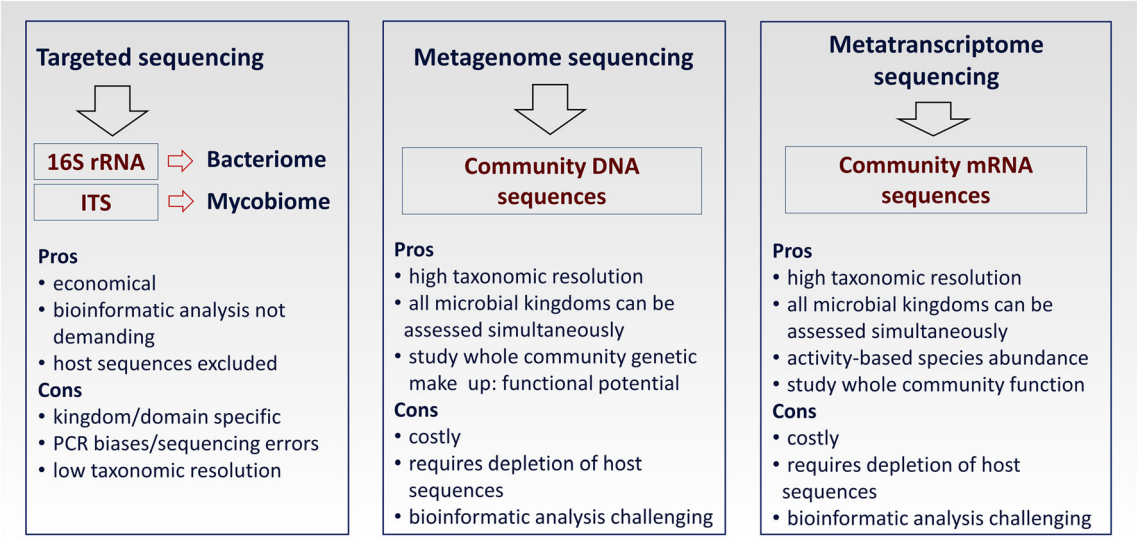

Fig. 1 Nucleic acid sequencing-based methods currently used to study the oral microbiome. In targeted sequencing, domain-specific primers are used to amplify a marker gene; the generated amplicons (libraries) are then sequenced. Typically, taxonomic profiles at the genus level are obtained. In metagenome sequencing, DNA from the entire microbial community (after depleting host DNA) is fragmented and sequenced. Proper analysis can provide strain-level profiles as well as the functional potential of the microbiomes. In metatranscriptome analysis, microbial mRNA (after depleting ribosomal RNA as well as host mRNA) is used to contract cDNA libraries that are then sequenced. Many sequencing platforms are available on the market. *cons* disadvantages (from "pro et contra" Latin "for and against"), *DNA* deoxyribonucleic acid, *ITS* internal transcribed spacer, *mRNA* messenger RNA, *PCR* polymerase chain reaction, *pros* advantages, *rRNA* ribosomal ribonucleic acid

cervical) squamous cell carcinoma in humans, as well as supportive animal studies [21], but a direct oncogenic role remains unsubstantiated [26].

Apart from human viruses, the oral cavity is also home to a complex community of bacteriophages that are thought to influence the ecology and pathogenicity of the oral bacterial community [27]. Whether or not this phage community plays a role in oral carcinogenesis has not been explored.

Emerging Role of *Porphyromonas gingivalis* and *Fusobacterium nucleatum*

In the 1990s, the relationship between bacteria and carcinogenesis was first established by demonstrating the causative role of *Helicobacter pylori* in gastric cancer [28], and since then, tremendous efforts have been invested in exploring the relationship between bacteria and cancer in sites elsewhere in the body. This has led to uncovering additional associations, such as that of *Chlamydia trachomatis* with cervical cancer [29], *Salmonella typhi* with gallbladder cancer [30], and *Bacteroides fragilis* with colon cancer [31]. As far as oral cancer is concerned, evidence is emerging, primarily from in vitro and animal studies, for the carcinogenicity of the periodontal bacteria *Porphyromonas gingivalis* (*P. gingivalis*) and *Fusobacterium nucleatum* (*F. nucleatum*). Interest in these two bacteria from the oral microbiome as potential carcinogens has been fueled by studies that implicated them in pancreatic and colorectal cancers (CRC), respectively [32–35]. The mechanisms by which they are thought to contribute to oral carcinogenesis are shown conceptually in Fig. 2 [11].

P. gingivalis has been shown to inhibit apoptosis at different levels, including activation of JAK1/STAT3 and PI3K/Akt signaling pathways [36, 37], suppression of proapoptotic BCL-2-associated death promoter [38], blocking activity of caspase-3 and caspase-9 [37, 38], upregulation of microRNA-203 [39], and prevention of ATP-dependent $P2X_7$-mediated apoptosis [40]. Both *P. gingivalis* and *F. nucleatum* activate cell proliferation through upregulation of kinases and cyclins [41–43], activation of the β-catenin signaling pathway [44, 45], and downregulating level of the p53 tumor suppressor [41]. They also have been found to enhance cellular invasion, primarily through upregulation/activation of matrix metalloproteinases, including MMP-1, MMP-9, MMP-10, and MMP-13, and inducing stemness and epithelial to mesenchymal transition (EMT) [43, 46–48]. In addition, *P. gingivalis* and *F. nucleatum* are believed to contribute to progression of oral cancer by inducing chronic inflammation via increasing production of pro-inflammatory cytokines [49–52]. Production of carcinogenic substances, such as acetaldehyde (from ethanol), may also play an important role, but has not been documented for these two species. Nonetheless, there is evidence for such production by other oral microorganism species, such as *Streptococci* [53], *Neisseria* [54], and *Candida* [55, 56].

Despite the convincing evidence from in vitro studies on the carcinogenicity of *P. gingivalis* and *F. nucleatum* just

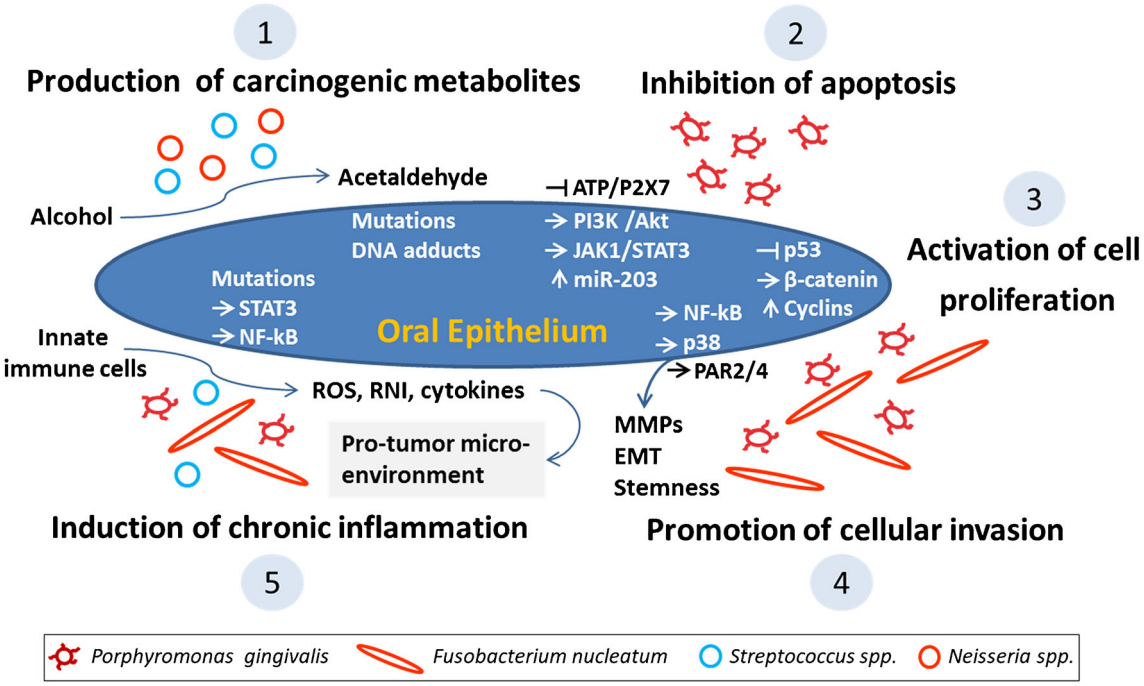

Fig. 2 Mechanisms by which specific oral bacteria may induce/contribute to oral cancer. The figure was reproduced with permission [11]. More details are described in the text. *ROS* reactive oxygen species, *RNI* reactive nitrogen intermediates, *MMPs* matrix metalloproteinases, *PAR* protease-associated receptor, *EMT* epithelial to mesenchymal transition

summarized, evidence for a direct carcinogenic role in oral cancer based on clinical studies is still lacking, as described in the following section.

The Bacteriome Associated with Oral Cancer: Clinical Studies

Variations in Composition of the Bacteriome Associated with Tumors

Clinical studies that assessed the association between bacteria and cancers of the oral cavity are summarized in Table 1. Most of these studies restricted inclusion to OSCC samples, but a number of them also included HNCs located at sites other than the oral cavity (parts of the pharynx, larynx, or esophagus) as well as lesions that were only potentially malignant. Only two decades ago, namely in 1998, Nagy and collaborators first demonstrated differences in composition of the microbial community colonizing the surface of OSCC and adjacent normal tissue, using culture techniques [57•]. Between 2000 and 2005, *Streptococcus anginosus* (*S. anginosus)* became implicated in the etiology of HNC, including OSCC, by research groups from Japan [58, 59, 61]. However, these studies suffered from lack of proper control samples: the high detection rates of the bacterium observed in HNC samples were simply because it is a normal colonizer of the oropharynx. Interest in *S. anginosus* thus faded quickly.

In 2006, Hooper et al. demonstrated, for the first time, the presence of a viable complex bacterial community *within the OSCC tissues* that is compositionally different from that found in the tumors' healthy margins [62•]. Since then, the vast majority of investigations have employed sequencing of the 16S rRNA (ribosomal RNA) gene to characterize the microbiomes in study samples, initially with the Sanger method and more recently with NGS chemistries [63, 65–70, 71•, 72–79, 80•, 81, 82, 83•, 84]. Nevertheless, there are significant methodological differences between these studies in terms of (1) the type of samples analyzed (saliva (stimulated or unstimulated), surface swabs, or biopsies); (2) the nature of control or comparison samples used (tumor-adjacent, clinically normal; contralateral to tumor; healthy subject; or benign lesions); (3) the hypervariable region of the 16S rRNA gene selected for sequencing (e.g., V1–V3 or V4–V5); and (4) the bioinformatic analysis method used for analysis of sequencing data (sequence quality control, reference database, and taxonomy assignment algorithm). The latter particularly affects taxonomic resolution, with only a few studies reporting species-level profiles and the rest limited to the genus level.

As displayed in Table 1, a number of bacterial taxa, particularly *Fusobacterium* spp., and to a lesser extent, *Campylobacter*, *Parvimonas*, and *Prevotella* spp., were repeatedly found to be significantly enriched in OSCC samples (Table 1), whereas *Streptococci* frequently were found in association with health. Nevertheless, a more careful examination of the results from these studies reveals there are

Table 1 Clinical studies assessing the association between bacteria and oral cancer (in chronologic order) (an expansion of Table 1 in the 2016 review by Perera et al. [11], with permission)

Study	Sample size (tumor/control)*	Technology used	Case sample	Control sample	Bacterial taxa associated with the tumors	Bacterial functions associated with the tumors
Nagy et al. 1998 [57•], Hungary	21/21 (self)	Cultivation; biochemical identification	Surface swabs: tumors	Surface swabs: contagious normal mucosa	*Fusobacterium*, *Porphyromonas*, *Actinomyces*, and *Propionibacterium* spp.	N/A
Tateda et al. 2000 [58], Japan	205/10 (self) OSCC 68 Others 137	Detection of *Streptococcus anginosus* by PCR and Southern-blot	Tumor tissue, gingival smears, and oropharyngeal swabs	Fresh contagious normal tissue	Authors concluded *S. sanginosus* was associated, but conclusion is not supported by the results (lack of proper control)	N/A
Morita et al. 2003 [59], Japan	OSCC 38/7 Esophageal 18/6	Detection of *S. anginosus* by q-PCR	Fresh tumor tissue	Fresh non-cancerous tissue	*S. anginosus* with esophageal, but not oral, cancer	N/A
Mager et al. 2005 [60], USA	45/229	Checkboard DNA-DNA hybridization	Unstimulated saliva	Unstimulated saliva	*Capnocytophaga gingivalis*, *Prevotella melaninogenica*, *Streptococcus mitis*	N/A
Sasaki et al. 2005 [61] Japan	42/7	Detection of *S. anginosus* by PCR	Fresh tumor tissue, dental plaque, and saliva	Fresh tissue: leukoplakia, lymphoma, and rhabdomyosarcoma	Authors concluded *S. anginosus* was associated, but conclusion is not supported by the results (lack of proper control)	N/A
Hooper et al. 2006 [62•], UK	20/12 (self)	Cultivation; isolates identified with 16S rRNA gene sequencing (Sanger)	Fresh tumor tissue: deep and superficial	Fresh contagious normal tissue	*Micrococcus luteus*, *Prevotella melaninogenica*, *Exiguo-bacterium oxidotolerans*, *Fusobacterium naviforme*, *Staphylococcus aureus*, *Veillonella parvula*	N/A
Hooper et al. 2007 [63], UK	10/10 (self)	16S rRNA clone sequencing (Sanger)	Fresh tumor tissue	Fresh contagious normal tissue	*Ralstonia insidiosa*, *Fusobacterium naviforme*, *Peptostreptococcus micros*, *Clavibacter michiganensis* subsp. *tessellarius*, *Capocytophaga* sp. oral strain S3, *Prevotella* sp. oral clone BE073	N/A
Katz et al. 2011 [64], USA	10/5	Immunohistochemical staining	FFPE gingival carcinoma tissue	FFPE normal gingival tissue	*Porphyromonas gingivalis*	N/A
Pushalkar et al. 2011 [65], USA	3/2	16S rRNA (V4–5) amplicon sequencing (454); DGGE	Stimulated saliva	Stimulated saliva	Genera *Streptococcus*, *Rothia*, *Gemella*, *Peptostreptococcus*, *Lactobacillus*, *Micromonas*, and *Porphyromonas*	N/A
Pushalkar et al. 2012 [66], USA	10/10 (self)	16S rRNA clone sequencing (Sanger); DGGE	Fresh tumor tissue	Fresh contagious normal tissue	*Parvimonas* sp. oral taxon 110, *Eubacterium infirmum*, *Eubacterium brachy*, *Gemella haemolysans*, *Gemella morbillorum*, *Gemella sanguinis*, *Johnsonella ignava*, *Peptostreptococcus stomatis*, *Streptococcus gordonii*, and *Streptococcus parasanguinis I*	N/A

Table 1 (continued)

Study	Sample size (tumor/control)*	Technology used	Case sample	Control sample	Bacterial taxa associated with the tumors	Bacterial functions associated with the tumors
Schmidt et al. 2014 [67], USA	16/8/6 (OSCC/"pre-cancer"/ healthy)	16S rRNA (V4) amplicon sequencing (454 and Miseq)	Surface swabs: tumors and "pre-cancer" lesions	Surface swabs: contralateral normal; normal mucosa from healthy subjects	Genera *Fusobacterium* and *Prevotella* (*Streptococcus* and *Rothia* showed inverse association)	N/A
Al-Hebshi et al. 2015 [68], Yemen	3/0	16S rRNA (V1–3) amplicon sequencing (454)	Fresh tumor tissue	None	*Bacteroides fragilis?*	N/A
Guerrero-Preston et al. 2016 [69], USA	17/25 OSCC 6 Oropharyngeal 11	16S rRNA (V3-V5) amplicon sequencing (454)	Oral rinse	Oral rinse	*Genera Streptococcus*, *Lactobacillus*, *Staphylococcus*, and *Parvimonas*—also *Fusobacterium* by G-test (*Haemophilus* and *Neisseria* among others showed inverse association)	N/A
Hu et al. 2016 [70], China	16/10/19 (OSCC/"pre-cancer"/healthy)	16S rRNA amplicon sequencing (Miseq)	Unstimulated saliva	Unstimulated saliva	Genera *Bacillus* (*Streptococcus* and *Abiotrophia* showed inverse association)	N/A
Al-Hebshi et al. 2017 [71•], Yemen/Saudi Arabia	20/20	16S rRNA (V1-V3) amplicon sequencing (Miseq)	Fresh tumor tissue	Deep epithelium swabs	*Fusobacterium nucleatum*, *Pseudomonas aeruginosa*, *Campylobacter* oral taxon 44, *Haemophilus influenza*, *Campylobacter showae*, and *Parvimonas micra* (*Streptococcus mitis*, *Rothia mucilaginosa*, and *Haemophilus parainfluenzae* among others showed inverse association)	As predicted with PICRUSt: bacterial mobility, flagellar assembly, bacterial chemotaxis, and LPS biosynthesis (DNA repair, glycolysis/gluconeogenesis and biosynthesis of amino acids were enriched in health)
Amer et al. 2017 [72], Ireland	36/32 (Leukoplakia/healthy) No OSCC	16S rRNA (V1–V2) amplicon sequencing (Miseq)	Surface swabs: leukoplakia patients	Surface swabs: contralateral normal; normal mucosa from healthy subjects	Genera *Fusobacterium, Leptotrichia*, and *Campylobacter*; *Rothia mucilaginosa*	N/A
Börnigen et al. 2017 [73], USA	112/242 OSCC 43 Oropharyngeal 64 Unknown 5	16S rRNA (V4) amplicon sequencing (Miseq)	Oral rinse	Oral rinse	Genus *Dialister* only (*Streptococcus* and *Rothia* showed inverse association)	Results ambiguous: discrepancies between the figure and text
Guerrero-Preston et al. 2017 [74], USA	As above + 154 subjects from the HMP	Same dataset above re-analyzed, along with HMP data, with Resphera Insight	Oral rinse	Oral rinse	*Fusobacterium nucleatum*, *Streptococcus mutans*, *Parvimonas micra*, and *Lactobacillus* spp.	N/A
Lee et al. 2017 [75], Taiwan	125/124/127 (OSCC/"pre-cancer"/healthy)	16S rRNA (V4) amplicon sequencing (Miseq)	Unstimulated saliva	Unstimulated saliva	Genera *Bacillus*, *Enterococcus*, *Parvimonas*, *Peptostreptococcs*, and *Slackia*	N/A
Mukherjee et al. 2017 [76], USA	39/39 (self) All mobile tongue cancer	16S rRNA (V4) amplicon sequencing (Ion Torrent)	Fresh-frozen tumor tissue	Fresh-frozen adjacent normal tissue	Genera *Streptococcus*, *Rothia*, *Actinomyces*, *Enterococcus*, and *Microococcus* (*Fusobacterium*, *Prevotella*,	N/A

Table 1 (continued)

Study	Sample size (tumor/control)*	Technology used	Case sample	Control sample	Bacterial taxa associated with the tumors	Bacterial functions associated with the tumors
					Parvimonas, *Campylobacter*, and *Porphyromonas* showed inverse association)	
Shin et al. 2017 [77], USA	34/24 (self) OSCC 4 Others 30	16S rRNA (V4) amplicon sequencing (Ion Torrent)	Fresh-frozen tumor tissue: primary tumor or/and metastatic	Fresh-frozen adjacent normal tissue	No significant difference for OSCC analyzed separately. When all tumors pooled: an increase in *Fusobacterium* and *Parvimonas*; a decrease in *Streptococcus*, *Veillonella*, *Actinomyces*, and *Rothia*	N/A
Wang et al. 2017 [78], USA	121/121 (self) OSCC 59 Others 62	16S rRNA clone sequencing (Sanger)	Fresh-frozen tumor tissue	Fresh-frozen adjacent normal tissue	Genus *Parvimonas* only (*Actinomyces* was inversely associated)	N/A
Zhao et al. 2017 [79], China	40/40 (self)	16S rRNA (V4–V5) amplicon sequencing (Miseq)	Surface swabs: tumors	Surface swabs: self-matched normal mucosa	Genera *Fusobacterium*, *Capnocytophaga*, *Alloprevotella*, *Treponema*, *Campylobacter*, *Parvimonas*, and *Dialister* (*Streptococcus*, *Veillonenlla*, *Lautropia*, and *Rothia* showed inverse association)	As predicted with PICRUSt: Translation, metabolism of cofactors and vitamins; metabolism of terpenoids and polyketides; replication and repair
Hayes et al. 2018 [80•], USA	129/254 OSCC 41 Oropharyngeal 30 Larynx 58 Nested case-control study in two larger cohort studies[a]	16S rRNA (V3–V4) amplicon sequencing (454)	Oral mouthwash samples (collected at baseline)	Oral mouthwash sample (collected at baseline)	Pooling all cases and controls: genera *Kingella* and *Corynebacterium* and species *Prevotella nanceiensis*, *Capnocytophaga leadbetteri* and *Selenomonas sputigena* were associated with decreased risk of HNSCC (*Parvimonas micra* and *Neisseria sicca* were associated with reduced risk of OSCC)	N/A
Lim et al. 2018 [81], Australia	52/21 OSCC 15 Oropharyngeal 37 Healthy subdivided into low and high risk	16S rRNA (V6–8) amplicon sequencing (Miseq)	Oral rinse	Oral rinse	Pooling all cases: genus *Oribacterium* only (*Rothia*, *Haemophilus*, *Corynebacterium*, *Paludibacter*, *Porphyromonas*, and *Capnocytophaga* were inversely associated)	N/A
Perera et al. 2018 [82], Sri Lanka	25/27	16S rRNA (V1–V3) amplicon sequencing (Miseq)	Fresh tumor tissue	Fresh tissue of fibroepithelial polyp	*Campylobacter concisus*, *Prevotella salivae*, *Prevotella loeschii*, and *Fusobacterium* oral taxon 204 (*Streptococcus mitis*, 6 other *Streptococcus* spp., *Rothia* spp. and *Lautropia mirabilis*	As predicted with PICRUSt: LPS biosynthesis, peptidases and carbon fixation in photosynthetic organisms among others (Base

Table 1 (continued)

Study	Sample size (tumor/control)*	Technology used	Case sample	Control sample	Bacterial taxa associated with the tumors	Bacterial functions associated with the tumors
					among others showed inverse association)	excision repair, glycolysis/gluconeogenesis, and biosynthesis of amino acids were enriched in health)
Yang C et al. 2018 [83•], Taiwan	197/51 (41 stage 1 + 66 stage 2/3 + 90 stage 4)[b]	16S rRNA (V3–V4) amplicon sequencing (Miseq)	Oral rinse	Oral rinse	*Fusobacterium periodonticum*, *Parvimonas micra*, *Streptococcus constellatus*, *Haemophilus influenza*, and *Filifactor alocis* (in contrast to decrease of *Streptococcus mitis*, *Haemophilus parainfluenzae*, *Porphyromonas pasteri*, *Veillonella parvula*)—strength of association with staging	As predicted with PICRUSt (in stage 4 vs. health): cytoskeleton proteins, methane metabolism, carbon fixation in photosynthetic organisms, restriction enzymes, others (amino acid synthesis and metabolism were enriched in health)
Yang S et al. 2018 [84], Taiwan	39/0 Cases clustered into 3 groups by mutational signature: MSC1 ($n = 9$) MSC2($n = 10$) MSC3 ($n = 11$)	16S rRNA (V4) amplicon sequencing (Miseq)	Unstimulated saliva	None	N/A (no control group to compare with: comparisons were made between the three patient clusters)	N/A (no control group to compare with: comparisons were made between the three patient clusters)
Yost et al. 2018 [85••], USA	4/4	Metatranscriptome (mRNA) sequencing (NextSeq 500)	Surface swabs: tumors	Surface swabs: adjacent normal mucosa from the cancer patient; tumor-matching and buccal sites from the healthy subjects	Genera *Fusobacteria*, *Selenomonas*, *Capnocytophaga*, *Dialister*, and *Johnsonella* (genus *Bacillus*; species *Porphyromonas catoniae*, *Kingella denitricans*, *Capnocytophaga gingivalis*, among others, were associated with healthy, tumor-matching sites)	Iron ion transport; tryptophanase activity; peptidases; superoxide dismutase; capsule biosynthesis; flagellum synthesis and assembly; chemotaxis; hemolysins and adhesins (base excision repair only was enriched in health)

* OSCC/healthy, unless otherwise indicated

DGGE denaturing gradient gel electrophoresis, *FFPE* formalin-fixed paraffin-embedded, *HMP* Human Microbiome Project, *N/A* not available, *HNSCC* head and neck squamous cell carcinoma, *LPS* lipopolysaccharide, *MSC* mutational signature cluster, *OSCC* oral squamous cell carcinoma, *PCR* polymerase chain reaction, *PICRUSt* phylogenetic investigation of communities by reconstruction of unobserved states, *pt(s)* patient(s), *rRNA* ribosomal ribonucleic acid, *sp(p)* species

[a] The American Cancer Society Cancer Prevention Study II Nutrition Cohort (CPS-II) ($n = 58$) + the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO) ($n = 71$)

[b] American Joint Committee on Cancer. AJCC cancer staging manual [86]

significant variations (and sometimes contradictions) in the composition of the bacteriome associated with tumors from one cohort to another, and even from one subject to another within the same cohort, especially at the species level. Overall, there is, therefore, not sufficient evidence to implicate specific bacterial species or any consortium thereof in the etiology of OSCC. Interestingly, only one of these studies identified *P. gingivalis* in association with oral cancer [64].

More Consistent Results Obtained with Functional Analysis

While the observed variation in composition of the bacteriome associated with oral cancer may, at least in part, be attributed to the methodological differences among studies described above, it may also be explained by the fact that different species frequently can serve the same functions in their communities and thus substitute for each other, a phenomenon called functional redundancy [87••]. That is, a microbial community with a certain subset of species enriched may perform the same function as another community with a different subset of overabundant species. This concept can be likened to player substitution in team sports like soccer. Indeed, functional analyses of bacteriomes associated with oral cancer produced more consistent results than those obtained with compositional profiling reflecting only the abundance/presence of specific individual members of the bacteriome. Some of the studies listed in Table 1 have used Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) to infer metagenomes from the 16S rRNA profiles obtained (functional prediction) [71•, 79, 82, 83•]. Furthermore, in 2018 and for the first time, Yost and team used metatranscriptome sequencing to explore the transcriptional activity (gene expression) of the microbiome associated with OSCC [85••]. In these studies, the tumor-associated bacteriomes possessed similar functional signatures despite variation in their compositional profiles. For example, enrichment of primarily pro-inflammatory features, such as LPS biosynthesis, flagella assembly, bacterial chemotaxis, and production of peptidases, were enriched in the tumors, while activities like glycolysis and gluconeogenesis, amino acid synthesis and metabolism, and DNA repair were enhanced in the healthy samples. The study by Yost and colleagues identified additional virulence factors associated with OSCC, including increased expression of tryptophanase, superoxide dismutase, hemolysins, and adhesins [85••]. More details about the microbiome functional features associated with OSCC identified in this study are shown in Fig. 3 [85••].

The Mycobiome Associated with Oral Cancer

The role of members of the oral microbiome other than bacteria, namely fungi, has been studied in conjunction with

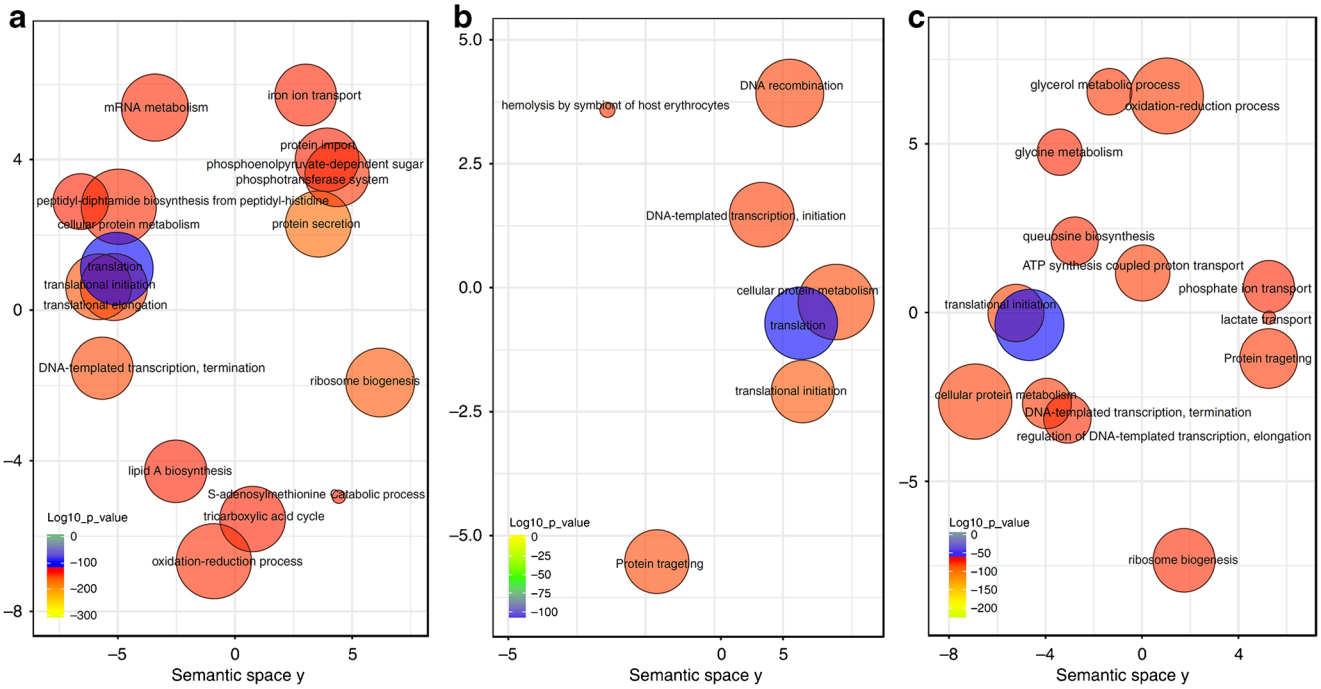

Fig. 3 Microbial pathways enriched in OSCC. The figure was reproduced with permission under Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/) [85••]. Bubbles show biological pathways that were overabundant in: **a** OSCC tumor sites vs. healthy control tumor-matched, **b** OSCC tumor sites vs. tumor-adjacent sites, and **c** OSCC tumor-adjacent sites vs. buccal sites from healthy control subjects

Table 2 Epidemiological studies assessing the association between fungi and oral cancer/potentially malignant lesions

Study	Sample size (tumor/control)*	Technology used	Case samples	Control samples	Key findings
Krogh et al. 1987 [88], Denmark	12/12 (self) Leukoplakia only	Cultivation for isolation HPLC for measuring nitrosation potential	Surface swabs: lesion surface	Surface swabs: adjacent normal mucosa	*Candida* strains with high nitrosation potential found in lesions with advanced "precancerous" changes
Rindum et al. 1994 [89], Denmark	53/100 Leukoplakia 32 CEOC 21 No OSCC	PAS stain for cytological examination Cultivation and biochemical assays for isolation, identification, and typing	Smears and swabs: lesion surface	Smears and surface swabs: adjacent normal mucosa and normal mucosa (cheek, tongue, and palate) from healthy subjects	Greater detection rates (predominantly *C. albicans*) in pathological lesions than in normal mucosa Rare types of *C. albicans* tended to be associated with dysplasia and non-homogenous leukoplakia
Barrett et al. 1998 [90], UK	4724 mucosal biopsies: OSCC 424 Dysplasia 597 Other lesions 3703	PAS stain for detection of fungal hyphae	Mucosal biopsy	N/A	Greater detection rates in moderate and severe epithelial dysplasia, median rhomboid glossitis, squamous papillomas, and progressive dysplasia No association with OSCC
Nagy et al. 1998 [57], Hungary	21/21 (self)	Cultivation; biochemical identification	Surface swabs: tumors	Surface swabs: contagious normal mucosa	*C. albicans*
McCullough et al. 2002 [91], UK	223 subjects: OSCC and dysplasia combined 103 Other mucosal lesions 120	PAS stain for detection of fungal hyphae; cultivation for isolation and CFU count	Mucosal biopsy; mouth rinse	N/A	Significantly higher prevalence and counts in subjects with OSCC and dysplasia Counts correlated with severity of dysplasia and presence of OSCC
Spolidorio et al. 2003 [92], Brazil	832 mucosal biopsies: OSCC 109 Dysplasia 315 Other lesions 408	PAS stain for detection of fungal hyphae	Mucosal biopsy	N/A	Greater detection rates in epithelial dysplasia, OSCC, and hyperkeratosis
Hebbar et al. 2013 [93], India	50 subjects: OSCC 9 Dysplasia 20 Other lesions 21	PAS stain for detection of fungal hyphae Cultivation for isolation and CFU count	Mucosal biopsy; mouth rinse	N/A	*C. albicans* prevalence (in both biopsies and mouth rinse) and counts correlated with severity of dysplasia and OSCC (lowest for mild dysplasia, highest for OSCC)
Berkovits et al. 2016 [94], Hungary	20/40	Cultivation for isolation and CFU count MALDI-TOF-MS for identification	Surface swabs: tumor	Surface swabs: adjacent normal mucosa and normal mucosa from healthy subjects	Greater average fungal burden (predominantly *Candida*) in OSCC vs. healthy and on tumor surface compared to the normal epithelium in the OSCC patients More diverse fungal community in association with OSCC: *Candida*, *Rhodotorula*, *Saccharomyces*, *Kloeckera*, and others
Mukherjee et al. 2017 [76], USA	39/39 (self) All mobile tongue cancer	ITS1 amplicon sequencing (Ion Torrent)	Fresh-frozen tumor tissue	Fresh-frozen adjacent normal tissue	Genera *Ophiocordyceps* and *Guignardia* significantly enriched in tumors (*Lysurus*, *Leptosphaeria*,

Table 2 (continued)

Study	Sample size (tumor/control)*	Technology used	Case samples	Control samples	Key findings
					Antrodiella, and *Boletus* more abundant in control samples)
Perera et al. 2018 [95•], Sri Lanka	25/27	ITS2 amplicon sequencing (Miseq)	Fresh tumor tissue	Fresh tissue from fibroepithelial polyp	*C. albicans, C. etchellsii*, and *Hannaella luteola*–like species enriched in OSCC (A *Hanseniaspora uvarum*–like species, *Malassezia* spp., *Aspergillus tamarii*, *Cladosporium halotolerans*, and *Alternaria alternata* more abundant in control samples)

* OSCC/healthy unless otherwise indicated

C. albicans Candida albicans, *CEOC* chronic erythematous oral candidosis, *CFU* colony-forming unit(s), *HPLC* high-performance liquid chromatography, high-pressure liquid chromatography, *ITS* internal transcribed spacer, *N/A* not available, *OSCC* oral squamous cell carcinoma, *PAS* periodic Acid-Schiff, *MALDI-TOF-MS* matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry, *spp.* species, *vs.* versus

OSCC as well as potentially malignant oral lesions (Table 2). Three decades ago, Krogh's group assessed the nitrosation potential of *Candida* strains isolated from oral leukoplakia lesions, and found strains with high potential for nitrosation to be associated with greater levels of dysplasia [88]. A series of studies published between 1994 and 2013 used periodic Acid-Schiff (PAS) staining and cultivation techniques to detect/isolate yeasts in mucosal swabs or biopsies of oral dysplasia or OSCC [89–93]. Two studies also performed culturing and counting of colony-forming unit (CFU) in oral rinse samples [91, 93]. Most of these studies, however, did not include healthy controls, but instead used for comparison other mucosal lesions, such as hyperkeratosis, benign fibrous growths, and lichen planus. Overall, these studies found the detection frequency and counts of yeasts (predominantly *Candida*) to be significantly greater in dysplasia and OSCC, and such elevation correlated with the severity of dysplasia.

Because recent studies that used NGS have demonstrated that the oral fungal community (mycobiome) is far more complex than previously thought [17, 18], a few studies have recently re-attempted to profile the mycobiome associated with OSCC, aided by novel technology. Using cultivation techniques coupled with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), Berkovits and team identified a more diverse OSCC-associated fungal community, comprising *Rhodotorula*, *Saccharomyces*, and *Kloeckera* in addition to *Candida* species [94]. Consistent with earlier studies, the fungal load was also greater in OSCC compared to healthy controls. In another study, Perera and colleagues used sequencing of the Internal Transcribed Spacer (ITS) to characterize the mycobiome within OSCC tissues compared to that in fibroepithelial polyps, and found a dysbiotic mycobiome dominated by *C. albicans* to be associated with OSCC [96]. Interestingly, Mukherjee's team also employed ITS sequencing to profile the mycobiome in tongue cancer tissues compared to adjacent normal tissues [76]. Surprisingly, however, despite identification of significant differences, *Candida* was not found to be differentially abundant, neither was it the most abundant genus.

"Passenger-Turning-Driver" Microbiome Model for Oral Cancer

Synthesizing all results from the studies presented, we developed a novel "passenger-turning-driver" conceptual model of the potential role of the microbiome in oral cancer as illustrated in Fig. 4. Unlike the bacterial "driver-passenger" model for colon cancer, in which tumorigenesis is initiated by driver species that subsequently are replaced in the tumor microenvironment by passenger species that can then either suppress or promote tumor progression [97], our model assumes the oral microbiome is

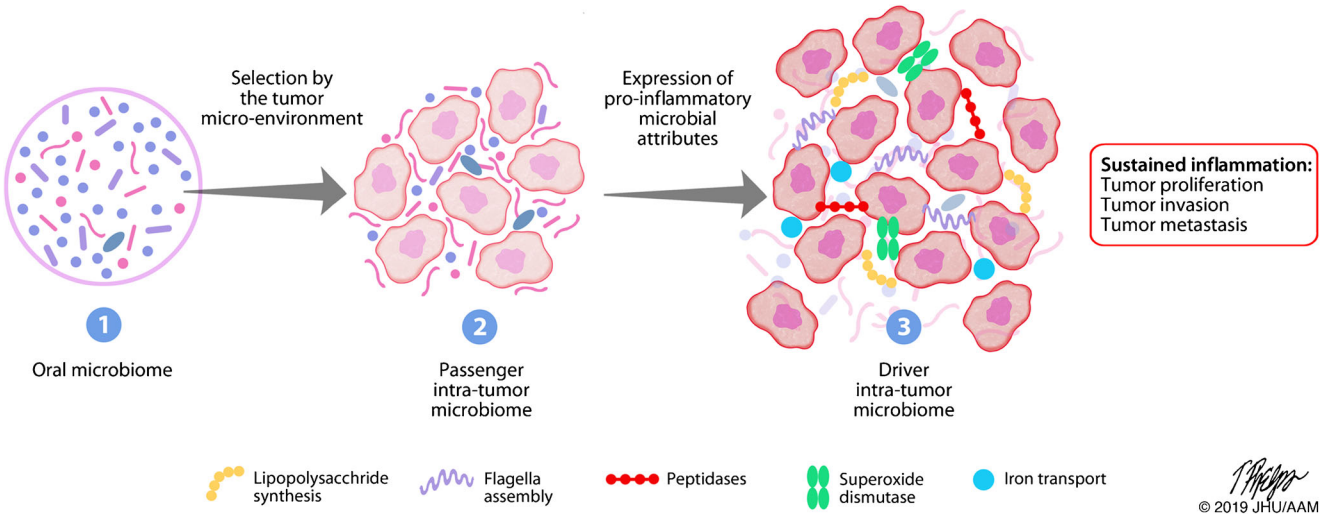

Fig. 4 "Passenger-turning-driver" conceptual model of the sustainable role of the oral microbiome in oral cancer. *1* The commensal oral microbial community associated with healthy mucosa: a mix of bacteria (Gram-positive and Gram-negative cocci, rods, and filaments) and fungi. *2* Formation of an initially "passenger" intra-tumor microbiome by enriching a subset of microbes that can adapt to the tumor microenvironment. *3* Expression of pro-inflammatory microbial features and virulence factors creates a functionally dysbiotic "driver" intra-tumor microbiome that enhances progression of oral cancer. Artwork by Tim Phelps, Department of Arts as Applied to Medicine, John Hopkins University School of Medicine, Baltimore, Maryland, USA. Reproduced with permission

not involved in initiation of oral cancer. Instead, we propose that the initial intra-tumor microbiome represents a passenger event that results from selection by the tumor microenvironment of a subset of species with competitive advantage, e.g., *Fusobacteria*, Gram-negative anaerobic rods (*Prevotella*, *Camphylobacter*, *Selenomonas*, etc.), anaerobic cocci (e.g., *Parvimonas*), and *C. albicans*. The particular species enriched can vary from one subject to another. As it matures, the intra-tumor microbiome expresses pro-inflammatory microbial features and virulence factors (LPS biosynthesis, flagella assembly, bacterial chemotaxis, peptidases, etc.), turning into a functionally dysbiotic, "driver" microbiome that enhances progression of oral cancer by sustaining chronic inflammation.

This conceptual model parallels the model for periodontitis pathogenesis proposed by Bartold and Van Dyke, which also is based on no particular, individual, specific, "pathogenic" bacterium being responsible for initiating the disease process [98, 99]. Rather, certain bacteria become overabundant due to the changes in the microenvironment caused by the initial inflammation and swelling of the gingival tissues that subsequently favor the bacteria that thrive in environments with less oxygen.

Conclusions

Based on existing evidence, we conclude there are significant variations in the composition of the microbiome associated with OSCC, and there are no specific species to implicate in its etiology—of course excluding oncoviruses that are associated with a special entity of oral cancer. Rather, it is the disturbed function of an initially "passenger" microbiome within the tumor microenvironment that is likely to contribute to progression of the tumor by overexpression of virulence factors and pro-inflammatory features. These attributes are common to many taxa that can substitute for each other in different subjects. Moving forward, therefore, a functional approach, particularly metatranscriptomics, is the right way to assess the role of the microbiome in oral cancer. In addition to characterizing the transcriptional activity of the microbiome within the tumor, metatranscriptomics also provides an opportunity to explore all microbial kingdoms (viruses, phages bacteria, archaea, fungi, and protozoa) present in the tumor simultaneously, and to study the interaction between the host and microbiome if the host transcriptome is sequenced in parallel. Further research in this direction will improve our understanding of the mechanisms by which the tumor's microbial community function contributes to oral carcinogenesis and influences the behavior of the neoplasm: this may open new avenues for the diagnosis, prevention and treatment of oral cancer.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- • Of importance
- •• Of major importance

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