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Review

The microbiome and oral cancer: More questions than answers

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ABSTRACT

Recent advances in DNA sequencing technology have facilitated rapid advances in the analysis of the human microbiome and its role in human disease. Several studies have now shown that OSCC and some oral premalignant conditions are associated with alterations in the oral microbiome. These studies raise questions regarding the role of the oral microbiome in the progression of oral malignancies and whether microbiome change is a significant risk factor in the development of oral cancer. This short review summarises current knowledge in the field and highlights questions that require further investigation.

Introduction

Oral squamous cell carcinoma (OSCC) accounts for over 90% of all oral cancers [1]. OSCC is generally considered the 8th most common cancer worldwide and is among the three most common cancers in South-Central Asia [1,2]. Despite therapeutic advances, the 5-year survival rate is approximately 50%, making OSCC one of the most devastating malignancies [1]. Recognised risk factors for OSCC include tobacco and betel nut use, alcohol consumption and age [1]. Tobacco is the most significant risk factor for the development of the disease with approximately 80% of OSCC occurring in smokers [2]. Epidemiological evidence indicates that smoking in combination with heavy drinking significantly increases the risk of developing OSCC [3,4].

However, many patients develop OSCC in the absence of these recognised risk factors. This has led to speculation about other possible contributory factors, including the role of the oral microbiome in the development of OSCC. More than 20 years ago, epidemiological studies indicated that poor oral hygiene and tooth loss were significantly associated with OSCC, providing the first indication that oral bacteria may play a role in the development of oral cancer [5,6]. A recent meta-analysis has demonstrated a significant association of periodontal disease with oral cancer [5]. These studies have resulted in the hypothesis that the inflammatory microbiota associated with periodontitis may play a role in the development and progression of OSCC. However chronic periodontitis and oral cancer have similar risk factors and to date, no causal relationship has been clearly demonstrated [6].

Probably the greatest driver of current investigations is the link between infection-driven inflammation and cancers at other sites in the gastrointestinal tract. It is well established that Hepatitis B and C are associated with hepatocellular carcinoma [7]. *Helicobacter pylori* has now been recognised as a carcinogenic agent by the WHO causing non-cardia gastric carcinoma and low grade B-cell MALT gastric lymphoma [8]. More recently *Fusobacterium nucleatum* has been implicated as a driver of the progression of colorectal carcinoma [9,10]. *F. nucleatum* is a commensal of the human oral cavity which is associated with subgingival plaque and the abundance of this organism increases with poor oral hygiene [11]. These factors have renewed interest in the oral microbiome and cancer and this short review is intended to summarise our current understanding and highlight the gaps in our knowledge.

The evidence for microbiome shifts in OSCC

In the last 5 years there has been a succession of studies characterising the oral microbiome associated with OSCC [12–20]. Studies that directly examine malignant tissue by swab or biopsy generally concur that these sites are enriched for Gram negative Fusobacteria and Bacteroidetes with reduced levels of *Streptococcus* and *Rothia spp.* [13,17,20] (Table 1). Earlier culture-based studies also showed that *F. nucleatum* and *Porphyromonas gingivalis* were enriched at these sites [21]. Some studies conclude that this enrichment provides evidence for their involvement in malignant transformation. However, an alternative hypothesis may be that this represents a microbiome acquired post-malignant transformation and possibly selected for by the inflamed tumour microenvironment. Definitive evidence that this microbiome shift is directly involved in the development of OSCC remains lacking. This, and several other outstanding questions discussed below, require

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Table 1
Bacterial genera associated with OSCC and healthy oral mucosa.

OSCC Associated	*Fusobacterium**
	*Campylobacter**
	Prevotella
	Pseudomonas
	Capnocytophaga
Oral Health Associated	*Streptococcus*
	*Rothia**
	Lautropia
	Haemophillus

* Oral leukoplakia associated.

further investigation before any potential OSCC-microbiome links can be verified.

Do microbiome changes occur during the development of OSCC?

Few studies have examined the microbiome during the early stages of oral carcinogenesis in order to determine if malignant progression is associated with the presence of specific microorganisms. Amer et al. recently investigated the microbiome in patients with oral leukoplakia (OLK), a lesion with malignant potential. They found that a significant proportion of leukoplakias were enriched with Fusobacteria, including members of the genera *Fusobacterium* and *Leptotrichia,* and *Campylobacter* species relative to contralateral normal tissue from the same patients [22]. Schmidt et al. also identified moderate enrichment for Bacteroidetes and Fusobacteria on precancerous oral lesions [20]. These studies suggest that the microbiome changes identified in studies of OSCC begin early in the process of malignant transformation, supporting a possible role for microbiome changes in the pathogenesis of the disease. Direct evidence that colonisation with Fusobacteria or other microbes increases the risk of malignant transformation is currently lacking. This will require further study to determine if microbiome shifts are associated with the degree of dysplasia in OLK, and longitudinal follow up studies of OLK patients to establish if those with microbiome shifts are at an increased risk of developing OSCC.

What drives microbiome changes in OSCC?

Changes in the oral microbiome during carcinogenesis may be driven by lifestyle factors and poor oral hygiene. Börnigen et al. examined oral rinses from oral and oro-pharyngeal cancer patients and showed that smoking and tooth loss were major drivers of oral dysbiosis, indicating that these factors may influence mucosal associated communities [23]. Smoking has been demonstrated to have a major impact on the salivary microbiome [24]. However, studies which examine diseased mucosa in comparison with contralateral healthy tissue from the same patient show that microbiome changes may be localised to the diseased site [20,22]. This suggests that site-specific mucosal changes may be the primary driver in the acquisition of an altered microbiome, probably due to changes in the expression of epithelial cell-surface receptors during carcinogenesis. Even tissues with low grade dysplasia exhibit loss of E-cadherin and Epithelial Membrane Protein 1 (EMP1) [25], indicating that the surface of dysplastic tissues are considerably different which may result in acquisition of an altered microbiome. However, the influence of poor oral hygiene, smoking and alcohol consumption on the wider oral microbiome may also be important as they will alter the reservoir of organisms that can colonise the mucosal surfaces. For example, Amer et al. showed in OLK patients that mucosal levels of *F. nucleatum* were generally greater in non-smokers relative to smokers [22]. This finding may be significant given that some studies identified non-smoking OLK patients as being at greater risk of progressing to OSCC than smokers [26].

Are the microbes found on OSCC drivers of malignant transformation?

In the case of *F. nucleatum,* transit of the organism to a non-oral site (i.e. the colon) may be involved in the pathogenesis of colorectal carcinomas. However, the significance of increased carriage of these organisms on oral mucosal surfaces is not known. Members of the phyla Fusobacteria and Bacteroidetes are normally low abundance commensals on mucosal surfaces and it is unclear how these organisms could transform to become drivers of malignant transformation. It is possible that their increased abundance might pass a critical threshold that triggers a damaging inflammatory response. The pro-inflammatory response of oral keratinocytes to *Candida albicans* has been shown to be dose dependent [27] and this may also be true for bacterial colonisation. The only experimental evidence that bacteria may induce malignant change in the oral cavity was presented by Binder Gallimindi et al. who showed that *P. gingivalis* and *F. nucleatum* could promote oral carcinogenesis in a chemically induced (4NQO) murine model of OSCC [28]. In addition to the bacterial microbiota, colonisation with the fungus *C. albicans* has also been implicated as a risk factor in OSCC [29]. Studies have shown a significantly higher degree of *C. albicans* colonisation in OSCC patients [30,31] and associations with the degree of dysplasia in OLK [30,32]. Studies have shown that these yeasts are genotypically different to carriage isolates from healthy patients [33]. This may have biological significance as *C. albicans* strains recovered from dysplastic tissue may have greater capacity to produce acetaldehyde and nitrosylating activity [34,35]. In a rat model of chemically induced carcinogenesis, *C. albicans* was also shown to induce neoplastic mucosal changes [36]. More recent studies of the mycobiome using Illumina sequencing technology have shown increased abundance of several fungal species including *C. albicans*, *C. etchellsii* and *Hanaella luteola* in OSCC. Although high-throughput sequencing technology allows unprecedented insight into the composition of the mycobiome, these studies will require tandem quantitative analysis of the fungal load in order to determine the significance of fungal carriage levels [37]. The impact of fungal colonisation on the bacterial microbiota may also be significant as Amer et al. [22] reported that *Candida* colonisation was associated with increased levels of *F. nucleatum*.

Studies of the oral virome have largely concentrated on HPV and EBV. In particular, oropharyngeal cancers are closely associated with HPV subtypes 16 and 18 which have the greatest oncogenic potential [38]. *In vitro*, HPV 16 has been shown to immortalise human oral keratinoctyes and this virus is the most prevalent type recovered from oropharyngeal tumours [39,40]. Epidemiological data on the prevalence of these viruses in oral tissues often vary considerably and this may in part be due to varying sensitivity of the methods used to detect viruses [38,40,41]. Although HPV is strongly associated with oropharyngeal cancers, particularly cancers of the tonsils, a definitive association with oral cancers has yet to be proven. Klozar et al. found that 70% of oropharyngeal tumours (including 80% of tonsillar cancers) were HPV positive whereas in the same study only 20% of oral cancers were HPV positive [42]. More recently a study of 409 oral SCC showed positivity of high risk HPV E6/7 oncogene expression in only 5.9% of cases [43]. Although a role for HPV in OSCC has not been definitively proven, the involvement of the HPV virus in a proportion of OSCC is possible. In addition, the role of different HPV subtypes or novel viral pathogens cannot be discounted and a thorough analysis of the virome in malignant and potentially malignant tissues is required.

What is the possible mechanism of microbiome-driven malignant transformation in the oral mucosa?

Initial mechanistic studies examined whether by-products of microbial metabolism could trigger malignant transformation. Saliva has been shown to possess low level genotoxicity as measured by the Ames test and this is significantly increased in saliva from smokers and alcohol drinkers and those with poor oral hygiene [44]. This genotoxic activity has been proposed to involve acetaldehyde (ACH) and *N*-nitrosamine compounds. Martilla et al. demonstrated that oral microbial cultures can generate carcinogenic ($> 100\,\mu M$) levels of ACH *in vitro* and that cultures from smokers produced significantly higher levels [45]. Significant levels of ACH can also be detected directly in saliva

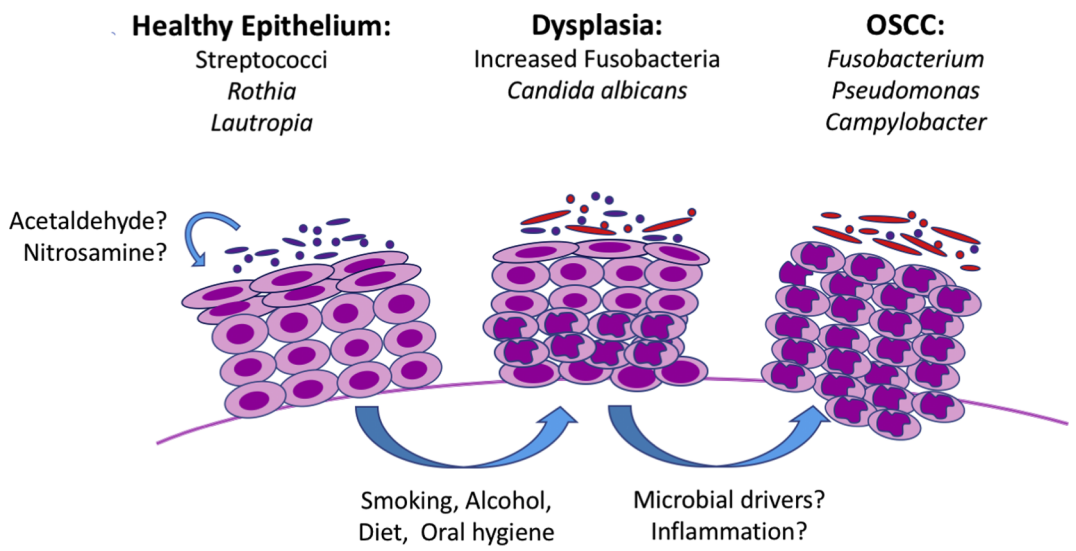

Fig. 1. A diagram outlining the possible mechanisms by which the oral microbiome could be involved in the aetiology of oral cancers. Normal healthy epithelium (left side) is colonised with higher levels of Streptococci, *Rothia spp.* and *Lautropia spp.* relative to malignant tissue. This normal healthy microbiome may play a role in producing acetaldehyde from alcohol and other genotoxic substances (nitrosamines), which could play a role in driving dysplastic change. Epithelium which exhibits dysplasia (center) has been shown to be colonised with increased levels of Fusobacteria and *Candida albicans*. This altered microbiome may play a role in malignant progression via inflammation or other mediators, contributing to the development of cancer. OSCC (right) has been shown to be colonised with a highly inflammatory microbiome consisting of *Fusobacterium*, *Pseudomonas* and *Campylobacter spp*.

following ingestion of alcohol and, interestingly, use of chlorhexidine for 3 days significantly reduced the amount of ACH produced [46]. *In vitro* characterisation of the oral microbiome suggests that *Neisseria* species and *Candida* species are the amongst the most potent microbial producers of ACH [35,47–49]. Paradoxically, current data indicates that smoking greatly reduces the abundance of *Neisseria spp*. in the oral cavity, which could theoretically reduce the ACH producing capacity of the oral microbiome [24]. The influence of OSCC risk factors on the carriage of ACH generating microorganisms requires further investigation.

Microbially derived *N*-nitrosamine compounds are another potential carcinogen. *N*-nitrosamines can be formed by commensal bacteria and *Candida spp. in vitro* from nitrite and secondary amines [50,51]. Community level metabolomics approaches are needed to determine whether disturbances in the normal microbiota can result in the production of these and other toxic metabolites.

Functional analysis of the OSCC metagenome indicates that this microbiota possesses enhanced inflammatory potential relative to healthy oral communities [17]. Activation of inflammatory driven cellular pathways of proliferation may have an important role in the progression of OSCC. Binder Gallimindi et al. demonstrated IL-6 activation of the STAT3 pathway in mice orally infected with *P. gingivalis* and *F. nucleatum* and hypothesised that this activation resulted in the increased levels of cyclin D1, MMP9 and heparinase observed in infected tissues [28]. *In vitro*, both *P. gingivalis* and *F. nucleatum* have been shown to invade oral keratinocytes and have been shown to interfere with cell signalling pathways providing plausible molecular mechanisms for their involvement in epithelial transformation (reviewed in [52]). *F. nucleatum* in particular has been shown to localise to precancerous tissue in the murine colon and has been suggested as a driver of malignant transformation, activating the Wnt signalling pathway through E-cadherin interactions via its FadA adhesin.

Conclusion

The role of the microbiome in malignant change in the oral cavity is still controversial and requires large cohort studies to provide evidence. OSCC generally requires the accumulation of multiple genetic lesions to manifest itself and microbial challenges are likely to be just one of many potential carcinogenic factors in the aetiology of the disease [40,53]. Our current hypothesis is that risk factors such as smoking or alcohol consumption could induce mutations resulting in epithelial dysplasia (Fig. 1). These initial dysplastic changes may involve the production of ACH or other carcinogenic agents by the normal oral microbiota. The resulting changes in the expression of epithelial cell surface markers may promote colonisation of the dysplastic tissue with an altered microbiome enriched with microorganisms such as Fusobacteria or *C. albicans* (Fig. 1). The levels of colonization on the dysplastic tissue may be influenced by oral hygiene. The presence of these organisms at high levels may then accelerate the progression towards OSCC, via inflammation, cell invasion or the production of genotoxic substances. Susceptibility to these mechanisms may be influenced by host factors such as age and diet. The result of these interactions is a highly inflamed tumour which is then colonised by a microbiome enriched for *Fusobacterium* and *Pseudomonas* amongst other Gram negative species. Conclusive evidence for that these organisms can induce malignant change in humans will require longitudinal studies to determine if high level carriage on mucosal surfaces increases the risk of OSCC.

Conflict of interest statement

None to declare.

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