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The bacterial small RNAs: The new biomarkers of oral microbiota-associated cancers and diseases

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Abstract: The oral microbiota is a vital part of the human microbiota that functions in various physiological processes and is highly relevant to cancers and other diseases. With the alterations of host immune competence, the homeostatic balance existing between the oral microbiota and host may be disturbed and result in the development of diseases. Numerous observations have suggested that small RNAs are key regulators of bacterial pathogenesis and bacteria-host interactions. Further, bacterial small RNAs are considered to be promising biomarkers for the development of novel, and efficacious therapies for oral dysbiosis. Mechanistic insights into how oral pathogens communicate with other bacteria or host cells in oral cancers via small RNAs are hot topics of research. Current studies also have begun to elucidate the key role of oral microbiota in the development of systemic diseases. This article discusses existing findings and nascent mechanisms governing the small RNA-based interactions between oral microbiota and associated diseases. The knowledge of such interactions is key in planning approaches to reverse dysbiosis to achieve health.

List of Abbreviations

OSCC	Oral squamous cell carcinoma
sRNA	Small RNA
P. gingivalis	*Porphyromonas gingivalis*
F. nucleatum	*Fusobacterium nucleatum*
OMV	Outer membrane vesicle
DSC2	Desmocollin-2
CRC	Colorectal carcinoma
A. actinomycete-mcomitans	*Aggregatibacter actinomycetemcomitans*
EPS	Extracellular polymeric substances
S. mutans	*Streptococcus mutans*
msRNAs	microRNA-size small RNAs
GCF	Gingival crevicular fluid
MetS	Metabolic syndrome
MAFLD	Metabolic-associated fatty liver disease
RA	Rheumatoid arthritis

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Introduction

The oral microbiota refers to the collection of various microorganisms that colonize the human oral cavity (Zhang *et al.*, 2015). This microbiota is receiving increasing attention from scholars and doctors given its relevant accessibility and complex interactions with cancers and many other diseases (Krishnan *et al.*, 2017). The complex structure and dynamic aspects of oral microbiota are pathogenic factors of oral diseases such as periodontitis and dental caries. They are also considered highly relevant to oral squamous cell carcinoma (OSCC) (Lamont *et al.*, 2018). Substantial evidence has implicated the mechanistic role of oral microbiota in inflammation and immune responses. As the unique microenvironment of the oral cavity, the majority of oral microbiota tend to form a biofilm against the mechanical action of mastication and fluid shear stress (Flemming *et al.*, 2016; Yin *et al.*, 2019). Within the biofilm, oral microbiota uses various means to communicate with one another and their eukaryotic hosts (Bassler and Losick, 2006). Bacterial small RNAs (sRNAs) are a significant number of RNA molecules between 40 and 500 nucleotides in length. They have been found to serve as post-transcriptional regulators that affect almost every field of

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bacterial physiology, including quorum sensing, environmental stimulus-response, and biofilm formation (Bossi and Figueroa-Bossi, 2016; Carroll *et al.*, 2016; Lei *et al.*, 2019; Merritt *et al.*, 2014). Many sRNAs are found to be formed through interactions with host cells and, in turn, affect their viability in the host (Papenfort and Vogel, 2010; Storz *et al.*, 2011). Therefore, bacterial sRNAs are considered novel biomarkers for oral microbiota-associated cancer therapy. The question of whether sRNAs function as the functional regulator or signal transduction in bacteria or host cells is an active area of discovery (Irfan *et al.*, 2020; Zhou *et al.*, 2019). Recent studies have explored the interaction of oral pathogens with host cells in cancers and oral diseases via sRNAs. This article discusses existing findings and nascent mechanisms governing the sRNAs-based interactions between oral microbiota and associated diseases.

The Role of Oral Microbiota in Oral Health

The oral cavity has distinct microenvironments where the oral microbiota located in the supragingival and subgingival communities vary dramatically in composition and spatial organization (Baker *et al.*, 2017; Lamont *et al.*, 2018). Oral microbiota in a healthy physiological state is considered a stable component of the ecosystem, which can compete with and resist exogenous pathogens and contribute to the development of normal tissues and the immune system (Graves *et al.*, 2019; Mira *et al.*, 2017; Willis and Gabaldon, 2020). However, with the alterations in host and environmental factors, the microbiome can shift to a state of dysbiosis and finally lead to oral diseases (Costalonga and Herzberg, 2014). In particular, it is reported that poor oral hygiene could lead to a remarkable increase in the relative abundance of potential cariogenic *Leptotrichia* and a decrease in *Streptococcus* (Belstrom *et al.*, 2018). Further, smoking appears to be a major environmental factor associated with oral disease progression (Yu *et al.*, 2017). Studies in recent years have shown that smoking may affect the balance of the microbiome by changing biofilm organization, local microenvironment, immune homeostasis, or direct contact with the microorganisms contained in it, which may be involved in the occurrence of diverse oral diseases (Huang and Shi, 2019). Thus, the oral microbiome is home to various microorganisms that have established mutually beneficial relationships with their hosts under healthy conditions. Additionally, the approaches that trigger a shift from the dysbiosis of oral microbiota and host to eubiosis are active areas of discovery.

Small RNA-Based Interaction of Oral Pathogens and Cancers

Oral squamous cell carcinoma

OSCC is the sixth most prevalent cancer worldwide and the most common head and neck malignant tumor with a poor prognosis and survival rate (Lafuente Ibanez de Mendoza *et al.*, 2020). Several studies have shown periodontitis to be a major risk factor for OSCC, and periodontal pathogens have similarly been associated with the lesions (Lamont *et al.*, 2022; Shin *et al.*, 2019). For example, the pattern is one of a dysbiotic microbial community enriched in potentially carcinogenic organisms such as *Porphyromonas gingivalis* (*P. gingivalis*) and with an underrepresentation of homeostatic commensals, contributing to the development of OSCC. A theoretical mechanistic framework for a bacterial contribution to carcinogenesis includes modulation of the balance of host cell proliferation and death, disruption of immune surveillance, and alteration of the metabolism of host-produced compounds, nutritional substrates, or drugs (Fitzsimonds *et al.*, 2020).

According to a report, *P. gingivalis* was shown to promote the development of OSCC and could modulate the expression of microRNAs (miRs) in host cells. *P. gingivalis* could increase OSCC cell proliferation by regulating cyclin D1 expression via the miR-21/programmed cell death 4 (PDCD4)/AP-1 negative feedback signaling pathway (Chang *et al.*, 2019). In another study, it was observed *P.gingivalis* worked as an enhancer of OSCC cell invasiveness by the dysregulation of the expression of the zinc-finger E-box-binding homeobox 1 (ZEB1)/CK13/miR-200 circuit (Sztukowska *et al.*, 2016). While *Fusobacterium nucleatum* (*F. nucleatum*) is prevalent in healthy microbiota, several studies have found that *F. nucleatum* is significantly enriched in patients with head and neck cancer (McIlvanna *et al.*, 2021). Further, Yost *et al.* (2018) profiled the RNA expression in the oral microbiome in OSCC and reported that *F. nucleatum* showed the highest upregulation of putative virulence factors for tumor sites (Yost *et al.*, 2018). Similar to *P. gingivalis*, it was found that *F. nucleatum* could upregulate the expression of miR-21 to promote the proliferation of cancer cells.

Recent evidence suggests that outer membrane vesicles (OMVs) released by *P. gingivalis* that contain differently packaged sRNAs may function in the development of OSCC. The OMV production can be found in all growth phases of bacterial culture, and is considered to play a significant role in the interaction between the bacteria and the host (Choi *et al.*, 2017b). For instance, it was reported that sRNA23392 was abundant in *P. gingivalis* OMVs and it promoted the invasion and migration of OSCC cells by targeting desmocollin-2 (DSC2). This study revealed a novel *P. gingivalis* OMVs/sRNA23392/DSC2 pathway-dependent tumor migration and invasion, whereby *P. gingivalis* promotes OSCC by delivering sRNAs into host cells (Liu *et al.*, 2021).

Other oral microbiota-associated cancers

Oral microbiota is located at the starting point of the digestive tract and plays an important role in regulating nutrient absorption, substance metabolism, and immune response (Gao *et al.*, 2018). Diseases or changes in the human environment often accompany changes in oral microbiota. Further, oral microbiota is also related to a greater risk of some gastrointestinal tumors. Landmark publications in 2012 from two independent groups reported that *F. nucleatum* infection was prevalent in human colorectal carcinoma (CRC) (Castellarin *et al.*, 2012; Kostic *et al.*, 2013). Additionally, *F. nucleatum* DNA was higher in

colorectal polyp tissue than in healthy tissue from controls. *P. gingivalis* and *A. actinomycetemcomitans* were confirmed to be associated with an increasing risk of pancreatic cancer (Fan *et al.*, 2018). Further, *Treponema denticola* and *Prevotella intermedia*, two common oral pathogens, were also reported to have distinctive enrichment in CRC in a case-control study (Yang *et al.*, 2019).

Indeed, microscopic imaging and molecular biology analyses have demonstrated that bacteria can deliver their sRNAs to and interact with eukaryotic cells via OMVs (Dauros-Singorenko *et al.*, 2018). Recently, using the method of small RNA Metagenomics by Sequencing (sMETASeq), a research team showed that bacterial sRNAs were expressed more widely and consistently than previously expected (Mjelle *et al.*, 2020). In the human body, bacterial sRNAs have been confirmed in plasma and serum, which has been reported in other tissues in more and more studies (Wang *et al.*, 2012). Up to now, secreted RNAs were confirmed in many bacteria, including *P. gingivalis*, *Streptococcus* sp., *Vibrio cholerae*, *Pseudomonas aeruginosa* and *Helicobacter pylori* (Ghosal, 2018; Koeppen *et al.*, 2016; Zhang *et al.*, 2020). The description of sRNA-based host-pathogen interactions has been carried out mainly for oral tumors (Pita *et al.*, 2020). The roles of such interactions in the pathogenesis of some oral microbiota-associated systemic cancers need to be explored in the future. The sRNA-based interaction of oral pathogens and cancers are depicted in Fig. 1.

Small RNA-Based Interaction of Oral Pathogens and Diseases

Dental caries

Dental caries is a multifactorial and dynamic disease with oral microorganisms as the initiating factor, which causes the staged demineralization and remineralization of dental hard tissues (Bowen, 2015; Pitts *et al.*, 2017). Early colonizers are commensal bacteria, such as *Streptococcus sanguinis* and *Streptococcus mitis*, which adhere more firmly to the teeth surfaces and grow faster than cariogenic bacteria, maintain microbial homeostasis and stability via antagonism against pathogens through various mechanisms (Bowen *et al.*, 2018; Maske *et al.*, 2017). Excessive dietary carbohydrate intake can promote the production of bacterial extracellular polymers substance (EPS) and acid metabolites, leading to the accumulation of acid-producing and acidophilic microorganisms (Tanner *et al.*, 2018). The formation of an EPS-rich biofilm accelerates acid production and protects the pathogenic microorganisms from rapid buffering by saliva (Bowen *et al.*, 2018; Marsh and Zaura, 2017).

As one of the recognized principal pathogens of caries, *Streptococcus mutans* (*S. mutans*) plays a vital pathogenic role in the production of extracellular polysaccharides and acid metabolites and a strong acid tolerance (Klein *et al.*, 2015). A great number of genes encode relative virulence factors and enable the competition mechanisms of *S. mutans*, which are linked with lipoteichoic acids (*dltABCD*, *SMU_775c*), exopolysaccharides (*gtfBCD*, *gbpB*, *dexA*, *vicRKX*) and extracellular DNA (*lytST*, *lrgAB*, *ccpA*) (Florez Salamanca and Klein, 2018; Lei *et al.*, 2018; Senadheera *et al.*, 2005; Smith and Spatafora, 2012). However, acquiring virulence genes is only the first step to disease development. Numerous studies have reported that sRNAs regulated the pathogenicity of cariogenic *S. mutans*. For instance, A novel group of microRNA-size small RNAs (msRNAs) was found in *S. mutans* where these msRNAs could down-regulate exopolysaccharides by directly binding to the 5′ untranslated region (UTR) region of the *vicR* gene (Lei *et al.*, 2020; Mao *et al.*, 2016). In another analysis, *S. mutans* were exposed to 1% or 5% sucrose, and bacterial RNA libraries were established to assess the expression levels of virulence-related sRNAs and target genes (*gtfB*, *gtfC*, and *spaP*). Several differentially expressed sRNAs were obtained, suggesting that sucrose can induce a series of sRNAs and might be involved in regulating biofilm formation (Liu *et al.*, 2019, 2017). Several other sRNAs taking part in the

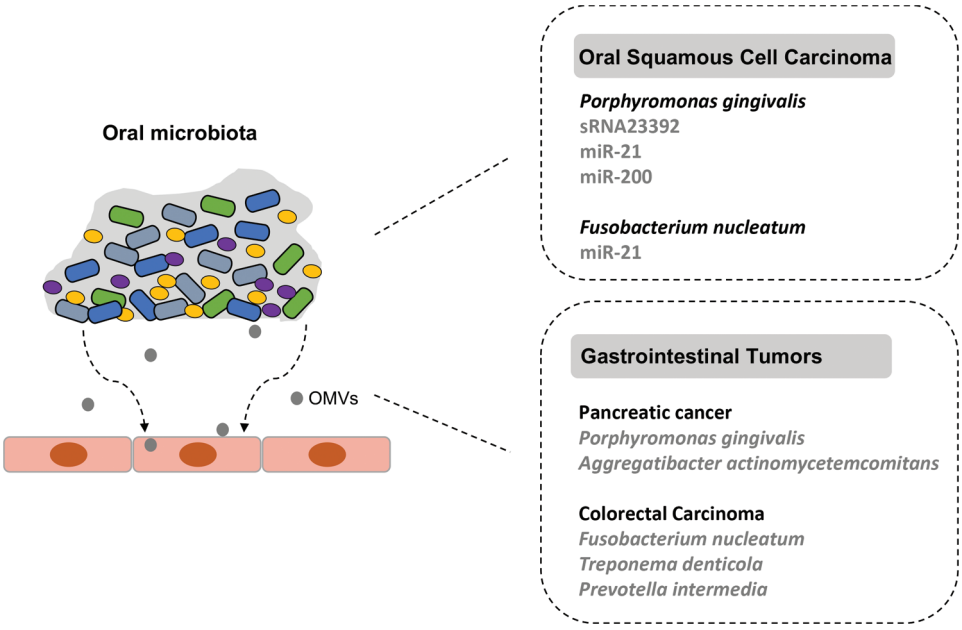

FIGURE 1. The small RNA (sRNA)-based interaction of oral microbiota with associated cancers. The dysbiotic oral microbial community is related to the development of oral squamous cell carcinoma (OSCC) and gastrointestinal tumors. The periodontal pathogen *P. gingivalis* can release outer membrane vesicles (OMVs) that contain different packaged sRNAs and function in the development of OSCC.

intricate regulatory networks of biofilm formation were identified by using deep sequencing. These include sRNA0426, which takes part in metabolic pathways, especially carbon metabolism (Yin *et al.*, 2020), and L10-Leader, which is related to growth and stress response (Xia *et al.*, 2012). In general, sRNAs of cariogenic *S. mutans* mainly function in the fine regulation of cellular processes that help it adapt to environmental changes and stress responses.

Periodontal disease

In periodontal disease, the polymicrobial community induces a chaotic and destructive host reaction through a complex mechanism, causing the progression of periodontal inflammatory disease (Ebersole *et al.*, 2017; Proctor *et al.*, 2020). The dysbiosis of oral microbiota is clearly related to the development of periodontal inflammation (Curtis *et al.*, 2020). It is certain that bacteria could cause gingivitis as their removal results in the reversal of inflammatory conditions (Huang *et al.*, 2014). The development of periodontitis is accompanied by dramatic changes in the composition of subgingival communities, with different Gram-negative species, rather than those that accumulate during gingivitis, gaining an ecological advantage over health-related groups (Abusleme *et al.*, 2013). *P. gingivalis*, *Tannerella forsythia*, and *Treponema denticola* (red complex) were recognized as such main pathogenic microorganisms (Holt and Ebersole, 2005). Changes in the host microenvironment may drive this dysregulation of the microbiome, and one possible driver of such changes is the host inflammatory response. In particular, inflammation exacerbates environmental dysfunction and tissue destruction, increases the flow of gingival crevicular fluid (GCF), carries degraded tissue collagen, blood, and other nutrients into the gingival crevicular canal. It also facilitates the growth and aggregation of pathogenic bacteria, especially those proteolytic and hydrolytic bacteria with iron acquisition ability. In contrast, health-related (symbiotic) species are less likely to survive in the new environment and are competed out (Diaz *et al.*, 2016). This imbalance leads to further ecological imbalances that exacerbate inflammation and ultimately lead to periodontitis in susceptible populations (Hajishengallis, 2015; Lamont *et al.*, 2018).

sRNAs play a critical role in post-transcriptional regulation, especially in rapid-changing environments such as periodontitis progression. For instance, it is reported that 20 Rfam sRNA families are overexpressed during periodontitis progression. These differentially expressed sRNAs are involved in various gene ontology activities, including carbohydrate metabolism, amino acid metabolism, ethanolamine catabolism, control of plasmid copy number, intron splicing, response to stress, and signal recognition particle-dependent cotranslational protein targeting to membrane (Duran-Pinedo *et al.*, 2015). These findings indicate that sRNAs are crucial regulatory elements of the dysbiotic process resulting in periodontitis. The newly identified PG_RS02100, a small conserved transcript, has been confirmed to regulate the survival of *P. gingivalis*, particularly against oxidative stress (Phillips *et al.*, 2018). Moreover, deep sequencing and bioinformatic analysis identified a group of msRNAs was also identified in the periodontal pathogens *Aggregatibacter asctinomycetemcomitans* (A.A_11134, A.A_20050, A.A_30457, and A.A_25585), *P. gingivalis* (P.G_45033, P.G_122, P.G_16418, and P.G_25037) and *Treponema denticola* (T.D_2161, T.D_16563, and T.D_15612). Additionally, msRNAs were detected to be secreted and delivered into host cells via bacterial OMVs, which suppressed the expression of certain cytokines in host cells (Choi *et al.*, 2017a). It has been proposed that sRNAs may serve as a new bacterial signaling molecule that mediates the interaction between bacteria and humans. The sRNA-based interaction of oral pathogens and oral diseases are depicted in Fig. 2.

Other systemic diseases

Many studies have shown the immense changes the oral microbiome has undergone in most oral diseases, such as

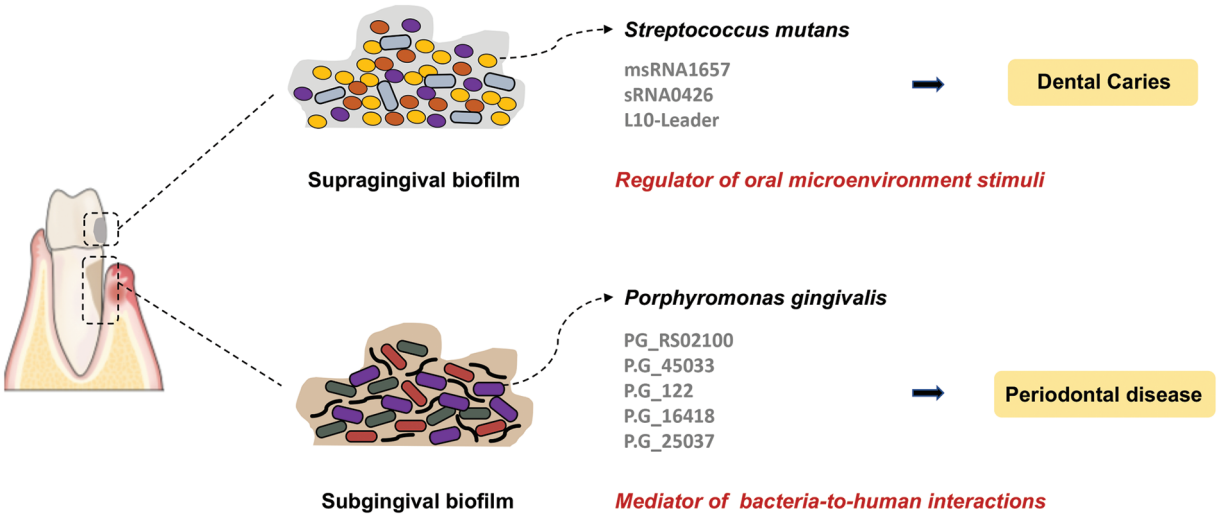

FIGURE 2. The small RNA (sRNA)-based interaction of oral microbiota with dental caries and periodontal disease. The dysbiosis of supragingival and subgingival biofilms leads to the development of dental caries and periodontal disease, respectively. Further, the small RNAs are key regulators of bacterial pathogenesis and bacteria-host interactions.

periodontitis and dental caries, which are also common in various systemic diseases. An analysis of the oral microbiome and metabolic syndrome (MetS) identified *Granulicatella* and *Neisseria* as distinctive genera in subjects with MetS and *Peptococcus* distinctive in the healthy controls (Si *et al*., 2017). High serum thyroid-stimulating hormone (TSH) levels are associated with many metabolic disorders. For example, a recent study reported that the salivary microbiome showed significantly higher diversity in individuals with higher TSH levels (Dong *et al*., 2021). Further, *Fusobacteria* and *Actinobacteria* were detected as significantly more abundant in subjects with diabetes, while *Proteobacteria* were less abundant (Matsha *et al*., 2020). It was also reported that the diversity of the oral resident microbes in metabolic-associated fatty liver disease (MAFLD) patients was significantly higher than in healthy individuals (Zhao *et al*., 2020). Furthermore, dysbiosis of oral microbiota was detected in rheumatoid arthritis (RA) patients and partially resolved after RA treatment (Zhang *et al*., 2015). There was evidence that periodontitis-related pathogens such as *P. gingivalis* and *A. actinomycetemcomitans* may promote autoantibody production and autoimmune exacerbation in RA (Maeda and Takeda, 2019). Indeed, substantial evidence has implicated the mechanistic role of the oral microbiome, including transmigration, cytokine release, molecular mimicry, toxin release, and translocation of oral bacteria to the gut in various systemic diseases (Read *et al*., 2021; Tonelli *et al*., 2023). Unlike oral diseases and cancers, the knowledge of whether sRNAs are involved in pathogenesis is not clear. It is worth studying whether the bacterial sRNAs can be considered promising biomarkers for developing novel and efficacious therapies for oral dysbiosis.

Conclusions

As an important part of the human microbiota, oral microorganisms are involved in various physiological processes, including the development of oral and systemic diseases. The pathological state affects the composition of oral microbiota, and then the dysbiosis of oral microbiota exacerbates the development of the disease. The mechanisms may involve inflammation and immune responses, where sRNAs may play an important regulatory role during this process. Numerous observations have suggested that sRNAs are key regulators of bacterial pathogenesis and bacteria-host interactions. Further research is required to explore the molecular mechanisms involved to illuminate the key roles of oral microbiota.

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