REVIEW ARTICLE

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Microbiome in Oral Squamous Cell Carcinoma: Mechanisms and Signaling Pathways

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Oral squamous cell carcinoma is part of head and neck squamous cell carcinoma which is the ultimate cause of morbidity and mortality in cancer. The alteration of microbial community in the saliva might act as a helpful marker for the prediction, detection and prognosis oral cancer, particularly the transition of cancer precursor lesion. There are three mechanisms of action of oral microbiota in cancer pathogenesis, chronic inflammation of bacterial stimulation, carcinogenesis by cytoskeletal rearrangements, and carcinogenic substances that produced by microorganisms. Changes in the composition of microbiota could therefore have the potential to be used as a significant oral biomarker to predict the pathological transition from oral epithelial precursor lesion to cancer.

Keywords: microbiome, oral cancer cellular proliferation, microorganism, oral cancer, oral squamous cell carcinoma

Introduction

Oral squamous cell carcinoma (OSCC), a part of head and neck squamous cell carcinoma (HNSCC), has a high recurrence rate with lymph node.[1] Most of the OSCC cases were diagnosed at late stage, hence the risk of secondary primary tumors was higher.[2-5] The 2019 death rate of oral and pharyngeal cancers was 10,860 cases in the United States of America, and tongue cancer was the main cause of death in oral cancer.[6] Recently, the rates of oral cancers have increased in developed countries such as Japan with an estimated of 7,000 new cases and 3,000 deaths with the 2:1 male-to-female ratio.[7] The 5-year cancer survival rate was 60-80%, but with early detection, the survival rate will rise to 90%.[6,7] Meanwhile in Indonesia in 2014, 319 oral cancer cases were diagnosed over the past 15 years at Dharmais National Cancer Hospital.[8] The Dharmais data showed that OSCC was most frequently found on the tongue, nearly 70% were advanced stage with bad prognosis and poor survival rates.[8]

Tobacco, betel quid, and alcohol are the primary risk factors for OSCC.[1] About 74% of OSCC cases are caused by tobacco chewing with or without betel nut. Risk factors such as tobacco smoking, alcohol consumption, and poor oral hygiene can inevitably make the case worse.[2] Not only chemical irritants such as cigarette and alcohol, but

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the dental prosthesis inflammation, human papillomavirus (HPV), and chronic periodontitis infection can also increase the risk of oral cancer.[1]

Chronic periodontal disease involves a pathological organism that will increase the risk of premalignant lesions and cancer.[6] According to some studies, oral pre-cancers and cancers affect the composition of the oral microbiome. In vice versa, chronic inflammation of bacterial infection induces cell proliferation, mutagenesis, oncogene activation and angiogenesis as well.[3] *Helicobacter pylori* is the first bacterium known as carcinogenic inducer.[4] Additionally, *Porphyromonas gingivalis* is another pathogenic bacterium that frequently found in OSCC. OSCC patients mostly suffer from chronic periodontitis, resulting in chronic inflammatory OSCC cells.[5] The relation between oral cancer-inducing factors and bacterial ecology is significant since alterations in oral microbiome have also been found in subjects consuming alcohol, betel quid and smoking.[9] Besides *P. gingivalis*, OSCC is strongly associated with bacteria such as *Streptococcus* sp., *Peptostreptococcus* sp., *Prevotella* sp., and *Capnocytophaga gingivalis*, while *Fusobacterium*, *Veillonella*, *Actinomyces*, *Clostridium*, *Haemophilus* and *Enterobacteriaceae* are associated with precursor lesions of the epithelium.[3]

Oral Microbiome in Healthy Individuals

Actinomyces, *Capnocytophaga*, *Eikenella*, *Eubacteria*, *Fusobacterium*, *Haemophilus*, *Lactobacterium*, *Leptotrichia*, *Neisseria*, *Porphyromonas*, *Prevotella*, *Propionibacterium*, *Peptostreptococcus*, *Streptococcus*, *Staphylococcus*, *Veillonella* and *Treponema* exist in healthy people's oral cavity. Other than that, *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Fusobacteria* are also prevalent oral microbial species in healthy individuals.[10] Those commensal species live with the host immune defense systems in equilibrium.[9] The host-microbiota co-dependence is preserved with host-provided nutrient-rich environment, while the commensal microbes secrete nutrient processing metabolites, avoid pathogenic microbe infections, and maintain homeostasis by stimulating immune responses.[10] *Streptococcus* is the predominant oral microbiome healthy genus.[4] Commensal and pathogenic bacterial organisms, by forming biofilm, circumvent the host immune response. Any disturbances of this equilibrium and reorganization of the microbial composition have been involved in various diseases such as, dental caries or periodontal diseases.[9] Most of these microorganisms live in a symbiotic capacity in our oral cavity, establishing mutually beneficial relationships with the host. Not only they are not harmful, but these commensal populations can also keep pathogenic organisms under control by not allowing them to adhere to mucosal surfaces.[11]

Commensal bacteria with proper inhibitors may improve the effectiveness of immunotherapy.[5] Compared with programmed death ligand 1 (PD-L1) specific antibody therapy, *Bifidobacterium* by oral administration controlled tumor growth with the same efficacy. Both the oral administration of *Bifidobacterium* and the PD-L1-specific antibody avoided tumor outgrowth. *Bifidobacterium* oral administration improves antitumor resistance and changes the effectiveness of control-point inhibitors as an immunotherapeutic agent by modifying the composition of intestinal microorganisms.[12]

Oral Microbiome in Cancer Patients

Oral microbial communities tend to vary from stable sites to malignant sites. For example, *Streptococcus anginosus* and *Treponema denticola* associate with multiple carcinomas of the upper gastrointestinal tract.[13] In general, anginosal infection may be involved in the carcinogenesis of HNSCC. The DNA of *S. anginosus* was found in specimens of carcinoma tissue but not in samples of lymphoma, rhabdomyosarcoma, or leukoplakia. Dental plaque may act as a dominant reservoir of this bacterium.[14,15]

In the pre-cancerous oral lesions, leukoplakia, *Bacteroidetes* and *Fusobacteria* are substantially enriched, compared to normal contralateral tissue in the same patients.[4,16] Meanwhile among patients with oral cancer, a substantial increase in the number of *C. gingivalis*, *P. melaninogenica*, and *S. mitis* among patients was reported.[17]

The incidence of cancer worldwide also refers to virus-related infections such as hepatitis viruses, HPV, and Epstein-Barr virus (EBV).[4] Nonetheless, in order to put viral infections in perspective, it has been estimated that at least six human viruses, EBV, hepatitis B virus (HBV), hepatitis C virus (HCV), HPV, human T-cell lymphotropic virus (HTLV-1) and Kaposi's associated sarcoma virus (KSHV) lead to 10-15% of all cancers worldwide.[18,19] HPV has been known recently as an etiological factor that cause oropharyngeal cancer, however, infection which caused by

HPV is not a main contributor to oral cancer, since the virus is infrequently found in oral cancers (only 2–4% of cases).[20]

Pathogenesis of Oral Microorganisms in Cancer Development

There are three mechanisms of oral microbiota in carcinogenesis. The first is the chronic inflammation bacterial stimulation. Such mechanism may results inflammatory mediators that may promote cells proliferation, angiogenesis activation and mutagenesis. With regard to the second mechanism, bacteria can influence carcinogenesis by cytoskeletal rearrangements, activation of nuclear factor-kappaB (NF-κB), and cellular apoptosis inhibition. The third mechanism is carcinogenic substances that produced by microorganisms.[21]

Stimulation of Chronic Inflammation

Porphyromonas, *Prevotella* and *Fusobacterium* as anaerobic species in oral cavity have significant role for periodontal diseases and chronic inflammatory mechanism. These bacteria induce inflammatory mediators production which affect epithelial and endothelial cells, fibroblasts, and extracellular matrix. These periodontal pathogens may affect the body to produce various cytokines such as interleukin (IL)-1β, IL-6, IL-17, IL-23, tumor necrosis factor (TNF)-α, and matrix metalloproteinases (MMP)-8 and MMP-9.[22]

In response to lipopolysaccharide (LPS), the key element of Gram-negative bacteria walls, the neutrophils, fibroblasts, and mast cells in periodontium tissues may synthesize IL-1β (Figure 1). The IL-1β induces the development of osteoclasts and bone resorption[23], resulting in alterations of sectional inflammatory in the periodontium. IL-1β also provokes the secretion of acute phase proteins, prostaglandins (PG), phospholipase A2, and proinflammatory cytokines such as TNF-α, IL-6, and MMPs.[5,24] Furthermore, IL-1β also encourages endothelial cells to produce Vascular Endothelial Growth Factor (VEGF) and many proangiogenic factors.[25] IL-1β was also associated with lower E-cadherin expression, which promotes cell migration.[26] Therefore, high level of IL-1β is associated to proliferation, more invasive and aggressive phenotype of a tumor.[25,27]

IL-6, which produced by the influence of LPS, IL-1β and TNF-α, also causes bone resorption and enhances the synthesis of PGE2, acute phase proteins, and chemokines.[1,28-32] IL-6 generates oxidative stress and may lead to a short-term aggregation of H_2O_2 in mitochondria, resulting in damage of mitochondrial.[33] IL-6 also affects the invasion and metastasis process by promoting MMPs expressions. Furthermore, IL-6 increases the release of Intercellular Adhesion Molecules (ICAMs) and Endothelial Leukocyte Adhesion Molecules (ELAMs), that promote adhesion of endothelial cells to tumor cells, thereby influencing the spread of tumors. Most genes activated by IL-6 are involved in the progression of the cell cycle and apoptosis suppression. IL-6 may influence the development of cancer by affecting anti-apoptotic pathways as well.[5,31,34]

TNF-α is secreted due to various elements, including bacterial LPS. Furthermore, TNF-α highly induces the progression of reactive oxygen species (ROS), PG, leukotrienes, and MMPs. TNF-α also decreases the production of osteogenic cells and fibroblasts. Unlike high doses of TNF-α that associated with tumor destruction, tumor development is related to low doses of TNF-α.[35,36] TNF-α induces tumor growth by activation of carcinogenic signaling pathways in epithelial cells, including Wnt and NF-κB.[28] TNF-α also has the ability of generating ROS to cause DNA damage. It has been shown that TNF-α affects motility and invasion processes by inducing MMPs to express and induce the development of various angiogenic factors such as IL-8 and VEGF.[37]

Anti-apoptotic Mechanisms

Epithelial cell responses to infection of *P. gingivalis* in both apoptosis and cell division. *P. gingivalis* can highly induce anti-apoptotic activity in primary cultures of gingival epithelial cells.[14] Role of *P. gingivalis* infection in tumor development remains to be determined, however, there is a solid proof of its inherent ability to influence many aspects of epithelial cell signaling. *P. gingivalis* promotes intracellular signaling of Jak1/Akt/Stat3, which lead to inhibition of mitochondrial apoptosis pathway[6], which has been targeted for inducing apoptosis in oral cancer cell.[38,39] It demonstrated the ability to lower the activity of p53 tumor suppressor.[40] *P. gingivalis* induces significant phosphorylation and inhibition of Bad in the membrane of mitochondrial as well as enhancing Bcl2/Bax ratios. In addition, *P. gingivalis* also suppresses caspases, important apoptotic machinery proteins which has been targeted for inducing apoptosis in oral cancer cell as well.[39]

P. gingivalis may also triggered adhesion of purinergic receptor P2X7-ATP, which plays a significant role in

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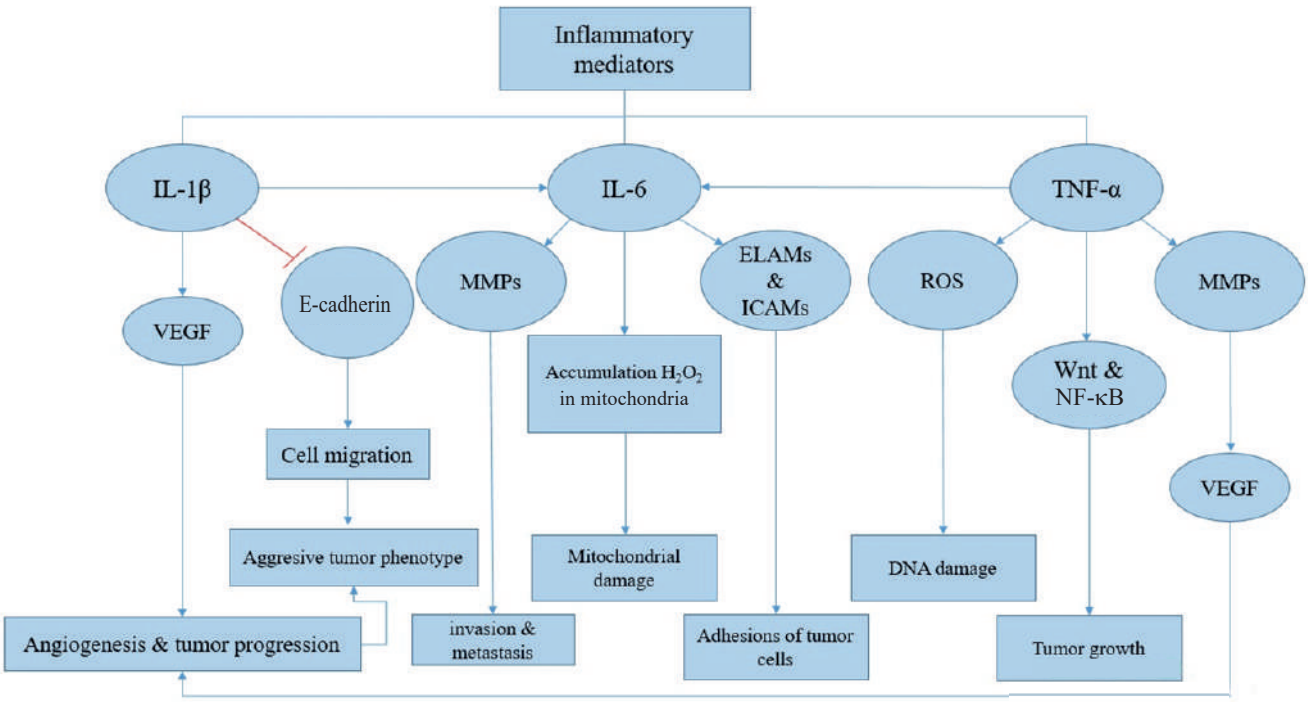

Figure 1. The mechanisms of chronic inflammation in carcinogenesis. Various inflammatory mediators induced by LPS bacteria may promote angiogenesis and tumor progression, aggressive tumor phenotype, invasion and metastasis, mitochondrial damage and adhesions of tumor cells.

neovascularization, metastasis, cell growth and secretion of inflammatory cytokines. This microorganism has shown its capability to release an anti-apoptotic enzyme nucleoside diphosphate kinase (NDK) that cleaves ATP and inhibits the activation of proapoptotic P2X7 receptor (Figure 2), thus facilitating the signaling of ATP/P2X7.[5] This NDK secretion may also regulate the cytosolic ATP-induced and ROS of mitochondrial which is an important mediator for the activation of transcription elements related to inflammation and the development of cancer.[5,41] In addition, *P. gingivalis* produces gingipains, which actually are cysteine proteinases, that can break the MMP-9 pro-enzyme into its mature active form.[5,21,40] Gingipains activation of MMP-9 induces degradation of the structure of the basal membrane, which encourages migration and invasion of cancer cells.[5,42]

Another oral microorganism, *Fusobacterium nucleatum*, may be the causative agent of oral squamous cell, pancreatic, and colorectal cancers. *F. nucleatum* infection modulates many anti-apoptotic pathways[43], including Toll-like receptor (TLR) activation and NF-κB signaling (Figure 3).[41] The most important part in the direct role of *F. nucleatum* in the carcinogenesis is the adhesin/invasin FadA, which adheres to E-cadherin on cancer cells and encourages the signaling of β-catenin. This pathway leads to increase Wnt transcriptional activity, pro-inflammatory cytokine activation, oncogenes, and cancer cells proliferation stimulation.[5,44] *F. nucleatum* also stimulates p38, leads to secretion of MMP-9 and MMP-13. Similar to MMP-9, MMP-13 also plays a significant role in tumor metastasis and invasion.[42]

Carcinogenic Effect Substances

Microorganisms play an important role in the metabolization of alcohol to acetaldehyde.[5] Many types of oral microorganisms including streptococci *S. Gordonii*, *S. Mitis*, *S. oralis*, *S. Salivary*, *S. sanguinis*, and *Candida* yeasts, have the alcohol dehydrogenase (ADH) enzyme, that metabolizes alcohol to acetaldehyde with subsequent potential for oral cancer growth.[45]

Lactic acid is produced by certain oral bacteria of the genus *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Bifidobacterium*, *Leuconostoc* and *Pediococcus*. Some species can produce more acids, for example *Peptostreptococcus stomatis* which produces acetic, butyric, isobutyric, isovaleric and isocaproic acids.[5] The majorities of bacteria isolated from the OSCC tissues were

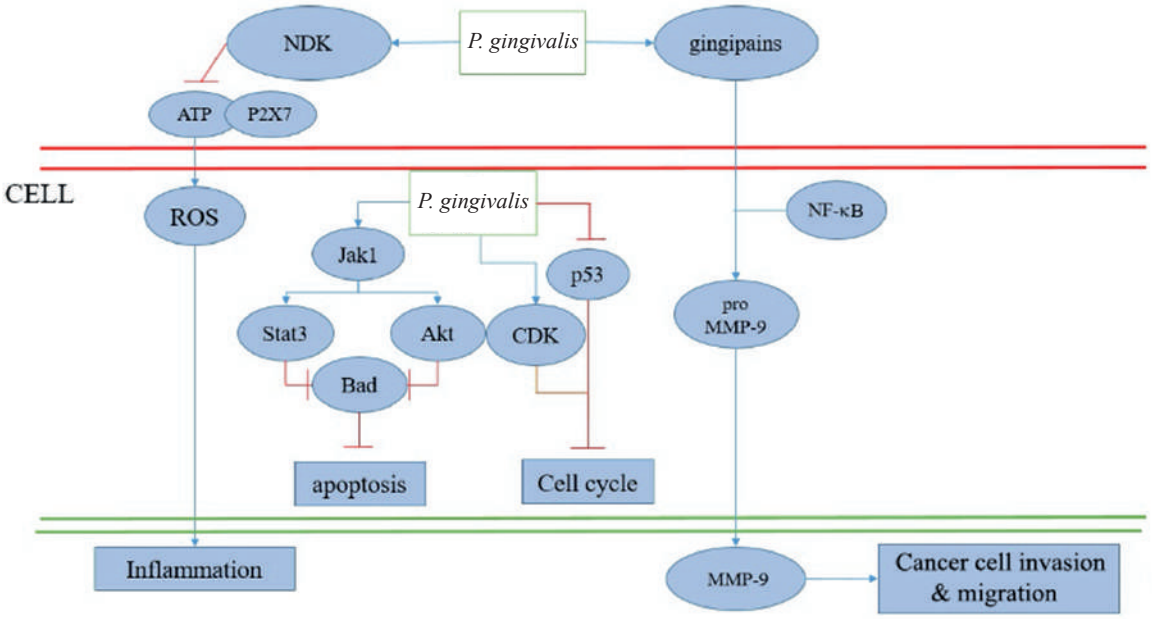

Figure 2. *P. gingivalis* can cause cell-cycle changes and apoptosis inhibition. Extracellular *P. gingivalis* may induce inflamation and promote cancell cell invasion and migration through gingipains proteinases. Intracellular *P. gingivalis* may inhibit apoptosis mechanism and disrupt cell cycle through activation of Jak1 and decrease the activity of p53.

saccharolytic and aciduric bacteria.[46] Such microorganisms may influence the pH reduction in the local environment by generating lactic acid. This acid production may enhance the acidic and hypoxic tumor microenvironment, thus increasing metastatic capability.[47]

Several oral microorganisms such as *P. gingivalis*, *Prevotella intermedia*, *Aggregatibacter actinomycetemcomitans*, and *F. nucleatum* contain volatile sulfur compounds (VSC), such as hydrogen sulfide (H_2S), dimethyl sulfide ($(CH_3)_2S$), dimethyl disulfide (CH_3SSCH_3) and methyl mercaptan (CH_3SH).[5] H_2S appears in saliva at the highest concentration, while CH_3SH is the dominant compound in the gingival pockets. H_2S is known as genotoxic agent and may result in cumulative mutations or genomic instability. Increased expression of various H_2S-producing enzymes in cancer cells has been observed, particularly in colon and ovarian cancers.[48]

It was also shown that H_2S promotes apoptosis in human gingival epithelial cells through mitochondrial pathway activation (Figure 4).[49] Upon cell exposure to H_2S, apoptosis was remarkably increased at 24 and 48 hours, ROS was enhanced, and depolarization of the mitochondrial membrane was disintegrated. Release of Cytochrome C, a small protein known to be related with the mitochondrial inner membrane involved in apoptosis initiation, increased significantly. Activities of Caspase-9 and -3 were reported highly stimulated, while the activity of caspase-8 remained low. In 48-hours the percentage of DNA-strand-break increased.[48,49]

Recent findings suggest that there are various mechanisms that lead to the effect of H_2S on cancer, consist of angiogenesis induction, control of mitochondrial bioenergetics, cell cycle progression acceleration, and anti-apoptosis functions.[50] Studies showed that H_2S has proangiogenic effects by enhancing the expression of VEGF in both kidney and ischemic tissues.[51] In addition, many evidence suggests that H_2S is an endogenous angiogenesis stimulator and promotes the growth and proliferation of tumor cells. Cystathionine β-synthase (CBS)-derived H_2S is used to induce angiogenesis and vasorelaxation, therefore promoting tumor growth in colon cancer.[50,52]

Growing evidence suggests that H_2S is involved in regulating mitochondrial function and cellular bioenergetics by direct donation of electrons and mitochondrial phosphodiesterases inhibition.[50] CBS co-locates with mitochondrial markers in ovarian cancer cells, and silencing CBS reduces the intake of mitochondrial oxygen accompany with an increase the production of ROS.[53] In

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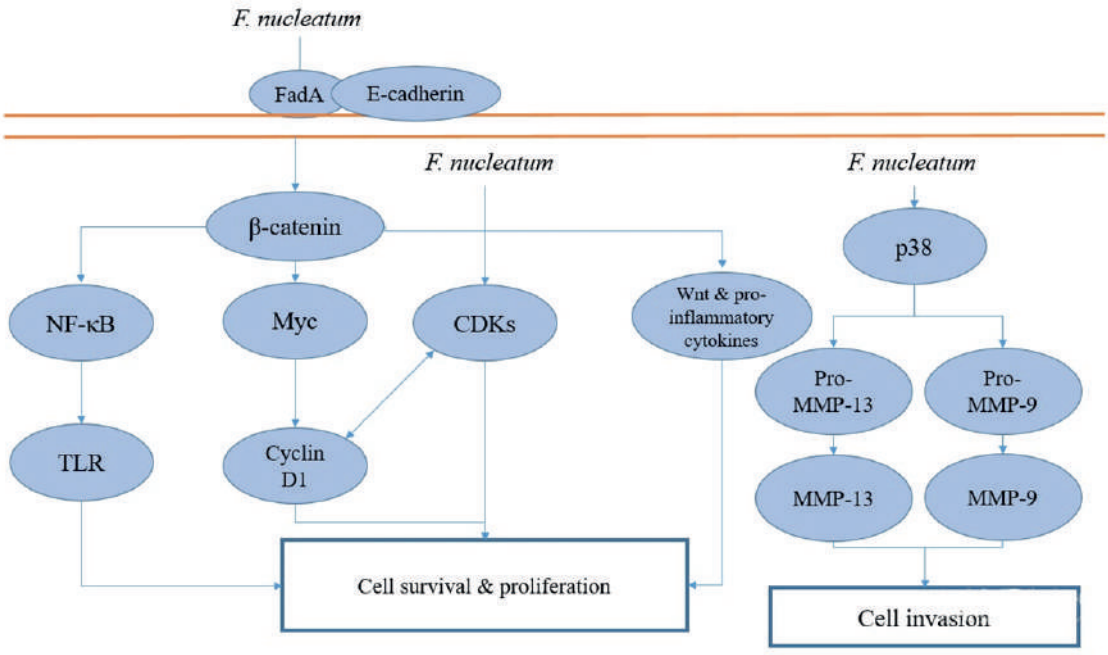

Figure 3. *F. nucleatum* can generate an oncogenic phenotype of epithelial cell. FadA will activating pathway of β-catenin, which leads to cell survival and proliferation. Intracellular *F. nucleatum* through p38 may cause MMP-9 and MMP-13 activation, which play an important part in cell invasion.

summary, CBS that generated from H_2S could sustain cellular bioenergetics in cancer cells, therefore promoting tumor growth and proliferation.[50] However, a recent study also shows that exogenous H_2S (NaHS, 200-500 μM) might serve as a proliferative induced factor that accelerates the progression of the cell cycle in oral squamous cell carcinoma by increasing phosphorylation of protein kinase B (PKB)/Akt and extracellular signal-regulated kinase (ERK).[50,54]

Microbiology Alteration as Biomarker for Oral Diagnostic

There are more than 500 species microorganisms in saliva of oral cancer patients, both cultivated and uncultivated varieties.[3] Compared to the approximately 50 strains observed using standard experimental methods, this large data set reflects significant improvement. Changes in the microbial community (*Peptostreptococcus*, *Bacillus*, *Parvimonas*, *Enterococcus*, and *Slackia*) in the saliva could act as a convenient marker for predicting, and detecting oral cancer, particularly the epithelial precursor lesion-cancer transition. At least nine bacteria, including *Alistipes*, *Bacteroids*, *Blautia*, *Clostridium*, *Dorea*, *Escherichia*, *Faecalibacterium*, *Megamonas*, and *Phascolarctobacterium*, showed positive associations in the epithelial precursor lesion and cancer groups. In addition, the normal group detected a very different positive/negative correlation network, suggesting community alteration in the disease group. Such associations may indicate the microbial community changes in saliva, which could identify many combinations of microorganisms associated with oral cancer and epithelial precursor lesions.[3] High salivary numbers of *Capnocytophaga gingivalis*, *Prevotella melaninogenica* and *Streptococcus mitis* can be used as OSCC diagnostic indicators. The involvement of on the salivary microbiota on OSCC has more severe effect than tobacco.[17] The surfaces of OSCC display significantly elevated levels of *Porphyromonas* and *Fusobacterium* relative to healthy mucosa.[55,56]

Conclusion

There are three mechanisms of action of oral microbiota in cancer pathogenesis, chronic inflammation of bacterial stimulation, carcinogenesis by cytoskeletal rearrangements, and carcinogenic substances that produced by microorganisms. The alteration of microbial community in the saliva might represent as a convenient marker for

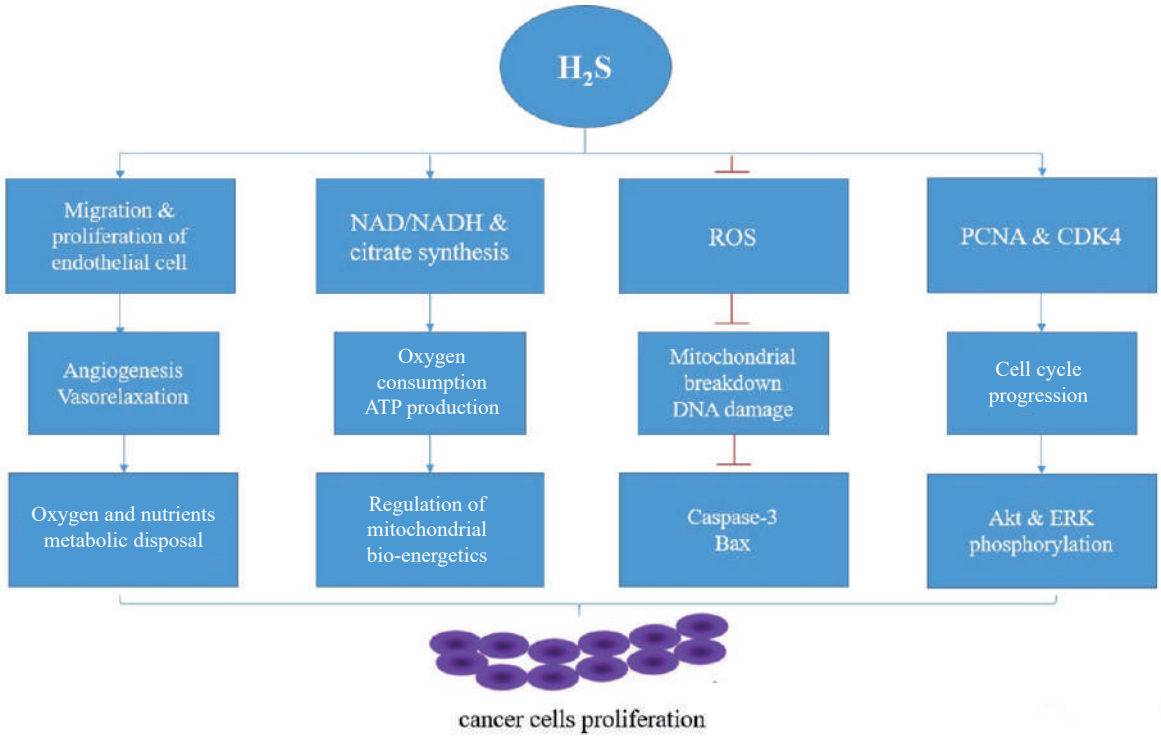

Figure 4. A schematic ilustration of H_2S in developing cancer cell proliferation. Induction of angiogenesis, regulation of mitochondrial bioenergetics, acceleration of cell cycle progression, and anti-apoptotic function contribute to the carconigenesis mechanisms of H_2S. H_2S: hydrogen sulfide; NAD: oxidized form of nicotinamide adenine dinucleotide; NADH: reduced form of nicotinamide adenine dinucleotide; ATP: adenosine 5'-triphosphate; ROS: reactive oxygen species; Bax: B-cell lymphoma gene-2 associated X protein; PCNA: proliferating cell nuclear antigen; CDK4: cyclin-dependent kinase 4; ERK: extracellular signal-regulated kinase.

the prediction, detection, and prognosis of oral cancer, especially the epithelial precursor lesion-cancer transition.

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