Porphyromonas gingivalis and *Fusobacterium nucleatum*

Mechanisms Leading to Development of Oral Cancer

A literature study

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Preface

In the past few decades, science and technology have made huge advancements especially in the field of medicine. Human life and the factors influencing it have always been of great interest to scientists to investigate prevalence of certain diseases and finding cure for it. In the past 30 years or so, epidemiological and experimental studies have put great emphasis into finding definitive causal relationship between cancer and its etiological factors. Recently, an increase in incidence and prevalence of oral cancer has been observed with more women and youngsters being affected. This focuses attention towards other contributory factors, which could be of great importance for prevention, early-detection and treatment of the disease.

The basis of this research actually stemmed from our passion towards microbiology and cell pathology. Therefore, we investigated bacterial mechanisms that could have potential oncogenic effects on normal cells. We hope that this research work can be beneficial for those who have interest in this topic.

This literature-based master thesis is a narrative review about oral cancer with focus on two important perio-pathogens *Porphyromonas gingivalis* and *Fusobacterium nucleatum.* This thesis aims and tries to explain theories about oral cancer and its etiological factors, putting a novel insight into potential oncogenic mechanisms of *P. gingivalis and F. nucleatum.*

We would like to thank our advisor førsteamanuensis Roger Simm from The Univerisity of Oslo, for good support and great guidance. Your guidelines and suggestions have been very helpful throughout the research work. Thank you for your professional support, trust and valuable time.

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Introduction

Cancer and its prevalence:

Cancer is a name given to group of related diseases characterized by growth and division of abnormal cells inside the body. Cells affected will grow in an uncontrollable fashion, and may develop characteristics that enable them to spread to other parts of the body. This process is referred to as metastasis (1). The body is composed of different types of cells that are renewed and removed in an orderly manner to keep up with performance and functionality. Cancer develops when uncontrollable proliferation of new cells takes place and the body is unable to remove old, damaged cells. Different types of cancers are found and named after their origin and appearance. For example, carcinoma is a type of cancer originating from skin and internal tissues whereas sarcoma originate from muscle, fat, cartilage and other connective tissues (2). Leukemia and lymphoma are types of cancer characterized by origin in bone marrow giving rise to abnormal blood cells. Leukemia originates in the bone marrow, whereas lymphoma mainly affects the lymphatic systems (2).

There is a difference between the term cancer and tumor. Tumor is mass of cells that is formed as a result of mutations of genes responsible for cell death (apoptosis) or/and cell proliferation and division, giving rise to abnormal cell properties. Many types of cancers form solid tumors that can be benign or malign. Benign tumors lack the ability to spread to other parts of the body. It will stay at its local site and provide often little harm. A malignant tumor, on the other hand, has the ability to metastasize and spread to other parts of the body via the blood or lymphatic systems causing functional destruction (1, 2).

Stages of cancer development

Tumor initiation, is the first process in cancer development, and starts by mutations of genes leading to atypical proliferation of one single cell. Atypical proliferation of cells give rise to a pre-neoplastic mass known as hyperplasia . As this cell divides, its daughter cells will also carry this mutation. As cell proliferations continues, and mutated cell population grows, additional mutations will occur. Those additional mutations may give rise to new characteristics in the growing cancerous cells thus increasing their aggressiveness and invasiveness giving rise to dysplasia. This stage is termed as *tumor progression* (1, 2).

Clone selection is the next stage where new mutations give rise to many different subpopulations. Not all subpopulations will survive, but the ones that do, have gained abilities that favors survival, invasiveness and the ability to metastasize to name a few. The next step is metastasis. Malignant tumors, for example, has the ability to invade and penetrate connective tissue to get access to the circulation/lymphatic drainage. This gives the tumor the opportunity to move through the body and find other parts to settle and to continue to grow and mutate (1, 2).

Oral cancer

According to the world health organization, one in six people on a global basis die due to cancer. Approximately 9.6 million people died of it in 2018, making it the second main cause of death worldwide. Because of its large scale mortality rate, understanding what cancer is and what causes it, will greatly help us understand ways to detected and treat it better (1). *Oral squamous cell carcinoma* (OSCC) is the most common type of malignant epithelial neoplasm occurring in the oral cavity. It originates from squamous cells inside the oral cavity and are often detected clinically in its later stages due to poor diagnostic markers. OSCC accounts for about 90% of all the cancers occurring in the oral cavity (3). Despite the advancements in the medical field, overall five year survival rate of oral squamous cell carcinoma patients is still around 50-60 % (3).

Mork et.al reports in an epidemiological study from 1998 that Norwegian males and females are at a low risk of developing oral and lip cancer as compared to other caucasion populations outside the Scandanavia. In the past 30 years, incidence of oral cancer have increased in the Norwegian society because of life-style changes and increase in number of immigrants(4). About 600 new cases of oral and lip cancer are being registered every year in Norway showing more prevalence in males than females (5). Surgical intervention together with chemo- and radiotherapy are often used for the treatment of cancer but diagnosis at advanced or later stages makes the effect of treatment minimal, decreasing the overall survival rate (3). Clinical detection and diagnosis of OSCC is confirmed by clinical and patho-histological tests. However, clinical features alone are not good enough to diagnose such a condition because many benign lesions can present themselves in the same manner as OSCC. Therefore

the clinical criteria is based upon the fact that, any white or red lesion existing for more than two weeks should be followed up and tested to exclude cancer (6).

Bacterial invasion Vs host

Epithelial and endothelial cells are affected by bacterial invasion, which causes activation of inflammatory cells such as monocytes, neutrophils and macrophages inducing release of pro-inflammatory cytokines. It is important to note that these bacteria react with cells by binding to membrane receptors, which recognize bacterial species as foreign substances. These receptors then send signals to immune system to respond to invading microorganisms. Pattern recognition receptors (PRRs) are present on epithelial cells, neutrophils, macrophages and dendritic cells, which recognize well-defined PAMPs. PAMPs are pathogen-associated molecular patterns such as certain lipopolysaccharides, fimbriae, lipoproteins, nucleic acids etc that are present on microbial membranes and are presented to toll-like receptors (7). Toll-like receptors are usually associated with immune cells such as macrophages or dendritic cells but they are also present on the oral mucosa. Location of toll-like receptors can be externally on the cell surface such as TLR3 or inside the cellular cavity such as NOD1 and NOD2 receptors (8). Activation of TLRs on antigen-presenting cells leads to innate immune response which is helpful in elimination and reduction of bacterial growth. Furthermore, bacterial invasion will also be responsible for inducing signals activating the adaptive immunity, which is another name for acquired immune system, which is important for removal of bacteria via humoral and cell-mediated response.

Etiology:

Cancer development is a multifactorial process where exposure to different risk factors and carcinogens play a significant role. A lot of research has been put into early detection of oral cancer by recognizing either the conditions or factors causing it or using diagnostic markers for its diagnosis. Many epidemiological studies discuss some well-defined risk factors that can increase the probability of getting cancer, for example, alcohol consumption, poor lifestyle choices, age, genetics, tobacco use, fungal infection, chronic viral infections and inflammation (9, 10). *Alcohol consumption, cigarette smoking* and *betel chewing* are known to be major risk factors for cancer development (3, 6). Other risk factors may include inability to repair damaged DNA

and metabolize carcinogens, deficiencies of vitamin A, E or C or other underlying immune defects (10).

Dietery and lifestyle factors:

- *Tobacco:* Risk of developing oral cancer is three times greater in smokers as compared to non-smokers.It is suggested that environment with cigarette smoke contains nitrosamines, benzopyrenes and aromatic amines that are thought to increase the risk of developing oral cancer in non-smokers by 87% as compared to people who live in non-smoky environment(11). Tobacco damages the antioxidant reparative mechanism in oral epithelial cells promoting damage. Cigarette smoking is also though to weaken the immune system that induces gingivitis, periodontitis and other systemic problems (11).
- *Alcohol:* Alcohol has both genotoxic and mutagenic affects both locally on the epithelial cells and systemically on liver (11, 12). Long term use of alcohol is associated with increased permeability of epithelial cells that destroys the lipid molecules of the cell membrane(13). Epithelial atrophy is the result of such a damage making it more prone to infections and harmful substances. Alcohol intake has some serious damaging effects on DNA repair thereby promoting mutagenic behavior (11).
- *Betel quid and areca nut:* Betel chewing is a popular common habit in Southeast Asia and reported to be strongly associated with OSCC. Prevalence of oral cancer in India is quite high i.e. 74%, which is a result of unhealthy lifestyle choices made by inhabitants (10). In-vitro studies on oral mucosal fibroblasts also shows that a betel liquid has a carcinogenic, mutagenic and genotoxic effect (14).
- *Diet and nutrition:* Some recent meta-analysis suggests a positive protective effect due to diet rich in fruits and vegetables (12). Fruits and vegetable are enriched with vitamins and minerals which provide us with a protective barrier, for example, vitamin A&C acting as antioxidants, vitamin E preventing tumor formation, vitamin A improving immune system (14).

Microbial factors:

- *Viral and bacterial infections:* They have gained attention for the last 30 years because they are known to be strongly associated with the development of cancer. Some viruses like human papilloma virus is known to have a carcinoogenic potential where they have a greater affinity for binding onto squamous epithelium cells. Many modulations and changes takes place in cell cycle machinery by such viruses and bacteria promoting formation of pre-cancerous or cancerous lesions (14).
- *Fungal infection:* Some fungal infections especially those caused by *Candida albicans* are reported to be involved in pathogenesis of oral premalignant lesions(14).
- *Volatile Sulphur compounds*: There is at present very little knowledge of carcinogenic substances produced by oral bacteria. *P. gingivalis* and *F. nucleatum* both produce volatile sulphur compounds (VSCs), such as, hydrogen sulphide (H_2S)(15). H_2S is a known genotoxic agent and its accumulation leads to genomic instability or cumulative mutations (15). Figure 2 shows one important possible relationship between bacterial infection and formation of oral cancer via production of such carcinogenic substances.

Environmental factors:

- Occupational exposure, dental factors, radiation to head and neck area are believed to be minor risk factors that may have a significant role in initiation and promotion of cancerous lesion (14). Exposure to ultraviolet rays, asbestos, poor oral hygiene and ionizing radiation have an impact on the well-being of oral health and therefore may be involved in harming normal cell replication and growth (14).
- Beryllium and arsenic are one of the few known chemical carcinogenic substances that are responsible for increasing the risk of cancer development by damaging DNA or altering cellular metabolism.
- On a worldly basis, carcinogens are responsible for one third of death from cancer (1).

Genetic and age-related factors:

- There have been some evidence that individuals carry a genetic predisposition for cancer and familial associations have also been found. However, population-based studies are difficult to perform because of existence of other co-factors like smoking and alcohol (14).
- One study by Nelson *et al.* reports that genetic mutations together with advancing age has been associated with increased occurrence of oral and pharyngeal cancer. Cells undergo biochemical-biophysical processes with advancing age and individuals who carry genetic pre-disposition for cancer can accumulate these mutations promoting tumourgenesis (12).
- Once the balance between cell proliferation and apoptosis is disturbed in an individual with predisposition for cancer, then there is a greater risk of transformation of a pre-cancerous lesion to cance. Some pre-neoplastic lesions such as, lichen planus (erosive/ulcerous type), leukoplakia and erythroplakia, are associated with development of oral cancer. Leukoplakia is a chronic inflammation of the oral tissue where the risk of transformation to malignancy is about 5-17% (12).

Although these risk factors are being presented independently but the initiation and progression of cancer is carried out by a combination these factors.

Periodontitis and oral cancer

Periodontitis is a multifactorial chronic disease of the oral tissue, which is characterized by gingival inflammation and bone loss. More than 20 years ago, epidemiological studies reported that poor oral hygiene and tooth loss were significantly associated with OSCC, providing the first indication that oral bacteria might play a role in oral cancer development (16). Tezal *et al.* reported that poor dental hygiene such as in the case of gingivitis and periodontitis are important risk factors for cancer (17) (figure 2). Inadequate poor oral hygiene is the primary cause behind periodontitis where accumulation of plaque and tartar stimulates production of inflammatory cytokines and prostaglandins to fight the infection. Colonization of pathological bacteria is strongly correlated with inflammation and cancer progression, states a study by Wang and Ganly in 2014 and Wang and Jia in 2016 (18, 19). The immune system recognizes bacterial and viral invasion in periodontal pockets as foreign organisms damaging

epithelial cells and attacks infected cells to remove the infection. Periodontitis is mainly caused by gram-negative anaerobic bacteria in dental biofilm which are responsible for secreting endotoxin substances destroying well-being of healthy epithelial cells (20). In periodontitis, cellular migration and proliferation increases together with increased production of inflammatory cytokines, growth factors, enzymes and some prostaglandins that are reported to be closely associated with cancer initiation and progression (20).

Evidence and association

Evidence indicates that chronic infection and inflammation are strongly associated with cancer (20). A number of case-control studies describes the role of oral health and head and neck cancer and some cohort studies have explored the relationship between periodontitis and other types of systemic cancer reporting a significant positive association.

One study conducted by Tezal *et al.* between years 1999 and 2005 showed that there is a direct association between chronic periodontitis and tongue cancer independent of smoking, age, gender etc. Following adjustments of these factors particularly age, smoking, loss of teeth etc provides with some interesting information i.e for every millimeter loss in alveolar bone there is 5.23 fold-increased risk of getting tongue cancer (21).

A case-control study conducted in Beijing by Meyer *et al.* in 2008 assessing the relationship between dentition and risk of oral cancer. This study recorded any missing tooth, gingivitis and periodontitis as a part of the oral exam. This study suggests that males and females suffering from periodontitis are more prone to develop oral cancer where males had a two to three-fold and females five to eight-fold increase in risk (22).

Another case-control study by Tezal *et al.* reported the effect of chronic periodontitis on head and neck squamous cell carcinoma (HNSCC). It was noted that each millimeter of alveolar bone loss was associated with more than four-fold increased risk of HNSCC where the greatest strength of association was found to be in the oral cavity. Poorly differentiated oral carcinoma was prominently associated with chronic periodontitis as compared to patients without periodontitis. This study claims that

chronic periodontitis is an individual risk factor for HNSCC and smoking modifies this association in some aspects (17).

Sadighi Shamami *et al*. in a review of epidemiological research articles defines a clear association between tooth loss, periodontitis and carcinogenesis. Nine out of ten-case control studies reported significant increase in the risk of oral cancer in patients with periodontitis thereby indicating that there is a possible link between cancer and periodontal disease when modifying factors such smoking and drinking were controlled (13).

Pathology

Periodontal bacteria and viruses work synergistically in periodontitis causing loss of alveolar bone and gingival inflammation (20). Many studies confirm that viruses such as *Cytomegalovirus*, *Epstein Barr virus* and *Human papilloma virus* are reportedly found in increased amounts in the saliva of patients diagnosed with periodontitis (20). Shifts in the oral microbiota occurs during periodontitis where anaerobic, gram-negative bacteria dominates making the environment more favorable for survival and disease progression. *Fusobacteria, P. gingivalis and Actinobacteria* are present in small amounts in a healthy oral cavity but the shift in the oral microbiota following an infection, causes these bacteria to increase in number. A bacterial specie which was beneficial at first now becomes opportunistic and potray unfavorable pathological behavior, hence providing a link between chronic inflammation and oral cancer (3). The detrimental effects brought out by viruses and bacteria can be viewed as direct or indirect effects based upon the origin of modulating factors:

Direct effect: Microorganisms release biological products such as endotoxins, enzymes and metabolic substances that can induce mutations in the tumor-suppressor genes and proto-oncogenes altering cellular signaling controlling proliferation and apoptosis.

Indirect effect: Microorganisms release products that activates host cells such as neutrophils, macrophages, lymphocytes, fibroblasts etc to produces toxic substances that interfere with DNA reparation or promotes anti-apoptotic activity. These

substances include reactive oxygen species, growth factors, matrix metalloproteases, cytokines etc.

The microbiome and oral cancer:

Background

Studies done 30 years ago, found a definite relationship between the bacteria *Helicobacter pylori* and gastric cancer. This discovery lead to increase interest in studying different bacterial species and their role in the development of other types of cancers, like cancer in the oral cavity (9, 15, 23). Many etiological factors have been identified as causative agents for the development of cancer, but cancer occurrence in patients without these risk factors have led to speculations about other possible contributory factors.

Periodontitis, as discussed earlier, is a multifactorial chronic oral disease which occurs as a result of shift in equilibrium of the oral microbiota causing inflammation in gums and loss of alveolar bone. It is been reported in many different studies that change in bacterial composition inside the oral cavity and non-optimal immune system response lead to periodontitis (9, 24, 25). Chronic periodontitis and oral cancer may have similar risk factors but further research is needed to investigate any causal relationship existing between them (16). Therefore, recent studies have been exploring a link between infection-driven inflammation and cancer (figure 2).

Some recent studies have found a strong link between some specific bacterial species and development of oral cancer. *Streptococcus sp., Peptostreptococcus sp., Prevotella sp., Fusobacterium sp., Porphyromonas gingivalis* and *Capnocytophaga gingivalis* are found to be strongly associated with oral cancer and epithelial precursor lesions (15). Moreover, two periopathogens *F. nucleatum* and *P. gingivalis* have been mentioned frequently in recent studies, that are not only found to be associated with OSCC but may contribute to the development of colorectal and pancreatic cancer (15). Recent studies using illumina sequencing technology suggests that some fungal and viral species such as, *Candida albicans*, *Epstein barr virus* and *Human papilloma virus* may have a possible association with OSCC but definitive data is lacking (16). However, epidemiological and experimental studies suggests that pathogenic microorganism may play a significant role in the development of oral cancer by

modifying cellular signaling, but other risk factors cannot be ignored and a bigger picture should be kept in mind while analyzing cancer characteristics and etiology.

Evidence and association

Higher complexity of the oral microbiota is seen in patients diagnosed with oral cancer suggesting a change that occurs as a result of sustained chronic inflammation or post-malignant tranformations (3, 16). The ratio of anaerobic to aerobic is increased on the surface of OSCC lesions, confirmed by some culture-based studies(3). Toxins from bacteria can give rise to DNA damage, thus normal cell division and apoptosis is disrupted (26, 27).

Microbiome shifts in OSCC

A case-control study by Perera *et al.* shows that analysis of malignant tissue or biopsy from the oral site shows higher levels of fusobacteria and bacteroidetes but reduced levels of streptococci and *Rothia spp* (16, 28).

An experimental study by Yang *et al.* investigated the microbiota of oral rinse from healthy patients and those diagnosed with OSCC. The microbiota was investigated using 16S rRNA V3V4 amplicon sequencing to study the composition and variety of the microbiota at different stages of OSCC development. This study suggests that a progressive shift in diversity and richness of microbiota is found at different stages of cancer development. In the ascending order, *Streptococcus, Veillonella, Neisseria, Haemophilus, Rothia* and *Fucobacterium* were found to predominant bacteria present in cancerous lesions. Disturbance of oral microbiota makes *F. nucleatum* levels to increase inside the oral cavity thereby inducing opportunistic pathological behavior. In addition to this, the study reported that *F. nucleatum* is found to continue increasing in number, as the lesion goes from being healthy to pre-cancerous stage and upto stage IV cancer. This also suggests that infection-driven inflammation plays a vital role in the destruction of epithelial tissues and promotion of tumorgenicity throughout the process of cancer developement (3).

A link between bacteria and oral squamous cell carcinoma was observed in a study where they examined the prevalence of periodontal bacteria in sub-gingival plaques of tissue from OSCC patients using high-throughput sequencing. The results show

increased existence of periodontal pathogens in cancer and precancerous tissues. This study also found the bacterial profile of these tissues to be similar to the profile of sub-gingival plaque. The bacteria that was most prevalent were, among others, *F. nucleatum* and *P. gingivalis*. The presence of these two commonly known periodontal bacteria was found in abundance in oral cancer tissue as well (23). Furthermore, many studies have suggested a strong link between *P. gingivalis* and *F. nucleatum* and oral squamous cell carcinoma (OSCC) (9, 15, 16, 29, 30). Co-existence of these bacteria is capable of inducing more damage to the tissue as compared to when they are found alone (31, 32).

P. gingivalis is a member of the oral microbiota and an important potential etiological agent causing gingival and periodontal inflammation (8). The oral cavity contains more than 700 bacterial species that live in harmony with the host (9, 26). The oral microbiome balance plays an essential role in maintaining the normal physiological environment (7). All of the commensal and opportunistic bacteria, virus and fungi live in symbiosis with one another and the host system ensuring safety from environmental and systemic exposures or stresses (7). External and internal factors may initiate imbalance of the oral microbiome resulting in activation of host defense mechanisms. Disturbance of the oral microbiota leads to domination of pathological species which in turn activates certain biological pathways promoting overexpression of inflammatory molecules. *P. gingivalis* is known to be a major oral perio-pathogen that increases in number following a disturbance in oral microbiota and leads to chronic periodontal inflammation inducing damage to oral tissues(9).

Amer et. al. and Schmidt *et al.* investigated separately the microbe of patients with pre-cancerous lesion such as leukoplakia and both found higher numbers of fusobacteria and Bacteroidetes in oral tissues, suggesting the fact that possible change in the microbiome begins at the start of malignant transformation (16). However, it should be noted that the direct evidence that colonization of fusobacteria and other microbes increases the risk of malignant transformation is still lacking (16). Further research in form longitudinal follow up studies of such patients is needed to provide with a definite answer.

Drivers of microbiome changes

The only experimental evidence that suggests that bacteria may induce malignant change in oral cavity was presented by Gallimindi *et al.* This experiment showed that *P. gingivalis* and *F. nucleatum* may induce carcinogenesis in chemically induced OSCCs (16, 31). Some studies have shown that smoking and tooth loss are major drivers of oral dysbiosis indicating that mucosal changes may be dependent upon these risk factors(16). Another case-control study explores diseased and healthy oral tissue from the same patient and concludes that site-specific mucosal changes can be accounted for primary drivers of an altered microbiome (16). It is also been stated that epithelial cell surface changes, such as lower expression of E-cadherin, occur quite early in the disease process (16). Dysplastic epithelial cell surface changes may also be held responsible for the wider changes in oral microbiota (16).Further investigation is needed to explore and find a definitive causal relationship between actual drivers and microbiota alteration.

Possible mechanism of microbiome-driven malignant transformation

One proposed idea is the production of metabolic products by microbial species that can lead to possible malignant transformation. Smoking and alcohol consumption can profoundly increase the genotoxicity level of saliva because of destruction of glandular cells which results in reduced saliva flow enhancing plaque formation and retention (16). Martilla *et al.* demonstrated that oral microbe can produce carcinogenic levels of acetaldehyde (ACH) in alcohol abusers as compared to non-alcoholic people (16). N-nitrosamine is another compound produced intraorally by microorganisms in smokers, that is recognized as a potential carcinogen and may play a role in promotion of cancer (16).

Another proposed theory indicates that microbiota during cancerous lesions possesses an enhanced inflammatory potential compared to normal oral tissue. Gallimindi *et al.* demonstrated that activation of particular inflammatory pathways could promote cancer progression. This study proposed that IL-6 activation of STAT3 is seen intraorally in mice infected with *P. gingivalis* and *F. nucleatum* which results in increased production of cyclin D1, MMP9 and heparinase. It is interesting to note that F. nucleatum when localized to pre-cancerous site acts as a driver for malignant transformation via activation of Wnt-like pathways through E-cadherin interactions via

its FadA adhesin. Detailed explanation of cellular mechanisms and their interaction with inflammatory molecules is described later in the context.

Another study shows that patients with OSCC are prone to develop chronic periodontitis. OSCC patients with periodontitis are therefore exposed to periodontal pathogens, and those pathogens will in turn modulate the behavior of cancer cells via inflammatory molecules (33). These molecules can activate transcription factors that regulate proliferation, angiogenesis, invasion and metastasis, making the tumor more aggressive (23).

Many epidemiological and experimental studies have found a potential association between bacterial infection, inflammation and OSCC, especially the link between periodontitis and OSCC . Bacterial infection can promote cancer in three different ways precisely, stimulation of chronic inflammation, disrupting apoptotic activity and production of carcinogenic substances as shown in figure 2 (15). We would like to further research bacterial mechanisms to understand the role of oral bacteria in cancer development and progression. Our master´s thesis will be focusing greatly on inflammatory processes and mechanisms promoting occurrence of cancer. As this knowledge might help to better understanding of the disease progression and perhaps be able to use the periodontal bacteria profile as a possible cancer screening in the future. Our master`s thesis is a review made on the previous known healthcare studies regarding the bacterial mechanisms of two popular pathogenic periodontal bacteria with known carcinogenic effect, *Porphyromonas gingivalis* and *Fusobacterium nucleatum*.

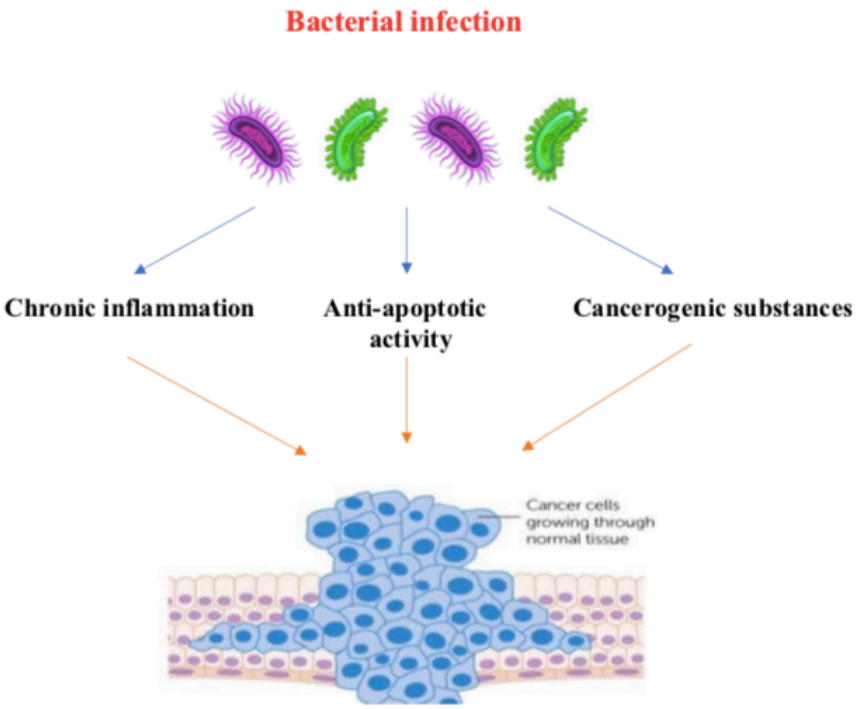

Figure 2. Influence of oral bacteria in the pathogenesis of cancer. Figure adapted from Karpinski TM (2019) (15).

Materials and methods

A PubMed search was made in august 2019 and april 2020 using combinations of the following keywords “mouth neoplasm” and “Porphyromonas gingivalis”, “mouth neoplasm” and “Fusobacterium nucleatum”, “oral microbiome” and “mouth neoplasm” and “periodontitis” and “cancer”. Titles and abstracts were examined in order to evaluate which articles to include and exclude in this review. We gathered in total twenty-nine articles at first (april 2020). Additional articles were selected from the references of the included articles from PubMed. Other relevant articles, books and websites were included as needed. Finally, sixty-three articles were included in this narrative review.

Porphyromonas gingivalis

Introduction

P. gingivalis is an obligate anaerobic gram-negative rod-shaped, nonmotile, pathogenic bacterium. The pathological role of P. gingivalis in periodontitis and OSCC has been proposed in different studies where *P. gingivalis* infection is mainly involved

in initiation and promotion of anti-apoptotic and infection-driven inflammatory pathways (7, 8).

Jak1/Akt/Stat3 pathway

P. gingivalis infection affects bodily functions by increasing cell proliferation and decreasing cell apoptosis. Two main effects after infection with *P. gingivalis* are changes to apoptosis and cell division (7, 9). Different mechanisms have been proposed in different experimental and epidemiological studies, which defines a clear association between bacterial infection, inflammatory response and development of cancerous lesions. Cell turnover is a normal physiological process where one parent cell is replicated into two identical daughter composing same genetic makeup. Apoptotic activity is a normal physiological process that takes place as a part of cell turnover or destroying mutated cells, causing the human body to get rid of old, damaged cells. Anti-apoptotic activity of *P. gingivalis* activates the Jak1/AKt/Stat3 signaling pathway which disrupts the mitochondrial apoptosis /pathway (7, 15) (figure 3). Mitochondria plays an important role in the apoptotic process and coordination of signals intra-and extracellularly. Activation of Jak/Stat cascade after extracellular stimulation leads to phosphorylation of various proteins within the cell inducing transcription of certain targeted genes (figure 3). In-vitro experiments on primary cultures of gingival cells shows that *P. gingivalis* is strongly anti-apoptotic and reduces chemically induced apoptosis thereby promoting oncogene phenotype (9). P. gingivalis causes phosphorylation of pro-apoptotic Bad through Akt at mitochondrial membrane hence inhibiting apoptosis process (7, 15) figure (3). This in turn enhances the ratio of Bc12 (anti-apoptotic) and Bax (pro-apoptotic), but Bax levels elevates in the start and then declines after 24h stimulation shows a study by Nakhjiri *et al.*(15). Enhancement of the ratio of Bc12 and Bax restricts the release of effector cytochrome c hence promoting anti-apoptotic behavior (7). This suggests that *P. gingivalis* plays a vital role in deactivation of apoptotic pathways while enhancing anti-apoptotic activity hence, promoting tumorigenic and invasive behavior of oral cells.

Adenosine triphosphate and P2X7

Intracellular receptors also play an important role in detecting pathogenic microorganisms and inducing needed response. Nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) are such receptors that are present in the

cytoplasm and that recognizes the intracellular invasion of pathogens and induces particular inflammatory response (8). On the other hand, Purinergic receptor (P2X7) present on plasma membrane are also activated by damaged cells that induces an assembly of inflammasome, a protein complex of caspase-1 and an adaptor protein ASC (8). The purinergic receptor is originally involved in cell death and apoptosis and secretion of inflammatory cytokines to fight intracellular infections (34). *P. gingivalis* also inhibits apoptosis of damaged gingival epithelial cells (GEC) which is originally induced by ATP-ligation of purinergic receptor P2X7, thereby promoting cell growth, neovascularization and metastasis (7, 15, 34). Adenosine triphosphate (ATP) is an organic compound present in all types of cells acting as an energy unit involved in basic functions of life such as muscle contraction, signal transition and many other. ATP is produced by metabolic processes such as electron transport chain inside the mitochondria of the cells which is then transported intracellularly to different organelles and extracellularly to other cells, accordingly. ATP is a unique compound that acts as both, an energy carrier and a neurotransmitter molecule, hence describing its diverse nature. Once secreted out of the cell, extracellular ATP plays an important role in inducing cellular apoptosis via intercellular signaling mechanisms.

GECs stimulation by *P. gingivalis* causes induction of IL-1β expression and is a part of the inflammatory response, IL-1β induces increased osteoclast formation and bone resorption in periodontium (15). *P. gingivalis* infection modulates extracellular ATP-induced cellular reactive oxygen species and oxidative stress pathways promoting inflammation and production of cytokines such as IL-1β (35). Furthermore, some studies confirm that stimulation of infected cells with ATP causes production and accumulation of inflammatory cytokine, IL-1β (8).

P. gingivalis also expresses an anti-apoptotic enzyme nucleoside-diphosphate kinase (NDK) homolog that inhibits the innate immune reaction, caused by stimulation with extracellular ATP(8, 34). NDK is an ATP-consuming enzyme which hydrolyses ATP extracellularly and prevents apoptosis mediated by P2X7 (34). In addition to this, *P. gingivalis* infection inhibits ATP-induced pro-apoptotic caspase-1 activation in GECs thereby modulating ATP/P2X7 signaling (8, 15). On the other hand, *P. gingivalis* cause modulation of ATP-induced cytosolic and mitochondrial ROS, as well as antioxidant gluthathione response generated through P2X7/NADPH-oxidase interactome (figure

3). ROS is known as an important key mediator, which damages DNA and activates transcription factors enhancing abnormal growth of cells.

P53 tumor-suppressor protein

Furthermore, it is known that *P. gingivalis* causes progression of pre-cancerous and cancerous cells by mutation of particular genes that are involved in cellular growth and progression. The cell cycle or cell-division cycle is a process that gives rise to daughter cells that are identical to each other and its parent cell. Cell cycle is composed of different phases through which somatic cells gets duplicated and bodily functions are maintained. *P. gingivalis*, in particular, reduces the level of p53 tumour suppressor protein via accelerating progression through S-phase of the cell (7, 15) (figure 3). This is brought out by manipulating the cyclin/CDK activity (15). It is known from some studies that one of the important p53 transciptional target genes is cyclin-dependent kinase inhibitor which is modulated here via P. gingivalis infection. One study shows that a fimbrial-deficient mutant of P. gingivalis does not show this activity suggesting the importance of FimA genotype for epithelial cell proliferation(9).

NF-κB1 and MAPK pathway

Excitement of different mechanisms and pathways takes place when bacterial species infect human cells. Some of these are initiated particularly after infection with *P. gingivalis* and F. nucleatum. Other bacterial species may also play an important role in initiating these pathways. Two most common known pathways are NF-κB1 and MAPK. Nuclear factor kappa-light-chain-enhancer of activated B cells **(**NF-κB1) is a protein subunit of the NF-kB protein complex found in all animal cell types, a transcription factor that regulates many immunological and inflammatory reactions(8). The pathway gets activated in response to stimuli such as cytokines, cell stress or bacterial LPS. It includes five subunits- RelA (p65), RelB, c-Rel, p50 (NF-kB1) and P52 that function as homodimers and heterodimers communicating with many other molecular species inside the cell (8). Irregular activation of this pathway will lead to unnecessary production of cytokines and is strongly linked to cancer development in some recent studies. After infection, related transcription factors are dissociated in the cytoplasm by the family of inhibitors of kB, IkBs (8). The IkB kinases induces activation and further phosphorylation and disintegration of IkB proteins which results in the activation of NF-kB modulating transcription and changes in cell growth (8, 36).

Activation of NF-kB pathway and some other pathways induces increased expression of promatrix metalloproteinase (proMMP-9) (7) (figure 3). Matrix metalloproteinases (MMPs) are enzymatic proteins secreted by mammalian cells in pro-forms that are processed into their active forms to perform adequate functions when needed (36). A culture-based experimental study by Inaba *et al.* reports that MMPs play a vital role in degradation of basement membrane and extracellular matrix promoting cellular migration and invasion (36). Subsequently, *P. gingivalis* produces cysteine proteinases named gingipains, which can cleave Matrix metalloproteinase-9-pro-enzyme (MMP9) changing it into its mature active form (7, 15) (figure 3). MMP2 and MMP9 are known to be strongly associated with cellular invasion and migration (36). This is NF- κB dependant process that promotes carcinoma cell migration and invasion (15).

Some other protein kinases regulates number of cellular activities such as managing cell stress and production of pro-inflammatory cytokines(8). Mitogen-activated protein kinases (MAPKs) are highly conserved family of Ser/Thr protein kinases that gets activated by infection(8). MAPK is a protein kinase that gets activated in response to cellular stress, heat shock and proinflammatory cytokines (37).These protein kinases are involved in controlling cellular functions such as gene expression, proliferation, differentiation, cell survival and apoptosis(37). Activation of surface receptors such as TLRs and PARs, as discussed earlier, leads to activation of NF-kB and MAPK pathways(8). The TLR family is responsible for activation of the adaptor molecule myeloid differentiation primary-response protein kinases 88 (MyD88), together with a shared adaptor protein of TLRs leading to downstream signaling of NF-kB and MAPK cascades. Study of MAPK pathway shows that higher responsiveness is seen with Jun K-terminal kinase (JNK) and p38 kinases(8) when stimulation occurs, it means that later named protein kinases have increased reaction to activation when compared to other kinases. Furthermore, infection via *P. gingivalis* LPS in human oral keratinocytes (HOKs) involves NF-kB and p38 MAPK signaling pathways including further members of MAPK family like MEK4 and MAPK4 and JNK1 (8).

Head and neck squamous cell carcinomas also show that stromal fibroblasts express collagenase-1 (matrix metalloproteinase (MMP)-1) in invasive malignant tumors which leads to activation of c-Jun NH2-terminal kinase and p38 MAPK together with

phosphorylation of c-Jun (8). Disruption to normal physiological functions leads to abnormality and uncontrolled production of cells inducing tumorigenic behavior.

E-cadherin and β-catenin

Epithelial-mesenchymal transition (EMT) is defined as when epithelial cells lose cell-cell adhesion and polarity promoting migration and invasive properties. EMT is a normal biological process that is necessary for development in embryogenesis and wound healing in the later stages of life (35). However, some recent studies suggests that early over-expression of EMT in primary oral epithelial cells is known to be associated with cancer initiation and metastasis.

In a recent in-vitro experiment by Lee *et al.* 2017, increased expression and activation of epithelial-mesenchymal-transition (EMT) is seen during long-term infection with *P. gingivalis* in primary epithelial cell (35). Furthermore, expression of EMT-assosiated transcription factors like Slug, Snail, Zeb1 increases significantly (35). This experiment also shows that cell migration increases somewhat when cells are co-infected with both bacteria, *F. nucleatum* and *P. gingivalis (35)*. In addition to this, many other biological molecules and substances responded to *P. gingivalis* infection. For example, expression for adhesion molecule E-cadherin decreases while matrix metalloproteinases 2, 7 and 9 increases both inducing cellular migration, motility and dysfunctionality. It should be noted that E-cadherin is a transmembrane protein that is important to keep the epithelial structure integrated (35). Hence, loss of E-cadherin will lead to migratory behavior and further possibility of metastatic potential increases. On the other hand, β-catenin, which is a dual protein molecule and a part of adhesion protein complex, stands responsible for cytoplasmic anchoring of E-cadherin (35). This molecule regulates cell-cell adhesion and gene transcription processes together with maintaining structural integrity. Therefore, loss of E-cadherin will lead to reduced membrane localization of β-catenin encouraging tumor progression (35). It is reported in the study that increased expression of Snail and Slug causes loss of E-cadherin (35). These changes are very well-known for playing a crucial role in promotion of invasiveness and tumorigenic behavior of cells confirming that *P. gingivalis* behaves as a potential risk factor for development for OSCC. Results of this study also confirmed that there is increase in cellular migration when the cells are co-infected by *F. nucleatum* as well.

Interleukin-1β

It is normal physiological immune response that IL-1β is produced in increased amounts during periodontal infection phase(15). High IL-1β activates endothelial cells to produce vascular endothelial growth factor (VEGF) and its higher content is associated with tumour invasiveness, migration and differentiation(15). In a study by Wang *et al.,* IL-1β is linked to lower expression of E-cadherin which leads to abnormalities in cellular functions, growth inhibition, apoptosis, cell cycle arrest and differentiation(15, 35). IL-1β causes induction of MMP-9 pathway which is involved in local extracellular matrix degradation and tumor invasiveness promoting transition of healthy epithelial cells from benign to cancer cells promoting dissemination and metastatic growth at remote sites(9, 15). Initiation of different cellular pathways along with growth-promoting signaling induced by inflammatory cytokines explains their important role exhibited in development of cancer and metastasis.

Interleukin-6

As discussed earlier, inflammatory cytokines are often released as a response to bacterial/viral infection. These cytokines together with other physio-chemical substances attack and kill the invading bacteria ensuring well-being of the host tissue. This process is complex and involves a lot of intra- and extracellular communication between different cells activating molecules and their particular functions. A very well-known pro-inflammatory cytokine is IL-6 that induces bone resorption and stimulates production of acute phase proteins, chemokines and PGE2(15). Different variations of prostaglandins (PGs) are produced normally in different organs of the body to maintain functions like digestion, urination and many more. Prostaglandin E2 released via stimulation from inflammatory cytokine IL-6 causes brain to induce fever as an integral response to infection or inflammation in body. IL-6 increases the expression of MMPs in addition to mitochondrial damage caused by oxidative stress in the cells(15). This cytokine also promotes adhesion of tumor cells to endothelial cells by increasing the expression of certain adhesion molecules such as endothelial leukocyte adhesion molecules (ELAMs)(15).

Tumor necrosis factor- α

TNF-α is another major inflammatory cytokine produced by macrophages, neutrophils, fibroblasts, lymphocytes and mast cells in response to stimuli such as bacterial LPS(15). TNF-α is responsible for the production of chemical substances such as reactive oxygen species (ROS), leukotrienes, prostaglandins, IL-8, VEGF, basic fibroblast growth factor and metalloproteinases, which enhances the tumor production and processes of motility(15). Moreover, induction of MMPs expression increases the invasiveness of tumour growth and differentiation(15). Additionally, it causes reduction of osteogenic cells and fibroblasts that play an important role in maintaining and sustaining cellular functions(15). An interesting fact is that high doses of TNF-α are known to cause tumour destruction in contrast to low-dosage which promotes tumour growth(15).

Other pro-inflammatory interleukins

Gingival epithelial cells (GECs) are first host cells providing protection against colonizing bacteria. As discussed earlier, *P. gingivalis* infection induces MAPK pathway, that is a signaling pathway involving chain of proteins inside the cells that communicates via phosphorylation when a receptor is activated via ligand/signal. GECs may also exhibit a functional NACHT, LRR and PYD domains-containing protein 3 (NALP3) which gets activated in response to damage/inflammation causing depletion of infection (8).

As discussed earlier, ATP is a basic unit of energy with diverse functions inside and outside of the cell hence playing an important role in maintaining proper cellular signaling. One in-vitro experiment shows that in fact treatment with ATP causes activation of caspase-1 in GECs. Caspase-1 is also known as a interleukin-1 converting enzyme that proteolytically cleaves proteins such as IL-1β and IL-18 into their mature forms performing an important role as an inflammatory response initiater. Caspase-1 activation initiates the production and release of pro-inflammatory cytokines IL-1β and IL-18 into mature active peptides enhancing the inflammatory effect in oral tissue (8).

One study reports that *P. gingivalis* activates signaling cascades that control transcription of target genes encoding for immune response and inflammatory

cytokines such as interleukin (IL)-1β, IL-6, IL-8 and tumour necrosis factor (TNF)-α in epithelial cells and interferon regulating factor (IRF) 6 in oral epithelial cells (27).

High-mobility group protein B1

Furthermore, *P. gingivalis* infection may modify high-mobility group protein B1 (HMGB1) release from GECs (8). HMGB1 is a pro-inflammatory danger signal/protein that is released into extracellular space after stimulation of GECs with ATP (8). High-mobility group box 1 (HMGB1) will be secreted into the cytoplasm firstly in response to injury, inflammation or infection as it is normally located in the nucleus (8). HMGB1 is a chromatin protein that supports and facilitates DNA formation and transcription factors inside the nucleus ensuring proper organization and regulation of DNA formation. The mobilization of HMGB1 to the cytoplasm is essential for activation of inflammasome and caspase-1 activation (38). HMGB1 once secreted out of the cell also acts as ligand and activates receptor for advanced glycation end products (RAGE), and molecules with activated advanced glycation end products (AGE) (26). Activation of such receptors are involved in promoting excessive proliferation and invasion of dysplastic or cancerous cells.

Human beta-defensins (HBDs)

Human beta-defensins (HBDs) are antimicrobial peptides controlling innate and adaptive responses providing resistance against microbial colonization on epithelial surfaces (39) . These proteins carry cationic electric charge and has affinity towards some gram-negative and gram-positive bacteria creating a pore complex in their cell wall/membrane thus inducing depolarization and cell lysis (39). Defensins are capable of strengthening adaptive immune system by enhancing phagocytic activity of macrophages (39).One investigation shows that treatment of macrophages with hBD3-3 peptide reduces LPS- induced production of nitric oxide synthase and nitric oxide (8). Nitrogenous compounds are known for their antibacterial action and reduction of these compounds promotes bacterial growth indirectly. This experiment also shows that there is a concentration dependent inverse relation to release of IL-6 and TNF-α (8).In a model study of lung inflammation, interstitial infiltration of neutrophils is reduced by human beta defensin 3-3 (HBD3-3) (8). This model study also shows that HBD3-3 was able to downregulate the nuclear factor-kappa (NF-κB) dependent inflammatory response via direct suppression of the phosphorylated-nuclear factor of kappa light

polypeptide gene enhancer in B-cells (8). These studies summarizes that HBDs are actually important in fighting the infection and inducing immune response that is needed to kill the invading bacteria. An experimental study reported that P.gingivalis culture supernatants are capable of partial or full degration of HBDs within 30 min of exposure. P.gingvalis produces proteases that are responsible for degrading protein structure hence down-regulating defensing related innate immune functions (40).

B7-H1 receptor

Another important receptor that modulates functions and molecules within immune system is B7-H1 receptor. This receptor belongs to B7-family exhibiting regulatory and functional properties which modifies cell-mediated immune reactions (8). Some studies shows that *P. gingivalis* induces B7-H1 expression in different carcinoma cell lines (SCC-25 cells, BHY cells) and as well as in primary gingival keratinocytes (7, 8). B7-H1 ligands bind to CD28/ CTLA-4 like programmed death-1 (PD-1) receptors that are present on T and B cells, monocytes and macrophages, endothelial and epithelial cells modifying their normal functions (8). This molecule being a member of immunoglobulin (IG) superfamily regulates T-cell activation and tolerance by inhibiting the function of activated T cells (7, 8). It is known that T-cells are involved in promoting immune-mediated cell death carried out by the support of CD8+ and CD4+ cells (7). Disrupting this function will induce more proliferation and increase in the aggressiveness of the tumor cells. Furthermore, other pro-inflammatory cytokines like interferon (IFN)-γ upregulates expression of B7-H1 somewhat enhancing its production. (8). On the other hand, blockage of B7-H1 ligation causes inhibitory effect on T regulatory cells that are important to control tissue damage caused by inflammation(8).

This immune system is a specialized memorial center that plays an important role in second activation process where an immediate immune response is brought about leading to speedy recovery from bacterial infection. (8). Desensitization is a process or phenomenon where antigen presenting cells do not recognize bacterium as a harmful specie and lacks to bring about an immune response. *P. gingivalis* infection involves induction of the expression of tolerogenic molecules like immunoglobulin –like transcript 3 (ILT-3) and B7-H1 in antigen-presenting cells (APCs) by desensitizing them against second activation (8). Activation of T-cells require co-stimulatory signal usually from APCs in order to perform their function. In this way, damage continues to

occur in oral tissue and host system response is not optimal to eliminate infection effectively.

Notch-signaling cascade

One recent in-vitro study from 2017 by Woo *et al.* claims that inflammatory mediators play a huge role in OSCCs to develop notch intracellular domain (NICD)-assosiated chemoresistance against Taxol medicine(41). This chemoresistance develops as a response to prolonged infection by *P. gingivalis* which induces activation of Notch 1 receptor(41). **Notch signaling cascade** increases malignant properties like migration, invasion, chemoresistance and stemness in OSCCs and have great influence on CSCs in particular(41). Inflammatory mediators for example **PGE2** are known to increase proliferation and maintainace of pluripotent cancer stem cells (CSCs) that have the capability of self-renewal, dedifferentiation and tumorigenicity(41). Some recent studies also presents the fact that CSCs expansion and metastasis is observed especially in the presence of PGE2 (41). Epidemiological studies statistics represent a high percentage of people getting affected from oral cancer when they have a persisting chronic periodontitis (41). Recent studies also show that *P. gingivalis* infection promotes migratory and invasive properties together with inducing higher secretion of MMPs (41). Notch inhibition theory can be a topic of interest when treating these cancer patients guiding us towards further research needed in pharmacological field. *P. gingivalis* is indeed involved in modulation of epithelial mesenchymal transition and tumorigenicity promoting distant metastasis and resistance to anti-cancer drugs(41).

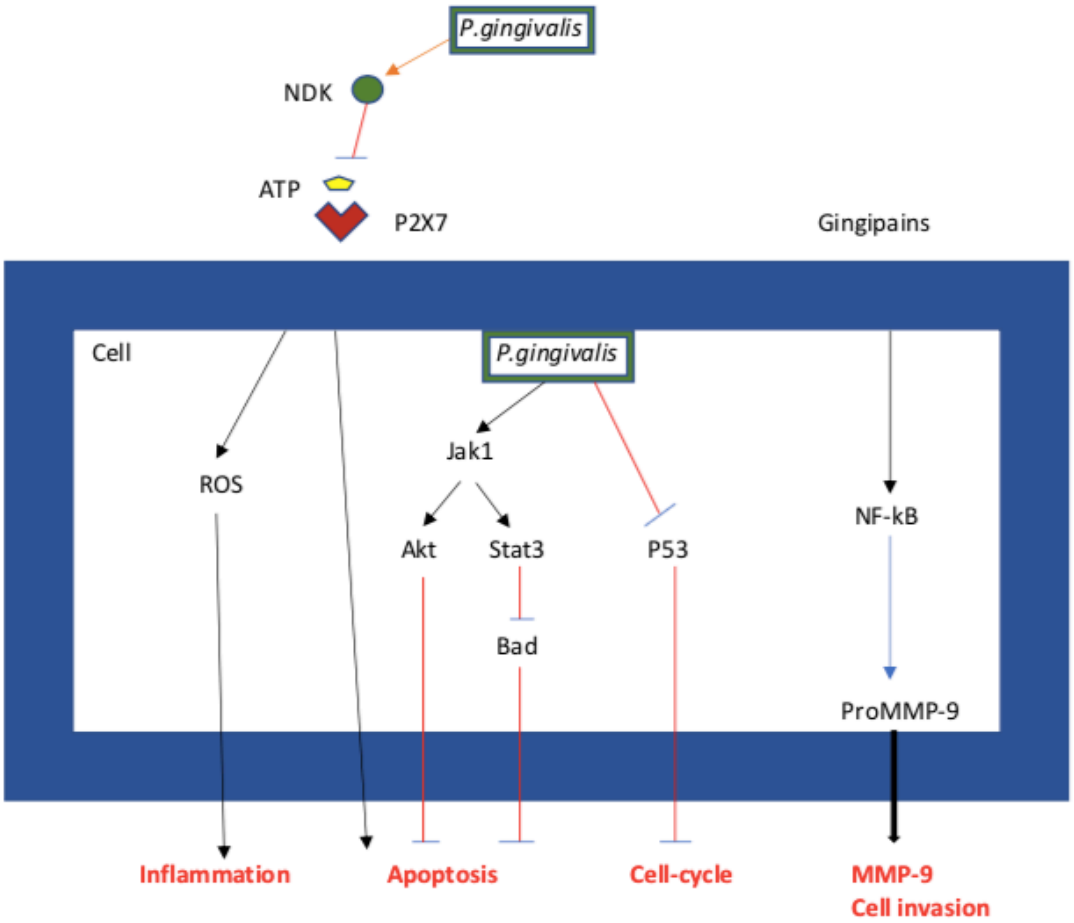

Figure 3. Oncogenic mechanisms of *P. gingivalis* in epithelial cells. Akt: protein kinase B; ATP: adenosine triphosphate; Bad: Bcl-2-associated death promoter; Jak1: Janus kinase 1; MMP: metalloproteinase; NDK: nucleoside diphosphate kinase; NF-kB: nuclear factor kappa B; P2X7: Purinergic receptor; Tumor protein p53 ; ROS: reactive oxygen species; Stat3: Signal transducer and activator of transcription 3.
Figure adapted from Karpinski (2019) (15).

Fusobacterium nucleatum

Introduction

Fusobacterium nucleatum (F.nucleatum) is an anaerobic, gram-negative spindle-shaped rod. *F. nucleatum* has a high invasive potential and has the ability to adhere and invade to human cells, like endothelial and epithelial cells (42, 43). It is one of the most common pathogen in human infections, like periodontitis (44). The role *F. nucleatum* plays in OSCC is more recognized as more recourse is done (23, 31). Observation of *F. nucleatum* in people with OCSS saw higher levels of these bacteria, compared to people with normal oral squamous epithelium (23, 45). Putative virulence

factors from *F. nucleatum* were the ones most expressed and upregulated in tumor sites, compared to other oral bacteria (46). *F. nucleatum* is also found to be involved in numerous diseases that are not associated with the oral cavity, like respiratory tract infections, gastrointestinal disease and many more (26, 47). *F. nucleatum* is found abundantly in colorectal cancer, suggesting its potential role in the disease mechanism (48-50).

The main carcinogenic mechanisms of *F. nucleatum* are related to its interaction with the host's immune system. That is its ability to induce chronic inflammation, the interaction of its surface proteins and host cells, evasion and suppuration of immune system (26, 45). *F. nucleatum* byproduct and LPS have the ability to create a microenvironment that shifts towards more inflammation. Those products increase inflammatory cytokines and chemokine, thereby facilitating tumor progression (26). LPS can penetrate oral cells and induce an acute inflammatory response, as well as being directly cytotoxic to epithelial cells and oral fibroblast to name a few (51). Chronic infection can activate transcriptions factors like STAT3 and NF-ƙB. These are part of a signaling pathway and promote tumor progression by maintaining an inflammatory state (23, 31). *F. nucleatum* induces gene expression alterations associated with the immune response and increases production of IL-1β, IL-6 and TNF- α (15, 26, 47).

STAT3 activation

Gallimidi *et al.* demonstrated that STAT3 activation is increased by more than 3-folds in presents of *F. nucleatum* in mouse tongue epithelium cells (31). STAT3 is a part of the STAT protein family involved in regulation of genes and are referred to as transcription factors. STAT3 regulates genes involved in cell growth, cell migration, apoptosis and modulate inflammatory responses to name a few (52). It has been suggested that STAT3 plays a role in cancer development, as it may increase cancer cell survival, while suppressing anti-carcinogenic mechanism of immunity (53, 54) . Infected epithelial mouse cells were compared to non-infected mouse cells, and an increase in anti-STAT3 antibodies was observed in the infected group. Epithelial mouse cells exposed to *F. nucleatum* (or *P. gingivalis* or a mixture *F. nucleatum* and *P. gingivalis*) showed increase IL-6 production, which is an important cytokine in the activation of STAT3. The epithelium cells were exposed to *F. nucleatum* multiple times, and a 3-fold increase in IL-6 mRNA was observed using RT-PCR analysis. The

overexpression of IL-6 and NF-ĸB is a result of TLR2 involvement by *F. nucleatum,* because TLR2 activation results in those products (31, 55). To emphasis *F. nucleatum* role in carcinogenesis they tested another type of bacteria that is commonly found in humans, *L. casei*, and it showed no effect when infected on the same epithelial mouse cells (31).

Toll-like receptor 2 activates inflammatory responses in the presence of F. nucleatum

This study demonstrated that epithelial cell model was able to differentiate between different bacteria, like *S. sanguinis* and *F. nucleatum* via TLR2 system (56). Toll-like receptors are able to recognize bacteria via pathogen-associated molecule pattern on bacteria membrane (55). It is responsible for the transcriptional and post-transcriptional of proteins involved in the immune system. Once TRL is activated, a host of proteins are activated, like NFkB, p38 and other interferon regulatory signaling factors (57). TLR2 activation gives rise to inflammatory responses in the presence of *F. nucleatum* but not *S. sanguinis,* as those responses were suppressed via TRL2 signaling. *S. sanguinis* is a gram-positive bacterium and exist as a normal part of the oral microbiota (56). In the presence of functional TLR2, *F. nucleatum* was able to induce hβD2 significantly (56). Upregulation of human beta defensing-2 (hβD2) occurs during infection or inflammation (58). *F. nucleatum* was also able to upregulate MMP-9, even in TRL-2 silencing, suggesting MMP-9 regulation is TRL-2 independent (56). This study demonstrated that in the presence of *F. nucleatum* in studied cell lines able to upregulate expression of genes for β-defensin, interleukin and MMP-9.This mechanism may play a part in *F. nucleatum* role in periodontal disease. However, this study did not look at potential role in cancer development.

DNA damage and impaired DNA repair

A study of the effect on DNA damage was done by Geng *et al.* were they infected squamous cell carcinoma cells from tongue tissue with *F. nucleatum*. *F. nucleatum* increased proliferation of cells in a time-dependent manner. This study infected cancer cells with MOI (multiplicity of infection) of 500, and observed an increase in ɣH2AX, as a measure of DNA damage (59). This protein is activated when there is DNA damage, like double strand breaks, and can be used as a marker for amount of DNA damage (60). The increase in ɣH2AX was not as high when MOI of 200 or no infection was applied. This study demonstrates that MOI of 500 caused a severe amount of DNA

damage and that the cells are unable to repair. Ku70 is a protein involved in repairing double strand DNA breaks, and initiates the repair mechanism *homologous recombination/nonhomologous end joining.* The expression and protein synthesis of Ku70 was reduced in presence of *F. nucleatum*. This indicates that the repair mechanism is impaired under infection of *F. nucleatum*. Reduction of expression of p53 was also observed. P53 is activated by ku70 once DNA damage occurs (59). This study demonstrates that *F. nucleatum* enhances DNA damage and depresses DNA damage repair mechanism (see figure 3). However, ɣH2AX as mention earlier, is an important protein in DNA damage as it is able to recruitment and activate DNA repair proteins (60). An increase suggest increase DNA damage, but should also increase DNA repair mechanism via this pathway.

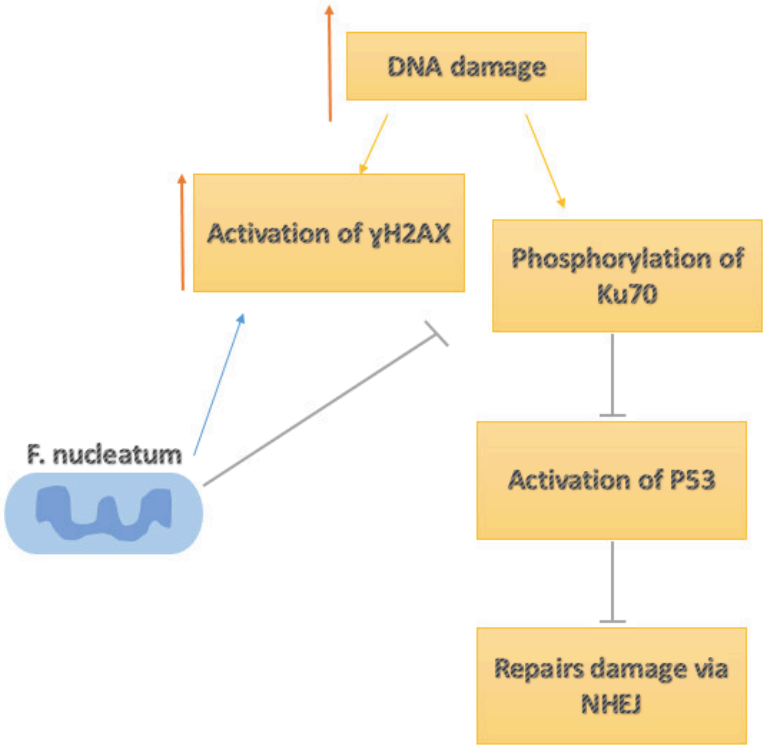

Figure 3: Simple illustration describing how *F. nucleatum* effects tongue cancer cells as described by Geng et al. (60). When cells are infected with *F. nucleatum,* an increase in number of ɣH2AX is seen, indicating an increase in DNA damage. DNA repair mechanism was reduced via the Ku70/p53 pathway, as those proteins were observed to be reduced in a time-dependent matter.

FadA interaction with E-cadherin promotes cell proliferation in colorectal cancer cells

Rubenstein *et al.* demonstrated that *F. nucleatum* is able to induce growth of CRC by promoting inflammatory and carcinogenetic responses (see figure 4) (43). As discussed earlier, *F. nucleatum* has the ability to adhere to cells and this adhesion is facilitated via the protein FadA. Adhesion is made possible by FadA binding to E-cadherin. E-cadherin is a adhesion molecule that connects epithelial cells to each other (61). This binding activated a cascade of pathways, like increased expression of transcription factors and inflammatory genes. The β-catenin pathway is activated, giving rise to NF-κB and Myc pathways responsible for cell survival and proliferation (43). This study demonstrated that decreased expression of E-cadherin would result in a decreased ability of *F. nucleatum* to attach and invade cells. With that, cells that *F. nucleatum* was able to attach to, showed increased proliferation. The cells with lower E-cadherin did not show a change in proliferation. Further, cells that had a FadA-deletion mutation, displayed similar lack of proliferation. This shows the importance of E-cadherin and FadA interaction in *F. nucleatum* ability to stimulate proliferation of CRC (43). Another study by the same another observed that *F. nucleatum* stimulates carcinogenesis via E-cadherin in already cancerous cells, but this mechanism was not present in non-cancerous cells (62). Although this study was conducted on colon cancer cells, it may also apply to oral cells, as they are both epithelial cells with E-cadherin. It is interesting to note, however, that one study found that the expression of E-cadherin is normal in well-differentiated OSCC but is reduced in moderate to poorly differentiated OSCC (63). Those findings may suggest that the *F. nucleatm's* role in FadA/E-cadherin interaction may be the beginning of cancer development, as E-cadherin is still expressed normally.

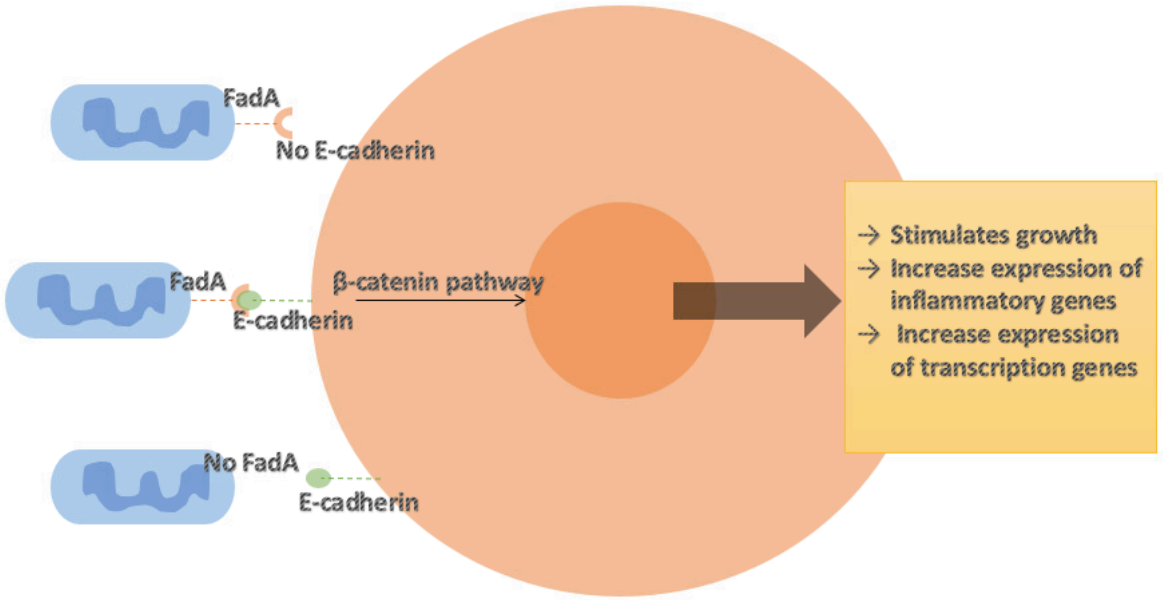

Figure 4: Simple schematic representation of mechanism demonstrated by Rubenstein et al. 2013 (43). *F. nucleatum* (blue bacteria) and a human cell with three possibilities. *F. nucleatum* in the middle has FadA of its surface. This receptor binds to its ligand, E-cadherin. This binding starts a pathway cascade that stimulates growth, increase expression of inflammatory genes and transcription factors. If either FadA or E-cadherin is non-present, then there will not be activation of this pathway. Those cells will lack proliferation abilities.

Co-infection with P. gingivalis and F. nucleatum

Li *et al*. found a link between *P. gingivalis* and *F. nucleatum*, where *F. nucleatum* has the ability to encourage and thereby increase the amount of *P. gingivalis* invasion by stimulating *P. gingivalis* adhesion. Co-infection of those bacteria gives them the ability to escape host immune system detection. Those bacteria can therefore survive more adequately (32). This mechanism is of benefit for these bacteria because they can execute their carcinogenic effect without disturbance.

Discussion:

Intriguingly, many epidemiological studies have found a significant relationship between periodontitis, OSCC and *P. gingivalis* as a potential etiological bacteria to enhance cancer mortality independent of periodontitis (35, 64, 65). Research by Katz et al. shows also higher presence of *P. gingivalis* in gingival carcinoma than in normal gingiva (35). Another retrospective study by Katz *et al.* showed that *P. gingivalis*

staining was found to be positive and more intense in gingival squamous cell carcinoma biopsy as compared to normal healthy tissue (66). It is interesting to note that a bacterium that can live inside the mouth with harmony and respect towards the host system can also cause devastating effects on cellular functions and growth once the microbial environment is disturbed.

Several mechanisms are known to prompt this cancer development where inflammatory mediators play an important role in the development. NF-ƙB and STAT3 are oncogenic transcriptions factors and can be activated by products of inflammation (65). As seen earlier, both *P. gingivalis* and *F. nucleatum* have the ability to activate those transcriptions factors hence increasing expression of certain biological molecules. Inflammation is able to suppress protective factors and create a pro-carcinogenic environment. NF-ƙB is an important molecule that is also thought to be the first, providing a link between carcinogenesis and inflammation. STAT3 plays a part in cell proliferation and survival and absence of STAT3 shows decreased tumor growth and production. NF-ƙB and STAT3 regulate production of cytokines and chemokines, like COX2, which gives prostaglandins further enhancing the inflammatory effect (64, 65). DNA damage is also an important factor that effects the development of carcinogenesis, as well as playing a significant role in the initiation phase of cancer. During cancer investigation, DNA damage by *F. nucleatum* has been very well documented (59). SCC cells become more aggressive when exposed to *F. nucleatum* and *P. gingivalis*, because those bacteria promote production of cytokines and other pro-inflammatory molecules (31). This finding suggest a role in progression of cancer, as well as those bacteria playing part in the initiation phase. *F. nucleatum* is both present in a healthy and diseased oral environment and may be part of that shift in environment (56). *F. nucleatum* is able to induce an inflammatory environment that favors tumor growth in host (50). It is suggested that *F. nucleatum* plays a role in the progression on cancerous cells, but does not have the same effect on non-cancerous cells (43, 62). Some mechanism appear to be involved in cancerous cells, driving them to become more aggressive and less differentiated, while another mechanism, like inflammation, may be involved prior to tumor formation.

P. gingivalis controls the transcription of target genes that are involved in downstream TLR, NF-ƙB and MAPK pathways responsible for pro-inflammatory processes in

primary and malignant epithelial cells (8). Infection with *P. gingivalis* affects mainly cell apoptosis and division disrupting normal cell-cycle and proliferating behavior of cells. Co-infection with *P. gingivalis,* makes *F. nucleatum* into an opportunistic pathogen. This causes a shift in hemostasis which leads to periodontitis (67). As we have described different mechanisms above, chronic infection and inflammation seems to play a significant role in cancer development. One may want to draw the conclusion that there is a link between periodontitis and oral cancer. *P. gingivalis* and *F. nucleatum,* two periodonal-pathogens could be potential microorganisms playing a significant role in cancer development via activating inflammatory processes during periodontitis. Can periodontitis be a risk factor for development of oral cancer? A case study presented with the following findings: sites with the most severe chronic periodontitis showed alveolar squamous cell carcinomas (68). Four different individuals with chronic periodontitis and alveolar squamous cell carcinomas were the participants. Although a small number of individuals, those findings may link periodontitis and OSCC. As presented in the introduction, Tezal *et al.* found an association between periodontitis and poor differentiated oral cancer. This study gives the explanation that constant cell proliferation because of chronic periodontitis may give rise to the carcinogenetic changes observed. This study argued that although further prospective studies is needed, early prevention of periodontitis may decrease occurrence of cancers in head and neck area (17).

Conclusion:

P. gingivalis and *F. nucleatum* both have attributes and characteristics consistent with a role in cancer initiation and development. Increased number of *P. gingivalis* and *F. nucleatum* are seen as the lesion progresses from pre-cancerous to cancerous stage. Our analysis shows that periodontitis may be major risk factor for the developement of oral cancer due to prolonged chronic inflammation promoting damage and destruction of gingival and bone tissue. At the same time, it should be remembered that cancer is a multifactorial disease where genetics play a vital role, exposing certain individuals at a higher risk of acquiring cancer. That's why changes in microflora and inflammatory insults may play a contributory, but not an exclusive role in cancer development (9).

These findings, linking *F. nucleatum* and *P. gingivalis* and OSCC can be used in the near future for screening cancer; however, there is a need for more studies in order to

see this opportunity. Longitudinal studies with follow-up on patients with pre-cancerous lesions can be of interest in this case where changes in oral microbiota can be studied at different stages of disease development, pouring light into any causal relationship between existence of specific type of bacteria and its oncogenic effects. The biochemical pathways in which these bacteria can effect cancer initiation and development are vast and complex. Further research is needed on a molecular level to find out carcinogenic roles of each specific oral bacteria in order to use them in diagnostics and pharmacological research.

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