A Narrative Review of the Risk Factors, Molecular Alterations and Epigenetic Dysregulation in Oral Squamous Cell Carcinoma

By

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A thesis submitted to the **Department of Mathematics and Natural Science** in partial fulfilment of the requirements for the degree of
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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing a degree at BRAC University.

2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.

3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.

4. I have acknowledged all main sources of help.

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Approval

The thesis/project titled "**A Narrative Review of the Risk Factors, Molecular Alterations and Epigenetic Dysregulation in Oral Squamous Cell Carcinoma**" submitted by

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of Spring, 2024 has been accepted as satisfactory in partial fulfilment of the requirement for the degree of Bachelor of Science in Biotechnology on 05.05.2024.

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Ethics Statement

I hereby state that the information contained in this review thesis, titled, "**A Narrative Review of the Risk factors, Molecular Alterations and Epigenetic Dysregulation in Oral Squamous Cell Carcinoma**" has been taken from publicly available database and research articles relevant to the topic. However, this review thesis itself has not been submitted for credit toward any other degree program at any other university or higher education institution.

This research was conducted under the supervision of Dr Fahim Kabir Monjurul-haque, Associate Professor in the Department of Mathematics and Natural Sciences at BRAC University, Dhaka.

Abstract

Oral cancers, generally referred to as Oral Squamous Cell Carcinomas (OSCC) or Head and Neck Squamous Cell Carcinomas (HNSCC), are a group of cancer that originates in the squamous cells in the mucosal surfaces of the head and neck. Although uncommon in developed countries, it is notoriously prevalent in developing and undeveloped countries, notably due to poor oral health. This disparity has contributed to the dearth of research in oral cancer, leading to poor diagnosis, prognosis, and mortality rates. Risk factors include tobacco, alcohol, and betel quid consumption. Bangladesh has the highest incidence and death rates, highlighting the poor state of oral cancer prevention and treatment, attributed to the cost of screening programs, limited localised research and literature, and prevalent of poor oral health. Dietary factors, infections, and genetic mutations also contribute to OSCC development. Epigenetic modifications, such as DNA methylation, histone acetylation, chromatin remodelling, and non-coding RNAs also influence OSCC development. Understanding these factors is vital for improving OSCC prevention, diagnosis, and treatment strategies globally.

Dedication

This work is dedicated to my good friends at BRAC who made me feel at home, thousands of nautical miles away from home; my mother, whose prayers guided me steadfastly through this journey; and the many teachers, who made my learning here, a wonderful experience.

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Chapter 1. Introduction

Cancer is one of the leading causes of death worldwide (World Health Organization, 2020). It arises due to the accumulation of mutations, mostly in genes regulating the cell division cycle and cell growth, either by inactivating the tumour suppressor genes (TSG) or activating the oncogenes (Hamid Teimouri et al., 2019). This causes the cell to enter a phase of uncontrolled cell division and proliferation, leading to the protrusion of a large neoplastic tumour mass. When this tumour is non-invasive, it is termed a benign tumour. However, if it is invasive and can spread to other parts of the body, then it is cancerous. Because of this association with dysregulated growth, cancer is more common among cell types with naturally high cell division rates, such as epithelial cells in the lung, intestines, prostate gland, or skin. In cells with low or no cell division, such as neural and muscle cells, cancer occurrence is rather rare. In fact, over 90% of cancers originate in epithelial cells (Coradini et al., 2011; Hinck & Näthke, 2014). Despite heavy investment in oncology research and development and recent improvements in diagnosis and treatment, curing cancer remains elusive as lifestyle factors continue to raise the future incidence rate. Among the most fatal cancer types is oral cancer, which has one of the worst survival rates among all cancer types and has seen a recent increase in the incident rate (Nath et al., 2022).

Oral cancers, generally referred to as Oral Squamous Cell Carcinomas (OSCC) or Head and Neck Squamous Cell Carcinomas (HNSCC), are a group of cancers that affect the squamous cells lining the mucosal surfaces of the head and neck (National Cancer Institute, 2021). Although uncommon in developed countries, oral cancer is notoriously prevalent in developing and undeveloped countries, notably due to poor oral health. This disparity has contributed to the dearth of research in oral cancer, leading to poor diagnosis, prognosis, and mortality rates. Most cases of oral cancers are caused by poor oral health and hygiene such as chewing tobacco, betel leaves, and smoking, as well as cancer-inducing oral infections like Human Papillomavirus infection (HPV) and Epstein-Barr virus infection (Irani, 2020; Kumar et al., 2016; Meurman, 2010). However, recent research has highlighted an interesting role of the oral microbiome in the development of oral cancers, with a link between oral cancers and oral dysbiosis (La Rosa et al., 2020; Panneerselvam et al., 2023). Unfortunately, while this includes many unanswered questions, recent experimental studies have not precisely defined the role of oral dysbiosis in oral cancer, particularly in its initiation stage (La Rosa et al.,

2020). Since oral dysbiosis is significantly influenced by oral hygiene, it is crucial to understand the complexity of the role of oral microbiome dysbiosis in oral cancer initiation and progression, the molecular pathways implicated by oral dysbiosis, and measures to implement to reduce the risk of OSCC from oral-health dysbiosis. This understanding can aid in the development of treatment and preventive measures aimed at reducing the incidence and mortality rate of oral cancers in affected regions.

1.1. Objective

This review aimed to introduce the concept of oral cancer, its epidemiology, and subtypes. It further delved into the molecular pathways and alterations implicated in oral cancer initiation, as well as the epigenetic alterations such as DNA methylation, histone modification, and non-coding RNA. Additionally, it critically explored the association between oral cancer and the oral microbiome, synthesized important comments and conclusions from relevant literature, and concluded with recommendations for further investigation and implementations to advance oral cancer prevention and treatment.

Chapter 2. State of Oral Squamous Cell Carcinomas

The epidemiology of oral cancer differs significantly and exhibits notable socioeconomic disparities. In fact, it is one of the few cancers with a high incidence in developing and underdeveloped countries, while showing a low incidence in developed countries. Furthermore, oral cancer is known to have one of the poorest patient outcomes, with the 5-year survival rate lower than that of most other cancers. This is largely attributed to late diagnosis and a lack of research in oral cancer.

2.1. Global Perspective

There is a noticeable and significant disparity in cancer incidence and outcomes between developed countries and the combined underdeveloped and developing nations. This discrepancy can be attributed to various factors, including late diagnosis, inadequate screening services, financial barriers, sociocultural and lifestyle factors, and general inaccessibility to cancer services (Awojobi et al., 2012; Babiker et al., 2017; Mishra et al., 2021; Saraswat et al., 2020; The World Bank, 2020). For instance, upon examining the incidence and mortality rates of oral cancers in the top ten countries, more than 50% of these countries fall into the category of developing or undeveloped nations based on socio-economic classification (see Table 4.1-2). Additionally, there is also a significant variation in the incidence rates and death rate between the male and female populations of the affected countries. In fact, the female-to-male ratio for both incidence and death is almost tripled for every country in the data collected by the World Cancer Research Fund International (2022). Interestingly, while many oral cancer patients acknowledge awareness of oral cancer screening prior to diagnosis, very few sought dental consultations or participated in such screenings, highlighting ignorance and negligence as potential contributors to the high incidence of oral cancer (Yasmin et al., 2023). A concise summary of the top ten countries with the highest incidence and mortality rates of oral cancer is presented in the table below:

Rank	Country	Incidence	*Age Standardised Rate (ASR) / 100, 000
	World	744, 994	8.0
1	Papua New Guinea	1, 480	25.7
2	Bangladesh	30, 583	21.0
3	Romania	5, 836	17.2
4	Hungary	3, 015	17.2
5	Cuba	3, 523	16.8
6	Slovakia	1, 492	16.2
7	India	219, 722	16.0
8	Sri Lanka	4, 481	15.3
9	Pakistan	24, 597	15.1
10	Moldova	918	14.8

Table 2.1.1: Incident rate for mouth and oral cancer as of 2020*. The data represent the top ten countries with the highest OSCC incidence. Overall, the oral cancer incidence is particularly prevalent in countries of South Asia such as Sri Lanka, Pakistan, India and Bangladesh (World Cancer Research Fund International, 2022).*

Rank	Country	Death Rate	**ASR / 100, 000
	World	364, 339	3.9
1	Bangladesh	16, 884	11.9
2	Papua New Guinea	588	11.1
3	Moldova	599	9.5
4	Pakistan	15, 127	9.4
5	Romania	3, 197	9.2
6	Hungary	1, 628	9.1
7	India	121, 096	8.9
8	Belarus	1, 401	8.8
9	Slovakia	802	8.6
10	Montenegro	97	8.1

Table 2.1.2: Death rate for mouth and oral cancer as of 2020*. Data indicate the top ten countries with the highest OSCC death rate. Cuba and Sri Lanka, despite being in the top ten countries with oral cancer, are replaced by Montenegro and Belarus in the top ten countries with leading mortality rates for oral cancer. This could be explained by better patient outcomes in Cuba and Sri Lanka, compared to the other countries. (World Cancer Research Fund International, 2022). Note that this data does not include nasopharyngeal and oesophageal cancers.*

*ASR = age-standardised rates: The ASR/100,000 figures represent an assessment of the disease rate per 100,000 persons in the country's population if it possessed a standard age composition. This standardisation is essential because countries' populations vary in age composition, and age is a significant risk factor in cancer, and can influence overall incidence and mortality rates in population studies. Hence, standardisation reduces the likelihood of age bias in the presented data (World Cancer Research Fund International, 2022).

2.2. Bangladesh

Bangladesh records an annual incidence of 60,219 cases of oral cancers, accompanied by 43,897 fatalities, resulting in a mortality-to-incidence ratio of 0.73 (International Agency for Research on Cancer, 2024). Over the period spanning from 1990 to 2019, for age-standardized incidence rate per 100,000 individuals, Bangladesh experienced an upward trend in males, while rates among females remained relatively stable, resulting in a minor overall decrease (Zhang et al., 2022). Despite an observable improvement in survival rates, as evidenced by trends in the mortality-to-incidence ratio, Bangladesh lags behind countries with similar populations in terms of survival outcomes (Zhang et al., 2022). The high incidence and mortality rate in Bangladesh is attributed to poor screening programs for oral cancer, a paucity of localized research and literature on the subject, and prevalent habits of tobacco and betel leaf consumption and smoking (Yasmin et al., 2023), with smoking and tobacco use identified as the most prominent risk factors among them.

Chapter 3. Risk Factors associated with oral squamous cell carcinomas

The risk factors for oral cancer can be broadly classified into environmental factors, genetic factors, age, and lifestyle/epigenetic factors (Aghiorghiesei et al., 2022). The impact of the risk factor in oral cancer initiation depends on population demographics. For example, in South Asian countries, betel leaves, tobacco, and smoking account for the most significant risk factors for oral cancer initiation. A notable example is Bangladesh, where, according to the 2020 data repository from the World Health Organization Global Health Observatory, 52% of males are smokers (The World Bank, 2020).

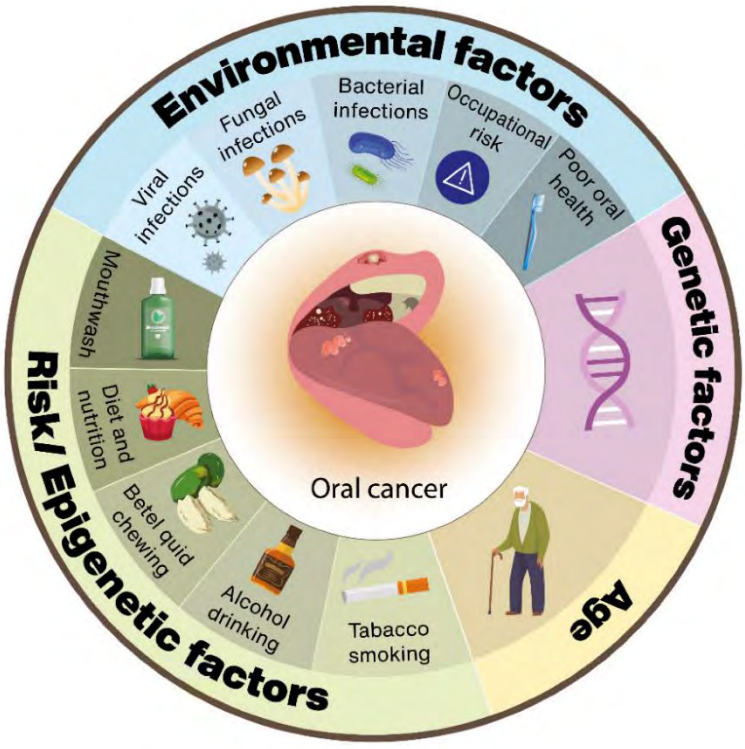

Fig 3.1. Risk factors of oral cancer: *Oral squamous cell carcinomas have a complex risk factor. Genetic factors include inherent DNA mutations or somatic mutations that arise as one ages due to errors in DNA replication and DNA damage repair. Environmental factors like occupational hazards, and oral cancer-inducing infections or lesions are also strong risk factors as they can transform normal squamous cells into malignant neoplasms. Diet, nutrition and lifestyle of an individual are also very important as they can either directly influence oral carcinogenesis, or accelerate its progression (Aghiorghiesei et al., 2022).*

3.1. Tobacco and Alcohol Consumption

Tobacco smoking and smokeless tobacco (betel leaves/nut chewing) are significant risk factors for oral cancer. Tobacco smoking has been shown to alter the structure of microbial biofilms, increasing susceptibility to periodontitis, a known predictor of oral cancer (Kumar et al., 2011; Börnigen et al., 2017; Hooper et al., 2009; Kumar et al., 2016; Michaud et al., 2008). Moreover, in vitro studies have demonstrated that tobacco smoke condensates can selectively promote the growth of certain species, such as Staphylococcus aureus, which induce the release of inflammatory cytokines implicated in oral carcinogenesis (Fujiki et al., 2004; Jeng et al., 2003). Smokers also demonstrate a highly pro-inflammatory response to bacteria colonisation due to alteration in host-bacterium response (Kumar et al., 2011). This is supported by other studies which reported only minor and rather insignificant disparity in microbial diversity between periodontitis and non-periodontitis subjects, attributing the periodontitis initiation instead to host immune response to microbial diversity, rather than the bacteria themselves (Van Dyke & Sheilesh, 2005).

However, studies investigating the subgingival microflora in periodontitis patients who smoke have yielded conflicting results. While some studies have reported higher risks of infection with pathogenic species in smokers compared to non-smokers (Shiloah et al., 2000; Winkelhoff et al., 2001), others found no significant differences between smoking and non-smoking cohorts (Apatzidou et al., 2005; Darby et al., 2000; Natto et al., 2005). Nevertheless, tobacco smoking remains a critical factor in increasing the risk of severe periodontitis (Gomes et al., 2006; Hujoel et al., 2003), driving changes in microbial diversity and increasing the likelihood of colonization by antibiotic-resistant bacteria (Kumar et al., 2003; Socransky et al., 1998; Ahmer et al., 1999; Aronson et al., 1982; Charlson et al., 2010; Gryczyńska et al., 1999; Itzhak & Alan E, 2005), which initiate cancer-inducing infections.

Alcohol consumption also influences the oral microbiota composition. For instance, a study by Morita et al. (2005) found higher levels of Streptococcus anginosus in the saliva of alcoholics compared to non-alcoholics. Furthermore, alcohol consumption, especially when combined with tobacco smoking, enhances the risk of oral cancer (Kumar et al., 2016). The carcinogenicity of polycyclic aromatic hydrocarbons (PAH) and tobacco-specific nitrosamines (TSNA) in tobacco, as well as the effects of ethanol

in alcohol, have been well-documented (Cheng, 2014). Additionally, alcohol consumption is associated with elevated levels of CpG hypermethylation in genes linked to oral cancers (Supic et al., 2011) and promotes tumourigenesis by causing DNA hypomethylation and demethylation of oncogene promoters (Irimie et al., 2018; Gasche & Goel, 2012). Ethanol-induced DNA damage also contributes to immune evasion in oral cancer, with mutations in Human Leukocyte Antigen (HLA) and Antigen Processing Machinery (APM) detected in oral squamous cell carcinoma samples (Gillison et al., 2019; de Ruiter et al., 2017; Ling et al., 2018).

3.2. Paan (betel quid)/ Areca Nut Chewing

Fig 3.2.1. Fresh betel leaves: *leaves are either chewed raw, or chewed with tobacco. In some cases, they are eaten with other condiments.*

Fig 3.2.1 Areca nuts: *Image adapted from IndiaMart online store.*

Betel quid (areca nut) chewing is a traditional practice prevalent among populations in South Asia, Taiwan, and the South Pacific Islands (Nair, 2004). However, this seemingly innocuous habit has been associated with numerous health risks, particularly concerning oral health. Chewers often develop clinically visible lesions such as reddish (erythroplakia) or whitish (leukoplakia), along with submucous fibrosis of the oral mucosa (Nair, 2004). Areca nut chewers exhibit endogenous nitrosation, which generates potentially carcinogenic nitrosamines, including 3-methylnitrosopropionitrile (Xie et al., 2016). The autoxidation of polyphenols in betel quid releases Reactive Oxygen Species (ROS), which when intensified by the alkaline pH of slaked lime (Nair, 2004), can stimulate hypoxic adaptation in OSCC cells (Jing et al., 2019).

Chewing betel quid with tobacco can release up to 1, 000 μg/day (Nair et al., 1999) of tobacco-specific nitrosamines (TSNAs) in the buccal mucosal tissues compared to just 20 μg released in tissues of smokers alone (Hoffmann & Hecht, 1985). These nitrosamines have been demonstrated to possess carcinogenic properties (Jeng et al., 2001; Ko et al., 2020) which undergo a series of metabolic activation by host endogenous enzymes to yield methyl and pyridylhydro butyl adducts in DNA (Hecht, 2003) which can lead to various forms of DNA damage, including DNA mutations and strand breaks. These alterations can disrupt the normal functioning of DNA, potentially leading to errors in DNA replication and transcription, and subsequently, the growth of abnormal neoplastic cells. Remarkably, cessation of betel quid chewing during therapeutic interventions may reduce or eliminate OSCC symptoms (Chaudhry et al., 2013; Peng et al., 2019; Shah, 2016).

Additionally, areca nut-specific nitrosamines (ASNAs) (Wenke et al., 1984; Nair et al., 1985; Nair et al., 1987; Stich et al., 1986; Prokopczyk et al., 1987), found in high levels in the saliva of betel quid chewers, can also cause damage to DNA. Aqueous extracts of betel quid have also been evidenced to possess the ability to generate superoxide anion and hydrogen peroxide at pH > 9.5. Particularly, the calcium hydroxide present in betel quid is the main catalyst responsible for the formation of reactive oxygen species (ROS) and the subsequent DNA damage in buccal mucosal cells that follows (Nair et al., 1990). Chromosome breaks have been reported in the oral exfoliated cells in chewers of betel quid (Nair Uj et al., 1991), as well as chromosomal aberrations in peripheral blood lymphocytes in the oral cells of betel quid (Dave et al., 1991; Desai et al., 1996).

3.3. Radiation and Occupational Exposure

Several studies have reported the correlation between socio-economic status, such as education level, income level, and occupation, and oral cancer development, with those with lower education and income levels possessing higher risks of oral cancer development (Auluck et al., 2016; Hung et al., 2020; Ndiaye et al., 2014; Warnakulasuriya, 2009). Therefore, occupational risks play a critical role in an individual's susceptibility to oral cancer development. For example, multiple and/or prolonged exposure to asbestos, wood dust, and polycyclic aromatic hydrocarbons increases the risks of oral cancer (Paget-Bailly et al., 2011; Smailyte, 2012).

Furthermore, excessive UV or solar radiation exposure can initiate oral cancer either directly by stimulating lip cancer or via actinic cheilitis condition, which has a high tendency to transform into oral squamous cell carcinoma (Kumar et al., 2016). The risks associated with this exposure have been found to be higher in low and middle-income countries due to lack of occupational self-protection, automatic monitoring equipment (Hashim & Boffetta, 2014), and dental visits (Adrien et al., 2014), perhaps due to the high cost of dental visits and absence of dental insurance coverage. For example, in a study in Bangladesh covering oral cancer patients, over 68.3% of the patients were from lower-income groups. Interestingly, despite the majority of these patients acknowledging awareness of oral cancer screening, very few of them participated in it in the past. Again, this could be attributed to the lack of dental insurance coverage and the high cost of dental visits (Yasmin et al., 2023).

3.4. Ancestry and underlying genetic causes

Oral squamous cell carcinoma, like other cancers, is not initiated by a single genetic, epigenetic, environmental, or lifestyle factor. Rather, it is induced by the accumulation of multiple aberrations in all these risk factors. Oral cancer genetic studies have revealed that DNA damage in tumour suppressor genes (TSGs) and proto-oncogenes can promote oral carcinogenesis. Mutations that drive suppression or under-expression of TSGs, as well as aberrations that promote over-expression of proto-oncogenes, are key drivers of oral cancer initiation (Hooper et al., 2009). Major studies have revealed the potential of the accumulated TSG mutations to release a stem cell from the normal cell cycle, allowing it to proliferate and prevent it from being killed by programmed cell death (Choi & Myers, 2008; Williams, 2000).

Numerous cytogenetic studies have evidenced alterations in the genome that increase the risks of oral cancers (Singh et al., 2015). Although several cases of chromosomal instabilities have been heavily linked to oral carcinogenesis, the primary driver aberrations are defects in the genome governing telomere stability, chromosomal stability, cell cycle regulation, and DNA damage repair (Reshmi & Gollin, 2005). Among the more than 63 karyotypes found in human oral cancer studies, it was observed that recurrent loss of chromosome 9, 13, 18, and Y deletions were predominant among patient samples examined (Jurel et al., 2014; Sidransky, 1995). In addition, frequent deletion of the chromosomal regions involved in arms off 3p, 7q, 8p,

11q, 13q, and 17p, as well as in the short arm of all acrocentric chromosomes, can potentially induce oral carcinogenesis (Jurel et al., 2014; Sidransky, 1995). In another experimental study, about 67% of all head and neck cancer cells investigated were found to contain a deleted region in chromosome 9p21-22 (Ah-See et al., 1994).

In addition to directly affecting oral carcinogenesis, genetic predisposition also influences an individual's susceptibility to cancer by other lifestyle factors. For example, the effect of smoking on oral cancer initiation and development is dependent on the individual's inherent genetics. Patients with null GSTM1 and GSTT1 genes would have an impaired ability to degrade ingested carcinogenic substances from tobacco compared to those with functional genes. Hence, they are at higher risk of oral carcinogenesis (Talukdar et al., 2013).

3.5. Others: Oral Health and Oral Cancer Inducing Infections, Oral Potentially Malignant Diseases (OPMDs), Gender, Diet and Nutrition.

Persistent HPV infection can also induce oral cancer development (Tumban, 2019), with HPV type 16 identified in approximately 90% of HPV-associated OSCCs (Chaturvedi et al., 2011; Fakhry et al., 2008; Nair & Pillai, 2005). Studies on oral lesions have evidenced the tumour suppressor protein p53 interactions with E6 protein of HPV (Agarwal, 1999; Nagpal et al., 2002; Pande et al., 2002; Ranju Ralhan et al., 2000). This suggests the possibility of HPV E6 targeting P53 for degradation by the ubiquitin-proteasome pathway. Epstein Barr virus (EBV) infection is also a strong risk factor for oral squamous cell carcinoma (Ching Yu Yen et al., 2009; Ram et al., 2011), evidenced by a meta-analysis study which reported a 2.5 odds ratio increased risk for oral cancer among infected EBV patients (de Lima et al., 2019). The latent member protein (LMP-1) from EBV is found to activate several growth-promoting cancer-relevant pathways such as NF-κB, JAK-STAT, JNK-p38, and PI3K-AKT (Eliopoulos & Young, 2001; Kis et al., 2009). SARS-CoV-2 infection has also been found to increase the risk of oral cancers (Mariz et al., 2020).

Oral carcinogenesis initiation is also associated with some bacterial and fungal infections. For example, Candida albicans infection independently raises the risk of oral cancer (Alnuaimi et al., 2015), while Porphyromonas gingivalis and Streptococcus anginosus have also been reported to be associated with oral carcinogenesis and metastasis (Galvão-Moreira & da Cruz, 2016; Groeger et al., 2011; Inaba et al., 2013;

Meurman & Bascones-Martinez, 2011; Rai et al., 2020). Other oral lesions and conditions, generally termed oral potentially malignant disorders, can also induce oral squamous cell carcinogenesis (Kumari et al., 2022).

Potential Carcinogenic Mechanism	Associated Oral Microorganism
Production of carcinogens	Candida spp.
Nitrosamine production	
Metabolism of pro-carcinogens	
Conversion of ethanol to acetaldehyde	Candida spp. Neisseria spp. Streptococcus spp. Other Gram-positive species
Induction of chronic inflammation	Periodontopathogenic bacteria Streptococcus species
Stimulation of pro-carcinogenic stimulators	
Direct influence of bacteria on human cell cycle/signalling	
Promotion of cellular proliferation	Periodontopathogenic bacteria
Inhibition of cellular apoptosis	Mycoplasma spp.

Table 3.5.1: Bacteria and Fungus Implication in Oral Carcinogenesis: *Both bacteria and fungus infection can initiate oral squamous cell carcinoma (Hooper et al., 2009)*

Citrus fruits, raw vegetables, and flavonoids decrease oral cancer risk by regulating the expression of cancer-relevant transcription factors (Bravi et al., 2013; Chuang et al., 2012; Nosrati et al., 2017; Pelucchi et al., 2011; Rossi et al., 2007; Toporcov et al., 2012). Another study reported that consuming red meat more than once weekly, compared to white meat, also increases oral squamous cell carcinoma risk (Gupta et al., 2017). Flavonoids like Epigallocatechin-3-gallate (EGCG) and glucoraphanin have also been documented to decrease OSCC risk. For example, one study reported reduced cell proliferation as well as increased apoptosis and autophagy in oral cancer cells by EGCG (Irimie et al., 2015). Another study reported detoxification of tobacco-specific carcinogens and NRF2-independent inactivation of pSTAT3, a pro-tumorigenic transcription factor, by glucoraphanin (Bauman et al., 2016). Further, high dietary fat intake (Peng et al., 2021), low iron intake (Rodríguez-Molinero et al., 2021), poor oral hygiene (Rosenquist et al., 2005), increased consumption of processed foods (Galvão De Podestá et al., 2019), and male sex (Green et al., 2020) all heighten the risk for oral carcinogenesis.

Chapter 4. Subtypes of OSCC

Most literature does not distinguish between head and neck squamous cell carcinoma (HNSCC) and oral squamous cell carcinoma (OSCC), while some classify OSCC as a subgroup of HNSCC. Due to similarity in origin, phenotypes, and treatment, and in line with existing data, both OSCC and HNSCC are considered similar, and all oral cancers that arise in the squamous cells lining the mucosal surfaces of the head and neck would be classified as such (National Cancer Institute, 2021). There are eight subtypes of OSCC. These include hypopharyngeal cancer, laryngeal cancer, metastatic squamous neck cancer with occult primary, paranasal sinus and nasal cavity cancer, salivary gland cancer, lip and oral cavity cancer, oropharyngeal cancer, and nasopharyngeal cancer. A brief overview of each subtype is discussed below, with emphasis on the origin of malignancy, profile of molecular alterations, and disease burden.

4.1. Hypopharyngeal Cancer

Hypopharyngeal cancer is a form of OSCC that occurs when malignant cells grow from the squamous cells in the tissue of the hypopharynx (see Fig 4.1.1). The cancer has the potential to extend to surrounding tissues such as the thyroid or trachea, the hyoid bone beneath the tongue, the larynx, or the esophagus. Additionally, it can migrate to the lymph nodes in the neck, the carotid artery, tissues near the upper spine, the chest cavity lining, and other areas of the body. Known risk factors include tobacco use, smoking, heavy alcohol use, iron-deficient nutrients, and any iron-deficient disease/condition such as anemia and Plummer-Vinson syndrome (National Cancer Institute, 2021). Annual absolute global incidence and mortality are reported as 86,257 and 40,902, respectively (International Agency for Research on Cancer, 2024).

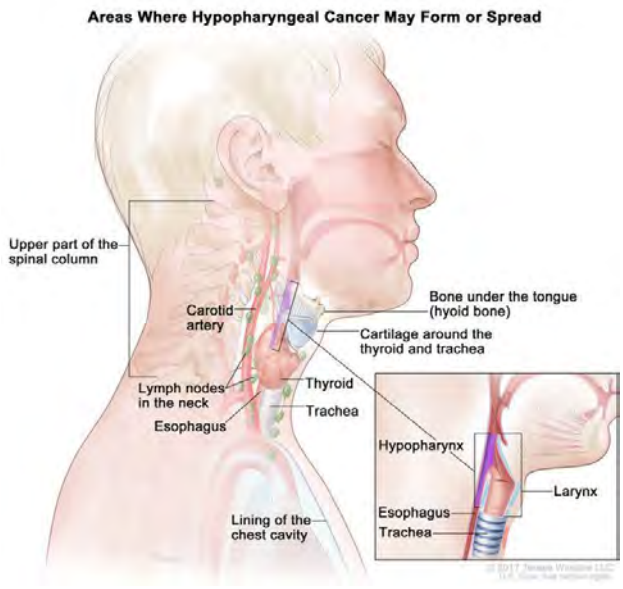

***Fig 4.1.1. Enlarged view of the Hypopharynx**: Hypopharyngeal cancer starts in the bottom part of the throat and can spread to nearby tissues like the thyroid cartilage, hyoid bone, thyroid, trachea, larynx, or oesophagus. It might also affect lymph nodes in the neck, the carotid artery, and tissues around the upper spine, chest lining, and other body parts. Figure adapted from (National Cancer Institute, 2021).*

The molecular alterations in hypopharyngeal cancer seem to be under-studied, with very few literature available on this topic. However, a study reported by Machnicki et al. (2022) identified TP53, FAT1, NOTCH1, KMTC2, and CDKN2A as the most frequent significantly mutated genes. It further went on to demonstrate in vitro using cancer cell lines the positive relationship between KMTC2 loss-of-function mutations and increased colony formation and proliferation, validating the KMTC2 gene as a core tumour suppressor protein in hypopharyngeal cancer. Another study revealed clusters of genes showing differential expression were found in specific chromosomal regions: 3q27.3, 17q21.2–q21.31, 7q11.22–q22.1, and 11q13.1–q13.3. Interestingly, these regions were previously identified through comparative genomic hybridization (CGH) as areas where gene amplification occurs. The study also reported that six genes (EIF4G1, DVL3, EPHB4, MCM7, BRMS1, and SART1) located in these regions are amplified in the hypopharyngeal cancer patient samples (Cromer et al., 2003). Hypomethylation of LINE-1 was also revealed to be positively correlated to recurrence, thus a positive biomarker for predicting tumor recurrence in hypopharyngeal cancer patients (Misawa et al., 2020). Contrary to popular publications, one study identified that over-expression of p53 increases the odds of recurrence in HPV-positive hypopharyngeal cancer (Perle Ernoux-Neufcoeur et al., 2010).

4.2. Laryngeal Cancers

Laryngeal squamous cell carcinoma is a relatively common oral squamous cell carcinoma subtype in the neoplastic cell forms in the tissues of the larynx (see fig 4.2.1). The annual absolute global incidence and mortality is reported as 189,191 and 103,359, respectively (International Agency for Research on cancer, 2024), indicating a poor mortality-to-incidence ratio.

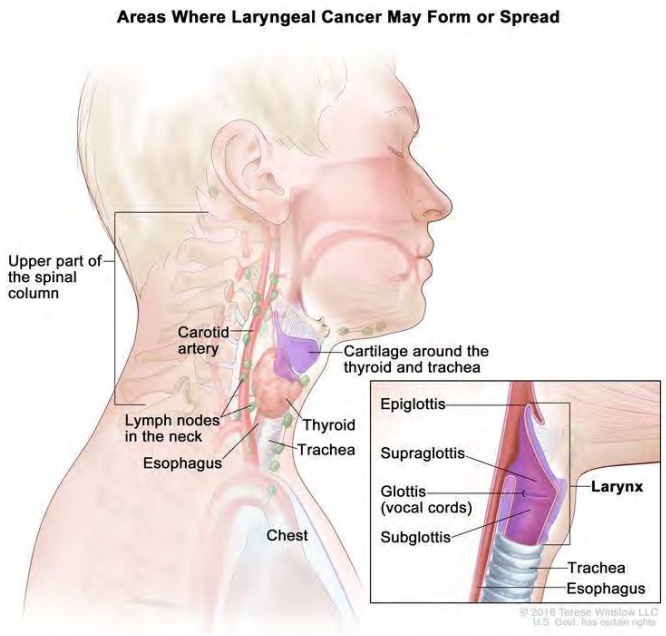

Fig 4.2.1. Enlarged view of the Larynx: *Laryngeal cancer occurs when cancerous cells develop in the tissues of the larynx, which is the part of the throat containing the vocal cords. The larynx consists of the supraglottis, glottis (vocal cords), and subglottis. The cancer can spread to nearby tissues like the thyroid, trachea, or oesophagus, as well as to lymph nodes in the neck, the carotid artery, upper spinal column, chest, and other body parts. Figure adapted from (National Cancer Institute, 2021).*

Several studies have identified key genes associated with the prognosis and tumourigenesis of laryngeal squamous cell carcinoma (LSCC). Mo et al. (2021) found that GATA6, HOXB13, and MAFF were risk factors in LSCC prognosis. Additionally, Zhang et al. (2019) proposed a five-gene signature consisting of EMP1, HOXB9, DPY19L2P1, MMP1, and KLHDC7B as an independent predictor of prognosis for laryngeal cancer. Ioachim et al. (2004) reported Cyclin D1 overexpression in laryngeal carcinoma. Furthermore, Falco et al. (2022) highlighted TP53, EGFR, CDKN1A, and NOTCH1 as key cellular pathways and genes dysregulated in LSCC tumourigenesis. Another study implicated genes such as TrkB, CTNNA2, CTNNA3, NOTCH, NAT1,

and NAT2, OGG1, PIK3CA, FGFR3, JAK3, MET, and FBXW7, PARK7, PTEN, p21 and p27, EGFR, Cyclin D1, Bcl-2, E-cadherin, and p53 in laryngeal squamous cell carcinomas. These findings collectively contribute to our understanding of the molecular mechanisms underlying LSCC development and prognosis. Cytogenetic examinations of LSCC have reported loss of chromosomes 3p, 5q, 8p, 9p, 18q and 21q are commonly identified as well, where loss of 18q could indicate poor prognosis of tumours. Loss of chromosome 9p21–22 appears to be most common (de Miguel-Luken et al., 2016).

4.3. Lip and Oral Cavity Cancer

Lip and oral cavity squamous cell carcinomas are subtypes of oral squamous cell carcinoma in which the neoplastic cell arises in the lips and around the mouth (see fig 4.3.1). The annual absolute global incidence and mortality for lip and oral cavity cancer are 389, 846 and 188, 438, respectively (International Agency for Research on Cancer, 2024), making it a relatively common cancer with poor patient outcomes.

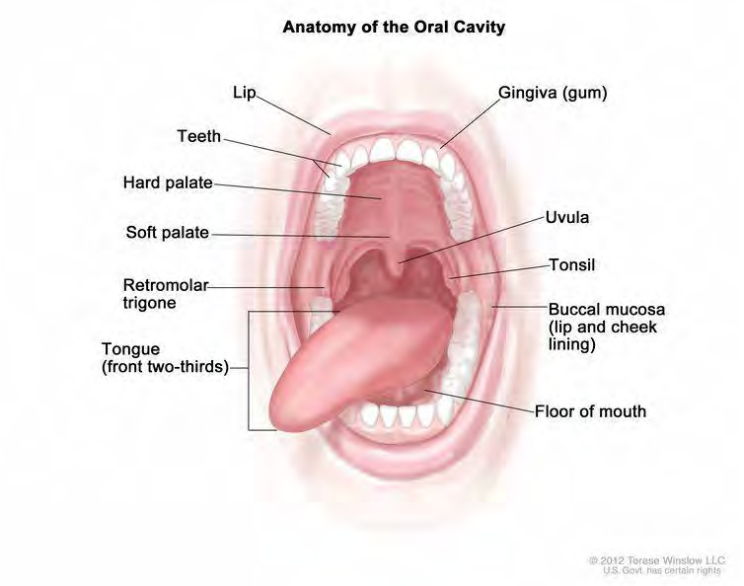

Fig 4.3.1. Anatomy of the oral cavity: *The oral cavity comprises the lips, hard palate (front part of the roof of the mouth made of bone), soft palate (back part of the roof of the mouth made of muscle), retromolar trigone (area behind the wisdom teeth), front two-thirds of the tongue, gums, inner lining of the lips and cheeks, and the floor of the mouth beneath the tongue. Figure adapted from (National Cancer Institute, 2021).*

Molecular alterations in lip and oral carcinogenesis seem to begin with microsatellite alterations in chromosome 9 in the early onset of carcinogenesis (A Mahale & D Saranath, 2000). VEGFA, IL6, MAPK3, INS, TNF, MAPK8, MMP9, CXCL8, EGF, *and* PTGS2 have also been reported to be implicated in lip and oral cavity carcinoma (Mathavan et al., 2021). miRNA also plays a critical role in lip and oral cancer pathobiology. For example, a study revealed miR-128a was significantly upregulated and miR-23a and let-7c were significantly downregulated in patients with lower lip cancer, validating their role as predictive and prognostic biomarkers (Ibrahim Bozgeyik

et al., 2022). XRCC1, ERCC1 and ERCC2 expression are also prognostic markers for lip and oral cavity cancer, and their high protein expression levels are associated with poor disease-free and overall survival rates (Wang et al., 2021). Damage to DNA repair genes are also one of the most dysregulated genes in lip and oral cancer (Toprani & Kelkar Mane, 2021)

4.4. Salivary Gland Cancer

Salivary gland squamous cell carcinomas is a rare subtype of oral squamous cell carcinoma in which the malignant cells arise in the tissues of the salivary glands (**see 4.4.1).** The annual absolute global incidence and mortality is reported as 55,080 and 23,942, respectively (International Agency for Research on cancer, 2024).

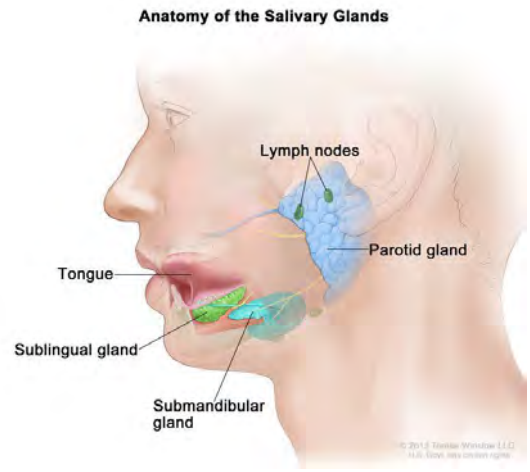

Fig. 4.4.1. Enlarged Anatomy of the salivary glands in the mouth*: The three main sets of salivary glands are the parotid glands, located near the ears, the sublingual glands beneath the tongue, and the submandibular glands beneath the lower jaw. Figure adapted from (National Cancer Institute, 2021).*

The most significant genetic alteration in salivary gland cancer is a chromosomal translocation t(11;19)(q21;p13), resulting in the fusion of CREB-regulated transcription coactivator 1 (CRTC1) with mammalian mastermind-like gene (MAML2) (Maroun Bou Zerdan et al., 2023). Genetic alterations have also been reported in TP53, PI3K, BAP1, PTEN, PIK3CA, HRAS, NF1, as well as the Cyclin-dependent pathway,

including aberrations in CCND1, CDK4/6, or CDKN2A/B (Maroun Bou Zerdan et al., 2023). Another study reported copy number variation in CRTC3-MAML2, HER2, EGFR, DCC, and SMAD4 (Yin & Ha, 2016). Salivary gland cytogenetic studies have revealed chromosomal instability characterized by copy number loss/deletions in 12q12-q13, 1p32-36, and gains at 22q12-q13, 8, 16p, and 17q, which contain a high number of cancer-relevant genes in these loci (Liu et al., 2011). Prognostic markers for salivary gland cancer include MDM2, MDM4, FGFR1, and FGFR3, all predicting poor outcomes (Ach et al., 2015).

4.5. Oropharyngeal Cancer

Oropharyngeal squamous cell carcinoma is a relatively common oral squamous cell carcinoma subtype where in the neoplastic cell forms in the tissues of the oropharynx, the middle part of the pharynx, located beneath the nasopharynx (**See fig 4.5.1).** The annual absolute global incidence and mortality values for oropharyngeal cancer is reported as 106,400 and 52,305 respectively (International Agency for Research on cancer, 2024).

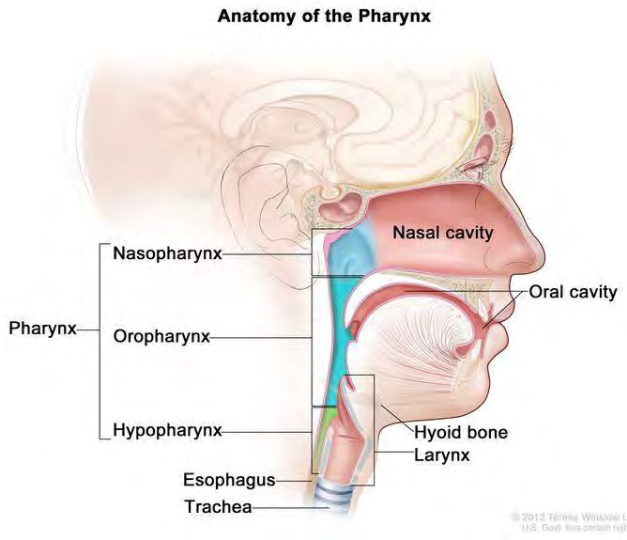

Fig.4.5.1. Enlarged anatomy of the oropharynx*: The pharynx is a muscular tube in the neck, starting from behind the nose and splitting into the larynx and oesophagus. It has three sections: the nasopharynx, oropharynx, and hypopharynx. Oropharyngeal cancer forms in the tissues of the oropharynx (National Cancer Institute, 2021).*

HPV-positive oropharyngeal cancer exhibits different molecular mechanisms from non-HPV subtypes. In the former, the E6 and E7 proteins from the virus target Tumour suppressor P53 and Retinoblastoma (Rb) proteins respectively, for ubiquitin-mediated proteasome degradation. This leads to loss-of-cell cycle checkpoint regulators and activation of lysine demethylase enzymes, which demethylate genes responsible for growth and proliferation. The Notch signalling pathway has also been reported to be dysregulated in oropharyngeal cancer (Ullah et al., 2023). The HPV oncogenes, particularly E6, also induce chromosome instability and aberrations in chromosome segregation in oropharyngeal cancer samples (Cosper et al., 2023). Interestingly, promoters of genes that have a function in the immune system, such as the EDARADD, GBP4, HAVCR2, HLA DPB1, IL12RB1, MARCO, and SIGLEC12 genes were found to be hypermethylated in oropharyngeal cancer samples (Anić et al., 2023), and this may explain why they seem to have a very poor response rate to immunotherapy.

4.6. Nasopharyngeal Cancer

Nasopharyngeal squamous cell carcinoma is a subtype of oral squamous cell carcinoma in which the neoplastic cell forms in the tissues of the nasopharynx, just above the oropharynx (see fig 4.5.1). The annual absolute global incidence and mortality for nasopharyngeal cancer is reported as 120,434 and 73,482, respectively (International Agency for Research on Cancer, 2024). This shows that it has an overall poor survival rate. Genetic studies reported loss of chromosomes in the 1p, 3p, 9p, 9q, 11q, 14q, and 16q regions and frequent copy number gains in chromosomes 1q, 2q, 3q, 4q, 6q, 7q, 8p, 8q, 11q, 12p, 12q, and 17q as common chromosomal aberrations in the early stage of nasopharyngeal cancer, with loss of 3p being the most common among them. Loss-of-heterozygosity was also reported in 3p21.3 and 9p21, affecting allele loss and functional dysregulation of the tumor suppressor genes in those loci, such as RASSF1A and CDKN2A (Siak et al., 2021). Aberrant PI3K/Akt signaling pathway (Wen et al., 2022), overexpression of EGFR gene (Siak et al., 2021), Hypermethylation of growth regulatory genes and hypomethylation of growth-promoting genes (Han et al., 2020) have also been observed in nasopharyngeal squamous cell carcinoma.

4.7. Paranasal Sinus and Nasal cavity cancer

Paranasal and nasal cavity cancer occurs when neoplastic cells arise in the tissues of the parasinuses and the nasal cavity **(see fig 4.7.1)**.

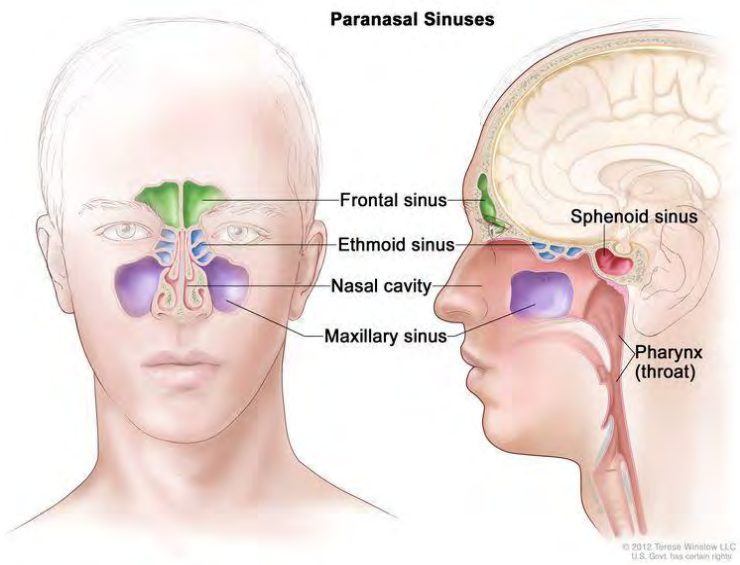

Fig.4.7.1. Anatomy of the parasinuses*: Figure Adapted from (National Cancer Institute, 2021)*

Paranasal sinus and nasal cavity carcinoma is very rare, representing just less than 0.2% of global cancer diagnoses annually (Bray et al., 2021), and approximately 3.67% of HNSCC cases (Muir & Weiland, 1995). Despite its rarity, it has several histological subtypes, categorized based on the cells of origin from which the cancer arises. These include keratinizing squamous cell carcinoma, cylindrical cell carcinoma, undifferentiated carcinoma, olfactory neuroblastoma, melanoma, lymphoma, and squamous cell carcinoma, which is the most common in the paranasal and nasal cavity (Miligi et al., 2019). Although it has a relatively low absolute risk, exposure to high-risk chemicals and occupational hazards heightens the overall risk. These risk factors include, but are not limited to, radiation, leather dust, chromium, nickel, formaldehyde, textile dust, woodwork and exposure to wood dust, and asbestos (Miligi et al., 2019). Paranasal and nasal squamous cell carcinomas exhibit complex chromosomal aberrations (Rushton et al., 2012; World Health Organization, 2013). DNA copy number gains are observed at chromosome 3q, 7p, 8q, 11q, 13, 17q, 19q, and 20q, while losses were also observed in 3p, 8p, 13q, 17p, 17q, and 18q (Miligi et al., 2019). Gene amplification of proto-oncogenes EGFR, CD44, CCND1/CTTN, and EBBR2 were also

observed, validly attributed to chromosome duplications in loci 7p12,11p13, 11q13, and 17q21 respectively (Miligi et al., 2019). Current treatments for paranasal and nasal cavity cancers include radiotherapy, chemotherapy, and targeted therapy (Miligi et al., 2019).

4.8. Metastatic Squamous Neck Cancer with Primary Occult

Besides the head and neck, squamous cells are also present in the tissues of the oesophagus, lungs, kidneys, and tissues lining the body cavities and hollow organs such as the uterus, blood vessels, and digestive tract. Sometimes, cancer can begin in squamous cells anywhere in the body and then metastasize through the lymphatic system or blood to other parts of the body. When these cancer cells spread to the lymph nodes in the neck or around the collarbone, and tests cannot identify a primary tumour, it is termed metastatic squamous neck cancer with occult (unknown) primary (National Cancer Institute, 2021). The occurrence of metastatic squamous neck cancer with occult (unknown) primary is very rare.

Chapter 5. Molecular Alterations and Pathways implicated in OSCC

Genetic alterations can either initiate tumourigenesis or drive the development of cancer. In oral cancer, several genetic alterations have been attributed to both development and progression of oral carcinogenesis. These include inactivating mutations in tumour suppressor genes and pathways, activating mutation of proto-oncogenes, and altered epigenetic mechanisms. Some of the most common genes implicated in oral cancer development and progression are discussed in this chapter.

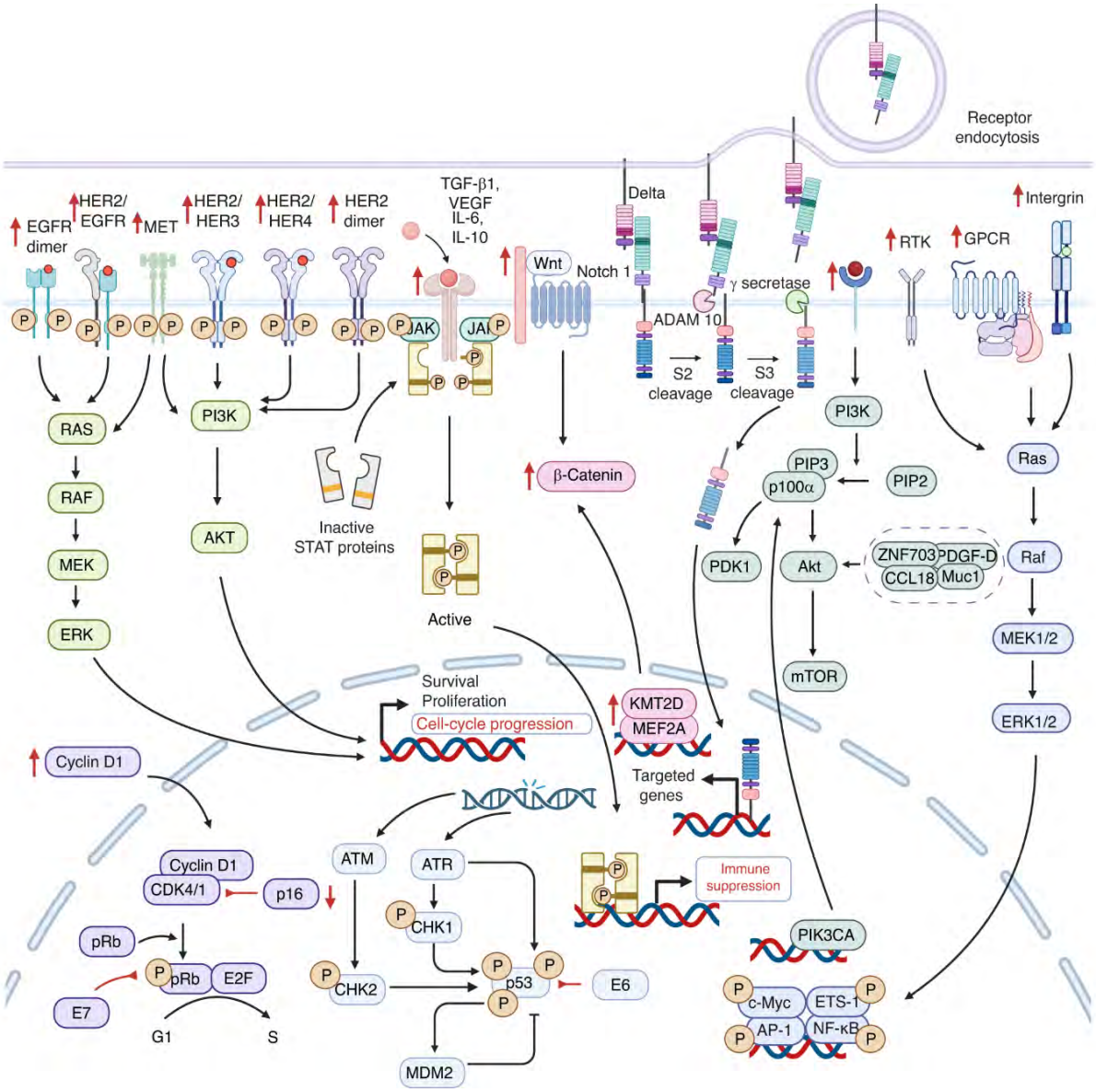

Fig 5.1.1 Genetic alterations in OSCC*: Genetic alterations play a significant role in the carcinogenesis and progression of oral squamous cell carcinoma. (OSCC). Several key pathways, including TP53/RB, p16/Cyclin D1/Rb, EGFR, Wnt/β-catenin, JAK/STAT, NOTCH, PI3K/AKT/mTOR, MET, RAS/RAF/MASK are all reported to be implicated in oral cancer. These alterations affect critical proteins like EGFR epidermal growth factor receptors, RB retinoblastoma, TP53 tumour suppressor protein as well as signalling molecules such as Janus-activated kinase (JAK), mitogen-activated protein kinase (MAPK), and signal transducer and activator of the transcription (STAT). Consequently, abnormal cellular behaviour ensues, contributing to the development and advancement of OSCC (Tan et al., 2023).*

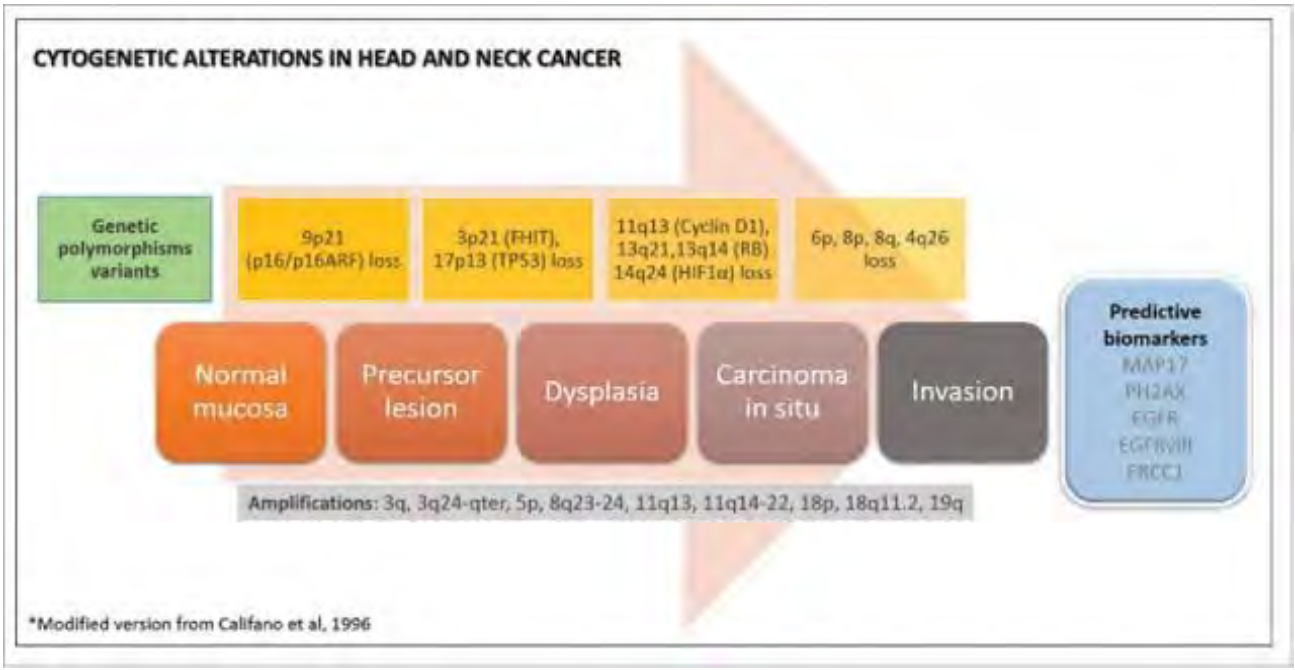

***Fig.5.1.2. Cytogenetic Alterations in OSCC**: In squamous cell carcinoma of the head and neck (SCCHN), various key genes, including Cyclin D1, a crucial proto-oncogene, and show amplification, particularly within the 11q13 region. This amplification of Cyclin D1 could serve as an indicator of progression in primary SCCHN. Additionally, there are observed increases in the number of certain other important genes, like those on 3q, 3q24-qter, 5p, 8q23–24, 11q14–22, 18p, 18q11.2, and 19q, among others (Callender et al., 1994)*

5.1. Activating Mutations

5.1.1. EGFR Pathway

The epidermal growth factor receptor (EGFR, ErB1/HER1) is a tyrosine kinase receptor proto-oncogene. EGFR activates several downstream signalling pathways involved in cellular growth, metabolism, proliferation and differentiation, such as PI3K/AKT/Raf/MAPK. PLC/PKC, and JAK/STAT (Wee & Wang, 2017). Over 80% of oral cancer patients express EGFR and are associated with poor treatment outcome (Tan et al., 2023). EGFR interacts with other receptors on the mucosal surface of the oral cavity, increasing its overall carcinogenic potential (Vouri et al., 2016). Somatic mutations in EGFR have been strongly linked to reduced apoptosis and increased growth in oral cancer cells, while also inducing metastasis and angiogenesis (Cassell & Grandis, 2010). In HNSCC, mutations in exons 18 to 21 of EGFR gene have been reported, with inframe deletion in exon 19 revealed to be the most common (PERISANIDIS, 2017). These mutations produce dysfunctional EGFR protein with

modified tyrosine kinase domain and ligand binding domain, the latter of which uncontrolled stimulation of EGFR, and subsequent overactivation (Wheeler et al., 2015). Overexpression of EGFR gene has also been reported in other cases of oral cancers (Hutchinson et al., 2020). Additionally, overexpression of distal-less homeobox 6 (DLX6) enhances proliferation and inhibits apoptosis in OSCC cells via the EGFR-CCND1 axis (Liang et al., 2020). Also, upregulated bone marrow stromal cell antigen (BST2) promotes tumour growth and confers resistance to gefitinib in OSCC patients by activating the EGFR pathway (Jin et al., 2019).

5.1.2. PI3k/AKT/mTOR Pathway

OSCC patients are associated with high frequency of somatic copy number alterations in genes encoding components of the PI3K/AKT/mTOR signalling network (Ghafouri-Fard et al., 2022; Marquard & Jücker, 2020; Peng et al., 2015; Simpson et al., 2015) and the most common mutational hotspots in PI3KCA in oral cancer, are in exon9 and exon 20 (Cai et al., 2017). Consistent activation of the PI3K-AKT pathway is mediated either by extracellular ATP (Zhou et al., 2022) or phosphorylation levels of AKT proceeding in either P2Y2-Src-EGFR axis or AKT-PDK1-mOR cascade respectively to stimulate cell metabolism, growth, and proliferation, which drive OSCC (Kupferman & Myers, 2006). Other factors that activate the PI3/AKT pathway to drive OSCC progression are the ITGB2 cancer associated fibroblasts (Zhang et al., 2020), ZNF703, (Wang et al., 2017), PDGF-D (Zhang et al., 2016), CCL18 (Jiang et al., 2016), and Muc1 (Li et al., 2015).

5.1.3. JAK/STAT Pathway

In both HPV^+ and HPV^-, abnormal activation of the Janus Activated Kinase/Signal Transducer and activator of transcription (JAK/STAT) pathway. STAT3 for example, aids OSCC immune suppression and evasion of cytotoxic T cells (Bu et al., 2017). STAT3 is also activated by other non-coding factors, such as miR-548d-3p and lncRNA p4713 which activates the JAK/STAT pathway using distinct mechanisms to stimulate OSCC metastasis and proliferation (Tan et al., 2019; Zhang et al., 2017).

5.1.4. Wnt/B-catenin Pathway

Aberrant Wnt/B-catenin signalling in OSC promotes activation of oncogenes via continuous B-catenin activation. For example, the DNA methyltransferase KmTD2

interacts with MEF2A resulting in increased transcription of B-catenin, which promotes cell proliferation (Chamoli et al., 2021; Wang et al., 2022). SNHG17/miR-384/ELF-1 axis upregulates expression of CTNNB1, a B-catenin transcription activator, subsequently stimulating the Wnt/B-catenin pathway to drive proliferation and metastasis of OSCC cells (Qiao et al., 2021). Because NOTCH and FAT1 regulate B-catenin function by suppressing its activity, inactivating mutations in NOTCH and FAT1 can lead to unregulated B-catenin activity (Huang et al., 2023). Overexpression of Wnt ligands, such as Wnt-7a and Wnt-7b, have also been reported to stimulate Wnt pathway and subsequent cell proliferation in OSCC (Shiah et al., 2014; Xie et al., 2020).

5.1.5. Ras/RAF/MAPK Pathway

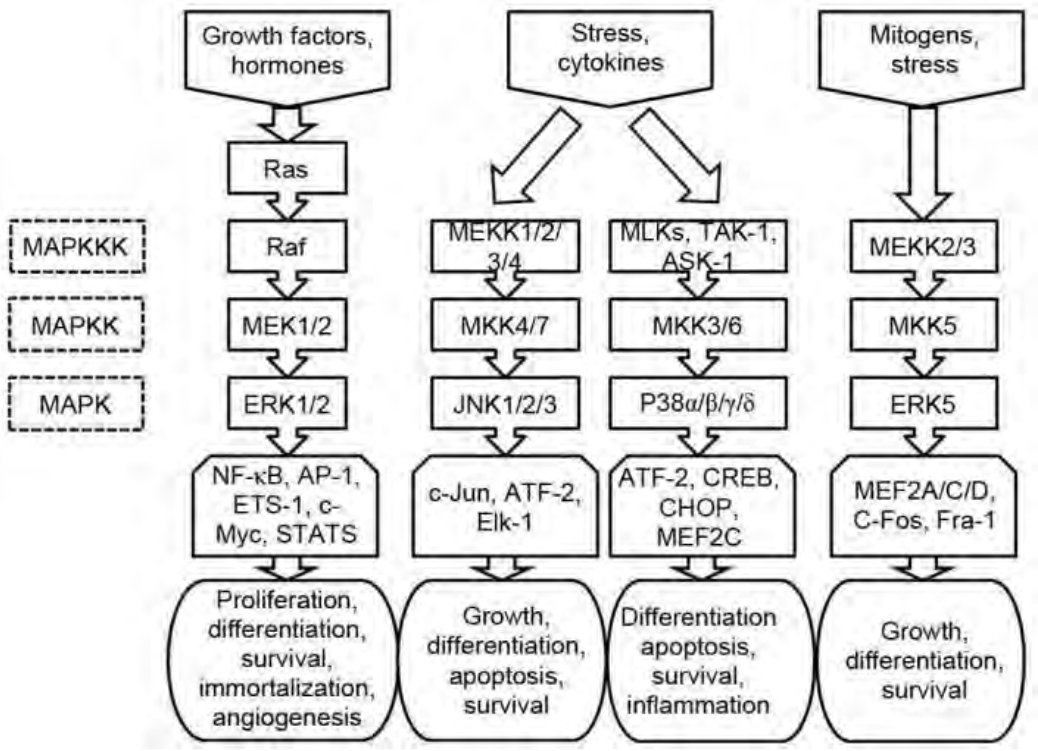

Fig 5.1.5.1: MAPK Pathway: *The MAPK pathway, illustrated schematically, involves MAPKs phosphorylating transcription factors, thereby regulating protein expression in response to external signals, crucial for cell growth, proliferation, and differentiation, with implications in diseases like cancer 5 (Peng et al., 2017).*

Rat Sarcoma virus (RAS) is a proto-oncogene with inherent GTPase activity. By cycling on and off between its GDP and GTP bound states, it regulates several downstream signalling cascades to control cellular proliferation, growth and invasion. In normal cells, RAS proteins remain relatively inactive until stimulated. However, in

oral cancer cells, due to mutations, they become dysfunctional and remain active, stimulating uncontrolled growth and proliferation (Krishna et al., 2018).

The mitogen-activated protein kinase (MAPK) regulates cell proliferation, cell death, angiogenesis, and dissemination. MAPK regulates four sub-pathways- the extracellular signal-regulated kinase (ERK1/2), c-JUN N-terminal kinase (JNK), p38 and ERK5) (see figure above). In OSCC cells, ERK1/2 is most significant in OSCC, and is activated by EGF binding (Endothermal Growth Factor), upon which it induces phosphorylation of transcription factors such as c-Myc, ETS,-1,AP-1,Nf-kB and others, that stimulate expression of genes promoting cell growth, immortalisation and angiogenesis (Peng et al., 2017) which collectively promote OSCC progression. Expression of mucosa-associated lymphoid tissue 1 (MALT1) inhibits ERK/MAPK activation to suppress cancer progression (Chiba et al., 2016).

5.2 Inactivating Mutations

TP53/RB, p16/Cyclin D1/Rb, and NOTCH are examples of suppressor signalling pathways. During the malignant transformation of OPMDs to OSCC, they become abnormally inactivated and downregulated. In other cases, lie HPV^+ oral cancer, the tumour suppressor genes p53, p16 and Rb become inactivated by the viral oncogenes E6 and E7 via the ubiquitin-mediated proteasome degradation (Tan et al., 2023).

5.2.1. TP53/RB

In oral cancer, the tumour suppressor protein TP53 modulates apoptosis, cell death and differentiation, thereby inhibiting carcinogenesis and OSCC progression. However, more than 80% of OSCC mutations were observed in the TP53 gene, with mutations in exon 4 or intron 6 as the most common sites in the early stage of carcinogenesis (N et al., 2014). In HPV^+ oral cancers, this TP53 mutation is more common due to its interaction with the HPV oncoprotein E6, which targets it for proteasome degradation. Retinoblasma (Rb), another tumour suppressor protein, is found to be implicated in oral cancer development and progression. In HPV^+ oral cancers, it interacts with the E7 oncoprotein which targets it for proteasome degradation, similar to the action of events leading to TP53 degradation by the E6 oncoprotein. In oral potentially malignant disorders, and other oral lesions, dysregulated Rb whether by HPV or not, increase the potential of the lesion turning into malignancy (Gipson et al., 2018).

5.2.2. P16/Cyclin D1 (CCND1)

The interaction of tumour suppressor protein p16 has been reported to disrupt cell cycle regulation, suppress senescence and lead to formation of neoplastic cells (Rayess et al., 2011). Cyclin D1 (CCND1) amplification has also been reported in oral cancer, usually in the advanced stage where it results in increased cell division and invasion among the OSCC cells. CCND1 alterations also have a poor prognosis (Ramos-García, Bravo, et al., 2018; Ramos-García, González-Moles, et al., 2018). The effect of CCND1 deficiency is also revealed in its inhibition of the Cyclin-dependent kinases CDK4 and CDK6. These proteins are involved in cell cycle progression functioning to dephosphorylate and inactivate Rb from firing the cell from the G1 phase to the S phase (Buchkovich et al., 1989).

5.2.3. NOTCH Pathway

Diverse effects of NOTCH signalling has been reported due to the variation of its mutational aberrations including nonsense mutations that produce truncated proteins, frameshift deletions, missense mutations with functional regions, and insertions. Common NOTCH1 mutations reported in oral cancer patients are missense that occur on or near the ligand binding domain (Mountzios et al., 2014). NOTCH1 and NOTCH3 are the most common NOTCH signalling genes implicated in oral carcinogenesis and the presence of nonsense mutations suggest a tumour suppressor role, since this could explain why its deficiency contributes to oral carcinogenesis (Nowell & Radtke, 2017). Interestingly, other studies show a tumour promoting role for NOTCh1. For example, a study in oral cancer patients reported NOTCH1 supports that NOTCH1 supports oral malignancy (Ishida et al., 2013), while another reports its contribution to promoting cancer relapse and migration via Wnt signalling, and sustain cancer stem cell characteristic due to overexpression of NOTCH1 in those cell states (Lee et al., 2016). As of 2023, it is still not clear what precise roles NOTCH1 plays in OSCC and whether its mutations are inactivating or activating mutations on its function (Tan et al., 2023).

6. Epigenetic Alterations in OSCC

While genetics can alter gene expression through mutations in the genetic code (nucleotides), epigenetics alter gene expression without any change in the DNA sequence. The mechanisms of epigenetics include DNA methylation, and histone modification, such as histone methylation, acetylation and phosphorylation, which remodel the chromatin to either suppress or promote gene expression. Several non-coding RNAs, such miRNAs, long non-coding RNA (lncRNA) and circular RNAs (cRNA) also play essential roles in epigenetics. In oral cancer, all of these have been reported to be implicated, with varying degrees of effect on malignancy **(see table 6.1)**.

Epigenetic Modification	Targets	Exhibition	Outcomes
DNA Methylation	p16 MGMT MLH1 $p15^{INK4B}$ E-cadherin PTEN APC $P14^{ARF}$ $P16^{INK4A}$ miR-137 miR193a	Hypermethylation	Oncogenic
	AIM2 CEACAM1 LINE-1 PI3 PTHLH	Hypomethylation	Oncogenic
Histone Acetylation	H3K9ac H3K4ac miR-154-5p	Downregulation	Oncogenic
	H3K27me3 HDAC6 HDAC8 HDAC1 HDAC2	Upregulation	Oncogenic
Chromatin Modification	SATB1 ZSCAN4 CSC factors RSF-1	Upregulation	Oncogenic

ncRNAs	miR-1246 miR-31 miR-214 miR-23a miR-372	Upregulation	Oncogenic
	miR-181a miR-17-92 cluster miR-329 miR-410 miR-211 TCF12 miR-214 miR-23a miR-372	Upregulation	
	lncRNA HOXA11-AS ZBTB7A miR-98-5p miR-214-3p circITCH	Downregulation	Oncogenic

***Table 6.1**: **Epigenetic Modification in OSCC**: DNA methylation, histone acetylation, chromatin remodelling and non-coding RNA (ncRNA) are known epigenetic mechanisms implicated in oral cancers. Although their effects vary, the majority has an oncogenic effect in the cancer cell progression (Tan et al., 2023).*

6.1. DNA Methylation

Both hypermethylation and hypomethylation affect oral malignancy. In oral cancer patients, smoking and betel quid correlates with global hypomethylation (Guerrero-Preston et al., 2009), while alcohol is associated with increased CpG hypermethylation in tumour suppressor genes (Gasche & Goel, 2012; Supic et al., 2011; Towle et al., 2013). In fact, hypermethylation has been reported in the promoters of regions encoding genes that regulate cell death, cell-to-cell adhesion, apoptosis and DNA repair (Mascolo et al., 2012). Smoking in particular has been found to increase p16 promoter methylation (Breitling et al., 2011; Lin et al., 2010). Abnormal methylation of CYGB, CCNA1, CDKN2A and CDKN2B has also been reported in some OSCC subtypes, such as salivary gland carcinomas (Demokan & Dalay, 2011). DNA methyltransferase (DNMT) transfers methyl group to the carbon-5 of cytosine, suppressing gene expression by steric hindrance and by recruiting transcription suppressor proteins that block access to the genes to transcription activator proteins. DNMT3a and DNMT1

overexpression also contribute to OSCC progression and poor prognosis (Daniel et al., 2010; Supic et al., 2017).

O-6-Methylguanine-DNA methyltransferase (MGMT), $P15^{INK4B}$ and mutL homolog1 (MLH1) are all tumour suppressor genes affected by DNA methylation. MGMT is a DNA repair enzyme that eliminates guanine DNA adducts to maintain genome integrity (Hema et al., 2017). DNA methylation silences MGMT, causing accumulation of carcinogens-induced DNA mutations, and a subsequent poor prognosis (Gasche & Goel, 2012; Guerrero-Preston et al., 2011). $P15^{INK4B}$ suppresses tumour progression by blocking cell proliferation and cell cycle progression at the G1 phase by rendering the cells insensitive to activators like TGF-β and IFN-α (Gasche & Goel, 2012). Methylation of $P15^{INK4B}$ inhibits its expression and therefore lowers its contribution to tumour suppression (Yeh et al., 2003). Other hypermethylated genes that contribute to OSCC are E-cadherin, P $P14^{ARF}$, $P16^{INK4A}$, miR-193A, AIM2. CEACAM1, LINE-1, p13, and PTHLH (Tan et al., 2023).

6.2. Histone and Chromatin Modification

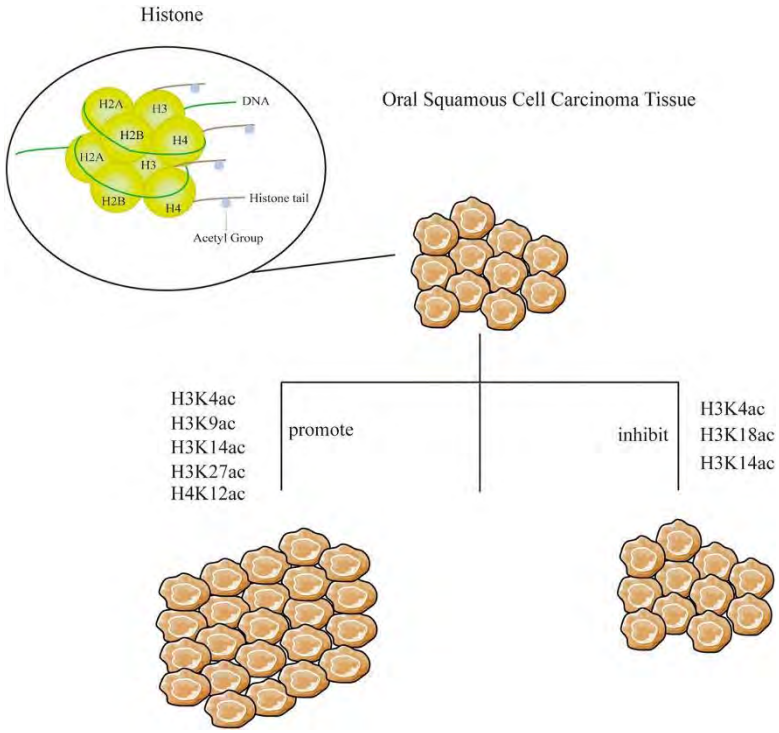

Fig.6.2.1 Histone acetylation in OSCC*: In oral cancer (OSCC), histone acetylation affects proliferation and metastasis either by promoting them, or suppressing them. Some modifications on the left promote these processes, while those on the right inhibit them (Li et al., 2023).*

The chromatin structure, comprising compact DNA with a dynamic topology, plays a crucial role in gene expression regulation within cells. During active gene expression, specific regions of chromatin adopt an open euchromatin state, facilitating access for transcription factors, RNA polymerase, and other transcription-related enzymes. Conversely, in the absence of gene expression requirements, chromatin assumes a tightly condensed heterochromatin state, restricting access to transcriptional machinery.

The organization of chromatin is primarily governed by histone enzymes, and dysregulation of histone modifications can disrupt the histone code, leading to aberrant gene expression patterns. Histone acetylation and methylation are two prominent histone modifications with contrasting effects on chromatin structure and gene expression. Generally, histone acetylation promotes chromatin relaxation, facilitating gene transcription, whereas histone methylation tends to compact chromatin, repressing gene expression.

Histone acetylation is catalyzed by Histone Acetyltransferase (HAT) enzymes, which add acetyl groups to histone proteins. Conversely, Histone Deacetylase (HDAC) enzymes mediate histone deacetylation, removing acetyl groups from histones. On the other hand, Histone Methyltransferase (HMT) enzymes are responsible for adding methyl groups to histones, while Histone Demethylase (HDM) enzymes remove methyl groups from histones, regulating histone methylation levels and thereby influencing chromatin structure and gene expression

In oral cancer, studies have revealed reduced histone H3k9 acetylation to be associated with chemoresistance via the NF-κB pathway and recruitment of cancer stem cells as well as promoting tumour proliferation via Epithelial-to-Mesenchymal Transition (EMT). Therefore, H3K9 may play a role in tumour suppression, possibly by enabling open chromatin in regions encoding tumour suppressor genes. Low levels of H3K4 acetylation have also been reported to be significantly associated with advanced stage oral cancer, poor survival and overexpression of histone deacetylase enzymes. H3K27 trimethylation was also identified to confer tumour progression and platinum resistance in OSCC (Chen et al., 2013). OSCC shows overexpression of HDAC1 which contributes to their progression by modulating the miR-154-PCNA signalling pathway (Lv et al., 2020). HDAC2 is also observed to be at higher levels in OSCC and OPMDs but there is not enough knowledge of their effects in them (Chang et al., 2009).

Besides histone acetylation, methylation and phosphorylation, chromatin remodelers also modify the chromatin, frequently by exchanging a histone subunit for a tighter or looser variant, to modify gene expression accessibility. SATB1 (AT rich sequence binding protein 1) is a 'writer' protein that modifies the chromatin to recruit chromatin remodelers to specific regions for gene expression regulation. In OSCC, high SATB1 expression has been reported to coincide with poor survival rates and metastasis, indicating a correlation between them. Another chromatin remodeler, protein, RSF-1, is also upregulated in OSCC and revealed to promote invasion, lymph node metastasis and advancement of OSCC and also chemotherapy resistance (Fang et al., 2011; Panchal et al., 2020). Another study reported the role of the zinc finger protein ZSCAN4. ZSCAN4 alters the chromatin topology in OSCC cells by promoting hyperacetylation of promoters that upregulate cancer stem cell factors such as OCT3/4 and NANOG promoters, indicating a link between ZSCAN4 activity and OSC development (Portney et al., 2020).

6.3. Non-coding RNAs

miNA are non-coding RNAs which alter gene expression by targeting corresponding mRNAs with complementary nucleotides for degradation. Hence, they are an efficient form of post-transcriptional gene silencing. Although ncRNA implication in OSCC include miRNAs, cRNA and long non-coding RNAs (lncRNA), miRNA has more extensive studies. In oral cancers, miRNAs have executed both oncogenic and tumour suppressor functions, OSCC miRNA profile transcriptomic have reported the implications of non-coding RNAs in oral cancer development and progression. For example, in laryngeal cancer alone, many ncRNAs have been reported to be implicated (de Miguel et al. 2016). Some miRNAs identified in oral cancer are miR-31, miR-34, miR-375, miR-138, miR-203, miR-200c, miR-222, miR-377, miR-30a-5p, miR-155, miR373-3p, miR-218, and miR-455-5p (Fang & Li, 2019).

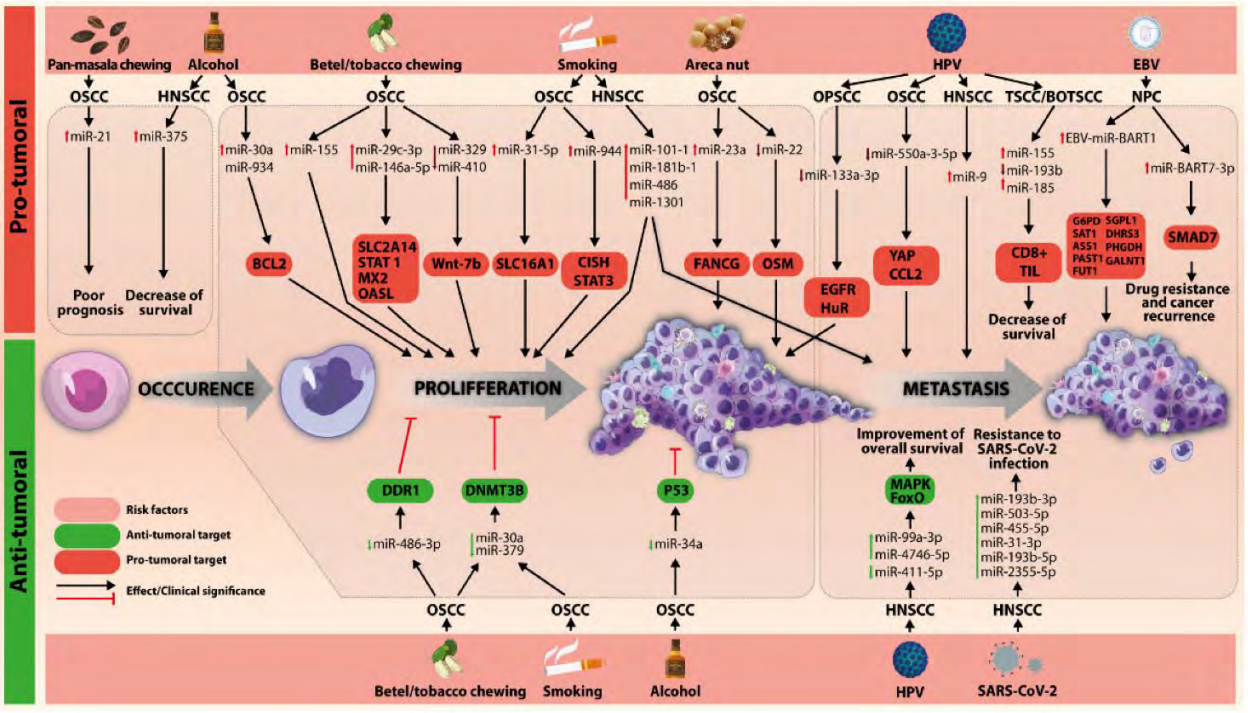

Fig 6.3.1. Interplay between miRNA and OSCC*: miRNAs play a crucial role in OSCC development and progression. As indicated, most of these miRNA functions are triggered by some carcinogens from risk factors such as alcohol, smoking, tobacco, betel quid, HPV and EBV infections. All miRNAs perform distinct functions and interact with different pathways. However, they collectively either function as OSCC tumour promoter (anti-tumoural) or tumour suppressor (pro-tumoural**) (Aghiorghiesei et al., 2022).*

miR-26, miR-137, and miR-203 have been found to be implicated in OSCC. miR-246 acts as an oncogene to facilitate cell mobility and invasion and is found at high levels in all stages of OSCC. miR-181a on the other hand, acts as a tumour suppressor to inhibit oral carcinogenesis. Similar results for miR-181a have also been found in miR-17-92 cluster, miR-410, miR-329, and miR-211, all with tumour suppressor functions. Besides miRNA, long non-coding RNA (lncRNA) and circular RNA (cRNA) have also been identified in OSCC (Tan et al., 2023).

7. Treatment

Like other cancers, the gold standard treatment for oral cancer is surgery and chemotherapy. Unfortunately, compared to other cancers, it has very limited treatment options outside this, despite its high mortality rate (<50% 5 years survival rate) (Usman et al., 2021). This can be attributed to it being uncommon in developed countries, underrepresentation in drug discovery research and development, or due to its heterogeneous nature. Existing targeted therapy for oral squamous cell carcinoma targets the TP53, EGFR, RAS, c-MET, MAPK and PI3K-AKT pathways (Usman et al., 2021). As with other cancers, drug resistance is a significant problem, and current research is actively pursued to identify the molecular mechanisms of the relevant drug resistance to improve future therapeutic interventions (see figure).

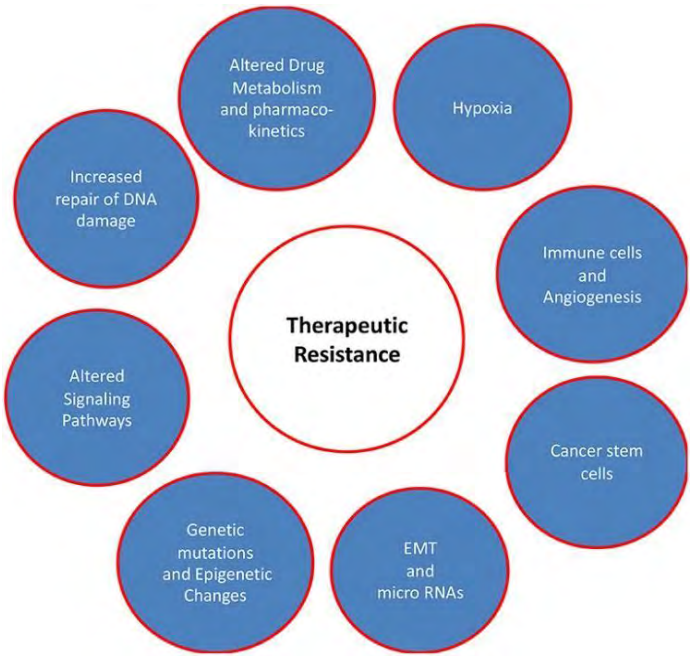

Fig 7.1.Factors responsible for therapeutic resistance in oral cancer patients: *The figure above describes the intricate interplay of multiple factors, such as genetic alterations, dysregulated signalling pathways, EMT/microRNA, hypoxia, drug metabolism, immune cells/angiogenesis, and hyperactive DNA damage repair systems, contributing to therapeutic resistance in oral cancer* (Usman et al., 2021).

8. Conclusion

Improving screening and early detection programs for oral squamous cell carcinoma (OSCC) is crucial, especially targeting individuals with oral-cancer inducing infections like H. pylori, HPV, and EBV, along with other Oral Potentially Malignant Diseases (OPMD) and lesions. Liquid biopsy techniques, such as detecting circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), extracellular vesicles (EVs), and microRNAs (miRNAs), show promise for prognostic and predictive purposes in head and neck cancer research. For instance, identifying ctDNA in virus-associated cancers like HPV-related OPC and EBV-positive NPC has shown significant prognostic value, advocating for its inclusion in future clinical trials. Multigene panel next-generation sequencing (NGS)-driven plasma ctDNA detection holds potential as a diagnostic tool, particularly in HPV-unrelated SCCHN. Integrating various liquid biopsy methods with imaging tests can enhance diagnostic accuracy for head and neck cancers (Cabezas-Camarero & Pérez-Segura, 2022).

The impact of histone methylation in OPSCC carcinogenesis and development remains understudied, highlighting a gap in current research. Investigating this area could uncover potential therapeutic targets and enhance understanding of histone modification's role in oral cancer progression.

While OSCC presents a high incidence and mortality rate, therapeutic interventions may be limited, potentially due to fewer cases in developed countries and limited interest from large biopharmaceutical companies. Developing computer-aided drug development programs in affected developing and underdeveloped countries could be a feasible solution, enabling the repurposing of drugs for OSCC. By bypassing the costs of wet lab bioassays, successful results from these programs could be advanced through clinical trials with the support of larger financial sponsors. Moreover, understanding the mechanism of drug resistance in current treatments is essential for identifying areas for improved drug development with increased efficacy.

Precision medicine efforts should consider the genomic diversity of OSCC patients, particularly in South Asian and Southeast Asian countries where the burden of OSCC is significant. Tailoring targeted therapy to the mutational profile of OSC patients in these regions can lead to more effective treatments.

Addressing awareness and accessibility issues is also crucial. While many OSCC patients are aware of oral health and cancer screening, the majority come from low-income groups, potentially neglecting screening due to cost barriers. Government subsidies for oral health and cancer screening could significantly improve early diagnosis and survival rates while reducing OSCC incidence rates.

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