ANTI CANCER EFFECT OF PADDY HUSK EXTRACTS IN HUMAN SALIVARY GLAND EPIDERMOID CANCER CELLS *IN VITRO* MODEL

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by

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LIST OF ABBREVIATIONS

FNA	Fine Needle Aspirations
MRI	Magnetic Resonance Imaging
CT	Computed Tomography
MUC7	Mucin 7
ACC	Adenoid Cystic Carcinoma
WHO	World Health Organization
PA	Pleomorphic Adenomas
WT	Warthin Tumour
MEC	Mucoepidermoid Carcinoma
HPV	Human Papilloma Virus
AJCC	American Joint Committee on Cancer
TNM	Tumour, Node and Metastasis
BID	BH3 Interacting Domain Death agonist
EGFR	Epidermal Growth Factor Receptor
FADD	Fas-associated Death Domain
TRADD	TNF receptor-associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
TNF	Tumour Necrosis Factor
BZW1	Basic leucine Zipper and W2 domains 1
HTB-41	Human submaxillary salivary gland epidermoid carcinoma cell line
HGF-1	Human Gingival Fibroblast-1
Bcl-2	B cell lymphoma 2
Apaf-1	Apoptotic protease activating factor-1
AIF	Apoptosis-inducing factor
IAPs	Inhibitor of Apoptosis Proteins
CDK	Cyclin-dependent kinase
MAPK	Mitogen Activated Protein Kinase
HNSCC	Head and Neck Squamous Cell Carcinoma
GABA	γ -Amino Butyric Acid
DMSO	Dimethyl sulphoxide
HMDS	Hexamethyldisilazane

%	Percentage
µm	micrometer
nm	nanometer
ml	milliliter
^{o}C	Celsius
g	gram
GC–MS	Gas Chromatography–Mass Spectrometry
DMEM	Dulbecco's Modified Eagle's Medium
PBS	Phosphate Buffer Saline
FBS	Fetal Bovine Serum
TBS	Tris Buffer Saline
APS	Ammonium Persulfate
TBEA	Trypan Blue Exclusion Assay
SEM	Scanning Electron Microscope
BSA	Bovine Serum Albumin
SDS-PAGE	Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis
PI	Propidium Iodide
SGCs	Salivary Gland Cancers
DOS	Dorsal Air Sac
NK	Natural Killer
VEGF	Vascular Endothelial Growth Factor
COX-2	Cyclooxygenase-2
5-LOX	5-Lipoxygenases

LIST OF APPENDICES

KESAN ANTI-KANSER EKSTRAK SEKAM PADI PADA SEL KANSER EPIDERMOID KELENJAR LIUR MANUSIA DENGAN MENGGUNAKAN MODEL *IN VITRO*

ABSTRAK

Pertanian global menghasilkan jutaan tan metrik sisa setiap tahun. Sekam padi merupakan sisa pertanian yang tidak dapat dimakan, dan diperolehi semasa proses pengisaran beras. Kajian sebelum ini melaporkan bahawa sekam padi mempunyai potensi kemopreventif yang disebabkan oleh kehadiran fitokimia berkaitan. Tujuan kajian ini adalah untuk mengkaji kehadiran fitokimia berkaitan anti-kanser daripada ekstrak sekam padi dan menilai kesan perencatannya terhadap sel karsinoma epidermoid kelenjar submaksilar manusia (HTB-41). Dua jenis pelarut telah digunakan untuk mengekstrak sekam padi iaitu air dan metanol akueus. Kandungan fitokimia ekstrak sekam padi dikenal pasti dengan menggunakan GC-MS. Aktiviti perencat dan analisis sitotoksik dikenalpasti dengan menggunakan Ujian Pemencilan Trypan Blue (TBEA). Analisis apoptosis dan kitaran sel dinilai oleh cytometer aliran, dan rawatan pasca morfologi sel dianalisis secara ultrastruktur, manakala Western blot dilakukan untuk analisis proteomik. Hasil kajian menunjukkan kehadiran vitamin E dan fitokimia lain dalam ekstrak sekam padi. Kedua-dua air dan ekstrak metanol akueus menunjukkan aktiviti perencatan pada sel HTB- 41 di mana dos IC_{50} ekstrak air (400 μg/ml) berjaya mengurangkan daya maju sel kepada 53.0 % dan dos IC_{50} ekstrak metanol akueus (200 μg/ml) berjaya mengurangkan daya maju sel kepada 51.12 % tanpa menunjukkan sebarang kesan sitotoksik yang ketara. Analisis apoptosis menunjukkan bahawa ekstrak air dan metanol akueus memberi kesan apoptosis ke atas HTB-41 dan hasil kajian ini disokong dengan analisis mikroskopi (pengecutan sel,

pelepasan membran dan badan apoptotik) dan pewarnaan Hoechst 33342 (pengecutan dan fragmentasi nuklens). Analisis flow cytometer menunjukkan bahawa ekstrak sekam padi merangsang populasi sel apoptosis yang signifikan dari 76.00% (tidak dirawat) kepada 47.86% (ekstrak air sekam padi) dan 43.13% (ekstrak metanol akueus sekam padi) serta menahan sel pada fasa S dari 19.90% (sample kawalan) kepada 36.90 % (ekstrak metanol akueus sekam padi) dan 27.86 % (ekstrak air sekam padi). Analisis Western blot merungkai kesan apoptosis melalui induksi laluan intrinsik caspase 3. Protein pro-apoptosis dan perencat tumor; Bax, p27kip1 diekspresikan lebih tinggi (P <0.05), manakala protein anti-apoptosis, Bcl-2 menurun selepas rawatan (P <0.01). Ini membawa kepada peningkatan ekspresi caspase 9 seterusnya mengaktifkan caspase 3 dan 7 untuk menghasilkan apoptosis sel. Kesimpulannya, kehadiran fitokimia dalam sekam padi terutamanya dalam ekstrak metanol akueus menunjukkan kesan perencatan dan anti-pertumbuhan yang lebih baik ke atas sel karsinoma epidermoid kelenjar submaksilar manusia (HTB-41), sambil bertindak secara selektif terhadap tumor tanpa mendatangkan perubahan yang signifikan ke atas sel fibroblas gusi manusia (HGF-1).

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ABSTRACT

Global agriculture produces millions of tons of waste yearly. Paddy husk is an inedible agriculture waste obtained during the process of rice milling. Studies reported that it has chemopreventive potential due to the presence of related phytochemicals. The aim of this study is to elucidate the presence of anti-cancer related phytochemicals from paddy husk extract and evaluate its inhibitory and anti-proliferative effects against human submaxillary salivary gland epidermoid carcinoma cells (HTB-41). Two types of solvent for paddy husk extract have been used; water and aqueous methanol. The phytochemical constituents of paddy husk extracts were identified using GC-MS. The inhibitory activity and cytotoxicity analysis was calculated using Trypan Blue Exclusion Assay (TBEA). Apoptosis and cell cycle analysis were evaluated by flow cytometer, and cell morphology post treatment was analysed ultrastructurally, while Western blot was performed for proteomic analysis. Our results showed presence of vitamin E and other phytochemicals in paddy husk extracts. Both water and aqueous methanol extracts demonstrated inhibitory activity on HTB- 41 cells where IC_{50} dose of water extract (400 μg/ml) managed to reduce cell viability to 53.0 % and IC_{50} dose of aqueous methanol extract (200 μg/ml) managed to reduce cell viability to 51.12 % without exhibiting any significant cytotoxic effects. Apoptosis analysis revealed that water and aqueous methanol extracts induce apoptosis effect on HTB-41 as supported with microscopic findings of cell shrinkage, membrane blebbing and apoptotic bodies, meanwhile, Hoechst 33342 staining showed nuclear shrinkage and fragmentation. Flow cytometry analysis demonstrated that paddy husk extracts promote a significant

amount of apoptotic cellular population from 76.00% (untreated) to 47.86% (paddy husk water extract) and 43.13% (paddy husk aqueous methanol) and arresting the cells at S-phase from 19.90% (control) to 36.90 % (paddy husk aqueous methanol extract) and 27.86 % (paddy husk water extract). Western blot analysis reveals that apoptosis was induced through caspase 3-mediated intrinsic pathway. Pro-apoptotic and tumour suppressor proteins; Bax, $p27^{kip1}$ expressed higher (P <0.05), while anti-apoptotic protein, Bcl-2 downregulated after treatment (P <0.01). This leads to increase of caspase 9 expression which in turn activate caspase 3 and 7 leading to cell apoptosis. In conclusion, the presence of phytochemicals in paddy husk especially in aqueous methanol extract successfully showed better inhibitory and anti-proliferative effects on the human submaxillary salivary gland epidermoid carcinoma cells (HTB-41), while it acted in a tumour-selective manner by not inducing any significant changes on human gingival fibroblast cell (HGF-1).

CHAPTER 1

INTRODUCTION

1.1 Research background

Salivary glands are essential for maintaining the oral cavity balance. They secrete saliva, a multifaceted fluid contains electrolytes, antimicrobial substances, and different type of enzymes to protect the surface of the teeth and the oral mucosa (Carpenter, 2013; de Paula et al., 2017). However, the salivary glands are susceptible to a variety of cancers. There are 10 benign and 20 malignant subtypes of salivary gland tumours recognised. The most typical malignant tumour affecting all salivary glands is mucoepidermoid carcinoma accounting for 10–15% of all salivary gland neoplasms and 30% of all salivary gland tumours. It typically appears as a painless, fixed, rubbery, or soft mass (Peraza et al., 2020). Mucoepidermoid carcinoma is considered as the most prevalent malignant tumour among aging men and female (Reinheimer et al., 2019; Sentani et al., 2019). On the other hand, there are few types of conventional treatment procedures performed for salivary gland cancer patients such as preoperative physical examinations, chemotherapies, computed tomography (CT) scans, radiologic evaluations, fine needle aspirations (FNA) and magnetic resonance imaging (MRI) (Pasick et al., 2020).

Although chemotherapies are the standard treatment against salivary glands cancer, it comes with side effects and in some cases, ineffective due to resistance including DNA/RNA damage repair, drug efflux and apoptosis inhibition (Kanno et al., 2021). Therefore, it is necessary for treatment to move towards targeting signalling pathways which involve the molecular aspects which in turn helps to develop innovative cancer therapeutics to overcome chemotherapy resistance. Numerous molecular targets in mucoepidermoid carcinoma have been identified by recent

research such as CDKN2A/B, PIK3CA, CRTC1/MAML2 genes (Bou et al., 2023; Stenman, 2013). A better prognosis is related to inducing caspase 3and 9 in a low-grade malignancy (Lee et al., 2016; Jang et al., 2019). A patient with mucoepidermoid carcinoma is also correlated with the presence of markers including Ki-67, CEA, p53, and Bcl-2 (Cros et al., 2013; Lopes et al., 2006; Sama et al., 2022; Wagner et al., 2018).

Patients with salivary gland cancer may experience dry mouth, painful oral tissue destruction, altered taste, oral ulcers, and other side effects following medicine and radiation or chemotherapy (Bascones-Martínez et al., 2015; Nieuw Amerongen & Veerman, 2003). Therefore, the need for new anti-cancer medications as an alternative therapy targeting mucoepidermoid carcinoma with less side effects is recommended.

Natural products can play an important role in providing alternative therapy for the treatment of salivary gland cancer through a variety of biological processes such as inducing apoptosis, decrease inflammation, and inhibit cell proliferation in cancer cells due to presence of some active phytochemicals, such as phenolic and flavonoid compounds which founded in paddy husk (Pratheeshkumar et al., 2012; Cabral et al., 2018; Sarker et al., 2020; Sun & Shahrajabian, 2023).More than 60% of today's anticancer drugs are derived from natural sources, including higher plants, terrestrial (and marine) microorganisms, and marine invertebrates (Demain & Vaishnav, 2011; Khazir et al., 2014; Cragg & Newman, 2018).

There are several advantages for using natural products in the drug discovery and development process in cancer therapy. They present several anti-cancer and anti-inflammatory phytochemicals that can become active compound for potential drug candidates against complex targets compared to synthetic drugs. Naturally derived ingredients phytochemicals provide synergistic effects which are not found in collections of synthetic chemicals. They can have complex two- and three-dimensional

structures and are still absorbed and metabolised in the body. Over the past two centuries, the global pharmaceutical industry has greatly benefited from biodiversity of plants throughout the world, which help in identifying new therapeutic targets for many major chronic diseases, especially in new drugs development (Dutra et al., 2016; Valli et al., 2018; Calixto, 2019) .

Natural products themselves greatly enhance the prognosis of salivary glands cancer patients' treatment. Recent studies revealed that certain natural compounds may exhibit anticancer effects by causing apoptosis and inhibiting the growth of different cancer cell types. The methanolic extract of Convallaria keiskei was found to significantly inhibit salivary gland cancer growth in vitro (Lee et al., 2016; Palasap et al., 2014; Ruan et al., 2006).

1.2 Relevance of the study

The development of salivary tumours is influenced by a number of environmental factors, including ionising radiation from medical procedures or dental x-rays, as well as various aspects of occupational aspects and personal habits, such as smoking and drinking. In the other hand, bad nutritional habits and diet may influence the carcinogenesis at many sites, according to laboratory and epidemiological findings (Actis & Eynard, 2000).

The potential anticancer activities of natural products have been investigated. In human clinical trials and research, active phytochemicals showed that dietary phytochemicals had cancer-preventive effects against carcinogens when ingested on a regular basis (Islam et al., 2022; Magrone et al., 2021; Mahadevappa & Kwok, 2017). Some active phytochemicals, such as phenolic compounds, flavonoids, and resveratrol, are possible bioactive agent alternatives in pharmaceutical and medical

fields that can improve human health and exhibit inhibitory and chemopreventive effects (Sun & Shahrajabian, 2023; Meiyanto et al., 2012; Cragg & Pezzuto, 2016).

Oryza sativa, known as paddy, is an organic substance that has been shown to possess chemopreventative agent against cancer, which remain as a primary food crop for more than half of the global population areas including Asia pacific, Middle East and African countries. A number of paddy by-products, such as husk, bran layer, straw, germ, and broken kernels are created during the paddy milling process (Henderson et al., 2012; Meharg & Jardine, 2003; Zhang et al., 2010).

Paddy by products were once considered as trash and were often fed to animals as grass hay or used in building. Paddy bran and paddy husk are agricultural by-product residues produced during rice milling process. Paddy bran has been extensively investigated since its chemoprevention potential was found, and it contains a variety of natural compounds that have anticancer properties (Moraes et al., 2014; Pappu et al., 2007; Tan et al., 2023). Nonetheless, many studies previously conducted have found that paddy husk contains considerable levels of phytochemicals such as flavonoids, tocopherol, phenolic acids, tocotrienols and momilactones, all of which have anticancer, antiangiogenic, and proapoptotic activities (Kalapathy et al., 2003; Ghasemzadeh et al., 2018; Saki et al., 2017; Sen et al., 2020; Yang et al., 2016).

Given that paddy husk can be a chemo preventative agent, the purpose of this research was to explore its phytochemical properties which may impact positively on salivary gland cancer using an *in vitro* model. Until now, there is still a knowledge gap regarding the mechanism of action of paddy husk against salivary gland cancer, especially in identifying the molecular pathway involved. Moreover, considering paddy husk is also easily obtained, this study will offer an efficient alternative to currently existing chemotherapeutic medicines. It may provide a new value to paddy

husk as a potential agent for treating human salivary gland cancer. Therefore, this study aims to evaluate the mechanism and role of paddy husk extracts, as well as their inhibitory and anti-proliferative activities through morphological changes and distribution pattern of cells using an in vitro model of human submaxillary salivary gland epidermoid carcinoma cell line, HTB-41.

1.3 Research aims and objectives

To explore the effects of paddy husk extracts on inhibitory and protein expression related to proliferation and apoptosis in human submaxillary salivary gland epidermoid carcinoma cells, HTB-41.

1.3.1 Specific Objectives

1. To investigate the inhibitory activity of the paddy husk in human salivary gland epidermoid carcinoma cells, HTB-41.

2. To investigate the effects of paddy husk on apoptosis, proliferation and ultrastructure in human submaxillary salivary gland epidermoid carcinoma cells, HTB-41.

3. To investigate the effects of paddy husk extracts on protein expression related to proliferation and apoptosis in human submaxillary salivary gland epidermoid carcinoma cells, HTB-41.

1.4 Research hypothesis

1. Paddy husk exhibit inhibitory activity in human submaxillary salivary gland epidermoid carcinoma cells, HTB-41.

2. Paddy husk induces apoptosis, proliferation and changes in ultrastructure in human submaxillary salivary gland epidermoid carcinoma cells, HTB-41.

3. Paddy husk extracts exhibit anti-cancer effects on protein expression through upregulation of pro-apoptotic and downregulation of anti-apoptotic proteins in human submaxillary salivary gland epidermoid carcinoma cells, HTB-41.

CHAPTER 2

LITERATURE REVIEW

2.1 Salivary glands

2.1.1 Development of salivary gland

As part of the embryonic development process, salivary glands develop from an epithelial placode (from E11 to E16 in mice and between 4th and 12th embryonic weeks in humans) (Porcheri & Mitsiadis, 2019). The initial placode develops a bud shape as it expands and spreads into the underlying mesenchyme. The epithelial bud stratifies as it grows, with concentric layers created by different cell types. As the salivary gland matures, additional, independent buds cleave and grow again forming an extensive arborization characteristic of the fully developed gland occurs during branching morphogenesis as depicted in Figure 2.1 (Tucker AS, 2007; Chibly AM et al., 2022; Holmberg & Hoffman, 2014).

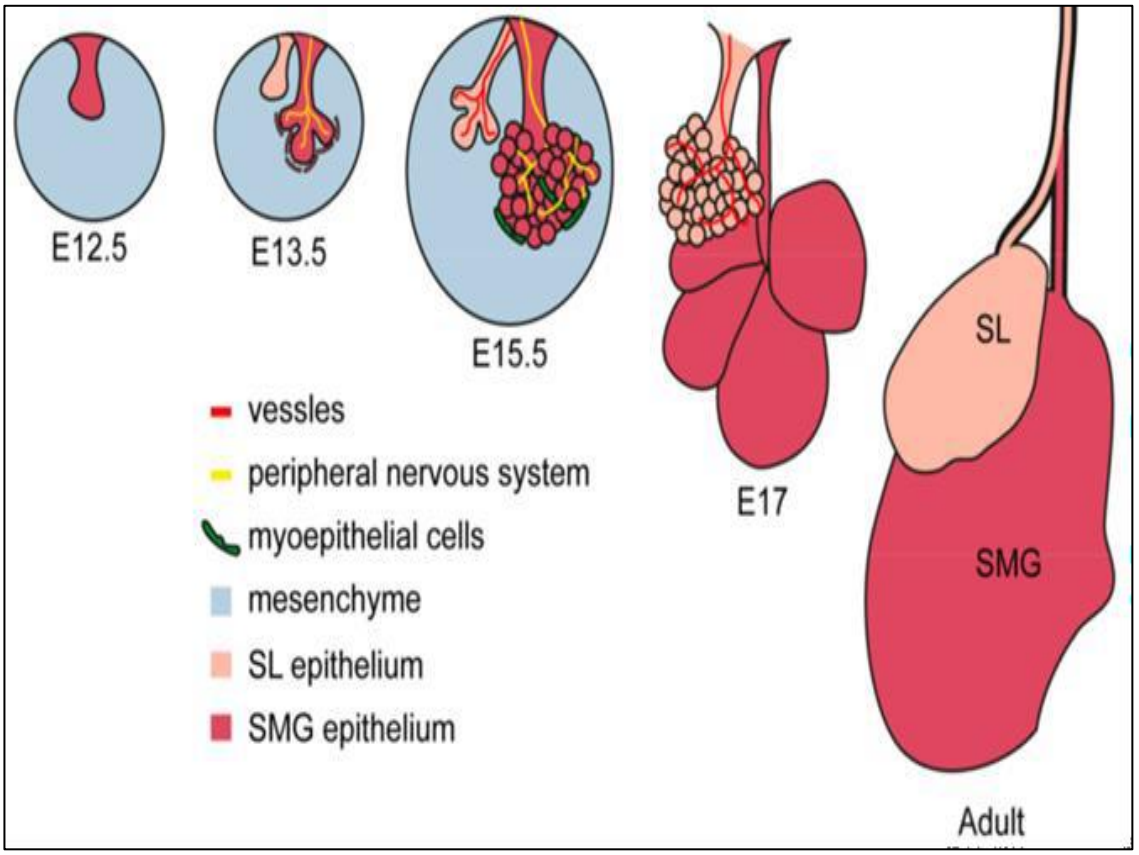

Figure 2.1 Embryonic development of murine submandibular gland and sublingual gland glands. Embryonic development starts with an epithelial placode, which, in turn, invades the mesenchymal layer and branches up. Innervation and vascularisation advance in parallel with the growth of salivary glands primordia and directly regulates its maturation. Myoepithelial cells arise from the external epithelial layer and develop into the contracting unit involved in the regulation of secretion. Image adapted from (Porcheri & Mitsiadis, 2019)

2.1.2 Structure of salivary gland

Major salivary glands are divided into three types of groups: parotid, submandibular, and sublingual glands; while minor salivary glands are about 600 to 1000 glands, which are found in the lingual labial, palatal, buccal and retromolar regions of the oral mucosa (Maruyama et al., 2019; Amano et al., 2012). The submandibular glands are ovoid in form and they are located inferior to the jaw, superior to the hyoid, and posterior to the digastric muscle's anterior belly. Their size is almost half that of the parotid gland. A tiny hole lateral to the tongue's frenulum allows ducts to enter the mouth's floor. These ducts are made up of both mucous and

serous cells, however, most of them are serious cells (Beale & Madani, 2006; Amano et al., 2012; Holmberg & Hoffman, 2014; Maruyama et al., 2019).

The parotid glands, which are located below and in front of the ear on each side of the face, are the biggest salivary glands. In the front, the parotid gland abuts the mastication muscles, and in the back, it goes behind the ascending ramus of the mandible. They are flat, well encapsulated, and mostly linked with facial nerve peripheral branches. On either side, ducts enter the mouth cavity opposite the second upper molar teeth. They are entirely made up of serous cells (Beale & Madani, 2006; Maruyama et al., 2019; Amano et al., 2012; Holmberg & Hoffman, 2014).

The sublingual glands are almond-shaped and consider as the smallest of the human major salivary glands. They are located behind the mucous membrane of the mouth in the sublingual fossa between the frenulum of the tongue and the teeth on the floor of the mouth. Short ducts enter the mouth near the submandibular ducts or along with them. They're mostly made up of mucous cells (Beale & Madani, 2006; Maruyama et al., 2019; Amano et al., 2012; Holmberg & Hoffman, 2014). The location of each major salivary glands is depicted in Figure 2.2.

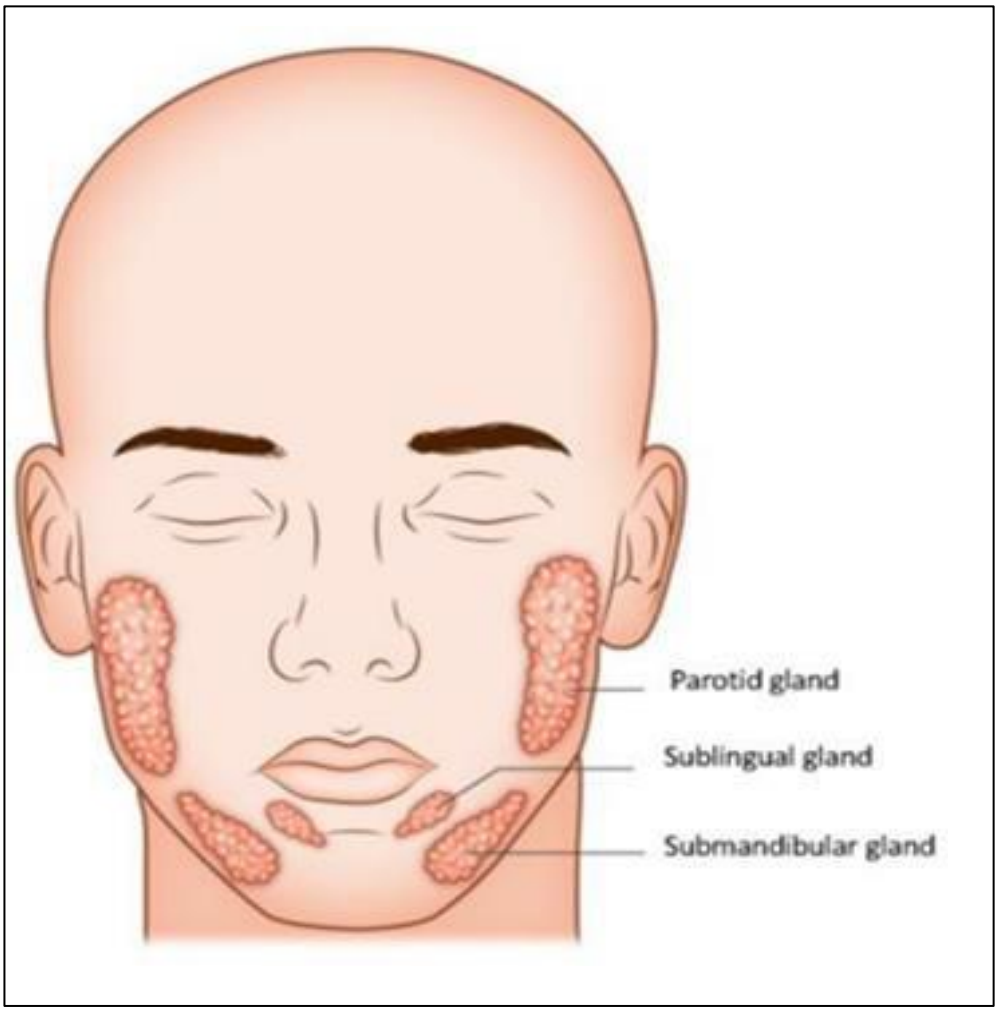

Figure 2.2 Schematic representation of types and location of major salivary glands. Parotid gland, sublingual gland and submandibular glands. Adapted from (Maruyama et al., 2019)

Major salivary glands are made up of parenchymal and stromal elements. The parenchyma is made up of secretory end pieces (acini) that produce a main fluid/saliva and is connected to a system of ducts (intercalated, striated, and excretory) that change it. An acinus is made up of mucous acinar cells or serous, as well as mucous cells covered with a serous demilune. The intercalated duct is formed of single layered cuboidal epithelium, leads to the striated duct, which made up of single layered columnar epithelium with multiple folds of the plasma membrane basally and mitochondria in between the folds.

The excretory duct (multi-layered columnar epithelium) is the final part of the duct system, leading the final saliva poured into the major excretory duct of oral cavity. As seen in Figure 2.3, acini and intercalated ducts are bordered by myoepithelial cells, which are contractile cells with a stellate structure. These myoepithelial cells are controlled by the autonomic nervous system and upon contraction they are believed to assist the flow of saliva by compressing the acini and the ducts and also to provide structural resilience to the parenchyma during secretion (de Paula et al., 2017; A. M. L. Pedersen et al., 2018; Roblegg et al.,2019).

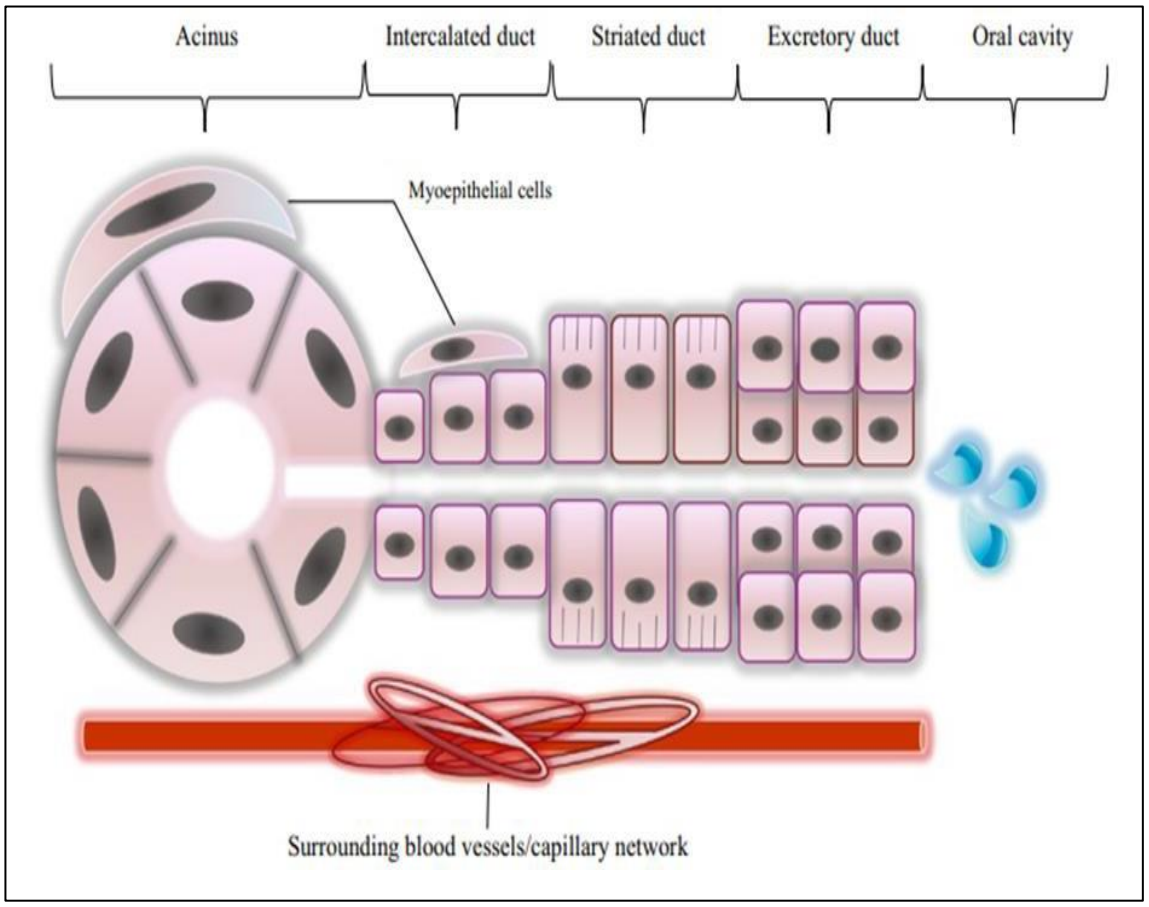

Figure 2.3 Secretory end piece (acinus) terminating into a duct system. Myoepithelial cells are the contractile cells with a stellate shape that surround the acini and intercalated ducts. Adapted from (A. M. L. Pedersen et al., 2018)

2.1.3 Function of salivary glands

Salivary glands generate saliva and are involved in many aspects of oral cavity homeostasis, including food digestion, taste, moisturization, and immunoreaction (Patel & Hoffman, 2014; A. M. L. Pedersen et al., 2018). The serous fluid and mucus of saliva, which contain water, salt, and protein, are secreted by acinar cells in the salivary glands. Duct cells, on the other hand, absorb salt and alter salivary output accordingly. The acini interposed portion is surrounded by myoepithelial cells, which are controlled by the nerve that governs salivation through a contraction (Sakai, 2016; A. M. L. Pedersen et al., 2018).

Hundreds of small, minor submucosal salivary glands, together with paired parotid, submandibular, and sublingual major salivary glands, release saliva (Tiwari M,2011). Saliva works as a protective, lubricating, mineralisation and renewing layer on mucosal and tooth surfaces of the oral cavity. Taste, mastication, and oral mechanoreception all stimulate saliva production (Tiwari M,2011; Proctor & Shaalan, 2021; Llena Puy& María Carmen,2006).

A layer of dense, well-hydrated mucus serves as one of the body's primary defence systems on wet epithelial linings, including those of the mouth, gastrointestinal tract, and lungs. Mucins, which are substantial glycoproteins that play an important role in host defence and preserving a favourable microbial habitat, are responsible for the viscoelastic qualities of mucus (Tabak LA ,1995; Pérez & Mabolo, 2007).

The two main mucins found in saliva are MUC7 and MUC5B. MUC5B, which is generated by mucous cells in the sublingual, submandibular, labial and palatine salivary glands, is the main gel-forming mucin in the mouth (Takehara et al., 2013). MUC7, the other mucin secreted, has no the capacity to form gels and is usually

detected as monomers or dimers. These two mucins have the ability to interact with salivary proteins to change their location and retention, potentially improving oral cavity protection. MUC7 and MUC5B can also interact with oral bacteria, making them easier to remove and/or less pathogenic (Brockhausen et al.,2009; Takehara et al., 2013; Frenkel & Ribbeck, 2015).

2.1.4 The salivary gland diseases

One of the three primary pairs of salivary glands is the submaxillary gland. It can exhibit obstructive pathologies like sialolithiasis, inflammatory pathology like sialadenitis, and benign or malignant tumour pathology (Torres-Gaya et al., 2020). The term "sialadenitis" describes inflammations and infections of the salivary glands that are either acute, chronic, or recurring.

Sialolithiasis is characterized by the formation of calcified formations in the salivary glands, particularly in the submandibular gland (Delli et al., 2014). When salivary stimulation is administered, sialoliths are typically accompanied by discomfort and swelling and are commonly linked to saliva retention (Wilsonet al., 2014). Radiographic examination, ultrasonography, sialo magnetic resonance imaging (MRI), sialography, computed tomography (CT) and sialo endoscopy scans can all be used to identify a sialolith (Wilson et al., 2014; Moore et al.,2023). The size of the sialolith largely determines how sialolithiasis is treated. While bigger sialoliths are often removed surgically, smaller sialoliths can be gently massaged toward the duct (Wilson et al., 2014; Roblegg et al.,2019; Moore et al.,2023).

An adenoid cystic carcinoma (ACC) is a rare malignant tumour of the salivary gland that occurs most often in the submandibular gland with prolonged clinical phase and a relatively short survival rate. However, it spreads to the lungs, bones, liver, and brain. Tubular, cribriform, and solid morphologic forms of the adenoid cystic

carcinoma exist, and they are frequently combined; the last type has a more aggressive clinical course (Coupland et al., 2014; Rafael et al., 2016).

The progression of adenoid cystic carcinoma is characterized by infiltrative perineural invasion and the early development of distant metastases (Coca-Pelaz et al.,2015). Radical tumour excision combined with adjuvant radiation still represents the gold standard of treatment (Coca-Pelaz et al.,2015; Thierauf et al., 2016). The low prevalence of adenoid cystic carcinoma has restricted current attempts to elucidate the molecular and genetic aspects of these tumours. Prognostic indicators are therefore urgently required to facilitate the development of innovative targeted therapies and to better identify patients at high risk of treatment failure (Thierauf et al., 2016; Fang, Y.et al.,2022).

2.1.5 Types of salivary glands tumors

Salivary gland tumours are rare and accounts for about 3% to 4% of all head and neck cancers. The World Health Organization's (WHO) recently divides them into more than 30 benign and malignant histologic groups. Parotid gland (70%) is the most common site for salivary tumours, followed by the submandibular gland, minor salivary glands, and sublingual gland. The most prevalent benign tumours are pleomorphic adenomas (65% of parotid tumours), followed by Warthin tumours (15–20%). while mucoepidermoid carcinoma considers the most frequent malignant tumour, accounting for 10% of salivary tumours and 30% of malignancies; half of these occur in the parotid gland (Abdel Razek & Mukherji, 2018; Reinheimer, A.et al., 2019).

Malignant salivary gland tumours grow in a variety of ways (Mengi, 2020). The most common types (adenoid cystic, low-grade mucoepidermoid carcinoma, and acinic cell carcinomas) grow slowly, sometimes so slowly that they are misdiagnosed

as benign or non-neoplastic lesions, particularly in the major salivary glands (Gatta et al., 2020; Cunha et al., 2021; Reinheimer, A.et al., 2019).

2.1.5(a) Pleomorphic adenoma

The term "pleomorphic adenoma" is obtained from the tumour's morphological complexity between glands and individuals. Pleomorphic adenoma has pathogenic histopathologic features including a single cell that differentiates into either myoepithelial or epithelial cells rather than just combined multiplication of carcinogenic epithelial and myoepithelial cells (Almeslet, 2020; Fu, H.et al.,2012; Heaton et al.,2013).

A fibrous capsule that separates the tumour from the adjacent salivary gland parenchyma may be strongly demarcated and/or encased by pleomorphic adenomas. In major salivary gland tumours, the capsule is thicker and more noticeable, but in small salivary gland tumours, the capsule is often poorly developed or non-existent (Lopes, M. et al.,2017; Dombrowski et al., 2019; Hernandez-Prera et al., 2021).

Pleomorphic adenomas (PA), which are especially common in the parotid gland, account for 70-80% of benign salivary gland tumours (Forrest, J. et al., 2008; Bokhari & Greene, 2022). The cause of pleomorphic adenoma is unknown, but the incidence of this tumour has increased in the last 15-20 years in relation to radiation exposure (Pan, S. Y.et al., 2017; Bokhari & Greene, 2022). Investigation of dietary variables, including eating vegetables and meat correlated with moderate decrease in the risk of the cancer, whereas consumption of sweets, dairy, and carbohydrates was associated with modestly elevated chances of this disease. Pleomorphic adenomas are the most common benign salivary gland neoplasm. It accounts for 45-75% of all salivary gland tumours, with an annual incidence of two to three and a half cases per 100,000 people. Pleomorphic adenoma can develop at any age; however, it is more

frequent in adults between the ages of thirty and sixty (Forrest, J. et al., 2008; Pan, S. Y.et al., 2017; Bokhari & Greene, 2022).

A complete head and neck examination is conducted as part of a physical exam to help in the diagnosis of pleomorphic adenoma. The lesion's location, size, and aetiology can all be discovered via magnetic resonance imaging. If the fine needle aspiration biopsy is unfavourable and it is decided that a pleomorphic adenoma is most likely the cause of the lesion, the patient is scheduled for resection with frozen section histopathologic analysis during the procedure. Resection is the best option for treating pleomorphic adenoma (Bentzen, S. M., 2006; Mendenhall et al., 2008).

After optional surgery, there is a greater than 95% local control rate. In the minority of patients with insufficient margins and/or multinodular recurrence, adjuvant postoperative radiotherapy increases the chance of local control. However, radiation activates a damage repair cascade in normal tissues (Kuo, Y. L et al., 2011; Mendenhall et al., 2008). According to tissue turnover rates and wound healing procedures, radiotherapy side effects can be divided into early and late reactions. Skin erythema, mucositis, nausea, and diarrhoea are among the early side effects of radiation treatment. While, atrophy, brain damage, vascular damage, radiation-induced fibrosis, endocrine and growth-related are among the later side effects (Kuo, Y. L et al., 2011; Barazzuol, L.et al., 2020).

2.1.5(b) Warthin tumour

Following pleomorphic adenoma, Warthin tumour has been noted as the second most common benign tumour of the salivary glands (Borsetto,D. et al., 2020). Warthin tumour is a bilayer columnar and basaloid oncocytic epithelial adenoma that develops several cysts with numerous papillae and is accompanied by a proliferation of lymphoid tissue that contains follicles. Warthin tumours (WT) commonly manifest

as a soft, gently expanding swelling or lump on the inferior pole of the parotid gland that may be painless or perhaps moderately painful (Borsetto,D. et al., 2020; Quer et al., 2021).

To diagnose Warthin tumour, ultrasound in conjunction with fine-needle aspiration biopsy (FNAB) is a precise and reliable approach. By doing so, it will be possible to learn more about the nature of the mass and determine whether more monitoring or surgery is necessary (Jechova et al., 2019). A study reported that the preferred therapies for Warthin's tumour are either superficial or complete parotidectomy with preservation of the facial nerve, and during long term follow-up, no cases of tumour recurrence were seen (Chulam et al., 2013). Despite this, surgery for Warthin tumour is linked to unfavourable side effects such postoperative infection, salivary fistula, and haemorrhage/hematoma (Schwalje AT et al., 2015; Espinoza S, et al., 2016; Ruohoalho J, et al., 2017). Additionally, patients with high variable Warthin tumour growth profiles will eventually require delayed parotid surgery, and more involved procedures have a higher risk of leading to facial nerve palsy and a probability of malignant transformation of less than 0.3% (Espinoza S, et al., 2016; Ruohoalho J, et al., 2017).

2.1.5(c) Mucoepidermoid Carcinoma

Mucoepidermoid carcinoma (MEC) is the most prevalent malignant salivary gland tumour, accounts for 15-30% of all malignancies of the salivary glands (Peraza et al., 2020; Pires et al., 2007; Xu et al., 2017). Mucoepidermoid carcinoma is a malignant glandular epithelial neoplasm having mucous, intermediate, and epidermoid cells, as well as columnar, clear cell, and oncocytic mucoepidermoid carcinoma characteristics, according to the World Health Organization (WHO), which was classified in 2005 and in 2017 based on clinical and histological characteristics

respectively (Auclair et al., 1992; Weinreb, I. et al., 2009). Similar to typical mucoepidermoid carcinoma, oncocytic mucoepidermoid carcinoma has a predominance of oncocytes. These cells exhibit dense granular eosinophilic cytoplasm, polygonal nuclei, marked nucleoli, and irregular in shape, centrally placed nuclei (Skálová, A. et al., 2020; Peraza et al., 2020).

Mucoepidermoid carcinoma often manifests as a soft, rubbery, variably fixed mass that causes no discomfort (Coca-Pelaz et al., 2015). Due to their superficial position, intraoral tumours might resemble a mucocele or other vascular tumours in the form of a blue-red-tinged swelling (Lanzel et al., 2016). Mucoepidermoid carcinomas often range in size from less than 1 cm to 3 cm. A complete surgical excision, with free margins and a focus on minimising postoperative morbidity, is the preferred treatment of action for anatomically accessible mucoepidermoid carcinomas in patients who have no evidence of. However, adjuvant radiation may enhance local control; even in instances with positive surgical margins, it may reach survival rates equivalent to those attained with total surgical excision metastases (Lanzel et al., 2016; Mimica, X. et al., 2021). For high-grade mucoepidermoid carcinomas and those with characteristics that point to a higher likelihood of recurrence, it is also suggested (Peraza et al., 2020; Mimica, X. et al., 2021).

2.1.6 Principle of carcinogenesis

A cancerous tumour is a change in the way cells are arranged in the body's tissues and an abnormal build-up of cells (Sonnenschein & Soto, 2016). Tumour development is a multistep process. The fundamental step in this process is the production of a mutant cell by genetic mutation of the genomic DNA, which is followed by the altered cell's selective growth (Sonnenschein & Soto, 2014; Sonnenschein & Soto, 2016). The growth may be induced by either a rise in the mutant

cell's rate of cell division or a fall in its rate of cell death (apoptosis) (Sonnenschein & Soto, 2016). Additional epigenetic and genetic alterations happen in the newly created lesion when the mutant cell multiplies (Klaunig & Wang, 2018).

Several human malignancies are associated with oxidative stress that results from it as both a cause and a modulator of those diseases (Milkovic, L.et al., 2014; Kruk & Aboul-Enein, 2017). Variations in antioxidant and oxidative DNA repair genes can change a person's vulnerability to developing cancer. Furthermore, oxidative stress and resulting oxidative damage can occur at multiple steps of the cancer process from the formation of the mutated cell (initiation) to the promotion of the mutated cell (cell proliferation; epigenetic effects) and eventual formation of the neoplasm (progression) Figure 2.4 (Milkovic, L.et al., 2014; Kruk & Aboul-Enein, 2017; Klaunig & Wang, 2018)**.** Oxygen species are overproduced from both endogenous and external sources. Organelles found inside cells as well as inflammatory sources are examples of endogenous sources, while radiation, medicines, and xenobiotics are examples of exogenous sources (Klaunig & Wang, 2018; Zahra et al., 2021).

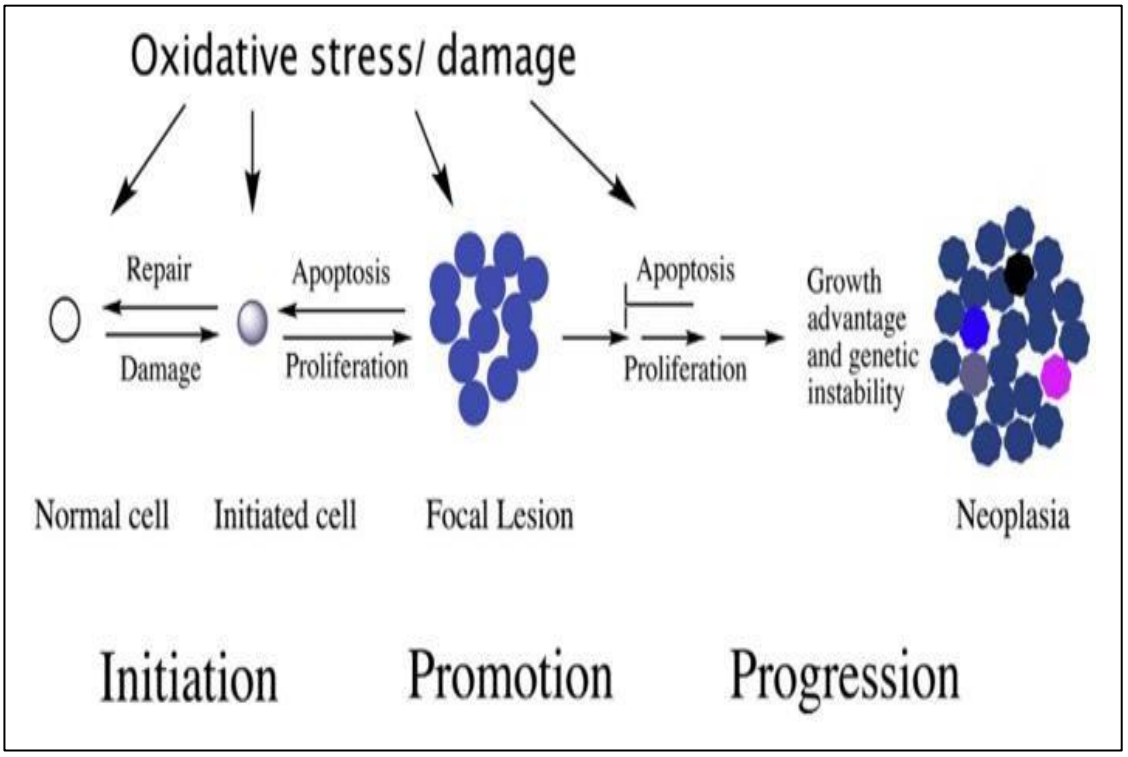

Figure 2.4 The cellular and pathologically visible phases of cancer which are divided into three main stages: initiation, promotion and progression (Klaunig & Wang, 2018)

The process of cancer development involves several stages. Berenblum and Schubik,1948 initially presented the idea of many stages of carcinogenesis and further research has since backed up their theory. Oncology now distinguishes between three crucial stages: initiation, promotion, and progression (Trosko, 2003; Devi., 2004; Oliveira et al., 2007).

Initiation is first step in carcinogenesis, where cellular genome mutations create the potential for neoplastic development. Exposure to a carcinogen initiates this process, which involves persistent cellular alterations known as oncogenes (Trosko, 2003; Klaunig & Wang, 2018; Rundhaug & Fischer, 2010). While numerous oncogenes are required for neoplastic transformation, the process can be initiated with a single hit. These mutations can have a significant impact on cellular behaviour and response, resulting in gene dysregulation in biochemical signalling pathways (Devi., 2004; Oliveira et al., 2007; Klaunig & Wang, 2018; Rundhaug & Fischer, 2010). The

full manifestation of neoplasia-causing mutations needs interaction with other gene alterations or changes in the cellular environment. Before a malignant lesion manifests, the altered cell divides continuously with fidelity to the modified karyotype and experiences additional mutations (Oliveira et al., 2007; Rundhaug & Fischer, 2010).

The promotion stage is preneoplastic cell proliferation and changes in gene expression resulting in more genetic damage and more mutations (Hanahan & Weinberg, 2000). The activation of cell proliferation, oxidative stress, chronic inflammation, and the maintenance of persistent hyperplasia are all critical components for promotion (Oliveira et al., 2007; Lopez et al., 2021). The changed (initiated) cell may still be benign unless stimulated by a tumour promoter to proliferate further, disturbing the cellular equilibrium (Lopez et al., 2021). Tumour promoters are chemicals that increase cell proliferation into a large number of daughter cells with the initiator's mutation. Specific promoters interact with known receptors on or in cells, whereas nonspecific promoters change gene expression without interacting with known receptors. Promoters bind to certain cell receptors that have been activated by an initiator in order to induce intracellular signalling pathways. Tumour development during promotion is dosage dependent, with starting cells requiring repeated exposure to a specific quantity of tumour promoters in order to develop stimuli (Lopez et al., 2021; Kontomanolis et al., 2020). Promoters will not directly induce structural genetic alterations, but will encourage the production of oxygen radicals, which impact chromosomes and DNA break. These actions could enhance cell development from promotion to progression (Kontomanolis et al., 2020).

The third stage, called progression, shows cellular and molecular alterations from preneoplastic to neoplastic state (Hawighorst et al. 2001; Oliveira et al., 2007). It is an irreversible stage characterized by chromosomal integrity disruption, altered

nuclear ploidy, and genetic instability (Klaunig & Wang, 2018). Progression is caused by subsequently occurring genetic alterations such as gene amplification and loss of heterozygosity (Hawighorst et al. 2001; Rundhaug & Fischer, 2010). Cell proliferation is unaffected by the presence of a stimulus during progression. However, angiogenesis is required for neoplastic development as an epigenetic event (Oliveira et al., 2007; Klaunig & Wang, 2018). The establishment of an angiogenic phenotype precedes the development of malignant features, and its suppression delays neoplastic development (Oliveira et al., 2007).

2.2 Salivary glands cancer

2.2.1 Epidemiology and risk factors

Salivary gland malignant neoplasms are exceedingly uncommon; estimates range from 0.05 to 2/100,000 people (Licitra et al.,2003; Guzzo et al., 2010). The population in Europe has the greatest incidence of malignancies of the salivary glands. In comparison to Africa, the ratio is 40:6, whereas in comparison to Asia, it is 40:1(Kordzińska-Cisek & Grzybowska-Szatkowska, 2018; Licitra et al.,2003). The parotid gland had the most malignancies identified, while the submandibular gland is the second-most frequent anatomical place with investigations carried out in Africa and Asia revealed a greater frequency of malignancy (To et al., 2012; da Silva et al., 2018; Kordzińska-Cisek & Grzybowska-Szatkowska, 2018). The majority of benign neoplasms, or 82.2 %, were pleomorphic adenoma tumours. However, mucoepidermoid carcinoma, which accounted for 33.3 % of all malignant tumour cases, was most prevalent followed by adenoid cystic carcinoma, which accounted for 22.5 % (To et al., 2012; da Silva et al., 2018; Young & Okuyemi 2022).

Salivary gland tumours were discovered in patients between the ages of 14 and 71 years throughout 10 years research in India with the mean age of occurrence being 45.01-16.3 years and a male to female ratio of 0.7-0.9 (Kumaran et al., 2019). Malignant salivary gland tumours were estimated to affect 0.4 to 2.6 individuals per 100,000 worldwide each year. It was calculated to be between 0.8 and 1.2 per 100,000 individuals in the USA. Australia, Canada, and the USA are the nation's most often afflicted by salivary gland malignancies. Among men, Whites and Filipinos are more likely to develop this neoplasm in the USA, Hawaii, and California than other ethnic groups from the same nations (García-Martín et al., 2019).

Oral cancer risk factors have been the subject of the most research include human papilloma virus (HPV), alcohol use, and tobacco use (Irani, 2020; Sankaranarayanan et al., 2015; Rao et al., 2013). Chemotherapy and radiation are only used on people who cannot tolerate surgery as the primary treatment for oral cancer. In addition, the microbial structure and development of oral squamous cell carcinoma are impacted by a number of other variables, including smoking, age, and food (D'souza & Addepalli ,2018; Mangalath et al., 2014; Sankaranarayanan et al., 2015). It is also believed that cytokines and other bacterial secretory mediators can cause cancer. Lastly, a growing body of research shows that those with poor oral hygiene have a greater prevalence of oral cancer (Argilés et al., 2019; Hou et al., 2022; Irani, 2020).

2.2.2 Pathology, diagnosis, and treatment

Mucoepidermoid carcinomas (22.4%), osteosarcomas (13.8%), and squamous cell carcinomas (12.1%) accounted for the most oral and maxillofacial tumour lesions in children and adult. The majority of the lesions, which were found to be asymptomatic, impacted a variety of regions, with the palate (19 %), the mandible

(13.8 %), and the maxilla being the most often afflicted (13.8%) (Vale et al., 2013; Amadeu et al.,2015; Arruda et al., 2017).

In both adults and children, mucoepidermoid carcinomas is the most prevalent primary salivary gland neoplasm. The majority appear as an asymptomatic lump or oedema (Chan et al., 2013; Vale et al., 2013; Bradley, 2016). Mucoepidermoid carcinomas are hard lesions that can be either fixed or mobile and are seen in the main salivary glands. However, early signs might include pain and paralysis of the face (Bradley, 2016; Israel et al., 2016). There are a variety of classification schemes, including low, intermediate, and high-grade disorders. In contrast to intermediate and high grade mucoepidermoid carcinomas, which exhibit aggressive malignant activity, low-grade mucoepidermoid carcinomas exhibit benign clinical behaviour (Bittar et al., 2015; Bradley, 2016; Israel et al., 2016).

The management of major and minor salivary gland tumours is difficult due to their heterogeneity. Surgery along with radiation and/or chemotherapy is still the preferred course of treatment for these tumours (Agulnik et al., 2008; To et al., 2012; Green et al., 2016). If the tumour is small in size, low grade, and accessible, surgical excision of a primary gland cancer can be curative in the majority of instances (Green et al., 2016; Cruz et al., 2020; Laurie & Licitra, 2006). After surgery, there is a substantial probability of locoregional failure for patients with high grade, advanced-stage (T3/T4), nodal involvement, and those with either narrow or positive margins (Green et al., 2016; To et al., 2012). Postoperative radiotherapy is likely to be advantageous for these individuals and advanced tumours involving the carotid or the skull base (Cruz et al., 2020; Laurie & Licitra, 2006). When alternative treatments are ineffective for patients with recurrence or metastatic illness, the use of systemic medicines is taken into consideration. Advanced salivary gland carcinomas have been