DIFFERENTIAL EXPRESSION OF MUCIN IN SALIVARY GLAND TUMOURS

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ABSTRACT

Introduction: Varied alterations in types of secreted mucin may affect the regulation of cell growth, immune response and adhesion of cell. These changes may indirectly contribute to the ability of tumour invasion and metastasis. However, the expression of mucins in salivary gland tumours has not been explored in depth. **Objectives:** To investigate expressions of mucin in the salivary gland tumour microenvironment and make comparisons between benign and malignant, and minor and major salivary gland tumours. **Methods:** Special stains were used to stain neutral mucin (Periodic acid Schiff), sialomucin (Alcian Blue) and sulfomucin (Aldehyde Fuschin) within tissues from 6 normal salivary glands and salivary gland tumours including 31 pleomorphic adenoma (PA), 27 mucoepidermoid carcinoma (MEC) and 15 adenoid cystic carcinoma (AdCC). Statistical analyses to compare mucin expression in these salivary gland tumours were done using Chi-square tests. **Results:** Sialomucin was the most expressed mucin in all salivary gland tumours regardless of origin. A significant difference was observed in mucin expression between benign and malignant salivary gland tumours, in which PA showed 3 times significantly higher expression of sialomucin compared to MEC and AdCC (p=0.028). PAs of major gland origin showed 42 times significantly higher expression of sialomucin compared to PAs of minor gland (p=0.000). **Conclusion:** Alcian blue was the best special stain to visualize mucin elements in salivary gland tumours. Sialomucin content in PA of major glands was vastly increased from that in minor glands. The degree of sialomucin expression may play a role in diagnosis of borderline salivary gland tumours.

Keywords : Pleomorphic adenoma, Mucoepidermoid carcinoma, Alcian blue, Sialomucin, Sulfomucin

ABSTRAK

Pengenalan: Pelbagai perubahan pada jenis musin yang dirembeskan akan mempengaruhi pengaturan pertumbuhan sel, perlekatan sel and tindak balas imunisasi badan. Perubahan ini secara tidak langsung akan menyumbang kepada keupayaan tumor untuk menceroboh dan metastasis. Walau bagaimanapun, ekspresi mucin pada tumor kelenjar air liur masih belum diterokai secara mendalam. **Objektif:** Objektif kajian ini adalah untuk menilai ekspresi musin dalam tumor kelenjar air liur tidak malignan dan malignan, dalam tumor kelenjar air liur kecil dan kelenjar air liur utama serta hubungan dengan parameter klinikal. **Kaedah:** Noda khas digunakan dalam pewarnaan musin neutral (Periodic acid Schiff), sialomucin (Alcian Blue) dan sulfomucin (Aldehyde Fuschin) dalam tisu dari 6 kelenjar air normal dan tumor kelenjar air liur termasuk 31 adenoma pleomorfik (PA), 27 karsinoma mucoepidermoid (MEC) dan 15 karsinoma sista adenoid (AdCC). Analisis statistic bagi membandingkan ekspresi mucin pada tumor kelenjar air liur dilakukan menggunakan ujian Chi-square. **Keputusan:** Sialomucin adalah mucin yang paling banyak didapati dalam kesemua tumor kelenjar air liur tanpa mengira asalnya. Perbezaan yang ketara diperhatikan pada ekspresi mucin antara tumor kelenjar air liur benigna dan malignan di mana PA menunjukkan ekspresi sialomucin sebanyak 3 kali ganda lebih tinggi berbanding MEC dan AdCC ($p=0.028$). PA kelenjar air liur utama pula menunjukkan ekspresi sialomucin sebanyak 42 kali lebih tinggi berbanding dengan PA kelenjar air liur kecil ($p=0.000$). **Kesimpulan:** Alcian blue adalah noda khas terbaik bagi memvisualisasikan elemen musin pada tumor kelenjar air liur. Kandungan sialomucin dalam PA kelenjar utama sangat tinggi berbanding kandungan kelenjar kecil. Tahap ekspresi sialomucin mungkin berperanan dalam diagnosis tumor kelenjar air liur terutama dalam kalangan kes-kes sempadan yang sukar diklasifikasikan.

Kata kunci : Adenoma pleomorfik, Karsinoma mucoepidermoid, Alcian blue, Sialomucin, Sulfomucin

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LIST OF ABBREVIATIONS

AB	Alcian Blue
AdCC	Adenoid Cystic Carcinoma
AF	Aldehyde Fuschin
BM	Buccal Mucosa
CT	Computed Tomography
FFPE	Formalin-fixed paraffin embedded
FNAC	Fine-Needle Aspiration Cytology
FOM	Floor of mouth
HID	High Iron Diamine
H&E	Hematoxylin and Eosin
IgA	Immunoglobulin A
MAPK	Mitogen-activated Protein Kinase
MC	Mucicarmine
MEC	Mucoepidermoid Carcinoma
MRI	Magnetic Resonance Imaging
PA	Pleomorphic Adenoma
PAS	Periodic Acid Schiff
PAS-D	Periodic Acid Schiff Diastase
pH	Potential Hydrogen
PLAG-1	Pleomorphic Adenoma Gene 1
PPUM	Pusat Perubatan Universiti Malaya
ROC	Receiver operating characteristic
SPSS	Statistical Package for Social Sciences

CHAPTER 1 : INTRODUCTION

1.1 Background

Salivary gland malignancies are rare, and thus deemed to be quite difficult to diagnose but nevertheless still sparks much interest for its pathology. This is due to several factors including their unpredictable clinical course, diverse histological presentation and differing opinions of pathologists based on their own experience and focuses on different aspects of the disease. Besides that, the diagnosis and management are also influenced by patients' presentation at the time of seeking treatment, accuracy and timing of imaging such as computed tomography (CT), magnetic resonance imaging (MRI) or fine-needle aspiration cytology (FNAC) as well as the behaviour of the pathology itself (Ambu, Ramalinggam, & Kaur, 2014).

The occurrence of salivary gland tumours amongst other neoplasms is less than 3% and most of them are benign in nature, accounting up to 75% of salivary gland tumours (Gleeson & Cawson, 2008). Majority of the tumours favour the parotid glands, constituting up to 80%, compared to the submandibular and sublingual glands (Guzzo et al., 2010). The causes of salivary gland tumours are widely unknown. It has been described to have a predilection for females in benign tumours with no racial predominance. Distant metastasis can occur in the lungs, bones and liver with each respectively accounting to 80%, 15% and 5% (Guzzo et al., 2010). Salivary gland neoplasms are more commonly diagnosed in adults and are quite rare in children with only 8% reported in peadiatrics head and neck tumours (Muenscher et al., 2009).

Although the exact aetiopathogenesis of salivary gland tumours are not well-known,

multiple studies have linked the presence of its secretory substance, mucin playing a major role in the disease progression (Hollingsworth & Swanson, 2004; Sushma Naag & Adi, 2010). Varied alterations in the type of secreted mucin may affect the regulation of cell growth, immune response and adhesion of cell (Huszno et al., 2012). These changes may indirectly contribute to the ability of tumour invasion and metastasis. However, studies on the expression of mucins in salivary gland tumours are still lacking with existing studies limited by sample sizes due to rare occurrences of these tumours or only focused on certain types of salivary gland tumours. There is also no study looking into the possibility of salivary gland origin as a confounding factor for mucin expressions in similar salivary gland tumours. Drawing inspiration from gastrointestinal diseases, the potential for classification of salivary gland tumours with regards to the nature of their mucin content has yet to be explored. In addition, special stains are affordable options as adjunctive investigative tools to provide indications of clinical behaviour in salivary gland tumours. This is in contrast to immunohistochemical stainings which have limited uses in salivary gland tumours but incur relatively higher costs when necessary.

1.2 Problem Statement

Varied alterations in types of secreted mucin may have an effect on the regulation of cell growth, immune response and adhesion of cell (Huszno et al., 2012). Thus these changes may indirectly contribute to the ability of tumour invasion and metastasis. However, the expression of mucins in salivary gland tumours has not been explored in depth.

1.3 Research Questions

1. Is there an association between mucin expression with clinical behaviour of benign and malignant salivary gland tumours?

2. Is there a difference in mucin expression within minor and major salivary gland tumours?

1.4 Aim & Specific objectives of the Study

Aim : To investigate expressions of mucin in the salivary gland tumour

Specific Objectives :

1) To identify types of mucin within salivary gland tumours

2) To compare mucin expression between benign and malignant salivary gland tumours
3) To compare mucin expressions between salivary gland tumours of minor and major salivary gland origin
4) To determine the association between mucin expression with clinical parameters of salivary gland tumours.

CHAPTER 2 : LITERATURE REVIEW

2.1 Salivary Gland

Salivary glands play a crucial role in the maintenance and protection of oral health. They have functions in food lubrication, the taste of food and also allow speech. Salivary glands produce saliva, which is secreted via acini. Functions of saliva include moisturization, lubrication and solubilization of food, in addition to maintaining homeostasis of the mouth and integrity of oral structures.

Salivary glands can be classified into major and minor salivary glands. There are 3 pairs of major salivary glands; parotid glands, submandibular glands and sublingual glands (Miletich, 2010). The parotid gland is situated just below the ear and drains into the mouth via the Stensen's duct and its secretions are mainly serous. Meanwhile, the submandibular gland is situated in the floor of the mouth and produces seromucous secretions which drain into the mouth via the Whartin's duct. The sublingual gland is also located underneath the floor of mouth (FOM) but drains via the Bartholin's duct and produces mainly mucinous secretions (Adams, Warner, & Nor, 2013). There are also around 600-1000 minor salivary glands in the oral cavity within the tongue, lips, FOM and cheeks (Miletich, 2010).

There are 3 types of acini and each is characterized by their cell compositions of either serous, mucous or mixed shown in **Figure 2.1**. Serous acini secrete saliva rich in several proteins but lacking in mucin proteins. Mucous acini secrete saliva rich in mucin proteins and are tied to carbohydrates (Miletich, 2010). Seromucous acini secrete a mixture of mucin and several proteins. After its release from these acini, mucin travels through intercalated ducts, small excretory duct and finally through a large

excretory duct that opens into the oral cavity (Miletich, 2010). Immunoglobulin A and lysozyme from the ductal cells are added into the secretions in the process. Myoepithelial cell contributes in contraction and enables the secretory cells to release saliva and also promotes salivary flow (Miletich, 2010).

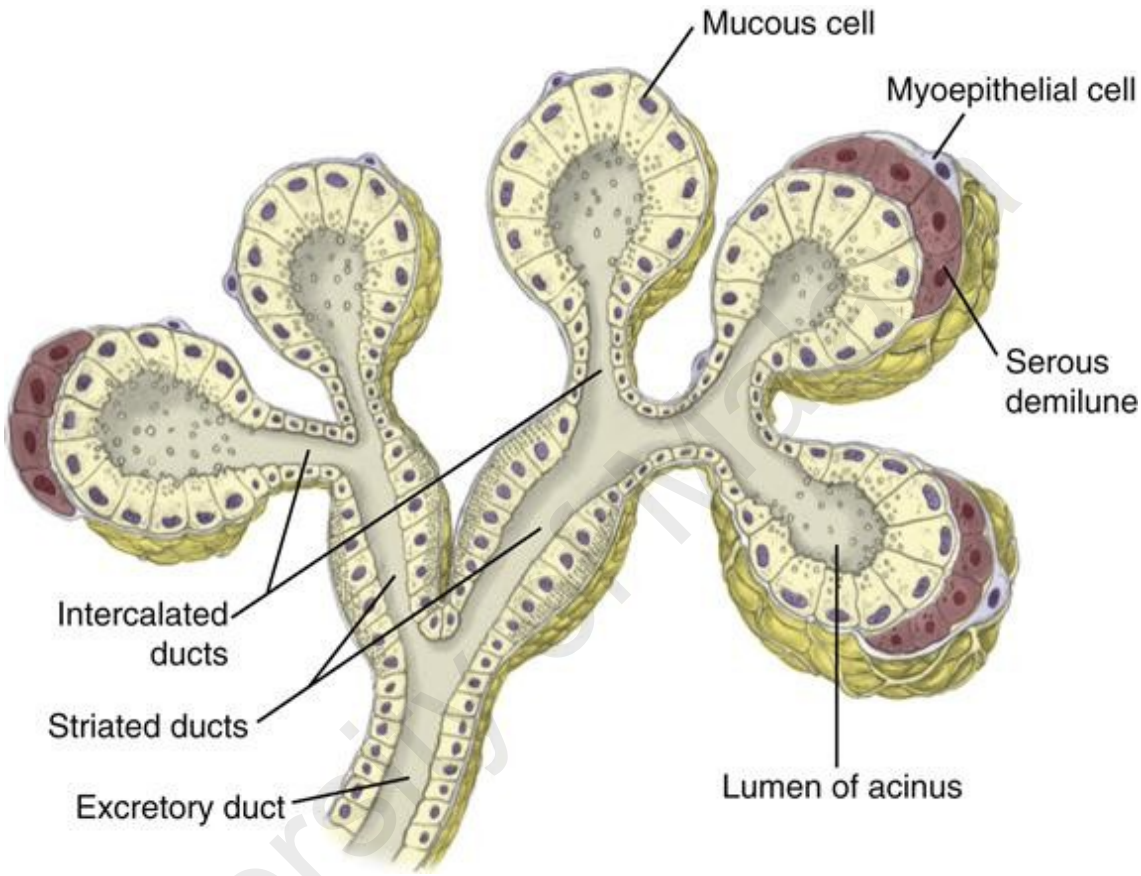

Figure 2-1 : Schematic diagram exhibiting types of salivary glands acini and ductal epithelium.
Source: Adopted from Pocket Dentistry in Head and Neck structures Chapter 11

2.2 Salivary Gland Tumours

Cancers in salivary glands are rare and account for 2-6.5% of head and neck cancers (Gillespie, Albergotti, & Eisele, 2012). These tumours can form within minor or major salivary glands with almost 80% of tumours originating from the parotid glands, 15% from submandibular glands and 5% from sublingual and minor glands (Bell & Hanna, 2012). Pathogenesis of salivary cancer is still unclear, however, some researches postulated that occupation-derived radiation and UV light exposure, tobacco or alcohol could be risk factors (O'Neill, 2009).

Although most salivary masses are benign at an incidence of 75%, the presentations are somewhat the same with malignancy thus posing a great challenge in diagnosis. It is also postulated that the heterogeneous nature of malignant salivary gland tumours also contributes to the significant challenge in diagnosis and treatment (Gillespie et al., 2012). It has been shown that salivary gland tumours have a slight predilection towards males compared to females with a 51% rate however both tend to develop within the 5th decade of life (Boukheris, Curtis, Land, & Dores, 2009). Based on the WHO classification of Salivary Gland Tumors 2017, they can be classified as shown in **Table 2.1**.

The most common benign salivary tumour is pleomorphic adenoma and the commonest salivary malignancies are mucoepidermoid carcinoma and adenoid cystic carcinoma (Bell, Luna, Weber, Kaye, & El-Naggar, 2008; Eversole, Sabes, & Sheldon, 1975).

Table 2.1 : WHO classification of Salivary Gland Tumors 2017

BENIGN TUMOURS	MALIGNANT TUMOURS
Pleomorphic adenoma Myoepithelioma Basal cell adenoma Warthin's tumor Oncocytoma Lymphadenoma Cystadenoma Sialadenoma papilliferum Ductal papillomas Sebaceous adenoma Canalicular adenoma and other ductal adenomas	Acinic cell carcinoma Secretory carcinoma Mucoepidermoid carcinoma Adenoid cystic carcinoma Polymorphous adenocarcinoma Epithelial-Myoepithelial carcinoma Clear cell carcinoma Basal cell adenocarcinoma Sebaceous adenocarcinoma Intraductal carcinoma Cystadenocarcinoma Adenocarcinoma, NOS Salivary duct carcinoma Myoepithelial carcinoma Carcinoma ex pleomorphic adenoma Carcinosarcoma Poorly differentiated carcinoma Neuroendocrine and non-neuroendocrine Undifferentiated carcinoma Large cell neuroendocrine carcinoma Small cell neuroendocrine carcinoma Lymphoepithelial carcinoma Squamous cell carcinoma Oncocytic carcinoma

2.3 Major and Minor Salivary Gland Tumours

Among major salivary gland tumours malignancies, 15-32% involves parotid glands, 41-45% involves submandibular glands and 70-90% involves sublingual glands. Around 40% of these tumours are quiescent and present as a slow-developing mass and with prolonged duration may involve the nerve and cause pain or paraesthesia. This can be seen in patients below the age of 40 years old (Ellis, 2009). Clinical signs and symptoms that should raise a red flag include rapid growth, pain, cervical lymphadenopathy and facial nerve involvement (Hocwald et al., 2001). Alternatively, patients may also present with trismus, skin ulcerations or fistulas and palatal or pharyngeal fullness.

On the other hand, among the many minor salivary glands within the head and neck region, almost half of these tumours of this origin are malignant in nature. The rate of occurrence within the palate is similar to those in the submandibular gland which is about 40-60% but may increase to almost 90% if the tongue, FOM and sublingual glands are involved (Lopes, Kowalski, Santos, & Almeida, 1999). Their signs and symptoms depend on the size, position and location. Most commonly they appear as submucosal swellings or bulge and the mucosal layer adheres to the mass with or without an ulcer. Those arising in the oropharyngeal region may present with a painless lump, dyspnea and hoarseness. If they occur within the nasopharynx region, features like facial pain, epistaxis and nasal obstruction may be present (Ellis, 2009).

2.4 Pleomorphic adenoma (PA)

Pleomorphic adenoma is a benign salivary gland tumour and accounts for almost 60% of all benign salivary neoplasms (Renata et al., 2013). It is made up of epithelial and myoepithelial cells originating from the intercalated ducts with a complex stroma. The mesenchymal tumour element commonly exhibits myxomatous, hyalinized, chondroid and areas of osseous metaplasia. PA has a diverse morphological pattern and the tumour cells can be arranged in tubular structures lined by bilayered epithelium. The ductal lumens normally present with eosinophilic coagulum and surrounding cells may appear plasmacytoid. These are embedded within a myxomatous, chondroid or hyalinized stroma. Ellis and colleagues, 2008 also showed that conventional PA lacks features of cellular atypia with low mitotic count and nuclear hyperchromatism but often contains stroma rich in glycosaminoglycans. Histogenesis of PA remains unclear but two main theories have been postulated; 1) it is the result of pluripotent cell clonal expansion and 2) they arise from a coordinated growth cell population that have the proliferative

capacity (Martins et al., 2005). However, the pluripotent clonal expansion theory is favoured as it has been demonstrated in many studies. A salivary gland neoplasm immunohistochemical study by Gurbuz et al, in 2006 also concluded that PA originates from stem cells.

The most frequent architectural pattern of growth is tubular followed by insular, trabeculae, fascicular, cystic, cribriform and pseudoangiomatous (Enescu, Enescu, Balasoiu, Ciolofan, & Capitanescu, 2014). Most PAs are easily treated via curative resections however some may recur after years of primary tumour removal. Besides, a small percentage of PA may turn malignant and thereafter diagnosed as carcinoma ex-pleomorphic adenoma which metastasizes and seeds to different sites (Laskawi, Schott, & Schroder, 1998). Studies have shown that tumorigenesis of PA is related to the activation of Pleomorphic Adenoma Gene 1 (Plag1), an oncogene which plays a vital role in its development within the salivary gland. Various studies verified this fact including one done by Shukun Shen and colleagues (Shen et al., 2011).

PA is managed surgically and removed by local excision or enucleation technique. It has been said that there is a 20-45% recurrence rate with this type of removal due to the microscopic tumour perforation of the capsule and is shed off yielding a subtotal removal. (Cappabianca et al., 2008).

2.5 Mucoepidermoid Carcinoma (MEC)

Approximately 30-35% of malignant salivary gland tumours are mucoepidermoid carcinoma (Eversole, Sabes, & Rovin, 1975). MEC can occur in both major and minor salivary glands. Cells are made up of mucous, epidermoid and intermediate types. Polygonal epidermoid cells show distinct keratinization and intercellular bridges.

Mucous cells consist of mucin proteins of varying sizes. Intermediate cells are often basal-like in appearance and function as progenitor cells for mucous and epidermoid cells. Extralobular tumours were believed to originate from excretory ducts (Akrish, Peled, Ben-Izhak, & Nagler, 2009).

MEC is diagnosed based on the presence of both, histological and cytogenetic abnormalities. It is categorized into 3 grades, which take into account the amount of cyst formation, degree of cytological mutation, and also the relative number of epidermoid, mucous, and intermediate cell types. Prominent cyst formation, an abundance of mucoid cells with small amounts of cytological mutations are specific features for low-grade tumours. On the other hand, squamoid and intermediate cells with increased mitosis are more pronounced in high-grade tumours. Intermediate grade tumours possess features in between the two. More alarming auxiliary histological features comprised of necrosis, perineural invasion, anaplasia and infiltrative pattern of growth (Bell, Holsinger, & El-Naggar, 2010).

Choice of treatment for these tumours would be surgical resections for low to intermediate-grades and neck dissection with radiotherapy for high-grade types (Bell et al., 2010). However even with successful removal, a minority of patients still have recurring lesions years later (Chen et al., 2007) and unfortunately, minimal treatment modalities are available to them as recurrent MEC is highly chemo-resistant (O'Neill, 2009). Chemotherapy is only then conducted for palliative care of patients despite being ineffective (Cai et al., 2011). Therefore an improvised understanding of the aetiopathogenesis of this tumour is needed in order for the development and design of targeted therapies that can provide better prognosis and further improve patient's quality of life.

2.6 Adenoid Cystic Carcinoma (AdCC)

Approximately 10-25% of most malignant salivary gland tumours are adenoid cystic carcinoma and it is the 2nd most common salivary masses (Tang et al., 2010). Neville 2015, noted that any salivary gland site can be affected but it favours the minor salivary glands with a 50-60% rate of occurrence. This tumour has a female predilection and is commonly found in the 5th to 6th decade of life (Mendenhall et al., 2004). It is postulated that they originate from intercalated duct reserve cells of each gland. Histologically AdCC is biphasic and therefore made up of both epithelial and myoepithelial cells (Akrish et al., 2009).

AdCC has 3 histological patterns namely tubular, cribriform and solid pattern. The cribriform pattern has been described as 'swiss cheese' in appearance due to the presence of numerous pseudocysts. In this pattern, the ductal areas are composed of basophilic mucoid material and the tumour cells are small with a basaloid presentation. The tumour cells in tubular pattern display the same type of cells but they differ in having smaller ducts lined by both myoepithelial cells and luminal ductal cells. Solid growth pattern, however, shows no tubules or cyst and is arranged in large tumour islands (Adams et al., 2013). Site of the primary tumour is an important prognostic factor as it has been shown that AdCC occurring in the tongue and maxillary antrum have poor prognosis while the worst prognosis is supposedly reserved for those within the submandibular gland (Spiro, Huvos, & Strong, 1979).

Poor prognosis has been linked to the extension of the tumour invading into the adjacent structures such as nerves, bones and muscle (Huang et al., 1977). The progression rate is slow but the long-term survival rate is poor as the 15 to 20-years survival rates are 35-

40% and 10% respectively. However, the 5-year survival rates are favourable at up to 70-90% (Tang et al., 2010). This low overall survival is due to the persistent growth, late recurrence prior to initial treatment, haematogenous spread, perineural invasion and seeding to neighbouring or distant structures (Xue, Zhang, Liu, Jing, & Ma, 2005). Many authors also consider that there is a close correlation between histological types and prognosis of the tumour. Increased recurrence, metastasis and mortality rates are seen most with solid pattern (Huang et al., 1977). AdCC has a low response to chemotherapy as their cells are fairly resistant. A partial response has been observed with the use of single agents or a combination of a drug (Rentchler, Burgess, & Byres, 1977). All grades of ACC are aggressive, therefore treatment requires a combination of both surgery and radiation (Seethala & Stenman, 2017).

2.7 Mucin and Tumour

Tumour cells growth and development have been associated with dysregulation of mucin protein core expression and its survival is the outcome of this favourable selection process (Hollingsworth & Swanson, 2004). Mucin also plays a role in tumour cells biological properties in several ways. It exerts an effect on control of the local environment, regulation of differentiation and proliferation of cells, tumour suppression, invasion, metastasis and regulation in inflammation and immune response. With regards to control of the local environment, it has been suggested that mucin is deployed in the same manner as epithelial cells by tumour cells, that is as protection and barrier which contributes to the ability to survive and proliferate in otherwise unfavourable conditions (Hollingsworth & Swanson, 2004). Besides that, the structure and composition of mucin that allow molecular discriminatory potential are also used by tumour cells in order to

enhance their survival and growth. This protection and barrier trait is believed to allow tumour cells to be shielded from toxic compounds, thus enabling them to be resistant towards acids, chemotherapy agents and cytotoxic compounds. This could explain the reason AdCC is reported to have a low response towards chemotherapy (Rentchler et al., 1977).

Mucin layer could also capture biologically active molecules within the matrix such as growth factors and cytokines that may affect the growth of a tumour. This may indirectly influence the regulation and interaction of the immune system, inflammatory response and stromal cells within the tumour (Hollingsworth & Swanson, 2004). Differentiation and proliferation of tumour cells are governed by cell surface mucin via the process of morphogenetic signal transduction and ligand-receptor interaction. It has been established that overexpression of mucin leads to signalling interaction through the mitogen-activated protein kinase (MAPK) pathway (Zrihan-Licht, Baruch, Elroy-Stein, Keydar, & Wreschner, 1994). This pathway is involved in response to external growth factors including the growth factors, cellular microenvironment and differentiation factors (Zrihan-Licht et al., 1994). Mucin has also been described to be involved in the metastasis of the tumour. This requires a molecular process that regulates anti-adhesion and adhesion effects in which mucin is involved. In adenocarcinomas, mucin is thought to contribute to the cells invasiveness and metastatic activity via the adhesive and anti-adhesive cell-surface properties of tumour cells (Spicer, Rowse, Lidner, & Gendler, 2005). Other than that, it may also block proteolytic activity and has the potential of autocatalytic proteolytic activity (Hollingsworth & Swanson, 2004).

Furthermore, mucin has been found to affect the immune response and inflammation process by directly interacting with cells that mediate this response (Linden & Varki, 2000). It has also been proven that tumour cells display several oligosaccharides that

bind to leucocyte. Therefore, this indirectly will affect the modulation of leucocyte activity such as adhesion, extravasation and motility and contribute to the increased survival opportunity of tumour cells (Kannagi, 2002). On another note, tumour cells that produce increased levels of soluble mucin and deposit them in localized areas of tumour or metastatic sites will create an obstacle blocking the action of leucocytes. Mucin has also been described to have the ability to induce apoptosis of activated T-cell (Hanson et al., 2001) and immunosuppressive effects on T-cell proliferation (Agrawal, Krantz, Reddish, & Longenecker, 1998).

2.8 Salivary Mucin

Secretory cells have varied morphology and distribution within the oral cavity. In the oral cavity, secretory cells of the salivary glands secrete mucin and its quality and quantity vary within its normal state and neoplastic counterpart (Sushma Naag & Adi, 2010). In an early study, mucin was demonstrated as the main component in saliva composed of oligosaccharides and glycoproteins attached to carbohydrates and amino acids (Ganga, 2003). This study was initiated on the basis that there would be a difference in the histochemical properties of salivary mucins in different mammalian glands and also in human salivary glands. This histochemical property is of utter importance in the investigation between normal and diseased conditions of these glands.

Mucins may present as a single type in a single tissue unit or a mixture of many different types. Histochemically they are divided into epithelial and connective tissue mucin. Epithelial cell components secrete neutral and acidic mucins while the mucins from connective tissue cells show more of acid mucosubstances such as chondroitin

sulphates, keratin sulphates, hyaluronic acids and also dermatan sulphates (Sushma Naag & Adi, 2010) as shown in Figure 2. Epithelial mucin is referred to as mucosubstances while connective tissue mucin is referred to as mucopolysaccharides. Epithelial mucins can be classified into neutral and acidic mucins. Acidic mucins are further categorized into sialomucin and sulfomucin as described by many authors (Totty, 2002). This is dependent on the presence of terminal sialic acid or sulphate groups of the oligosaccharide chain (Filipe & Branfoot, 1974).

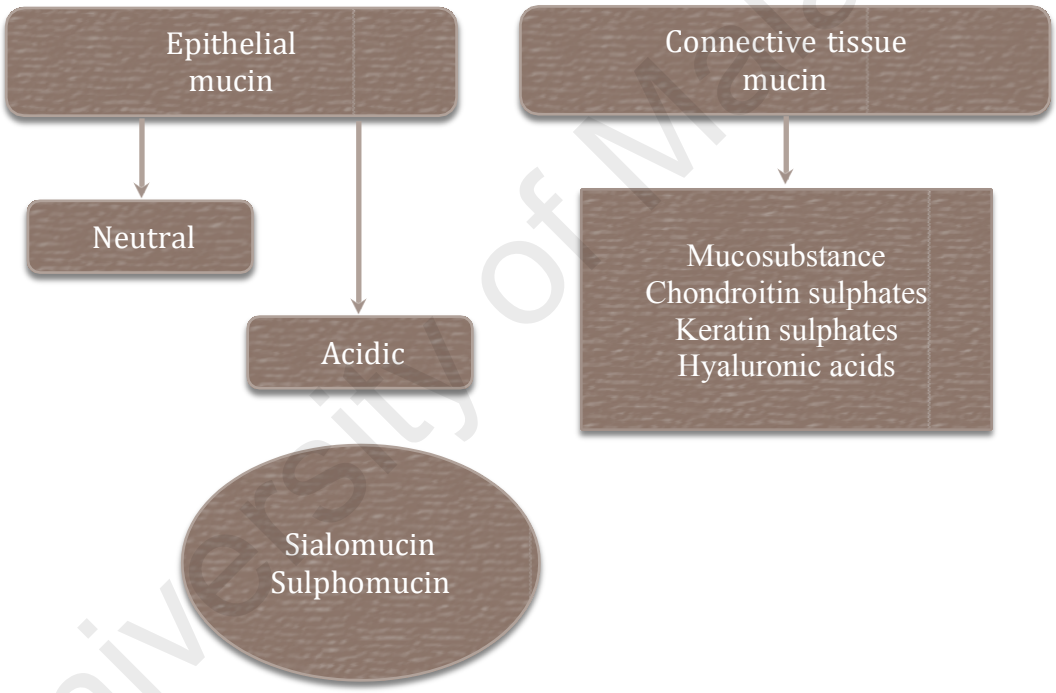

Figure 2-2 : Diagrammatic representation of the types of salivary mucin
Source: Modified from various sources

Totty (2002) showed evidence that mucin synthesis begins in the rough endoplasmic reticulum and completed in the Golgi apparatus. Mucin carbohydrate composition accounts for 90% of its molecular weight. This so-called dense 'sugar coating' allows them satisfactory water retention capacity and enables them to be resistant towards proteolysis thus contributing to its enhancement as a mucosal barrier. The

polysaccharide chains also vary from neutral to weakly and strong acidic mucins (Bancroft, Stevens, & Turner, 1990).

Neutral mucins are composed of hexosamines and hexose unit without a free acidic group. There is an elevated amount of uncharged monosaccharides such as mannose, galactose and galactosamine. It is found in higher concentration within gastric mucosa surface.

Mucin varies chemically and the composition is determined by the type of cells origin. For example, sialomucins are formed of terminal sialic acid molecules on the oligosaccharide chain of polypeptide and they contain neuraminic acid derivatives (**Figure 2.3**). They are found in mucous glands such as the bronchial submucous glands and goblet cells of the salivary glands (Habib et al., 1986b). Meanwhile, sulfomucin consists of sulphate esters linked to hexosamines such as glucosamine. Sialomucins are the simplest forms and are present in small and large bowel. On the other hand, sulfomucin are more complex and are only present in large bowels (Subbuswamy, 1971). Masson and colleagues also demonstrated that sialomucin is essential for its antiviral and antibacterial property as it contains secretory immunoglobulin A (IgA) and is important within the various digestive system. Sulfomucin was described as having anti-

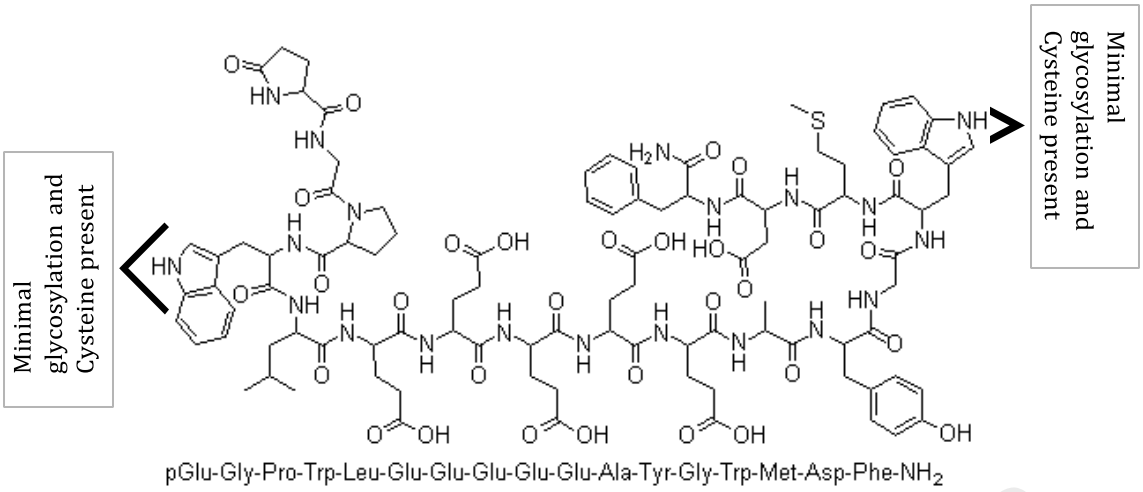

Figure 2-3 : Basic mucin structure

Mucin contains a centre of 10-80 repeating sequence of amino acids and at both terminal ends marked < > are where there is minimal glycosylation occuring and presence of cysteine that is important to prevent mucins from binding together.
Source: Adopted from Chemnet.com and modified

2.9 Mucin Staining

Mucin can be stained with different single stains and also a combination of stains thus enabling them to be classified respectively. Both epithelial and connective tissue mucins can be stained for identification. Stains available for mucin include Periodic Acid Shiff (PAS), Alcian blue (AB), Mucicarmine (MC), High Iron Diamine (HID), Aldehyde Fuchsin (AF), Hale Colloidal Iron and Toluidine blue staining. Epithelial neutral mucin can be stained with PAS while epithelial acidic mucin can be stained with PAS, MC and AB. For the distinction between the two types of acidic mucin, sialomucin can be stained with PAS, MC, AB (pH2.5) and hale colloidal iron while sulfomucin can be stained with AB (pH0.5), AF and HID. Connective tissue mucin can be highlighted with AB and Hale colloidal iron (Rekthman & Bishop, 2011). A summary is shown in **Figure 2.4**.

Leepi et al described the method to identify intracellular sulfomucin with the usage of AB at low pH and HID (Spicer, Hanson, & Floravanti, 1967). A comparison of the reliability of methods was done to evaluate mucin composition within lung adenocarcinomas using Mucicarmine (MC), PAS, AB-PAS and AB-AF in resected specimens. They found that Mowry AB-PAS combination showed the greatest proportion of positive results and together with AB-AF, enabled the appreciation of both acidic mucins; sialomucin and sulphated mucin (Culling, Reid, Worth, & Dunn, 1977).

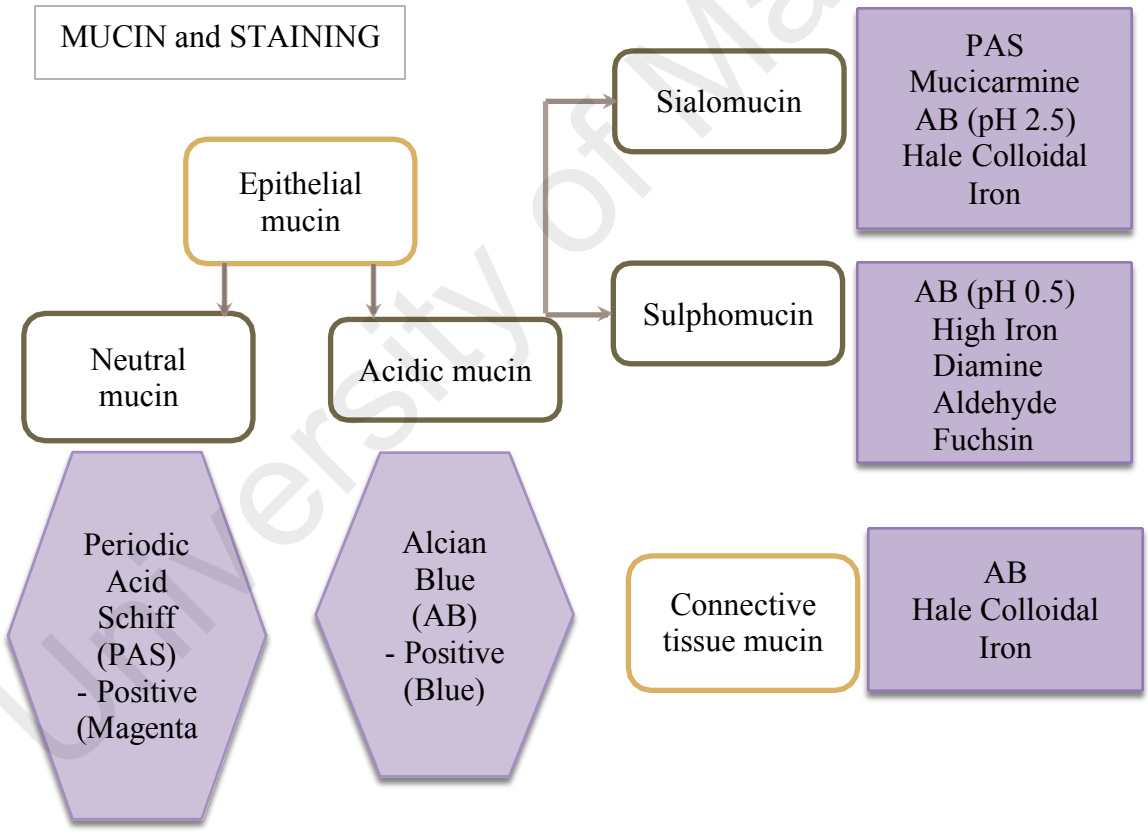

Figure 2-4 : Diagrammatic representation of mucins and their corresponding stains
Source: Modified from various sources

Furthermore, it was demonstrated that in tumours of colon, lungs and ovaries, AB/PAS had a better contrast for identification of neutral and acidic mucin as compared to HID/AB for sialomucin and sulfomucin (Ullah, 2012). Within salivary gland tumours, the presence of mucin was highlighted with PAS, AB, MC and AF. In ACC of salivary gland tumours, the pseudocystic spaces demonstrated a strong to moderate staining with AF (Sushma Naag & Adi, 2010).

Hence, for this study we will be using a combination of PAS, AB and AF stains to achieve better contrast. PAS/AB stain will be used to identify neutral and acidic mucin, while AB/AF will be used for identification of acidic mucin. AB at pH 2.5 is specific to highlight the presence of sialomucin while AF is specific for traces of sulfomucin.

2.10 Mucin in Normal Salivary Glands

Our salivary glands, as mentioned, are divided into major and minor salivary glands. Rohini, Avinash, Rajesh, & Umarji (2014) have shown that within the parotid glands there is a predominance of neutral mucin and only traces of sialomucin and sulfomucin. Thus, it would stain intensely with PAS with the negative areas of staining may be due to the presence of enzymes and traces of acidic mucin. There would also be a minimal reduction in the intensity of the magenta colour after diastase digestion. The predominance of neutral mucin within the parotid gland also indicates that it is rich in enzymes (Rohini, Avinash, Rajesh, & Umarji, 2014). Serous acini in parotid glands would also show strong positivity to AB with pH 2.5 compared to AB pH1 and AF. This again highlights that there are the presence of both neutral and acidic mucin but sulfomucin would only be in traces (Rohini et al., 2014).

For submandibular and sublingual glands, it was demonstrated that there is a mixture of both neutral and acidic mucin. This is due to the heterogeneity nature of the acini (Hamada et al., 2004). However, Yarington et al (1972) showed that the submandibular gland is composed of stronger acidic mucosubstances compared to the sublingual gland. It has also been shown that sulfomucin within submandibular gland is increased within the 3rd trimester and resembles mucin staining as in premalignant transformation. Thus, this may aid in the early detection of cancer (Ganga, 2003).

Besides that, Yarington and colleagues also successfully demonstrated that there is a difference in the secretory capacity between the parotid gland as they progress from normal to pathologic state. Other than that, it was also demonstrated that the secretion is more acidic at the periphery of the salivary gland acini (Yarington & Omaha, 1972). On another note, staining highlighted within the ducts is due to presence of goblet cells or other secretory apparatus within the ductal epithelium whilst staining at the periphery of the acinus are considered as cellular staining (Yarington & Omaha, 1972).

2.11 Mucin in Various Tumours

Mucins are high molecular weight glycoproteins that are produced by glandular epithelial cells. Multiple comparative studies were done to study the amount of mucin within inflammatory, premalignant and malignant states in various areas of the body such as the bronchial glands (Lamb & Reid, 1970) intestinal mucosa (Subbuswamy, 1971), gaster (Mandal, Chakrabarti, Ray, Chattopadhyay, & Das, 2013), colorectal (Ionila, Margaritescu, Pirici, & Mogoanta, 2011) and large intestine (Filipe & Branfoot, 1974). In large polyps and well-differentiated carcinomas of the colon, sulfomucin was found to be increased whilst sialomucin can be found in moderately differentiated and

undifferentiated carcinomas (Gad, 1969).

In gastrointestinal tumours, tumour grading was done based on the mucin production (Huszno et al., 2012). Huszno et al. showed that malignant tumours had a predominance of sulfomucin, however as the differentiation progressed sulphate mucin decreased and neutral mucin increased. Well-differentiated carcinoma reportedly produces more sialomucin as compared to sulfomucin and poorly differentiated carcinoma releases higher amounts of neutral mucin (Gad, 1982). Another study showed that PAS-D globules were useful in differentiating benign and malignant breast carcinoma (Panicker, Jariwala, Buch, & Joshi, 2012). In colorectal carcinoma, the mucinous variants showed a strong predominance of acidic mucin and a major increase of neutral mucin compared to its normal counterparts as the tumour progressed (Jain, Mondal, Sinha, Mukhopadhyay, & Chakraborty, 2014).

Filipe et al, 1974 also suggested that there is a direct relationship between the extent of mucinous change with the invasiveness of carcinoma within the large intestine. It was shown by a marked increase of sialomucins noted in the more extensive tumour (Filipe & Branfoot, 1974). In the prostate gland, it was also shown that there was an increase in acidic mucin within well-differentiated adenocarcinomas. This study also successfully demonstrated that AB (pH2.5) staining technique is useful in differentiating benign prostate hyperplasia with well-differentiated prostate adenocarcinoma especially in questionable malignant cases (Agrawal, Deshpande, Sudhamani, & Zawar, 2014).

2.12 Mucin in Salivary Gland Tumours

The role of mucin histochemistry in identifying mucin-producing tumours has long been established (Cook, 1982). It has been proven to be useful in identifying intestinal metaplasia based on the mucin components. As metaplasia may have potential malignant changes, it allows screening of patients for early detection of cancer (Subbuswamy, 1971). Therefore, this study is to explore mucin histochemistry within various salivary gland tumours.

In salivary gland tumours, it has been shown that there was a predominance of sialomucin within benign tumours and a combination with sulfomucin in the malignant types. This is shown in PA whereby there is a predominance of sialomucin and neutral mucin (Sushma Naag & Adi, 2010). Meanwhile, higher traces of sialomucin and sulfomucin were found in MEC of salivary glands compared to MEC of the oesophagus (Lam, Loke, & Ma, 1993). In AdCC was also shown more presence of sialomucin and focal areas of neutral mucin (Toida et al., 1985). Another study demonstrated that the presence of periodic acid Schiff diastase (PAS-D) positive granules was useful in differentiating various salivary gland neoplasms (Panicker et al., 2012).

CHAPTER 3 : METHODOLOGY

3.1 Study design

This is a descriptive histochemical study to investigate expression of salivary mucins within salivary gland tumours. This study has been approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya (DF OS1911/0044(P)) and the Medical Research Ethics Committee, University Malaya Medical Centre (20200622-8793). This work was financed by Research University Grant, Faculty of Dentistry, University of Malaya (GPF008E-2019).

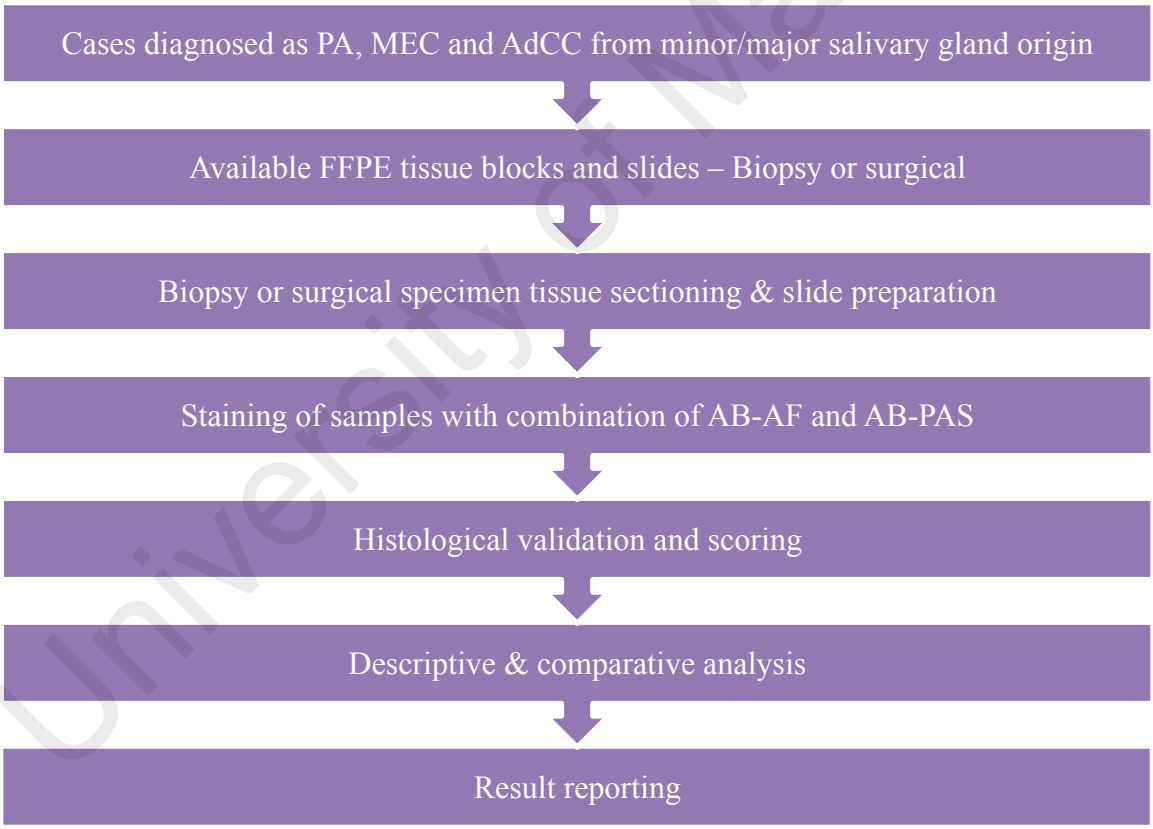

Figure 3.1: Flow chart showing important stages in the research

3.2 Materials

3.2.1 Samples

The samples were sourced from the archives of Oral Pathology Research and Diagnostic Laboratory, Department of Oral and Maxillofacial Clinical Science, Faculty of Dentistry, University of Malaya and the Archive of Anatomical Pathology Division of Department of Pathology, University Malaya Medical Centre between the years of 1995 and 2019. Selected cases were diagnosed based on the World Health Organisation Histological Classification of Tumors of Salivary Glands 2017 (Seethala & Stenman, 2017). A total of 76 formalin-fixed embedded (FFPE) blocks comprising of 32 cases of pleomorphic adenoma (PA), 28 cases of mucoepidermoid carcinoma (MEC) and 16 cases of adenoid cystic carcinoma (AdCC) were retrieved after examination of the haematoxylin and eosin (H&E) sections. Control group tissues were obtained from intraoral mucocele cases (normal salivary tissue only) and normal submandibular glands. Normal submandibular glands were obtained from surgical specimens with neck dissections.

Sample size calculation was done using G*Power software, version 3.1.9.4 for this study. Based on the effect size of 0.25 and power of 0.86, the estimated sample size was 32 for each group. Unfortunately, 5 PA cases, 9 MEC cases and 7 AdCC cases from minor salivary glands had to be replaced because initial selected blocks were eaten by rats. After staining, 1 MEC case from minor salivary gland had to be discarded due to insufficient tumour tissue. Two more cases (1 PA and 1 AdCC) from major salivary gland were rejected as the wrong tissue blocks were provided. At final count, the sample collection was as tabled in **Table 3.1**.

Table 3.1: Details of sample collection

Sample groups	Tissue origin		Total
	Major salivary gland	Minor salivary gland	
PA	15	16	31
MEC	12	15	27
AdCC	3	12	15
Control (Normal salivary gland)	3	3	6
Total	33	46	79

Inclusion criteria:

1. Samples that were diagnosed histologically as PA, MEC and AdCC
2. Availability of tumour tissue paraffin block
3. Adequate intratumoral and invasive front of tumour tissue

Exclusion criteria :

1. Salivary gland tumour tissue samples from patients without adequate clinical and follow-up data.
2. Insufficient intratumoral and invasive front of tumour tissue

3.2.2 Staining

Three mucin stains; Periodic acid Schiff (PAS), alcian blue pH 2.5 (AB) and aldehyde fuschin (AF) were identified for the histochemical staining (refer **Table 3.2**).

Table 3.2 : Staining reagents/kits used in this study

Staining reagents/kits	Source
Sigma Aldrich Schiffs Fuschin sulfite reagent suitable for detection of glycoproteins- 500ml	Lab Chem Sdn Bhd
Periodic acid for analysis, Merck	Lab Chem Sdn Bhd
Alcian blue (pH2.5) 100test/kit Mfr: Hermburg Germany	Premier Diagnostics
Gomori's paraldehyde fuschins 1 kit (100 tests)	Premier Diagnostics
Hematoxylin	Premier Diagnostics

3.3 Methods

3.3.1 Haematoxylin and eosin staining procedure

Five micron thickness of sections were prepared from the retrieved FFPE blocks to be stained by routine H&E (refer to Appendix 1 for details).

3.3.2 Histochemistry staining procedure

Histochemical staining was done on 5µm-thick sections mounted on poly-L-slides (Superfrost Plus, Thermofisher). PAS, AB (pH2.5) and AF stainings were performed for each sample. The histochemical staining procedures are described in Appendix 2 and 3.

Table 3.3 : Histochemical staining interpretation

Stains	Colour/Interpretation
Periodic acid Schiff	Magenta : Neutral mucin
Alcian Blue pH 2.5	Blue : Sialomucin (acidic mucin)
Aldehyde Fuschin	Purple : Sulfomucin (acidic mucin)

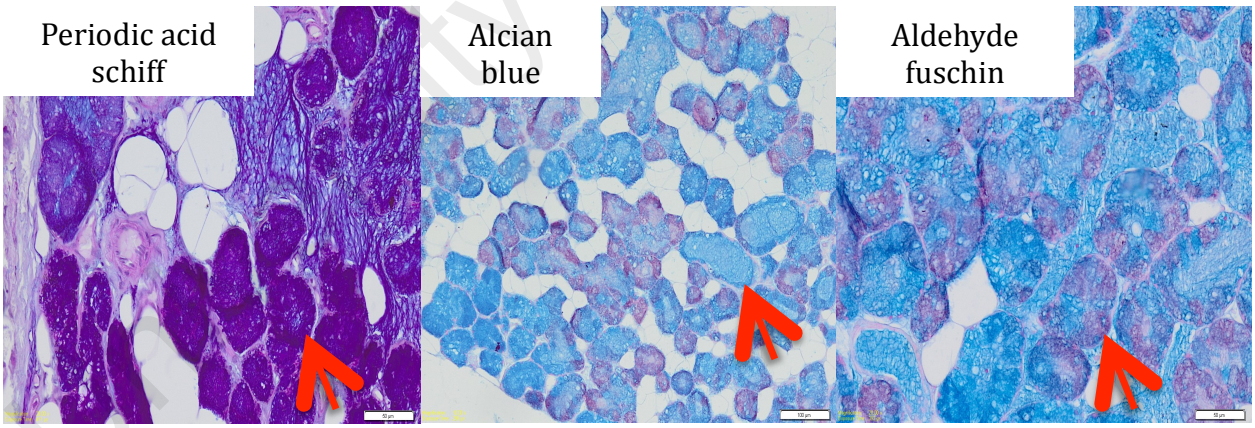

Figure 3.2: Photomicrographs of control tissue with respective stainings

3.4 Histochemistry assessment

3.4.1 Descriptive / Qualitative

The localizations of AB, PAS, and AF were examined under virtual microscope. The distribution of the stains within the tumour microenvironment were assessed within the ductal lumens and stromal components. The findings were recorded in the format of (+) indicating presence of stain and (-) indicating absence of stain as in **Table 3.4**.

Table 3.4: Template to record presence of stains within ductal and stromal components of samples

	PA		MEC		AdCC	
AB	Ductal +/-	Stromal +/-	Ductal +/-	Stromal +/-	Ductal +/-	Stromal +/-
AF	Ductal +/-	Stromal +/-	Ductal +/-	Stromal +/-	Ductal +/-	Stromal +/-
PAS	Ductal +/-	Stromal +/-	Ductal +/-	Stromal +/-	Ductal +/-	Stromal +/-

3.4.2 Semi-quantitative

A semi-quantitative approach was then used to further evaluate expression of AB, PAS and AF in ductal lumens of all samples. For each sample, the entire surface area of the tumour microenvironment was examined under 20X to 100X magnifications to assess percentage of stains within lumens of ductal structures. However areas with intense inflammation and necrosis were avoided due to possibility of inaccurate scoring. The extent of staining was categorized as in **Table 3.5**.

Table 3.5 : Positivity of surface area

Score	Presence of staining
0	<25%
1	25-50%
2	50-75%
3	>75%

3.4.3 Calibrations

The calibration performed in this study were intra-examiner calibration and inter-examiner calibration. Intra-examiner calibration was carried out individually by analyzing 10 cases of AB-PAS staining and 10 cases of AB-AF staining within the time frame of one week between first and second data scoring. Inter-examiner calibration was performed between 2 supervisors and 1 trainee specialist during which 10 cases of AB-PAS staining and 10 cases of AB-AF staining were randomly examined. In the event of great discrepancy, a consensus is achieved via discussion among all examiners. Kappa values for each stain are as shown in **Table 3.6**.

Table 3.6 : Kappa values for PAS, AB and AF stains

Stain	Kappa
PAS	0.89-1
AB	0.75
AF	0.75

3.4.4 Result analysis

The result analysis was performed using IBM Statistical Package for Social Sciences (SPSS) version 26. Expression of AB, PAS and AF were subjected to Chi-square test. Comparative analysis was performed using Chi-square test and Fisher's exact where applicable, for mucin expression (AB, PAS and AF) in relation to minor or major

salivary gland tumours and benign or malignant salivary gland tumours. Scores of 0-1 were further collapsed into "low" and scores of 2-3 into "high" to fulfill requirements for Chi-square statistical analysis. Attempts to determine cut-off values with receiver operating characteristic (ROC) curve were unsuccessful as values acquired did not meet the required standards. Association between mucin expression (AB, PAS and AF) and clinical parameters were examined using Chi-square test. For all the statistical analyses, *P* Value <0.05 was indicates statistical significance.

CHAPTER 4 : RESULTS

4.1 Demographics of minor and major salivary gland tumours

Demographic presentation of salivary gland tumours in this study is shown in **Table 4.1.** All 3 types of tumours of either origin showed a wide range of age presentation ranging from 15 to 81 years of age. Benign tumours like PA occurred significantly more often in younger patients while malignant tumours often occurred among elderly patients. A female predilection was seen in all salivary gland tumours. In terms of ethnicity, Chinese were generally most affected in the occurrence of all salivary gland tumours of either origin.

Among minor salivary gland tumours, the palatal region was a common site for PAs and MECs. On the other hand, major salivary gland tumours with the exception of AdCCs, showed significant involvement of the parotid region compared to other reported sites. Other reported sites for minor salivary gland tumours included the buccal mucosa (BM), lip, floor of mouth (FOM), auricular region, submandibular gland, antrum and alveolar ridge region. Other sites involved in major salivary gland tumours included the infra-auricular area, submandibular gland, neck, upper gums and parapharyngeal region.

Table 4.1 : Demographic data of salivary gland tumours in this study

	PA n=31	MEC n=27	AdCC n=15	*P* value[c]
Age*				
<47 years, n(%)	23 (74)	9 (33)	3 (20)	[γ]**0.000**
>47 years, n(%)	8 (26)	18 (67)	12 (80)	
Gender				
Female, n(%)	23 (74)	15 (56)	9 (60)	0.310
Male, n(%)	8 (26)	12 (44)	6 (40)	
Ethnicity				
Chinese, n(%)	17 (55)	13 (48)	8 (53)	0.618
Malay, n(%)	8 (25)	11 (41)	7 (47)	
Indian, n(%)	6 (20)	3 (11)	-	
Site				
[α]Palate, n(%)	11 (36)	7 (26)	2 (13)	0.281
[α]Non-palate, n(%)	20 (64)	20 (74)	13 (87)	
[β]Parotid, n(%)	22 (71)	11 (41)	2 (13)	[δ]**0.000**
[β]Non-parotid, n(%)	9 (29)	16 (59)	13 (87)	

Significance level p=0.05 c= Pearson Chi-square test

*Median age of 47 years was used as cut-off point for age binary measurement

[α] Sites within minor salivary glands

[β] Sites within major salivary glands

[γ] Pearson Chi-square test for age showed significant difference between PA and MEC (p=0.003) and between PA and AdCC (p=0.001) but no significant difference between MEC and AdCC (p=0.611). Post-hoc analysis (Binary logistic regression) for MEC and AdCC occurred 6 times and 12 times higher in age >47 compared to PA (p=0.037).

[δ] Pearson Chi-square test for parotid site showed a significant difference between MEC and AdCC (p=0.037). Post-hoc analysis (Binary logistic regression) for MEC occurred 0.09 times higher in parotid compared to AdCC (p=0.035).

4.2 Mucin in normal minor and major salivary glands

Minor salivary glands are composed predominantly of mucous acini and when stained with AB-PAS, showed a dominance of AB-positive regions with traces of PAS present. When stained with AB-AF, a dominance of AB-positive regions were also observed over AF. These findings are illustrated in **Figure 4.1**.

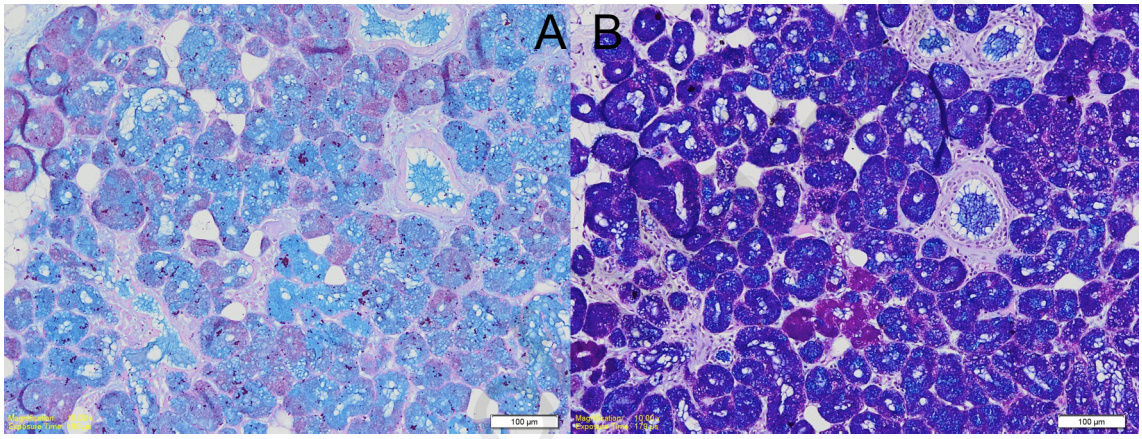

Figure 4.1 : Photomicrograph shows staining in normal minor salivary gland. (A) Staining with AB-AF. (B) Staining with AB-PAS. (Magnification x100)

Major glands, however, can be composed of either serous acini, mucous acini or both. In our study, the submandibular gland was used as our normal control for major glands. When stained with AB-PAS, the acini showed a predominance of PAS-positive regions with focal areas of AB-positive. On the other hand, AB-AF showed a uniform distribution of both stainings. The findings are illustrated in **Figure 4.2**.

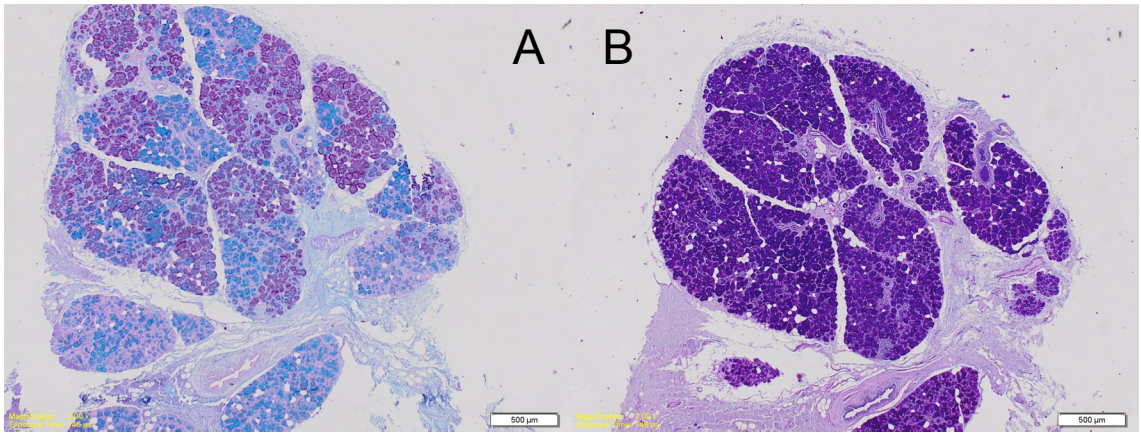

Figure 4.2 : Photomicrograph shows staining in normal major salivary gland. (A) Staining with AB-AF. (B) Staining with AB-PAS. (Magnification x 20)

4.3 Mucin in minor salivary gland tumours

Table 4.2 : Mucin expression within luminal structures of PA, MEC and AdCC of minor glands

Stain	AB		AF		PAS	
	<49%	>50%	<49%	>50%	<49%	>50%
PA (n=16)	13 (81)	3 (19)	15 (94)	1 (6)	15 (94)	1 (6)
MEC (n=15)	8 (53)	7 (47)	15 (100)	-	12 (80)	3 (20)
AdCC (n=12)	8 (67)	4 (34)	12 (100)	-	12 (100)	-

*<49% of expression are considered low expression and >/=50% are considered high expression

All 3 types of mucins were present in PA, MEC & AdCC but in variable amounts. Histochemical staining of PA sections showed presence of both neutral and acidic mucins within the luminal components. However, higher expression of AB within these areas (**Table 4.2**) indicated high amounts of sialomucin in the ductal structures (**Figure 4.3 A&B**). The myoepithelial cells were negative to all staining.

MEC frequently showed higher expression of AB within the lumens (**Table 4.2**). AB was strongly highlighted within the lumens while the epidermoid cells were negative to all staining (**Figure 4.3 C&D**). This showed a complete dominance of acidic mucin, specifically sialomucin within this malignant salivary neoplasm.

In AdCC sections, most of the luminal spaces were dominantly AB-positive (**Table 4.2**), consistent with an intense presence of sialomucin as shown in **Figure 4.3 E&F**. AF and PAS were still present in trace amounts. Therefore, this showed a predominance of sialomucin with trace amounts of neutral and sulfomucin for this tumour.

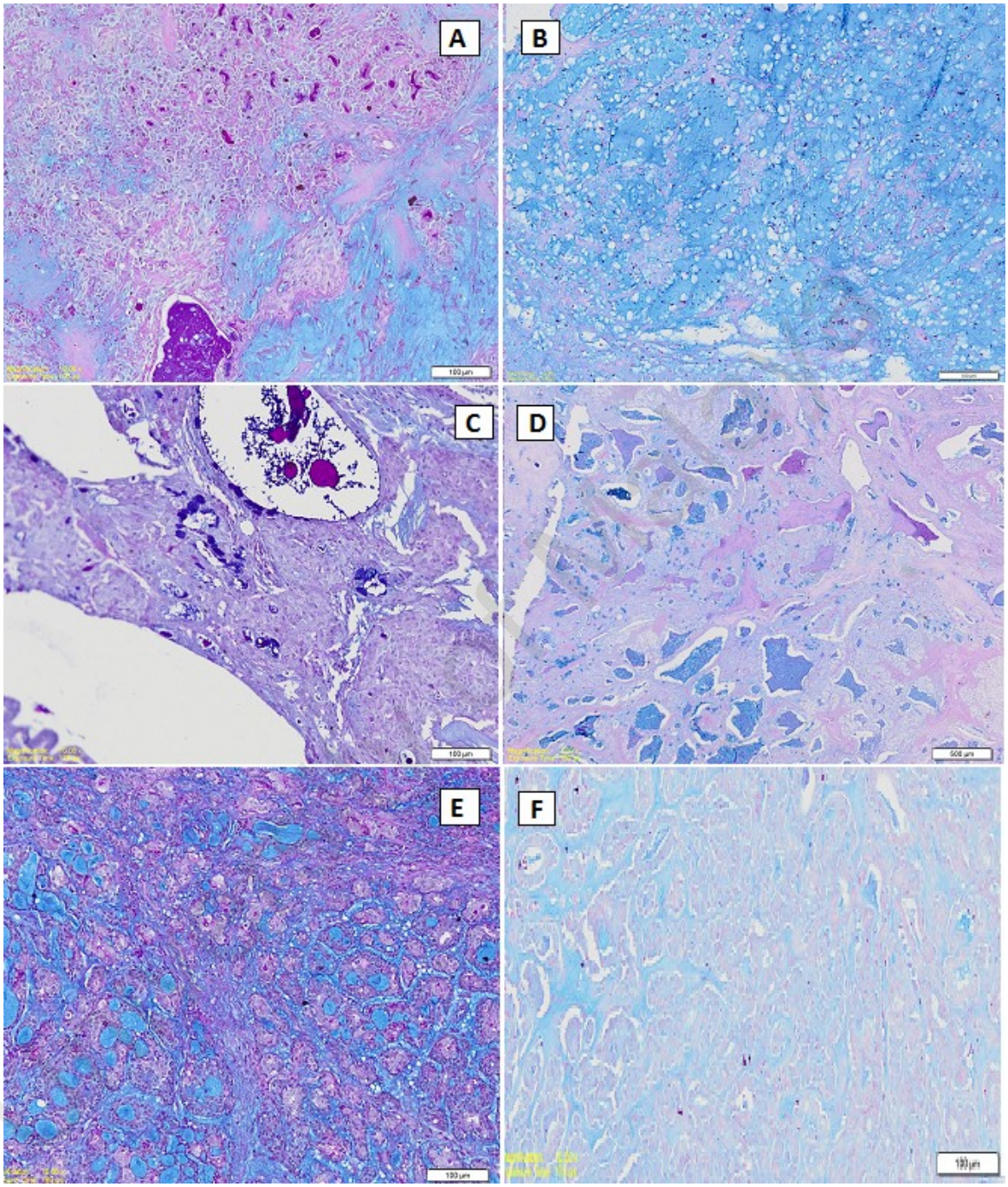

Figure 4.3 : Photomicrograph shows staining in PA (A & B), MEC (C & D) and AdCC (E & F) of minor salivary gland. (A) Chondromyxoid areas strongly AB positive with AB-PAS staining (B) Predominance of AB staining within the lumens with AB-AF staining. (C) AB and PAS presence within lumen and mucous cells with AB-PAS staining. (D) Dominance of AB within lumen and stromal with AB-AF staining. (E) Dominance of AB within the pseudocystic spaces with AB-PAS staining. (F) Dominance of AB with trace amounts of AF observed within the pseudocystic spaces with AB-AF staining. [Magnification X100]

Table 4.3 : Mucin expression within stromal components of PA,MEC and AdCC in minor glands

Stain	PA (n=16)		MEC (n=15)		AdCC (n=12)
	AB	PAS	AB	PAS	
AB-PAS (n,%)	13(81)	-	7(47)	3(20)	-
	AB	AF	AB	AF	
AB-AF (n,%)	13(81)	-	13(87)	-	-

The stromal components of PA and MEC exhibited more expression of AB compared to AF and PAS. This suggests a plentiful amount of acid mucopolysaccharide presence. For PA, the positive stromal areas were usually the chondromyxoid areas. Hyalinized areas were usually highlighted by PAS stains. Our samples did not show other variations of stroma usually present in PA. Stromal components in AdCCs did not express any positive staining for AB, AF and PAS. (**Table 4.3**).

4.4 Mucin in major salivary gland tumours

Table 4.4 : Mucin expression within luminal structures of PA,MEC and ADCC of major glands

Stain	AB		AF		PAS	
	<49%	>50%	<49%	>50%	<49%	>50%
PA (n=15)	2 (14)	13 (86)	15 (100)	-	15 (100)	-
MEC (n=12)	8 (66)	4 (34)	12 (100)	-	9 (75)	3 (25)
AdCC (n=3)	1 (33)	2 (67)	3 (100)	-	3 (100)	-

*<49% of expression are considered low expression and >/=50% are considered high expression

In major salivary gland tumours, all the PAs, MECs & AdCCs demonstrated presence of neutral mucin. Histochemical staining in PA sections showed all mucin components within the lumens but the majority was AB-positive, indicating greater presence of sialomucin (**Figure 4.4 A&B**). In MEC sections, there was greater expression of AB and PAS within the luminal structures compared to AF (**Figure 4.4 C&D**). In AdCC sections of major glands, all mucin components were present but the luminal spaces showed affinity for AB than AF and PAS (**Figure 4.4 E&F**). This indicated a high volume of sialomucin presence with trace amounts of neutral mucin and sulfomucin. **Table 4.4** summarizes these findings.

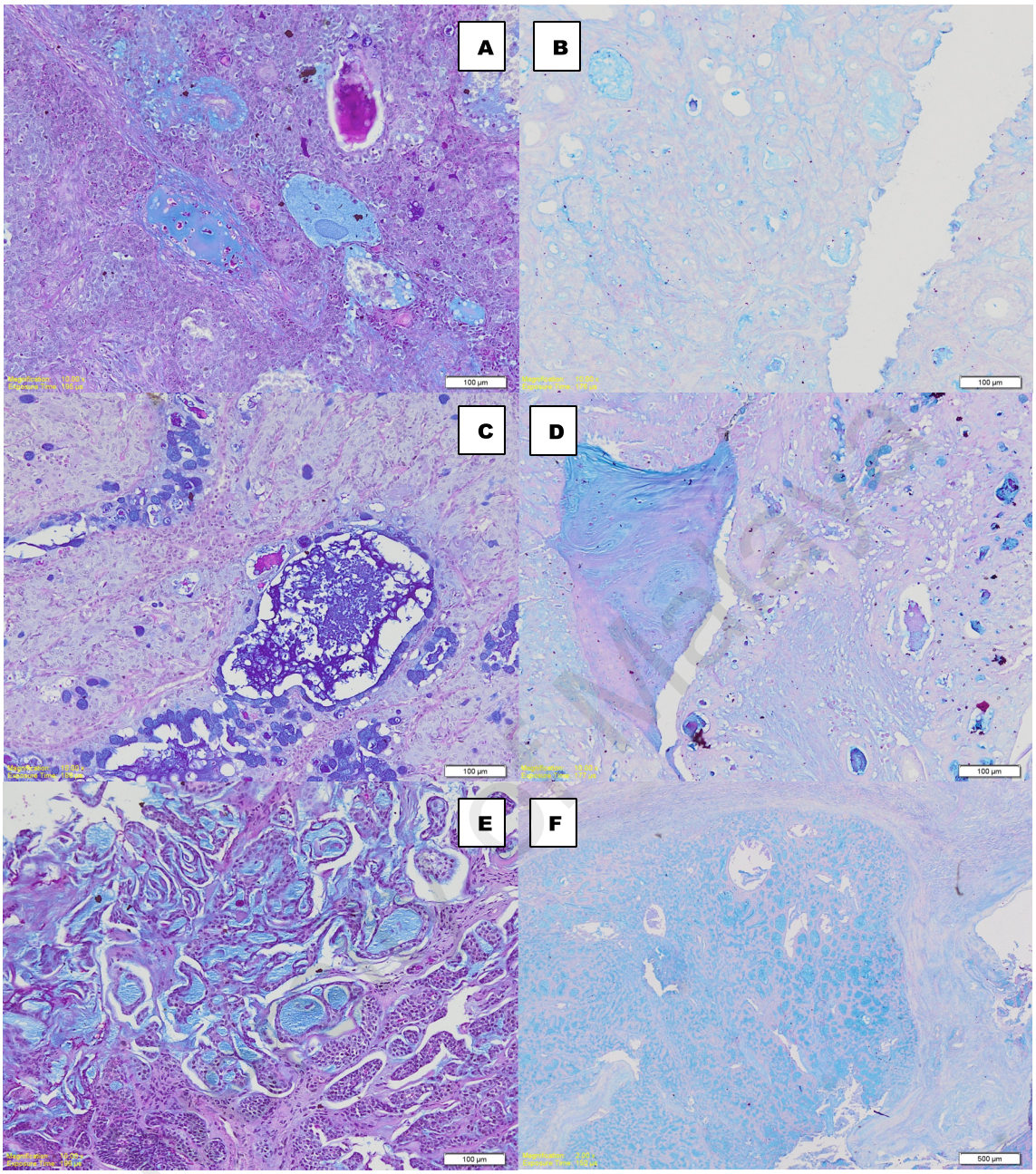

Figure 4.4 : Photomicrograph shows staining in PA (A & B), MEC (C & D) and AdCC (E & F) of major salivary gland. (A) Majority of luminal areas were AB-positive with AB-PAS staining. (B) Dominance of AB within luminal areas with AB-AF staining. (C) Luminal areas showing PAS-positivity with AB-PAS staining. (D) Dominance of AB within luminal and stromal areas with AB-AF staining (E) Dominance of AB within pseudocystic spaces with AB-PAS staining. (F) Dominance of AB with traces of AF within pseudocystic spaces in AB-AF staining. [Magnification X100]

Table 4.5 : Mucin expression within stromal components of PA,MEC and AdCC in major glands

Stain	PA (n=15)		MEC (n=12)		AdCC (n=3)
	AB	PAS	AB	PAS	
AB-PAS (n,%)	12(75)	1(6)	3(25)	4(33)	-
	AB	AF	AB	AF	
AB-AF (n,%)	12(75)	-	8(67)	-	-

Similar to minor salivary gland tumours, the stromal components for PA showed a strong dominance of AB within the chondromyxoid areas and PAS-stained hyalinized areas. For MEC, despite the slightly higher expression of PAS with AB-PAS staining, a dominant AB-positivity was noted with AB-AF staining. Therefore, again demonstrating sialomucin predominance with minimal amounts of neutral and sulfomucin presence. Thus, suggesting a high mucopolysaccharide content presence (**Table 4.5**). Stromal components in AdCCs did not express any positive staining for AB, AF and PAS.

4.5 Overview of mucin expressions in all study samples

Table 4.6 : Summary of mucin expressions in luminal spaces

Origin/Tumour	Minor glands	Major glands
Normal	> sialomucin	*Mixture of neutral and acidic mucin
PA	> sialomucin (19%)	> sialomucin (86%)
MEC	> sialomucin (47%) and neutral mucin (20%)	> sialomucin (33%) and neutral mucin (25%)
AdCC	> sialomucin (34%)	> sialomucin (67%)

* Submandibular gland

4.6 Comparative Analyses

A significant difference was observed between the expression of AB within benign and malignant salivary gland tumours (**Table 4.7**). There was greater presence of AB within the benign tumours compared to malignant tumours. **Table 4.9** shows that there will be 3 times more expression of AB within benign salivary gland tumours compared to malignant salivary gland tumours. However, there was no significant difference in AF and PAS expression between benign and malignant salivary gland tumours.

A significant difference between the expression of AB within PA of minor and major salivary gland was also seen (**Table 4.8**). Major PA contains greater amount of sialomucin. **Table 4.9** shows that there was an independent association of AB expression in PA of major salivary gland in which there will be 42 times higher expression of AB within PA of major salivary gland origin compared to PA of minor gland origin. Meanwhile, no significant difference of AB, AF and PAS expression was observed for MEC and AdCC of either origin.

Table 4.7 : Comparison of mucin expression between benign and malignant salivary gland tumours

Stain	Benign n=31		Malignant n=42		*P* Value[c]
	Low	High	Low	High	
AB (n,%)	12(39)	19(61)	29(69)	13(31)	**0.016**
AF (n,%)	31(100)	-	41(98)	1(2)	0.387
PAS (n,%)	28(90)	3(10)	38(90)	4(10)	0.982

Significant level: p=0.05 c: Pearson Chi-square test
Low: <50%, High: ≥50%
Benign – PA, Malignant – MEC & AdCC

Table 4.8 : Comparison of mucin expression between minor and major salivary gland tumours

Stain	PA n=31		*P* Value	MEC n=27		*P* Value	AdCC n=15		*P* Value
AB	Minor	Major		Minor	Major		Major	Major	
Low	13 (81)	2 (13)	[c]**0.000**	8 (53)	8 (67)	[c]0.484	9 (75)	1 (33)	[f]0.242
High	3 (19)	13 (87)		7 (47)	4 (33)		3 (25)	2 (67)	
AF									
Low	15 (94)	15 (100)	[f]1.000	15 (100)	12 (100)	[α] -	12 (100)	3 (100)	[α] -
High	1(6)	-		-	-		-	-	
PAS									
Low	15 (94)	15 (100)	[f]1.000	12 (80)	9 (75)	[c]0.756	12 (100)	3 (100)	[α] -
High	1(6)	-		3 (20)	3 (25)		-	-	

Significant level p=0.05 c: Pearson Chi-square f: Fisher's Exact test
Low: <50%, High: ≥50%

α p-values were not computed as figures did not meet statistical test requirements

Table 4.9: Binary logistic regression showing characteristics, p-values, odds ratio and confidence interval for mucin expression of AB

Characteristics	*P* Value	Hazard ratio	95% Confidence Interval
Benign tumours	***0.028**	3.395	1.140
Major PA	***0.000**	42.25	5.146

*Binary logistic regression shows statistical significance for mucin expression of AB ($p<0.05$)

Table 4.10 : Association of mucin expression within salivary gland tumours with clinical parameters

Stain	AB n(%)		*P* Value[c]	AF n(%)		*P* Value[c]	PAS n(%)		*P* Value[c]
	Low	High		Low	High		Low	High	
Gender									
Female	27 (55)	22 (45)	0.794	48 (98)	1 (2)	0.481	44 (90)	5 (10)	0.799
Male	14 (10)	58 (42)		24 (100)	-		22 (92)	2 (8)	
*Age									
<47	18 (49)	19 (51)	0.190	36 (97)	1 (3)	0.321	32 (87)	5 (13)	0.248
>47	23 (64)	13 (36)		36 (100)	-		34 (96)	2 (6)	
Ethinicity									
Indian	3 (30)	7 (70)	0.188	10 (100)	-	0.400	9 (90)	1 (10)	0.917
Chinese	23 (62)	14 (38)		37 (100)	-		33 (89)	4 (11)	
Malay	15 (58)	11 (42)		25 (96)	1 (4)		24 (92)	2 (8)	
Tumour site									
Parotid	13 (46)	15 (54)	0.410	28 (100)	-	0.332	25 (89)	3 (11)	0.966
Palate	14 (64)	8 (36)		22 (100)	-		20 (91)	2 (9)	

Significant level: p=0.05 c: Pearson Chi-square test

*Median age of 47 years was used as cut-off point for age binary measurement

Statistical analysis with Chi-square tests (Pearson Chi-square test) showed no significant association between mucin expression and clinical parameters such as age, gender, ethnicity and tumour site (**Table 4.10**).

CHAPTER 5 : DISCUSSION

Mucin is a complex molecule rich in protein and polysaccharide components, and bounded by covalent bonds (Hand, Pathmanathanl, & Field, 1999). They are usually present in various mixtures thus, a single application of stain is insufficient for proper identification. Mucin is generally divided into epithelial and connective tissue mucin. Epithelial cell components secrete neutral and acidic mucins while the connective tissue cells express more of acid mucosubstances (Sushma Naag & Adi, 2010). It has been shown that mucin components are altered from normal to pathological state hence, various studies were conducted to delineate its biological characteristics for better classifications (Azzopardi & Smith, 1959; Ganga, 2003).

Histochemistry staining of salivary gland tumours is useful in determining the mucosubstances present within the microenvironment. Even though this histochemistry role was identified by Cook (1982) especially for gastrointestinal tumours, comparable results were expected in salivary gland tumours.

5.1 Demographics of salivary gland tumours

A female predilection was observed among the salivary gland tumours in this study, comparable to other studies whereby a slight female predilection was demonstrated by both benign and malignant salivary gland tumours (Shareef, Abd Rahman, Zainudin, & Pohchi, 2011; Tilakaratne, Jayasooriya, Tennakoon, & Saku, 2009)There were also studies (Ito, Ito, Vargas, de Almeida, & Lopes, 2005) reporting a predilection for males within salivary gland tumours, but our samples only showed this trend in MEC and AdCC of major salivary glands.

A wide age range was seen with PA occurring significantly more often among those less than 47 years of age while MEC and AdCC were more frequently encountered among those above 47 years of age. This age distribution is consistent with the reports of peak incidence for benign salivary gland tumours from 3rd to 6th decade and malignant tumours from 4th to 8th decade of life (Ito et al., 2005). This finding also coincides with earlier studies showing malignant tumours affecting the older age group compared to benign tumours (Eveson & Cawson, 1985; Foote & Frazell, 1953; Pires, Pringle, de Almeida, & Chen, 2007).

Both minor and major salivary gland tumours were seemingly more prevalent among the Chinese population. This is in agreement with a local study (Mustafa, Jalil, Pauline, & Kiong, 2015) which demonstrated a peak among Malaysian Chinese for intraoral salivary gland tumours. However, we did not find any occurrence of AdCC among Indians. Our results differ from another study demonstrating a Malay ethnicity dominance. However, this study was conducted among the Malay-dominant population around the Hospital of University of Science Malaysia (Shareef et al., 2011). Our findings are also inconsistent with findings by Ambu et al. (2014) who reported a Malay majority, followed by Chinese and Indians. This study focused more on salivary gland tumours involving the parotid glands specifically (Ambu, Ramalinggam, & Kaur, 2014).

5.2 Site of salivary gland tumours

For minor salivary gland tumours, the palate has been identified as the most common site with a percentage of 42-75 of cases (Pires et al., 2007). This is reflected in our study as the palate was the most favoured site for PA, MEC and AdCC. This finding agrees with an earlier report by Waldron et al. (Waldron, El-Mofty, & Gnepp, 1988) Tilakaratne et al. (2009) demonstrated that malignant minor salivary gland tumours had a high predilection for the FOM which was the second most common site in our study. They also reported that benign minor salivary gland tumours were common on the lip region, which was a commonly implicated site in our study (Tilakaratne et al., 2009).

For major salivary glands, benign tumours are found frequently in the parotid gland (Eveson & Cawson, 1985; Spiro, 1986). Malignant tumours tend to affect the parotid glands, followed by minor salivary glands and submandibular glands in decreasing order. (Eveson & Cawson, 1985; Ito et al., 2005). In our study, benign and malignant major salivary gland tumours were significantly more common in the parotid glands. We had no salivary gland tumours arising from the sublingual gland. This is in concordance with Ito et al. (2005) who reported parotid gland as the most common site and sublingual gland as a rarely involved site.

5.3 Mucin profile in normal minor salivary glands

We observed the presence of a majority of mucinous acini in minor salivary glands, which is in concordance with Hand et al. (1999) who reported substantial secretory cells with a flattened nucleus against the basal lamina and central cytoplasm that were filled with pale granules less likely picked up by H&E staining. Our normal minor salivary gland samples showed a high content of acidic mucin and only traces of neutral mucin. This is consistent with the presence of mucinous acini prevalent in minor salivary glands which usually contain acidic mucin. (Ganga, 2003; Quintarelli & Robinson, 1961)

Minor salivary glands also contain serous and seromucous components, usually manifesting as demilunes capping the mucous acini ends or tubules. However, individual serous cells may be observed within mucous acini but infrequently (Hand et al., 1999). It was also demonstrated that different sites displayed different compositions of minor salivary gland acini. The labial glands were found to be lacking in serous demilunes but instead contained light secretory granules with a serous-like configuration (Tandler, Denning, Mandel, & Kutscher, 1970). Similarly, the labial glands in our study had predominantly mucous acini with a focal presence of the light secretory granules. However, the carbohydrate compositions between these cells are different. (Eversole, 1972) The serous demilunes are present in other sites including the buccal, palatal, minor sublingual glands and anterior and posterior lingual glands. (Tandler, Pinkstaff, & Riva, 1995) The present study also noted the presence of serous demilunes at the periphery of the mucous acini in minor salivary glands situated on the palate, FOM, BM and alveolar ridge. However, minor glands occurring on the angle of the mandible and auricular region were found to be composed mainly of serous acini.

5.4 Mucin profile in normal major salivary glands

Major salivary glands refer to the parotid glands, submandibular glands and sublingual glands. Microscopically, the parotid gland is composed of a mixture of tubular and acini gland structures with lobules separated by septae. It is made up of 95% serous acini and 5% mucous acini (Rohini et al., 2014). The submandibular gland is also a compound tubuloacinar gland containing both serous and mucous acini with serous cells dominating (Dhabale, 2014). The sublingual gland predominantly comprises mucous tissue but is also regarded as a seromucous gland. Histologically it is unencapsulated and contains a mixture of both seromucous acini (Adams et al., 2013; Hamada et al., 2004). The submandibular gland reportedly demonstrated more composition of acidic mucosubstances compared to sublingual glands (Yarington & Omaha, 1972). In the present study, we could only secure normal submandibular glands since they were usually a part of neck dissections for surgical cases while the other two glands were rarely sacrificed during surgery. The submandibular glands in this study are predominantly composed of mucous acini with outer serous demilunes, similar to that in other reports (Dhabale, 2014). The chemical composition is heterogenous with a mixture of both neutral and acidic mucins. Our findings coincide with studies by Hamada et al. (2004) and Yarington et al. (1972). Some authors elaborated that mucous acinar cells in submandibular gland demonstrate a neutral carboxyl and sulfomucin presence (Eversole, 1972; Sushma Naag & Adi, 2010). Within the ductal epithelium and lumen, again there was a mixture of neutral and acidic mucins. This is in agreement with findings demonstrated by Dhabale et al. (2014). In contrast, parotid gland composition allegedly demonstrates the dominance of neutral mucosubstances with minimal amounts of sialomucin and sulfomucin (Rohini et al., 2014). Indeed, these findings go to show that salivary glands have complex structures and compositions with

unique features in each type.

5.5 Mucin profile in minor and major salivary gland tumours

From our study, we observed that PA of minor and major salivary glands secreted more sialomucin within the luminal spaces. Further analysis showed that expression of sialomucin within major PA was 42 times significantly higher than minor PA. In comparison, Sushma Naag & Adi (2010) found elevated amounts of neutral and sialomucin acidic content within PA of major salivary glands. Chondromyxoid areas in PA of either origin were also dominantly positive for sialomucin suggesting an acid mucopolysaccharide presence. Even in neoplastic state, the mucin content within the stroma in PA was similar to the mucin produced by normal connective tissue cells (Sushma Naag & Adi, 2010). The acidic stromal content is consistent with a study attributing the increased proportions of acidic mucins to the lack of differentiation in PA of myxoid variants (Satpathy, Spadigam, Dhupar, & Syed, 2014). The stromal content can be explained by the matrix-synthesizing ability of myopithelial cells that are prevalent in PA. Neoplastic myoepithelial cells produce abundance of basement-membrane and non-basement membrane elements. Chondroitin sulfate proteoglycan is the most dominant component of the non-basement matrix. It stains positive for alcian blue and presents as bluish-gray myxochondroid material microscopically (Shah, Mulla, & Mayank, 2016).

MEC of either origin expressed more sialomucin and less neutral mucin in our study. This differs slightly from a study which reported a mixture of sialomucin and sulfomucin in MEC of major glands but with relatively higher sialomucin content (Lam et al., 1993). Statistical analysis did not show any significant difference in mucin expressions between MEC of minor and major salivary gland origin. The stromal components in MEC contained mostly sialomucin and sometimes neutral mucin content.

We had only 3 AdCC cases from major salivary glands which made a direct comparison to AdCC of minor salivary glands not very reliable. However, we did note that the AdCC samples in our study generally showed elevated sialomucin content within pseudocystic spaces, which is in agreement with previous studies ("Histochemical Study of Salivary Gland Tumors," 1981). Our results are also consistent with Sushma Naag & Adi (2010) and Toida et al. (1985) which demonstrated AB dominance with focal areas showing faint positivity for PAS. A combination of the presence of sulfomucin and sialomucin has also been reported in the pseudocystic spaces of AdCC (Peel, 2001). We did find focal areas with sulfomucin expression in our samples.

To the best of our knowledge, there is no similar research done specifically on minor salivary glands. The objective of this comparison is to ensure that mucin profiling done on salivary gland tumours are not compromised by their origin. From our results, the mucin expressions were not significantly different for MEC and AdCC in terms of origin. However, this finding is likely more applicable to salivary glands of similar variants. This is because we noted that most MEC samples in our study were of low-

grade (cystic) variants and tended to express more mucin compared to the few solid variants. This observation also applies to AdCCs of tubular variants compared to solid variants.

5.6 Mucin profile in benign and malignant salivary gland tumours

Both benign and malignant salivary gland tumours demonstrated the presence of neutral and acidic mucin. Our study showed a significant difference between the expression of sialomucin between benign and malignant salivary gland tumours. There would be 3 times more expression of sialomucin within benign tumours compared to malignant tumours. Taken at face value, sialomucin or sialomucin-producing cells may have a role in the progression of the clinical behavior of salivary gland tumours. On the other hand, we noted that minor PA contained slightly higher sulfomucin content compared to the malignant minor salivary gland tumours. The opposite was true for major PA and malignant major salivary gland tumours. Then again, the staining for sulfomucin content in all the salivary gland tumours were underwhelming overall. These findings are not completely the same as previous studies, whereby a predominance of sialomucin within benign salivary gland tumours and a combination of sialomucin and sulfomucin in malignant salivary gland tumours were demonstrated (Spicer et al., 1967; Sushma Naag & Adi, 2010). Lam et al. (1993) also demonstrated elevated amounts of sialomucin and sulfomucin in MEC of salivary glands compared to oesophageal MEC. However, one study demonstrated that in PA, epithelial mucin is rich in neutral glycoproteins while the myoepithelial mucin is rich in sulfated and non-sulfated glycosaminoglycans (Lombardi et al., 2014). Since myoepithelial mucin is more likely present in PA that MEC, this might explain the increased sulfomucin content in PA in our study.

In our literature review, we found mucin studies in salivary gland tumours severely lacking for further comparisons. This is most likely because researchers tend to gravitate towards the more expensive and precise immunohistochemical studies. There were other mucin studies but these mainly involved gastrointestinal tumours. Mucin has been used for grading of gastrointestinal tumours and the postulated theory is that there will be a predominance of sulfomucin in malignancy and a significant increase in neutral mucin with progressive differentiation of the tumour (Filipe & Branfoot, 1974; Gad, 1982). Studies have shown that malignant tumours had a predominance of sulfomucin, however as the differentiation progressed sulphate mucin decreased and neutral mucin increased (Huszno et al., 2012; Jain et al., 2014). Although insignificant, there was a greater extent of neutral mucin content among MEC compared to PA in our study. Clearly however, the concept of differentiation cannot be applied in this situation. Even among gastrointestinal mucin studies, the results were not very consistent. According to Gad (1982), well-differentiated carcinomas expressed more sialomucin compared to sulfomucin. Marked increase of sialomucins were also noted in more extensive tumours in another paper (Filipe & Branfoot, 1974).

There have been instances where mucin expression was linked to histological tumour grade and prognosis of MEC (Alos et al., 2005). Our findings suggest that AB staining may play a role in distinguishing benign salivary gland tumours from malignant ones. Just as Agrawal et al. (2014) has successfully proven the benefit of using AB (pH2.5) to distinguish between benign prostate hyperplasia and well-differentiated prostate adenocarcinoma especially in questionable malignant cases, we recommend the same with AB for borderline salivary gland tumours.

5.7 Association of mucin expression with clinical parameters

There was no significant association observed between mucin expression with clinical parameters such as age, gender, ethnicity and tumour site within salivary gland tumours in our study. Mucin expression has no correlation with the clinical profile of these patients but is more likely to be closely related to the varied histological features of the salivary gland tumours.

CHAPTER 6 : CONCLUSION

From the present study, we found that sialomucin was the most expressed mucin in all salivary gland tumours regardless of origin. Neutral mucin was expressed rather frequently in MECs but sulfomucin was surprisingly absent or lowly expressed in all tumours. This would imply that alcian blue is the best special stain to visualize mucin elements during microscopic examination of salivary gland tumours.

In addition, there was a significant difference in mucin expression between benign and malignant salivary gland tumours. Benign salivary gland tumours expressed 3 times more sialomucin content compared to malignant salivary gland tumours suggesting a role in borderline salivary gland tumour diagnosis. It would also be interesting to explore the role of sialomucin or sialomucin-producing cells in the evolution of benign to malignant salivary gland tumour. This could be better explored if the study is repeated on a sample of PA and carcinoma ex-PA.

We also observed that expression of sialomucin within PA of major glands was 42 times significantly higher than in minor glands. On the other hand, mucin expressions in MEC and AdCC were not significantly altered due to their origin. Therefore for future research of similar nature, the sampling of these tissues may be from either group.

6.1 Limitation of study

There were several limitations in this study. Firstly, although the sample size was larger than previous studies, there was an unequal distribution within each type of salivary gland tumours especially for AdCC. Secondly, the varied histological features of each type of salivary gland tumour could influence the amount of mucinous content

highlighted by the special stains. This problem was particularly prominent when minute or absence of mucinous composition was noted within solid tumour variants. This study could be improved by selecting salivary gland tumours of the same variants. We also had issues with the Aldehyde Fuschin kit stain because the reagents given as counterstains for nuclei were red while AF was violet and these colours were of similar intensity, thus making interpretation a laborious procedure. Lastly, histochemistry is only limited to portraying the presence of mucosubstances but does not allow depiction of the refined element of these mucosubstances.

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