EXPRESSION OF BRAF, EGFR AND CD10 IN AMELOBLASTOMA: THEIR POTENTIAL ROLE IN LOCAL TUMOUR INVASIVENESS

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DEPARTMENT OF ORAL AND MAXILLOFACIAL CLINICAL SCIENCES, FACULTY OF DENTISTRY
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UNIVERSITY OF MALAYA

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Their Potential Role in Local Tumour Invasiveness

Field of Study: Oral and Maxillofacial Surgery

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ABSTRACT

Objectives: The aim of the present study is to investigate the expression of BRAF, EGFR and CD 10 in ameloblastoma and determine the impact of these pro-invasive biomarkers on the biological behavior of different ameloblastoma subsets. **Methods:** BRAF, EGFR and CD10 expression were examined with immunohistochemical techniques in 39 cases of paraffin-embedded ameloblastoma [19 unicystic ameloblastoma (UA) and 20 solid/multicystic ameloblastoma (SMA)]. Semi-quantitative score method was used to evaluate the immunoexpression which classified into pre-ameloblast-like cells (PA-cells), stellate reticulum-like cells (SR-like) and stromal cells (ST-cell) in ameloblastoma**.** The study was approved by the Medical Ethic Committee, Faculty of Dentistry, University of Malaya [Ethics DF OS1502/0011(P)]. **Findings:** The pro-invasive markers were significantly expressed in all three localisation for both UA and SMA ($P<0.05$). Statistically significant differences in the expression of these markers between epithelial components and stromal cells were observed ($P<0.05$). Immunoreactivity of EGFR in SMA was associated with ethnicity ($P<0.05$). **Conclusion:** BRAF, EGFR and CD10 were significantly expressed in SMA and UA which indicating their active local bone activity in ameloblastoma. These findings suggest their potential roles as prognostic markers of ameloblastoma and targeted therapy could be considered to treat the advanced unresectable ameloblastoma. (PPPC/C1-2015/DGJ/02)

ABSTRAK

Objektif: Tujuan kajian ini adalah untuk menyiasat ekspresi BRAF, EGFR dan CD10 dalam ameloblastoma. Ia juga untuk menentukan kesan pro-invasif biomarker-biomarker ini pada kelakuan biologi dalam subset ameloblastoma yang berbeza. **Kaedah:** Ekspresi BRAF, EGFR dan CD10 telah diperiksa dengan teknik immunohistokimia dalam 39 kes ameloblastoma [19 ameloblastoma uni-sista (UA) dan 20 ameloblastoma pepejal bersista (SMA)]. Kaedah skor separa-kuantitatif telah digunakan untuk menilai ekspresi-immune biomarker dalam lokasi sel-sel yang berbeza seperti sel pra-ameloblast (sel PA), sel reticulum stelat (sel SR) dan sel stroma (sel ST). Kajian ini telah diluluskan oleh Jawatankuasa Etika Medikal, Fakulti Pergigian, Universiti Malaya [DF OS1502 / 0011 (P)]. **Penemuan:** Petanda pro-invasif biomarker-biomarker telah ternyata dengan ketara dalam ketiga-tiga lokasi sel untuk UA dan SMA ($P<0.05$). Perbezaan yang signifikan dalam ekspresi ini di komponen epitelium dan sel-sel stroma diperhatikan ($P<0.05$). Immunoreaktiviti EGFR di SMA dikaitkan dengan keetnikan ($P<0.05$). **Kesimpulan:** Ekspresi BRAF, EGFR dan CD10 yang ketara dalam UA dan SMA menunjukkan aktiviti biomarker-biomarker ini pada tulang setempat mereka yang aktif dalam ameloblastoma. Penemuan ini menunjukkan potensi peranan biomarker-biomarker ini digunakan sebagai penanda ramalan untuk ameloblastoma supaya terapi sasaran boleh dipertimbangkan. (PPPC/C1-2015/DGJ/02)

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Akt	:	Protein Kinase B
AME-1	:	Ameloblastoma Cell Line 1
BRAF	:	V-raf Murine Sarcoma Viral Oncogene Homolog B1
CD10	:	Neprilysin
CT	:	Computed Tomographic Scan
Cyclin-D1	:	A Protein Encoded by the CCND1 gene
ECM	:	Extracellular Matrix
EGFR	:	Epidermal Growth Factor Receptor
ErBb	:	Receptor Tyrosine Kinases Related to EGFR
ERK	:	Extracellular Signal-Regulated Kinases
FAK	:	Focal Adhesion Kinase
FGF2	:	Fibroblast Growth Factor 2
G Protein	:	Guanine Nucleotide-binding Protein
GF	:	Growth Factor
GSK3	:	Glycogen Synthase Kinase 3
H&E	:	Haemoatoxylin and Eosin
JAK	:	Janus Kinase
MAPK	:	Mitogen-activated Protein Kinases
MDM2	:	Mouse Double Minute 2 Homolog
MEK	:	Mitogen-activated Protein/ Extracellular Signal-Regulated Kinase
MMPs	:	Matrix Metalloproteinase

mTOR	:	Mechanistic Target of Rapamycin
NFκB	:	Nuclear Factor Kappa-Light-Chain-Enhancer of activated B cells
OPG	:	Osteoprotegerin
P	:	Phosphorylation
P14ARF	:	Alternate Reading Frame Protein 14 Tumour Suppresor
P53	:	Tumour Protein p53
PBS	:	Phosphate Buffered Saline
PI3K	:	Phosphatidylinositol 3-Kinase
PTEN	:	Phosphatase and Tensin Homolog
RANK	:	Receptor Activator of Nuclear Factor κB
Ras	:	A GTPase
Rho	:	A Kinase belonging to family of serine-threonine kinases
ROCK	:	Rho-associated Protein Kinase
RTK	:	Receptor Tyrosine Kinase
SMA	:	Solid/Multicystic Ameloblastoma
STAT3	:	Signal Transducer and Activator of Transcription 3
STAT5	:	Signal Transducer and Activator of Transcription 5
TNF	:	Tumor Necrosis Factor
UA	:	Unicystic Ameloblastoma
WHO	:	World Health Organization

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

Ameloblastoma is a slow growing, benign but locally invasive odontogenic epithelial neoplasm which accounts for approximately 1% of all oral tumours (Eckardt et al., 2009). It has no gender or race predilection and occurs in all age groups with a peak incidence in the 3rd through 4th decades of life (Mendenhall et al., 2007). The presentation of ameloblastoma is usually asymptomatic and is occasionally associated with a slow growing mass, loose teeth, change in occlusion, paraesthesia and pain. This neoplasm is often found within jaw bones (intraosseous ameloblastoma) especially in the posterior mandibular region. It can also be presented in the soft tissue as a peripheral (extraosseous) ameloblastoma.

Follicular, plexiform, acanthomatous, granular cell, basal cell and desmoplastic type are amongst the commoner histological variants for ameloblastomas (Barnes et al., 2005; Mendenhall et al., 2007). In general, these histological variants can be broadly grouped into solid/multicystic ameloblastoma and unicystic ameloblastoma. Solid/multicystic subtypes tend to occur more frequently and are more aggressive in clinical behaviour. The treatment modalities for ameloblastomas include marsupialisation, enucleation and curettage, marginal resection and radical surgery, depending on their types (de AC Almeida et al., 2016). The reported recurrence rates after resection vary from 0% to 25%, with solid/multicystic type being higher.

The most controversial behaviour of ameloblastoma is its invasiveness into the surrounding bone, despite its benign nature. The exact mechanism of bone resorption remains unclear. However, a few studies have shown evidence that the ameloblastomas express different types of markers and this has shed some light into our understanding of the tumour pathogenesis, progression and behaviour. These tumour markers are

believed to be responsible in promoting bone resorption, extracellular matrix degradation, cell signalling, cell adhesion, cell migration, cell proliferation and cell apoptosis. Insights into the underlying biological traits of these neoplasms may pave way for new treatment approaches by modulating the activities of these specific molecular targets.

Research Questions:

1. Are there any associations between the immunoprofile of BRAF, EGFR and CD10 protein markers with the behaviour of different subsets of ameloblastoma?
2. Can the expression levels of these BRAF, EGFR and CD10 markers of different subsets of ameloblastoma be used as reliable/independent prognostic parameters?
3. Can the understanding of immunoprofile of BRAF, EGFR and CD10 markers provide new directions for clinical therapy of ameloblastoma?

Aim:

To examine various types of ameloblastoma for the presence of pro-invasive protein markers.

Objectives:

1) To conduct an immunohistochemical investigation for BRAF, EGFR and CD10 markers in ameloblastoma.
2) To compare the immunoprofile of these BRAF, EGFR and CD10 markers between unicystic and solid/multicystic ameloblastoma.
3) To determine the impact of these BRAF, EGFR and CD10 markers on the biological behavior of the different ameloblastoma subsets.

Null hypothesis:

Ameloblastoma do not express pro-invasive markers

Clinical relevance of study:

Ameloblastomas express BRAF, EGFR and CD10 markers and their immunoexpression patterns might show correlation with the different subsets and their behaviours. This could potentially then be the basis of new treatment strategies or new treatment modalities for the various types of ameloblastoma.

CHAPTER 2: LITERATURE REVIEW

2.1 Odontogenic Tumours

Odontogenic tumours are lesions of interest among oral and maxillofacial surgeons and oral pathologists because of their diverse aetiopathogenesis, diagnosis and treatment modalities. They therefore form a heterogeneous group of diseases that may range from hamartomas to benign and malignant neoplasms with different degrees of aggressiveness and metastatic potential. Histogenetically, they may take origin from epithelial, ectomesenchymal or mesenchymal components of the tooth apparatus. The relative rarity and wide variety of these tumour entities poses a great diagnostic and therapeutic challenge (Barnes et al., 2005).

A French physician and professor of pathology and clinical surgery, Pierre Paul Broca was the first to propose the classification of odontogenic tumours using the odontome concept in the year 1869 (Philipsen & Reichart, 2006). This was followed by Thoma and Goldman's classification (1946) that divided the odontogenic tumours into tumours of ectodermal, mesodermal and mixed origin (Thoma & Goldman, 1946). In 1958, Pindborg and Clausen (1958) proposed a classification which was based on the pathogenesis of odontogenic tumours resulting from reciprocal epithelial-mesenchymal tissue interaction. The first authoritative World Health Organization (WHO) guide to the odontogenic tumours and cysts classification was published in 1971 followed by a second edition in year 1992 (Kramer et al., 1992; Philipsen & Reichert, 2006). In 2003, WHO started work on a volume on Head and Neck Tumours including a chapter on Odontogenic Tumours and Bone Related Lesions which was published in 2005 by IARC, Lyon (Barnes et al., 2005). The following Table 2.1 shows the 2005 WHO histological classification of odontogenic tumours (Barnes et al., 2005).

Table 2.1: WHO histological classifications of odontogenic tumours (Barnes et al., 2005)

MALIGNANT TUMOURS **Odontogenic carcinomas** Metastasising (malignant) ameloblastoma Ameloblastic carcinoma- primary type Ameloblastic carcinoma- secondary type (dedifferentiated), intraosseous Ameloblastic carcinoma- secondary type (dedifferentiated), peripheral Primary intraosseous squamous cell carcinoma- solid type Primary intraosseous squamous cell carcinoma derived from keratocystic odontogenic tumour Primary intraosseous squamous cell carcinoma derived from odontogenic cysts Clear cell odontogenic carcinoma Ghost cell odontogenic carcinoma **Odontogenic sarcoma** Ameloblastic fibrosarcoma Ameloblastic fibrodentino- and fibro-odontosarcoma **BENIGN TUMOURS** **Odontogenic epithelium with mature, fibrous stroma without odontogenic ectomesenchyme** Ameloblastoma, solid/multicystic type Ameloblastoma, extraosseous/peripheral type Ameloblastoma, desmoplastic type Ameloblastoma, unicystic type Squamous odontogenic tumour Calcifying epithelial odontogenic tumour Adenomatoid odontogenic tumour Keratocystic odontogenic tumour	**Odontogenic epithelium with odontogenic ectomesenchyme, with or without hard tissue formation** Ameloblastic fibroma Ameloblastic fibrodentinoma Ameloblastic fibro-odontoma Odontoma- complex type Odontoma- compound type Odontoameloblastoma Calcifying cyst odontogenic tumour Dentinogenic ghost cell tumour **Mesenchyme and/or odontogenic ectomesenchyme with or without odontogenic epithelium** Odontogenic fibroma Odontogenic myxoma/myxofibroma Cementoblastoma **Bone related lesions** Ossifying fibroma Fibrous dysplasia Osseous dysplasia Central giant cell lesion (granuloma) Cherubism Aneurysmal bone cyst Simple bone cyst **OTHER TUMOURS** Melanotic neuroectodermal tumour of infancy

2.2 Ameloblastoma

2.2.1 Aetiopathogenesis

Ameloblastoma is the most frequently encountered benign but locally invasive tumour originating from odontogenic epithelium. It was first recognised in 1827 by Cusack then described by Broca in 1868 and designated as adamantinoma by Louis-Charles Malassez in 1885. Ivy and Churchill have proposed the standardisation of the term "ameloblastoma" in 1930 and it has been used until now (Ivy & Churchill, 1930; Reichart, Philipsen & Sonner, 1995). The ethymology of "ameloblastoma", derived from the French word "amel" means enamel and the Greek word "blastos" means germ.

WHO histological classified ameloblastoma as a benign tumour of odontogenic epithelium with mature, fibrous stroma without odontogenic ectomesenchyme. The variants of ameloblastoma include solid/multicystic, unicystic, desmoplastic and extraosseous/peripheral (Barnes et al., 2005). Nevertheless, ameloblastoma can be subclassified into two main groups clinically: the solid multicystic variant; and the unicystic variant. The solid/multicystic ameloblastoma (SMA) is slow growing but locally invasive benign tumour with a high rate of recurrence but rarely metastasis if inadequately removed. Unicystic ameloblastoma (UA) usually presents as a cyst that resembles a dentigerous cyst clinically and radiologically, has a better prognosis after limited surgical procedures, and is associated with a low recurrence rate.

In the past decade, several studies have identified molecular and genetic alterations which are responsible for the tumorigenesis of ameloblastoma. Diverse molecular processes are implicated in the ameloblastoma development and growth, including those involved in apoptosis, bone remodelling, tooth development, cell proliferation, cells signalling, cell adhesions, tumour suppressor genes, extracellular matrix related proteins, clonality pattern and others (González et al., 2014; Jhamb & Kramer, 2014).

The positive staining pattern of Ki-67 in the peripheral ameloblastoma-like cells of the solid ameloblastoma and basal cells of unicystic ameloblastoma indicates that the cellular proliferation and growth of ameloblastoma are concentrated in the peripheral areas (Jaaskelainen et al., 2002). Several studies showed higher Ki-67 staining in SMA with a follicular pattern, and a higher cellular proliferation index in recurrent ameloblastoma (Piattelli et al., 1998; Sandra et al., 2001; Gomes et al., 2010)

Apoptosis is programmed cell death initiated intrinsically or extrinsically. Apoptosis plays a role in balancing the cell division and tissue homeostasis. Caspase 3 is a family of intracellular protease that cleave the interleukin-1β, which is required for apoptosis. Luo et al. (2006) found that 61.5% of ameloblastomas were positive in immunohistochemistry staining for caspase-3 (a cysteine-aspartic acid protease family) which as diffusely distributed in the central area of ameloblastoma. Additionally, the consistent strong staining for type 1 membrane protein (Fas) may suggest that Fas is capable of inducing alternative caspase-independent cell death pathway in ameloblastoma (Luo et al., 2006). B-cell lymphoma 2 (Bcl-2) is an anti-apoptotic protein which is usually found in the periphery basal layer of ameloblastoma. The higher levels of apoptosis-inhibiting protein at the peripheral layer compared to the apoptosis-modulating protein at the central layer in ameloblastoma may play a role in the survival of neoplastic cells (Sandra et al., 2001).

The tumour suppressor gene p53 is a transcriptional factor that regulates the expression of genes involved in programmed cell death or cell cycle arrest in response to genomic damage or cell stress (Ko & Prives, 1996). The mutated p53 gene is frequently associated with cellular proliferation and malignant transformation across all type of cancers (Gomes et al., 2010). According to a study by Kumamoto et al. (2004) p53 acts as a tumour suppressor gene in the p53-MDM2-p14^{ARF} complex. Ameloblastoma exhibited different immunohistochemical reactivity for p53 among

various histological types. Interestingly, p53 expression was significantly higher in ameloblastoma than in enamel organs and dental lamina (Kumamoto et al., 2004). Besides, plexiform variant has higher expression of p53 compared to follicular variant while no expression of p53 was found in acanthomatous and granular cell variants; and only scanty reactivity was found in basal and desmoplastic variants (Kumamoto et al., 2004). Kumamoto and co-workers also found that the expression of MDM2 and $p14^{ARF}$ was higher in ameloblastoma and malignant ameloblastoma than in tooth germs which suggest that these upstream regulators of p53 are involved in oncogenesis or malignant transformation of odontogenic epithelium (Kumamoto et al., 2004).

The receptor activator of nuclear factor κB (NFκB) belongs to the tumour necrosis factor (TNF) superfamily which exhibit pro-inflammatory activities through activation of the transcription factor nuclear factor κB. RANK is present on the osteoclast surface and is activated by RANK ligand (RANKL) which is essential for osteoclast activities in bone remodelling. Osteoprotegerin (OPG) is a soluble receptor that binds to RANKL and disrupts RANKL/RANK interaction (Kearns, Khosla, & Kostenuik, 2008). OPG, RANK and RANKL are expressed in both SMA and unicystic ameloblastoma (Kumamoto et al., 2004; da Silva et al., 2008). Da Silva et al. (2008) reported that elevated levels of RANK at stromal cells of SMA compared to UA and the ratio of RANKL to OPG positive cells is higher in SMA and UA suggesting net bone resorption (da Silva et al., 2008). In contrast a study by Siar et al. (2015) showed a distinctly low/negative RANKL expression pattern demonstrated by recurrent SMA and overexpression of OPG and RANK which appeared to be important factors determining the osteoclastogenesis in ameloblastoma (Siar et al., 2015).

Cell signalling pathways can be activated by both intracellular and extracellular signals in various cellular processes including differentiation, metabolism and proliferation. BRAF is a mitogen-activated protein kinase (Ras/MAPK) pathway

intermediate and is a potent activator of MEK which is identified in numerous human cancers (Cantwell-Dorris, O'Leary, & Sheils, 2011). The missense mutation of BRAF at residue 600 that substitutes a glutamine for valine allows activation of this signalling cascade in the absence of any extracellular stimuli and allowing the cell to become self-sufficient in growth signals within the cells. In the study by Kurppa et al., (2014) two-thirds of ameloblastoma-derived epithelial cells expressed $BRAF^{V600E}$ mutation which plays a role in tumour progression and proliferation (Kurppa et al., 2014).

Epidermal growth factor receptor (EGFR) signalling is important in cell development and regulation of cell survival in both normal and neoplastic cells. EGFR is a cell surface transmembrane receptor kinase which when phosphorylated and triggers downstream mitogenic signalling via both the MAPK and PI3K pathways (Abdel-Aziz & Amin, 2012). Abdel-Aziz and Amin (2012) observed that all ameloblastoma exhibited EGFR immunoexpression with no identified relation to recurrence. On the contrary, a study by Oikawa et al. (2013) showed no significance difference in the expression of EGFR in both odontogenic epithelium of dental follicles and ameloblastoma. Conversely, EGFR expression is slightly higher in recurrent ameloblastoma than in primary lesion; higher HER4 expression in follicular variant than plexiform variant of ameloblastoma (Oikawa et al., 2013). Even though the results are inconclusive, the EGFR and HER4 might have a role in tumorigenesis and prediction of the outcome of ameloblastoma.

The extracellular matrix (ECM) surrounding cell has a complex role in modulating the behaviour of cells that are in direct contact with it. ECM plays a role in cell survival, migration, development, proliferation and function (Jhamb & Kramer, 2014). Matrix metalloproteinase (MMPs) are zinc-dependent endopeptidases that promote invasion and proliferation of neoplastic cells (Ribeiro et al., 2009). Overexpression of MMP-1, MMP-2 and MMP-9 in ameloblastoma putatively promotes osteoclastogenesis and

mediates tumour invasion by degradation of the ECM (Ribeiro et al., 2009).

2.2.2 Epidemiology

Ameloblastoma is the commonest locally aggressive epithelial odontogenic benign neoplasm of the jaw bone. It comprises 1% of all the oral tumours and about 11-18% of odontogenic tumours (Sciubba, Fantasia, & Kahn, 2001; Siar et al., 2012). Besides odontoma, the incidence of ameloblastoma was almost equalled all the others odontogenic neoplasms combined. The distribution of ameloblastomas varies among different geographical and racial groups. Reichart et al. (1995) has reported the relative frequency of ameloblastoma ranged between 24.8% among Caucasians to 38.4% among Asians. The age series from their report ranged from 4 to 92 years similar to other reports (Reichart et al., 1995; Kim & Jang, 2001; Dhanuthai et al., 2012).

The peak incidence varies between third decade of life (Asian) and fifth decade of life (North America) (Dhanuthai et al., 2012). However, UA tends to occur in younger age groups of early twenties as reported by Kim and Jang (2001). Patients with desmoplastic ameloblastoma tend to occur in older age group (Luo & Li, 2009). The distribution of ameloblastoma among male and female gender were relatively equal (Reichart et al., 1995; Dhanuthai et al., 2012) with exception of selective studies which demonstrated either male or female predominance (Lu et al., 1998; Kim & Jang, 2001; Zhang et al., 2010). The incidence of ameloblastoma globally has been estimated at 0.5 cases per million person-years diagnosed between ages 30 and 60 years (McClary et al., 2015).

2.2.3 Clinico-radiographic characteristic

The common presentations of ameloblastoma include slow painless hard bony swellings of the jaws, numbness, delayed tooth eruption, soft tissue growth, discharging

sinus, tooth mobility, tooth displacement and non-healing socket. Nevertheless, 79% of ameloblastoma cases reported in Brazil were asymptomatic as well as most of the ameloblastoma cases in China and Egypt (Avelar et al, 2008; Siar et al., 2012). In the series of 60 cases reported by Becelli et al. (2002) up to 35% of their lesions identified as incidental findings on imaging (Becelli et al., 2002). Pain is uncommon but it may occur in cases of haemorrhage following fine needle aspiration or biopsy. Rarely, paraesthesia is reported when perineural invasion occurs in desmoplastic ameloblastoma (McClary et al., 2015).

Average duration of clinical symptoms is ranged from 13.6 months to 27 months (Reichart et al., 1995; Siar et al., 2012). Patients from industrialised countries were reported to have a shorter duration of symptoms (24.5 months) compared to developing countries (32.6 months) (Reichart et al., 1995). The explanation for this difference may reflect the reduced accessibility in developing countries to health care services, and lower socioeconomic status (Dhanuthai et al., 2012).

The mandible is affected in majority of cases in comparison to maxilla. The ratio of maxilla to mandible ranged from 1.0:2.2 to 1.0:13.2. This difference is due to the geographical factor as suggested by Reichart et al., (1995) and Dhanuthai et al., (2012). Most cases (SMA or UA) had predilection for posterior region of the mandible. However, Siar et al. (2012) has reported that desmoplastic ameloblastoma was commoner in the anterior jaw segment; similar to the finding by Luo and Li (2009). Maxillary ameloblastoma, on the other hand, commonly occur in posterior molar region.

The radiographic features of ameloblastoma ranged from unilocular radiolucency, multilocular radiolucency to mixed radiolucent-radiopaque appearance. The study by Reichart's et al. (1995) showed almost equal finding of unilocular (51.1%) and multilocular (48.9%) radiographic appearance of ameloblastoma which was similar to

the review of Dhanuthai and co-workers (2012). These findings can be associated with embedded tooth, root resorption, cupping and opacity (Reichart et al., 1995). In contrast, Kim and Jang reported that multilocular radiolucent ameloblastoma outnumbered unilocular radiolucent ameloblastoma in Asia, except in Korea (Kim et al., 2001). The unilocular radiolucency with scalloped margin which is often associated with impacted molar is seen in unicystic ameloblastoma. The classic "soup bubble" appearance is mostly found in the multicystic ameloblastoma. However, histologically verified unicystic ameloblastoma can present either as unilocular or multilocular radiolucency on imaging (Reichart et al., 1995).

Philipsen et al. (1992) suggested that the metaplastic bone formation was responsible for mixed radiolucent-radiopaque radiographic appearance in desmoplastic ameloblastoma, while Li and colleagues proposed that the typical appearance was attributed to the different density of compressed odontogenic epithelium with desmoplastic stroma and adjacent bone (Philipsen et al., 1992; Li et al., 2011).

Plain x-ray lacking the sensitivity and specificity to give a complete evaluation of the bony extension and soft tissue involvement in ameloblastoma, thus computed tomography (CT scan) is the best diagnostic imaging modality to overcome it. CT scan is useful to identify the extension of bony involvement of lesion and aid in surgical planning while Magnetic Resonance Imaging (MRI) shows more information on the soft tissue and marrow extension beyond the bony margin particularly in maxillary ameloblastoma. PET-CT is useful for staging of metastatic ameloblastoma (McClary et al., 2015).

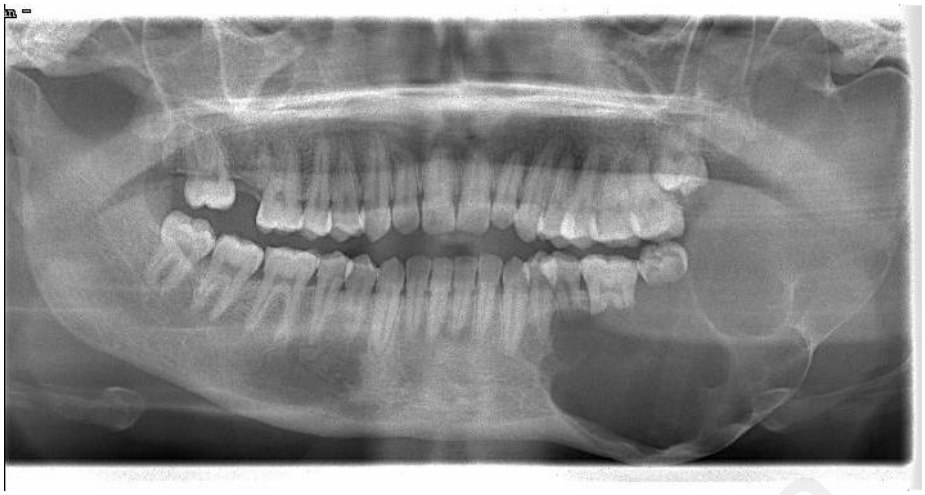


Figure 2.1: Panoramic tomogram showing 'soap-bubble appearance' in SMA at left side of mandible.

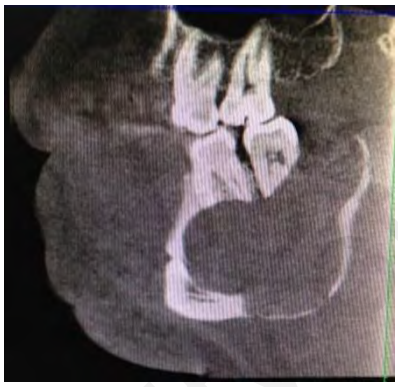
A.  B. 

Figure 2.2: Sagittal (A) and axial (B) CT scan views of SMA at left side of mandible.

2.2.4 Histopathological features

The histological subtypes of ameloblastoma are follicular, plexiform, acanthomatous, granular, basal cell and desmoplastic. According to Hong et al, the histopathology of an ameloblastoma is significantly associated with its potential for recurrence (Hong et al., 2007).

2.2.4.1 Follicular subtype

The follicular variant of ameloblastoma is the commonest and most readily identifiable type, with the presence of all the core features of ameloblastoma. It grows mainly as islands of epithelial cells in a connective tissue stroma. The cords and strands

of cells might be present, too. The peripheral layer of epithelial cells is tall columnar cells with palisaded, hyperchromatic nuclei, reversed polarity and vacuolated cytoplasm. The central loosely arranged polyhedral cells resemble the stellate reticulum. The islands of ameloblast-like tumour cells can enlarge to sufficient size as to induce central cystic degeneration (Kessler, 2004).

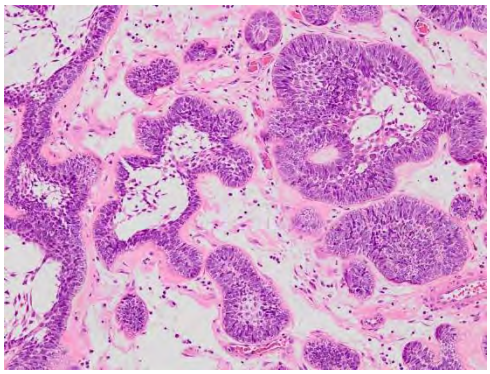
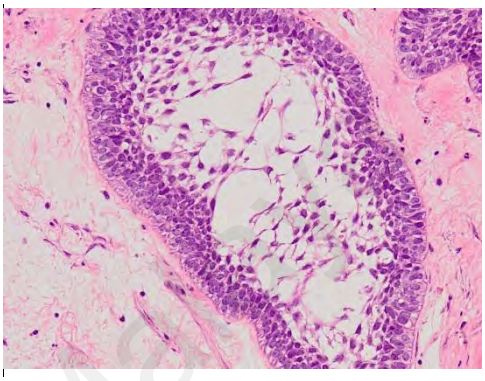
A.  B. 

Figure 2.3: Histopathological features of follicular pattern ameloblastoma. (Original magnification Ax40; B x100; H&E stain)

2.2.4.2 Plexiform subtype

The plexiform ameloblastoma lacks of the core histological features of ameloblastoma. It shows sparse fibrous connective tissue stroma with a myxoid appearance. Plexiform ameloblastoma is composed of strand-like interconnecting tumour epithelial cells surrounding loosely arranged stellate reticulum-like cells (Kessler, 2004).

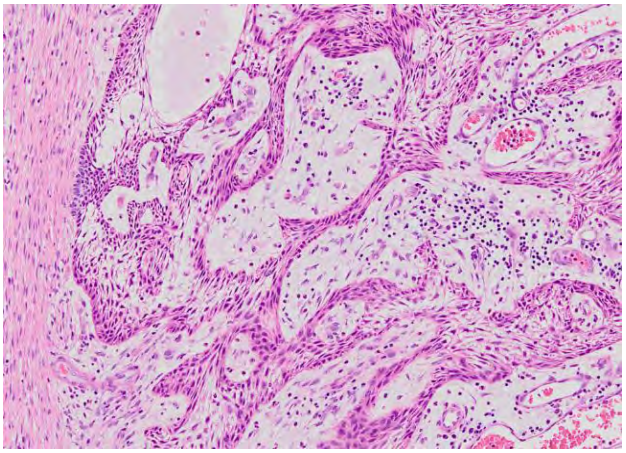


Figure 2.4: Histopathological features of plexiform ameloblastoma. (Original magnification x40; H&E stain)

2.2.4.3 Acanthomatous subtype

Acanthomatous ameloblastoma resembles the follicular ameloblastoma, and grows in island-like pattern. The central cells in acanthomatous ameloblastoma are squamous cells rather than stellate reticulum-like cells which tend to keratinisation known as keratinising pearls in the most central part of tumour and produce central cystic changes in large tumour islands.

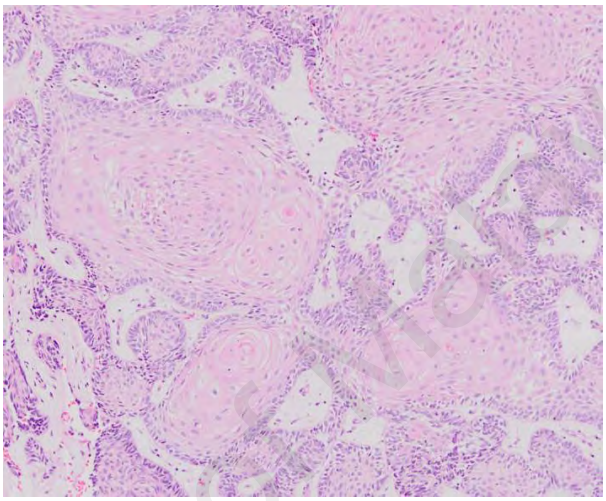


Figure 2.5: Histopathological features of acanthomatous ameloblastoma with keratinising pearls. (Original magnification x40; H&E stain)

2.2.4.4 Granular cell subtype

The granular cell ameloblastoma is a relatively rare subtype of ameloblastoma and is often found as mixtures of other subtypes especially follicular type. It has all the core histological characteristics of ameloblastoma. However, the presence of granular cells in the central part of epithelial islands is the distinctive feature of granular cell type ameloblastoma. The large oval to polyhedral shaped of granular cells often have poorly demarcated membranes and the cytoplasm of adjacent cells merge imperceptibly.

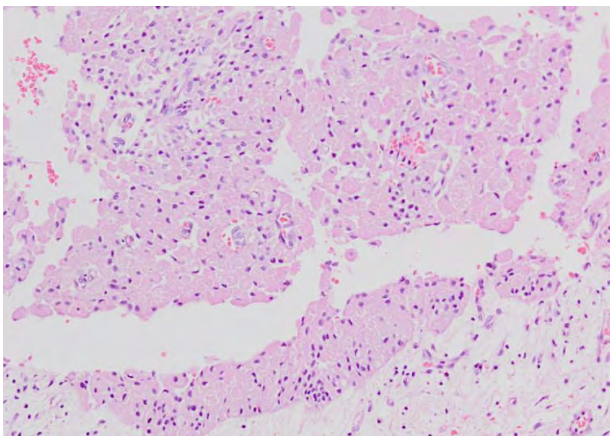


Figure 2.6: Histopathological features of granular ameloblastoma. (Original magnification x100; H&E stain)

2.2.4.5 Basal cell subtype

Basal cell ameloblastoma is the rarest variant of ameloblastoma which has been reported more in peripheral locations than intraosseously. The central portion of tumours is occupied by polyhedral or spindle shaped basaloid-appearing cells rather than the typical stellate reticulum. The peripheral low columnar or cuboidal cells show hyperchromatism and palisading nuclei, without reverse polarity or subnuclear vacuole formation. This histological subtype of ameloblastoma highly resembles basal cell carcinoma. Thus, Kessler suggested that the reported cases of intraoral basal cell carcinoma were most likely to be basal cell ameloblastoma (Kessler, 2004).

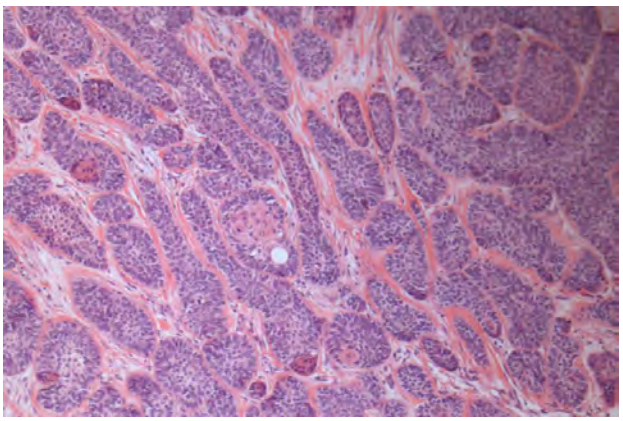


Figure 2.7: Histopathological features of basal cell ameloblastoma. (Original magnification x40; H&E stain)

2.2.4.6 Desmoplastic subtype

The desmoplastic ameloblastoma often grows predominantly as islands, but also in thin strands and cords of epithelium with dense, hyalinised and hypocellular collagen stroma. The reverse nuclear polarity and subnuclear vacuoles are difficult to identify in the flattened cuboidal epithelial cells. Myxoid changes of the juxtaepithelal stroma are often found (Kessler, 2004; Barnes et al., 2005).

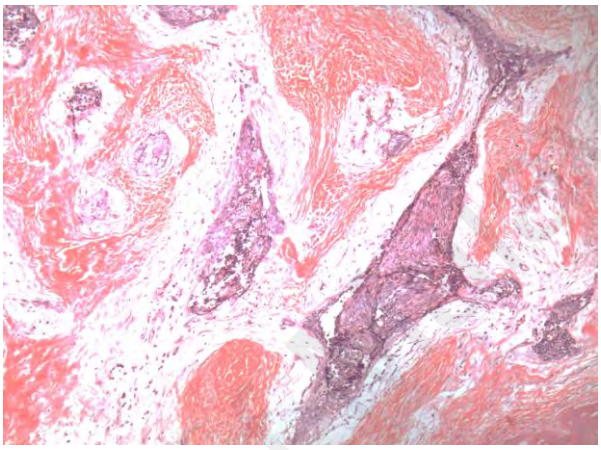


Figure 2.8: Histopathological features of desmoplastic ameloblastoma. (Original magnification x100; H&E stain)

2.2.5 Treatment and recurrence of ameloblastoma

The treatment of ameloblastomas is primarily divided into surgical and non-surgical. There has been controversy and debates regarding the most appropriate treatment modality to eradicate ameloblastoma with minimal facial disfigurement and low recurrence rate. These treatments ranged from conservative to invasive procedures. The conservative treatments include curettage, enucleation or marsupialisation with adjuvant therapy such as cryosurgery, chemical cauterisation, tissue fixatives (Carnoy's solution) and cautery (McClary et al., 2015). The invasive surgical option is marginal or radical resection of ameloblastoma with 1-2cm of grossly disease-free bone margin followed by immediate reconstruction for a better function of speech and swallowing (Carlson & Marx, 2006). The non-surgical options include targeted biological therapy, radiotherapy and chemotherapy (McClary et al., 2015). The selection of appropriate treatment option

is greatly dependent on the histological variant of ameloblastoma, its site and size, and age of the affected individual (Reichart et al., 1995).

Lau and Samman (2006) identified the highest recurrence rate among the different treatment modalities was for enucleation alone (30.5%). The main reasons were inadequate removal of the cystic lining, especially in areas of difficult surgical access, such as in maxillary antrum, and the possibility of the invasion of the tumour cells into cancellous bone. A recurrence rate of 16% was reported for enucleation of ameloblastoma followed by the application of Carnoy's solution for 3 to 5 minutes. Treatment by surgical resection in unicystic ameloblastoma showed the lowest recurrence rate of 4% (Lau & Samman, 2006).

Reichart et al. (1995) reported the recurrence rate of unicystic ameloblastoma was lower (13.7%) compared to multicystic ameloblastoma (22.7%). Even though the unicystic ameloblastoma believed to be less aggressive and enucleation alone may be sufficient for the tumour eradication. However, Zhang et al. (2010) showed unicystic ameloblastoma with intraluminal or intramural invasion was aggressive and needed radical treatment as in solid ameloblastoma. When locally aggressive solid ameloblastoma is treated with only conservative management, it could lead to recurrence rates as high as 75-90% (Zhang et al., 2010). The study conducted by de AC Almeida et al. (2016) showed three times greater recurrence risk when conservative treatment (40%) was performed in primary ameloblastoma compared to radical treatment (12%). Thus, the initial incisional biopsy or frozen section during surgical procedure was crucial in identify the histological type of ameloblastoma before the selection of definitive treatment.

Close post-operative follow up was mandatory as more than 50% of recurrence cases were reported within 5 years of treatment while the more common recurrence time was 2-5 years as reported by Zhang and co-workers (2010).

2.2.6 Biological pathway in invasiveness of ameloblastoma tumour markers

Tumour cells invasion into the adjacent healthy tissues remains the essential step in tumour progression. Although ameloblastoma is benign in nature, many studies have been carried out to identify the invasive activities of ameloblastoma to assist in the prediction of their biological behaviour treatment, outcomes and possible targeted therapy.

2.2.6.1 Introduction to BRAF

The identification of RAS-RAF-MEK-ERK (MAPK) pathway acts as a signal transducer between the extracellular environment and the nucleus. This pathway is a conserved kinase cascade involved in the regulation of cell proliferation, differentiation and survival in response to extracellular signalling eg. hormones, cytokines and various types of growth factors. Active RAS activates and recruits the RAF proteins to the cell membrane while BRAF signals activate ERK through MEK. This downstream signalling induces a range of processes including cell differentiation, proliferation, growth and apoptosis (Cantwell-Dorris et al., 2011).

BRAF is a Ras/MAPK (Ras/mitogen activated protein kinase) signalling pathway intermediate and is an activator of MEK. BRAF mutation activation is found in 5-7% of human benign and malignant tumours (Capper et al., 2011). Mutation of BRAF is characterised by a missense substitution of valine by glutamic acid at amino acid position 600 which induces a constitutive change of the kinase activity of BRAF and consecutive phosphorylation of downstream target (Wan et al., 2004). High $BRAF^{V600E}$ mutation rates have been detected in papillary thyroid carcinoma (40-70%), pleomorphic xanthoastrocytomas (60-70%), melanoma (30-70%), ameloblastoma (46-63%), colorectal carcinoma (5-10%) and others (Cohen et al., 2003; Schindler et al., 2011; Fisher et al., 2014; Kurppa et al., 2014; Sweeney et al., 2014). In ameloblastoma, the presence of activating BRAF mutations suggests the involvement of a hyperactive

RAS-RAF-MAPK pathway in the pathogenesis of highly oncogenic ameloblastoma (Kurppa et al., 2014). The mutated BRAF V600E protein in the RAS-RAF-MAPK pathway is also known for down-streaming the epithelial growth factor receptor (EGFR) which causes the resistance to anti-EGFR therapy in colorectal carcinoma (De Roock et al., 2010). The detection of BRAF mutations is believed to be an important biomarker with diagnostic, prognostic, targeted therapeutic and predictive potential in clinical implications. It is highly suggested BRAF to play a role in tumour invasiveness of ameloblastoma (Kurppa et al., 2014; Fregnani et al., 2016).

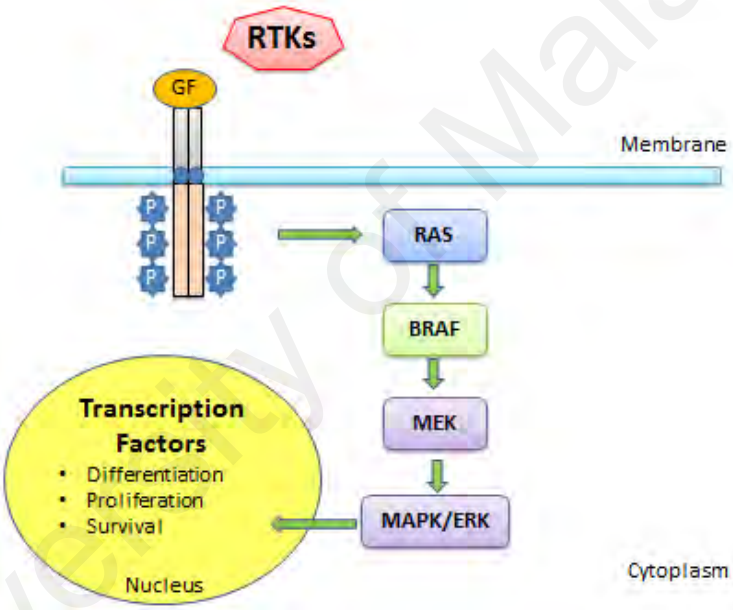


Figure 2.9: Schematic of the mitogen activated protein kinase (MAPK) signalling pathway. RTK: receptor tyrosine kinase; MEK: mitogen activating kinase enzyme; ERK: extracellular signal-regulated kinases.

2.2.6.2 Introduction to EGFR

Several studies have analysed the expression and functions of growth factor in ameloblastoma which suggested that the cell regulatory system modulated by EGF/EGFR may have a role in invasive behaviour of ameloblastoma (Vered, Shohat, & Buchner, 2003; da Rosa et al., 2014).

EGFR (Epidermal Growth Factor Receptor) is a cell surface transmembrane receptor tyrosine that belongs to the ErbB family. It activates downstream mitogenic signalling via Ras-Raf-MAPK, STAT3, STAT5 and P13K-Akt-mTOR pathway (Sibilia et al., 2007). These pathways are known to be involved in controlling embryogenesis, proliferation, differentiation, proto-oncogene expression, pro-survival and anti-apoptotic pathways (Sibilia et al., 2007; da Rosa et al., 2014). Overexpression of EGFR reaches 100% in human head and neck tumours while ameloblastoma is the commonest epithelial odontogenic tumour derived from EGFR-expressing odontogenic epithelial sources (Vered, Shohat, & Buchner, 2003; Sibilia et al., 2007). In the study of da Rosa et al. (2014), the primary cell line derived from human ameloblastoma (AME-1) had expressed high EGF-EGFR level, and related to up-regulation of MMP2 and MMP9 which strongly suggested its influence in the migration, invasion and protease activity of ameloblastoma. Furthermore, EGFR expression in follicular and plexiform variants of ameloblastoma was observed in the study of Abdel-Aziz and Amin (2012). Oikawa et al. (2013) reported that although there was no significant difference in the EGFR expression between normal epithelium of dental follicles and ameloblastoma, but its expression is slightly higher in recurrent ameloblastoma than in primary lesions. Thus, it is highly suggestive that EGFR plays a strong role in the invasiveness of ameloblastoma.

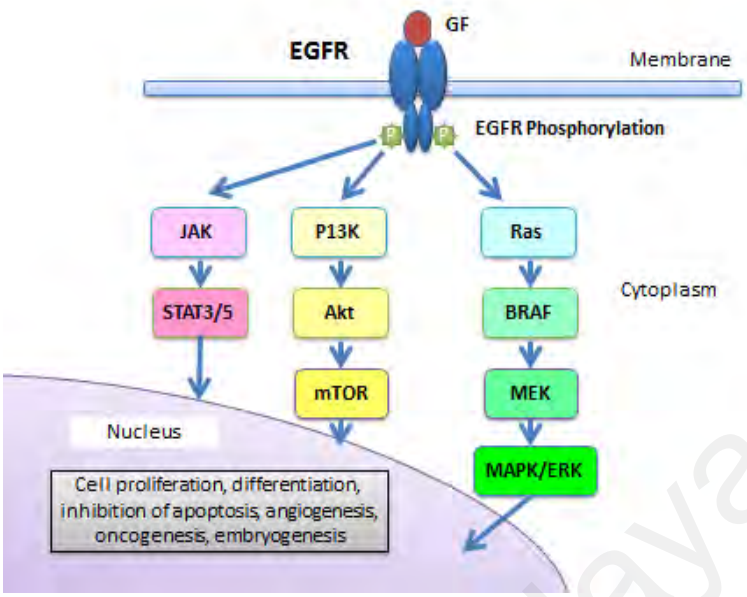


Figure 2.10: EGFR Signalling Pathway. (Adapted Normanno et al., 2006)

2.2.6.3 Introduction to CD10

CD10 is a 90-110kd cell surface zinc dependent metalloendoprotease glycoprotein with endopeptidase activities, which can also be found on the surface of different cell types. CD10 (aka neutral endopeptidase, membrane metalloendopeptidase or neprilysin) takes part in the cleavage and inactivation of certain neuropeptide and peptide hormones important for signal transduction. CD10 is not specific to hematopoietic malignancies but it is also expressed by normal cells as in foetal liver, bone marrow, spleen and brain and other solid tumours as detected in renal cell carcinoma, transitional cell carcinoma, prostatic adenocarcinoma, pancreatic adenocarcinoma, malignant melanoma and endometrial stromal sarcoma (Iezzi et al., 2008; Maguer-Satta, Besancon, & Bachelard-Cascales, 2011). Controversial results have been reported in bladder carcinomas, which CD10 down-regulation in progressive tumours or inversely upregulation associated with invasion and metastasis (Maguer-Satta et al., 2011). Both Abdel-Aziz and Amin (2012) and Iezzi et al. (2008) have identified that CD10 expression was apparent in most of the recurrent ameloblastoma and could be an indicator for the aggressiveness of ameloblastoma. Iezzi and colleagues (2008) also reported that solid ameloblastoma

showed strong intensity of immunoreactivity for CD10 in peritumoral stromal cells compared to unicystic ameloblastoma and peripheral ameloblastoma variants. Several other studies showed that CD10 might be a good marker for differentiating between the primary tumours and metastases and this is useful as a prediction tool for tumour progression (Jhamb & Kramer, 2014). Therefore, CD10 is used as a prognostic tool to predict the tumour invasiveness and high recurrence rate. The use of CD10 molecular target for treatment has been suggested.

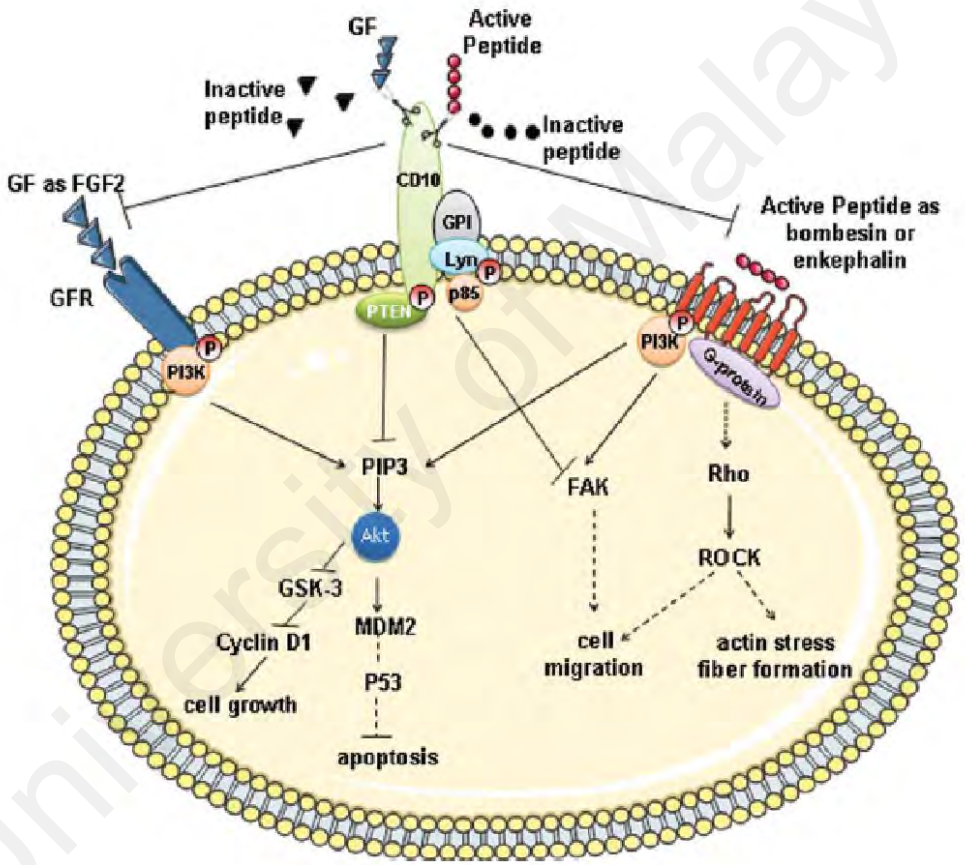


Figure 2.11: CD10 signalling pathway. (Maguer-Satta et al., 2011)

CHAPTER 3: MATERIALS AND METHODS / METHODOLOGY

3.1 Materials

3.1.1 Samples

A total sample of 39 formalin-fixed paraffin-embedded blocks of ameloblastoma cases were selected from the tissue biopsy record of Oral Pathology Diagnostic and Research Laboratory, Faculty of Dentistry, University of Malaya. These cases were retrieved after reviewing the haematoxylin and eosin-stained sections. The selected samples comprised of 20 solid/multicystic ameloblastoma (SMA) and 19 unicystic ameloblastoma (UA) cases diagnosed according to the classification described in the World Health Organization Histological Classification of Odontogenic Tumours (Barnes et al., 2005).

The inclusion criteria for the selected samples were:

1. Sample met the histological criteria established by WHO Histological
2. Classification of Odontogenic Tumours 2005 for SMA and UA;
3. Sample with sufficient tissue representative of SMA/UA.

The exclusion criteria for the selected samples were:

1. Sample with evidence of malignant transformation;
2. Sample with insufficient tissue representative of SMA/UA
3. Sample from the same patient.

The demographic and clinical characteristics data were collected from the available histopathological report. This study was approved by the Medical Ethic Committee, Faculty of Dentistry, University of Malaya [Ethics DF OS1502/0011 (P)].

3.1.2 Antibodies

The primary antibodies used were rabbit monoclonal [EP152Y] to BRAF, rabbit monoclonal [EP774Y] to EGFR and mouse monoclonal [56C6] to CD10. All of these

three antibodies were sourced from Abcam PLC Cambridge, UK. The immunohistochemical staining process for these antibodies using Dako REAL™ Envision Kits (Dako Corporation, Glostrup, Denmark), Dako REAL™ Peroxidase-Blocking Solution (Dako Corporation, Glostrup, Denmark), Dako REAL™ Antibody diluents (Dako Corporation, Glostrup, Denmark) and target retrieval solutions were sourced from BitaLife Sciences Sdn Bhd.

3.2 Methods

3.2.1 Specimens preparation

All samples of formalin-fixed and paraffin-embedded blocks were cut in serial sections of 4 micrometer thickness with a microtome. Each section was mounted on silanised glass slide which incubated overnight at 60°C for deparaffinisation.

3.2.2 Haematoxylin and Eosin (H&E)

The 39 samples were stained with Haematoxylin and Eosin using the standard protocol as shown in Appendix A. The H&E stained samples were assessed for the adequacy of representative tissue for SMA/UA.

3.2.3 Immunohistochemistry

The optimisation of immuno-markers was processed to establish the optimum dilution for each immuno-marker prior to immunohistochemical procedures. All the samples were processed according to the manufacturer recommended immunoperoxidase Envision technique (Dako Envision™ Kit, Dako Cytomation, Copenhagen, Denmark) using the Decloaking Chamber™ (Biocare Medical) for the immunohistochemical studies. The protocol for immunohistochemistry process is described in detail in Appendix B. Immunohistochemistry staining using phosphate buffered saline in place of the primary antibody will be used as a negative control. Positive controls will be performed according to the manufacturer's recommendations.

The following table shows the summary of the primary antibodies used in this study.

Table 3.1: Summary of the primary antibodies used for immunohistochemical studies.

Primary Antibody	Source	Dilution	Antigen Retrieval Buffer (pH)	Incubation Period and Temperature	Washing Buffer (pH)	Control Tissue
Anti-BRAF antibody ab33899 Rabbit monoclonal [EP152Y] to B Raf	Abcam®	1:50	Citrate Buffer (pH6.0)	2 hours at room temperature	Phosphate Buffered Saline (pH7.4)	Breast cancer
Anti-EGFR antibody Rabbit monoclonal [EP774Y] to EGFR (phosphor Y1092)	Abcam®	1:250	Citrate Buffer (pH6.0)	Overnight at 4°C	Phosphate Buffered Saline (pH7.4)	Breast cancer
Anti-CD10 antibody ab951 Mouse monoclonal [56C6] to CD 10	Abcam®	1:25	Citrate Buffer (pH6.0)	Overnight at 4°C	Phosphate Buffered Saline (pH7.4)	Tonsil

3.2.4 Interpretation of results

3.2.4.1 Descriptive

All of the immunohistochemically stained sections were examined with virtual microscope (Olympus BX51 Microscope, Olympus Imaging Inc., Tokyo, Japan). Digitalized images were captured using the same system (Olyvia Dotslide Virtual Slide System, Olympus Imaging Inc., Tokyo, Japan).

3.2.4.2 Semiquantitative methods

The expression of BRAF, EGFR and CD10 biomarkers for SMA and UA was evaluated using a semiquantitative method. Four representative field areas from the tumour centre (SMA and UA) and another four fields from the advancing front of both variants in the immunostained specimens were systematically examined at x400 magnification (x40 objective and x10 ocular). Immunoreactive positive cells identified were categorised as pre-ameloblast-like (PA) cells, stellate reticulum-like (SR) cells and stromal cells (ST), and showed brown colour-staining irrespective of the staining. Localisation of markers was identified in both cytoplasmic and membranous area for each positively-stained cell. The regions with artefact were avoided to prevent the occurrence of false positive result. The level of expression for BRAF, EGFR and CD10 were quantified according to the percentage of immunoreactive tumour epithelial cells and stromal cells present as summarised below:

Score	Level of Immunoreactivity
-	Negative when none of the tumour epithelial cells were positively stained in the cytoplasm or on the cell membrane
+	Mild when staining is present in focal areas (<25%)
++	Moderate when staining is evident in significant areas of the tumour (25%-50%)
+++	Strong when staining is present in predominant areas of the tumour (>50%)

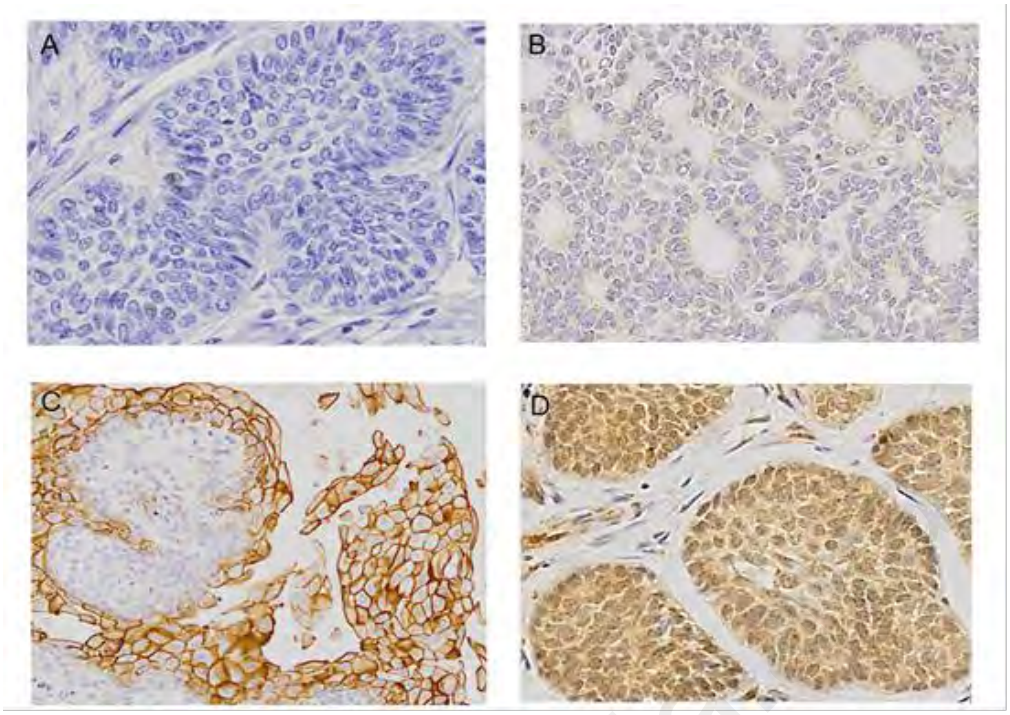


Figure 3.1: Photomicrograph examples of immunoreactivity and scoring guide. A. Negative immunoreactivity for all cellular localisations; B. Mild to moderate intensity staining mainly cytoplasmic (Score: PA-like cells=2, SR-like cells=1, stromal cells=1); C. Strong staining intensity in tumour epithelial cells (Score: PA-like cells=3, SR-like cells=0, stromal cells=0); D. Strong intensity in the nucleus, cytoplasm and membrane (Score: PA-like cells=3, SR-like cells=3, stromal cells=3). [Original magnification x 400]

3.2.4.3 Calibration

Cohen's kappa coefficient statistic test was used to determine the consistency in intraobserver and interobserver scoring of the immunoreactivity staining. The intraobserver calibration was conducted by author personally within 2 weeks between the first and the second data scoring while the interobserver calibration was carried out between the author and her supervisor. There was strong agreement within intraobserver and interobserver in immunoreactivity staining scoring with Kappa value 0.85 and 0.90 respectively.

3.2.4.4 Statistical analysis

All data was analysed statistically using IBM SPSS version 20. The Friedman test was used to compare the expression of BRAF, EGFR and CD10 between PA-like cells, SR-like cells and stromal cells in both UA and SMA. Comparison of the cellular

localisation in expression of different markers in UA and SMA was performed using Wilcoxon Signed-Ranks test. Mann-Whitney U test was performed for comparative analysis of BRAF, EGFR and CD10 in relation to ameloblastoma subsets within different staining localisation (PA-like cells, SR-like cells and stromal cells). The association between BRAF, EGFR and CD10 with different clinical parameters in UA and SMA were tested using Fisher's exact test. All statistical analysis performed with *P* value of <0.05 being considered significant.

CHAPTER 4: RESULTS

4.1 Demographic Characteristics

The demographic characteristics of these 39 cases of ameloblastoma are summarised in Table 4.1. There are 19 cases of UA and 20 cases of SMA. In both ameloblastoma subsets, male was slightly more predominant compared to female with a ratio of 1:0.9. The majority of the cases were Chinese (43.5%) followed by Malays (38.5%), Indians (10.3%) and others (7.7%). The mean age in SMA was 38.75 years which was older than UA (22.89 years). Ameloblastoma mainly occurred in the mandible for both SMA (80%) and UA (89.5%). A painless swelling was the most common symptom experienced by 13 (72.2%) SMA and 15 (83.3%) UA patients. The mean duration for presenting symptoms was 40 months in SMA and 17 months in UA. The most common known clinical diagnosis made was ameloblastoma for both SMA (90%) and UA (68.4%).

Table 4.1: Demographic characteristics of patients with solid/multicystic (SMA) and unicystic ameloblastoma (UA)

Variables		**SMA** N = 20	**UA** N = 19	Total
Gender, n (%)				
	Male	11 (55.0)	10 (52.6)	21
	Female	9 (45.0)	9 (47.4)	18
Age (years)				
	Mean	38.75	22.89	
	SD	38	16	
	Range	15 to 67	8 to 58	
Race, n (%)				
	Malay	8 (40.0)	7 (36.8)	15
	Chinese	8 (40.0)	9 (47.7)	17
	Indian	3 (15.0)	1 (5.3)	4
	Others	1 (5.0)	2 (10.5)	3

Table 4.2: Clinical characteristic of patients with solid/multicystic (SMA) and unicystic ameloblastoma (UA)

Variables		**SMA**	**UA**
Total		20	19
Presenting complaints, n (%)			
	Painless swelling	13 (72.2)	15 (83.3)
	Pain and swelling	4 (22.2)	3 (16.7)
	Others	1 (5.6)	0
Known site, n (%)			
	Maxilla	4 (20.0)	2 (10.5)
	Mandible	16 (80.0)	17 (89.5)
Known duration, n (%)			
	0-6 month	5 (33.3)	9 (64.3)
	7-11 month	1 (7.7)	1 (7.1)
	12-24 month	2 (13.3)	2 (14.3)
	>24 month	7 (46.7)	2 (14.3)
Known clinical diagnosis, n (%)			
	Ameloblastoma	18 (90.0)	13 (68.4)
	Dentigerous cyst	1 (5.0)	5 (26.3)
	Lateral periodontal cyst	1 (5.0)	0
	Radicular cyst	0	1 (5.3)
Known treatment, n (%)			
	Enucleation	4 (36.4)	2 (33.3)
	Segmental Resection	7 (63.6)	4 (66.7)

4.2 Expression of BRAF in ameloblastoma

Immunoreactivity of BRAF in both ameloblastoma subsets is summarised in Figure 4.1. A significantly strong intensity of BRAF is found within SR-like cells for both SMA and UA. However, higher expression of BRAF at SMA compared to UA at SR-like cells. Their immunoreactivity was observed mainly in the cytoplasmic region with some at the nucleus. In UA, majority of the PA-like cells and stromal cells are mild to moderate in staining. Their staining was mainly localised within the cytoplasmic area rather than the nucleus. Figure 4.2 illustrates the distribution pattern of BRAF in SMA and UA.

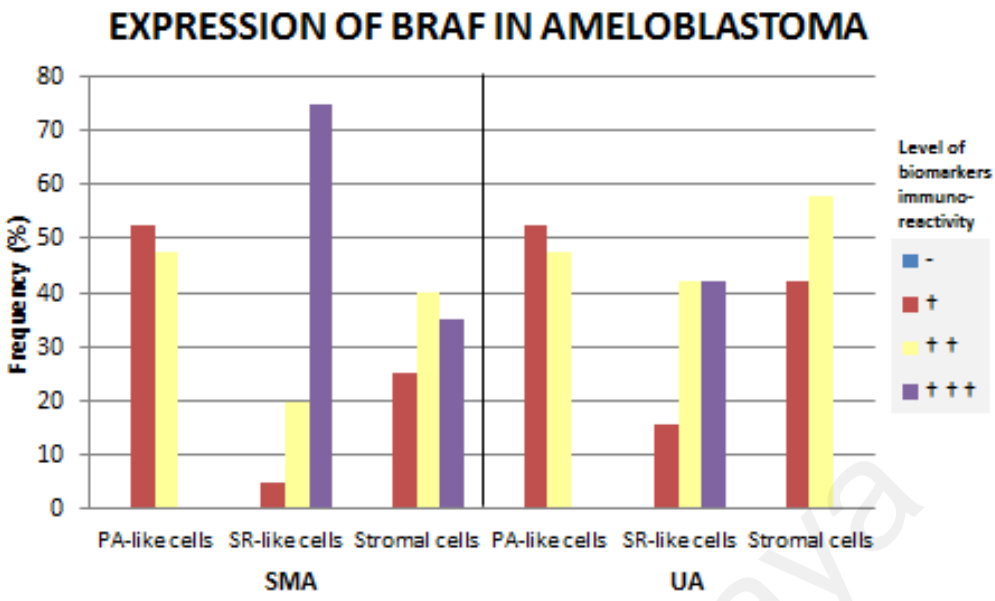


Figure 4.1: **Expression of BRAF in solid/multicystic ameloblastoma (SMA) and unicystic ameloblastoma (UA). A significantly higher expression of BRAF was detected in SR-like cells compared to PA-like cells or stromal cells in both SMA and UA (*P*<0.05)**

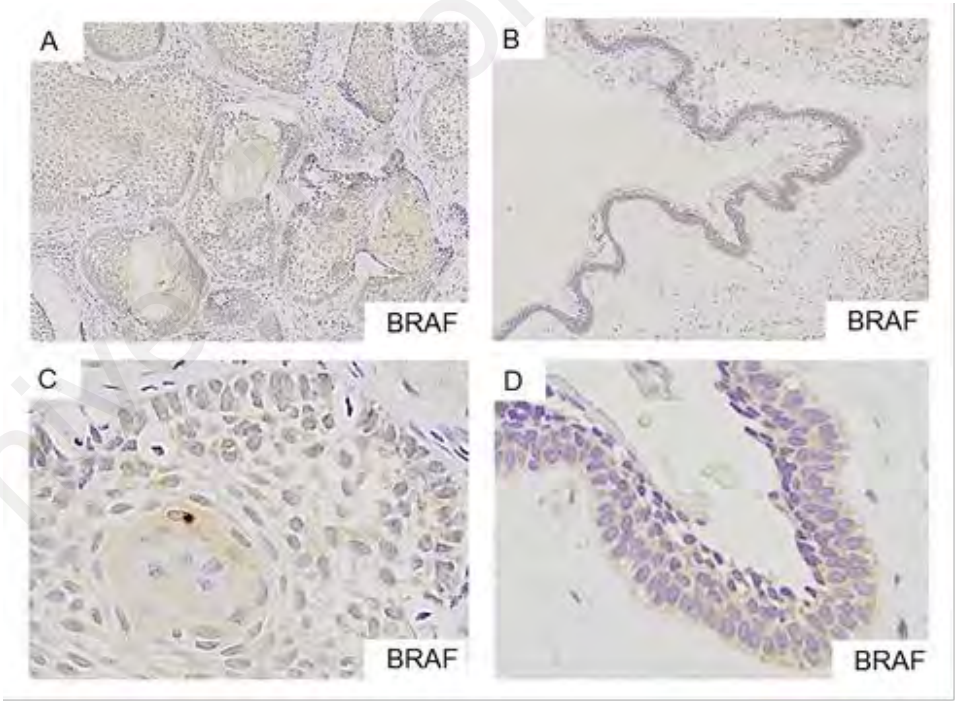


Figure 4.2: **Differential expression of BRAF in the pre-ameloblast-like, stellate reticulum-like and stromal cells of solid/multicystic ameloblastoma (A, C) and unicystic ameloblastoma (B, D). (Original magnification A, B x40, C, D x200).**

4.3 Expression of EGFR in ameloblastoma

Expression of EGFR was markedly strong in both SR-like cells and stromal cells in both SMA and UA. The strong immunoreactivity of EGFR was equally distributed within the cytoplasmic and nucleus regions. EGFR expression in SMA was higher than UA for all three cell types. More than 80% of the PA-like cells are found to have moderate intensity of EGFR staining in both SMA and UA. None of the sample showed negative immunoreactivity toward EGFR. Figure 4.3 summarised the expression of EGFR in PA-like cells, SR-like cells and stromal cells in both ameloblastoma subsets.

Differential expression of the EGFR in the representative section of each ameloblastoma subset is illustrated in Figure 4.4. Both cytoplasm and nucleus in PA-like cells, SR-like cells and stromal cells demonstrated an even staining intensity. Overall stronger immunoreactivity of EGFR was found in SR-like cells and stromal cells.

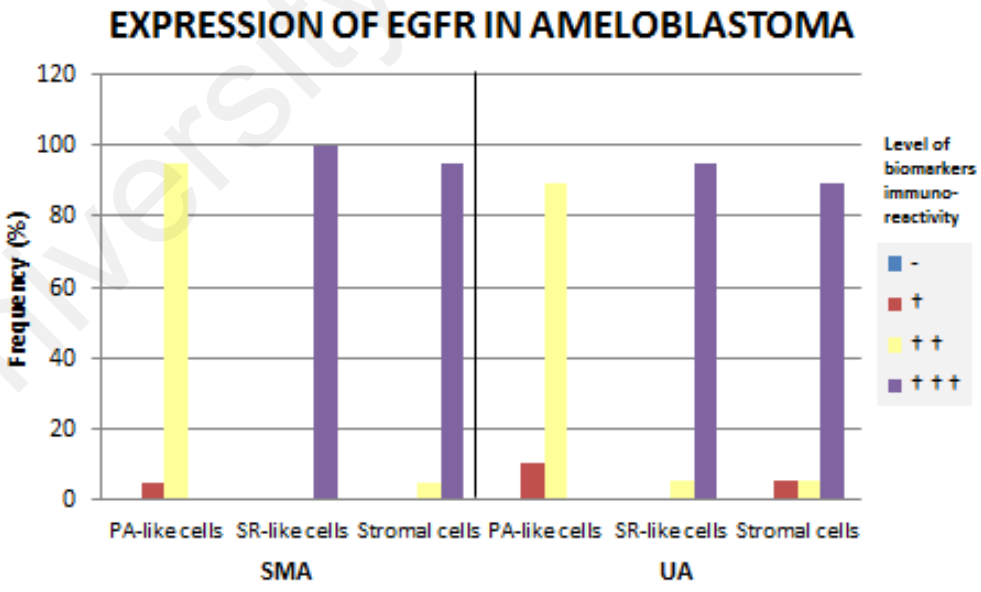


Figure 4.3: **Expression of EGFR in solid/multicystic ameloblastoma (SMA) and unicystic ameloblastoma (UA). A significantly higher expression of EGFR was detected in SR-like cells and stromal cells compared to PA-like cells in both SMA and UA ($P<0.05$)**

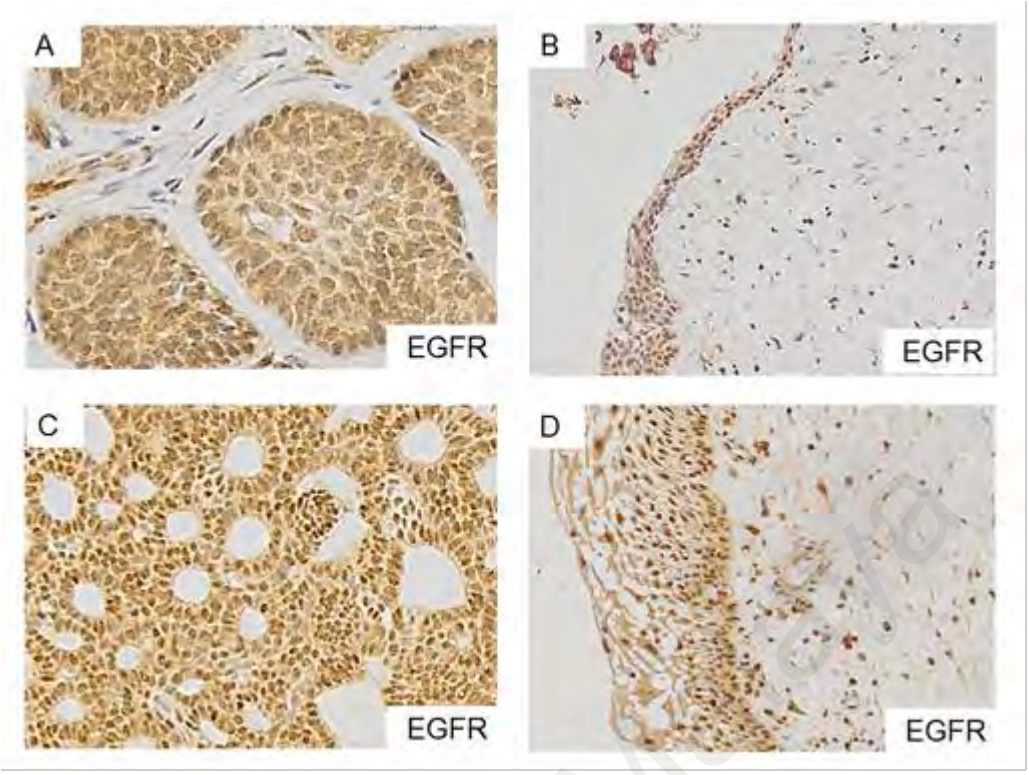


Figure 4.4: Differential expression of EGFR in the pre-ameloblast-like, stellate reticulum-like and stromal cells of solid/multicystic ameloblastoma (A, C) and unicystic ameloblastoma (B, D). (Original magnification B x40, C, D x100; A x200).

4.4 Expression of CD10 in ameloblastoma

The expression of CD10 intensity in SMA and UA is summarised in Figure 4.5. All PA-like cells show negative immunoreactivity in expression of CD10 marker in both subsets of ameloblastoma. None of them show strong immunoreactivity in stromal cells. Majority of the intense immunoreactivity of CD10 are observed at SR-like cells in SMA (80.0%) and UA (42.1%). Exclusive membranous staining seen at SR-like cells with focal cytoplasmic staining at stromal cells. Figure 4.6 illustrates the differential expression of CD10 in SMA and UA.

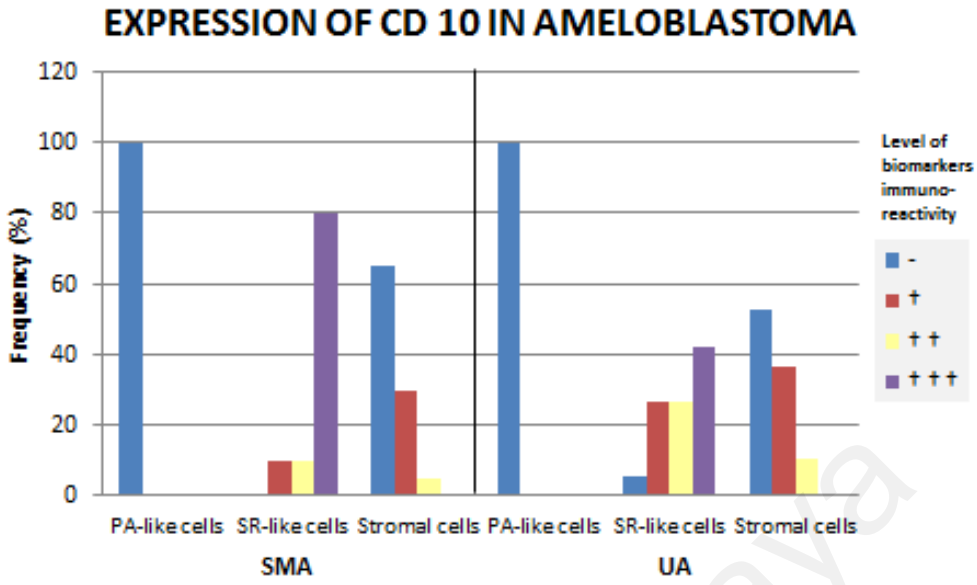


Figure 4.5: Expression of CD10 in solid/multicystic ameloblastoma (SMA) and unicystic ameloblastoma (UA). A significantly higher expression for CD10 was detected in SR-like cells compared to PA-like cells or stromal cells in both SMA and UA (*P*<0.05).

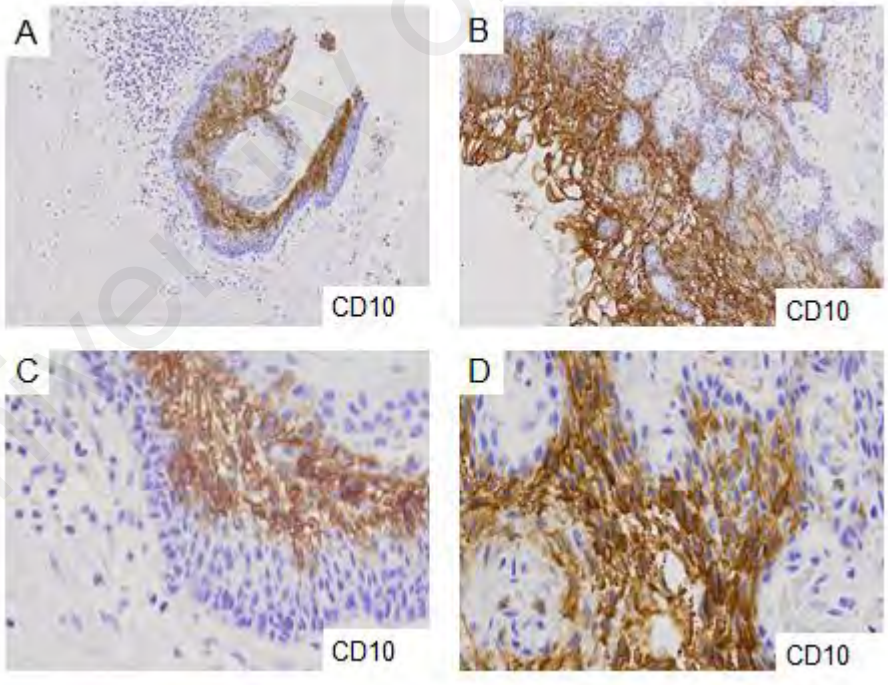


Figure 4.6: **Differential expression of CD10 in the pre-ameloblast-like, stellate reticulum-like and stromal cells of solid/multicystic ameloblastoma (A, C) and unicystic ameloblastoma (B, D). (Original magnification A, B x40; C, D x200).**

4.5 Statistical Analysis

4.5.1 Comparative analysis of immunoreactivity for BRAF, EGFR and CD10 in different cellular localisations between SMA and UA

Friedman Test was used for analysing the immunoreactivity of the three different markers in relation to PA-like cells, SR-like cells and stromal cells in SMA and UA. Both subtypes of ameloblastoma showed significant differences in their immunoreactivity for BRAF, EGFR and CD10 within PA-like cells, SR-like cells and stromal cells ($P<0.05$) as described in Tables 4.3 and 4.4. Post-hoc analysis with Wilcoxon signed-rank test (Table 4.5) was conducted and Bonferroni correction applied, resulting in a significance level set at $P<0.016$. There is a statistically significant expression of BRAF in SR-like cells compared to PA-like cells and stromal cells; expression of EGFR in PA-like cells compared to SR-like cells and stromal cells; expression of CD10 in all three cellular localisations.

Table 4.3: Comparison between the expression of BRAF, EGFR and CD10 in PA-like cells, SR-like cells and stromal cells in solid/multicystic ameloblastoma

Marker	n	Cellular localisations	Mean (SD)	χ^2 statistic (df)	*P* value*
BRAF	20	PA-like cells	1.75 (0.444)	21.900 (2)	**.000**
		SR-like cells	2.70 (0.571)		
		Stromal cells	2.10 (0.788)		
EGFR	20	PA-like cells	1.95 (0.224)	38.100(2)	**.000**
		SR-like cells	3.00 (0.000)		
		Stromal cells	2.95 (0.224)		
CD 10	20	PA-like cells	0.00 (0.000)	37.284 (2)	**.000**
		SR-like cells	2.70 (0.657)		
		Stromal cells	0.40 (0.598)		

*Friedman test was performed. Bold value indicate significant difference

Table 4.4: Comparison between the expression of BRAF, EGFR and CD10 in PA-like cells, SR-like cells and stromal cells in unicystic ameloblastoma

Marker	n	Cellular localisations	Mean (SD)	χ^2 statistic (df)	*P* value*
BRAF	19	PA-like cells	1.47 (0.513)	20.042 (2)	**.000**
		SR-like cells	2.26 (0.733)		
		Stromal cells	1.58 (0.507)		
EGFR	19	PA-like cells	1.89 (0.315)	33.655 (2)	**.000**
		SR-like cells	2.95 (0.229)		
		Stromal cells	2.84 (0.501)		
CD 10	19	PA-like cells	0.00 (0.000)	28.677 (2)	**.000**
		SR-like cells	2.05 (0.970)		
		Stromal cells	0.58 (0.692)		

*Friedman test was performed. Bold value indicate significant difference

Table 4.5: Comparison between the cellular localisation of expression of BRAF, EGFR and CD10 in solid/multicystic ameloblastoma (SMA) and unicystic ameloblastoma (UA)

Ameloblastoma subset	Localisation comparison	Marker [Z statistic (*P* value*)]		
		BRAF	**EGFR**	**CD10**
SMA (n: 20)	PA-like cells & SR-like cells	-4.146 (**.000**)	-4.379 (**.000**)	-4.176 (**.000**)
	PA-like cells & Stromal cells	-1.69 (.090)	-4.264 (**.000**)	-2.53 (**.011**)
	SR-like cells & Stromal cells	-2.762 (**.006**)	-1.000 (.317)	-3.993 (**.000**)
UA (n: 19)	PA-like cells & SR-like cells	-3.638 (**.000**)	-4.264 (**.000**)	-3.78 (**.000**)
	PA-like cells & Stromal cells	-0.816 (.414)	-4.025 (**.000**)	-2.81 (**.005**)
	SR-like cells & Stromal cells	-2.968 (**.003**)	-0.816 (.414)	-3.132 (**.002**)

* Wilcoxon Signed-Rank test was performed. Bold values indicate significant difference.

4.5.2 Comparative analysis of BRAF, EGFR and CD10 in relation to ameloblastoma subsets within different localisations

Mann-Whitney U test was used for comparing the expression of BRAF, EGFR and CD10 within PA-like cells, SR-like cells and stromal cells between SMA and UA (Tables 4.6 to 4.8). Expression of BRAF was significantly different between SMA and UA within SR-like cells and stromal cells with higher mean rank value observed in SMA. The analysis also revealed that expression of CD10 was significantly different between SR-like cells between SMA and UA. All the BRAF, EGFR and CD10 showed 100% epithelial-stromal immunoreactivity in both ameloblastoma variants (Table 4.9).

Table 4.6: Comparison of expression of BRAF, EGFR and CD10 within pre-ameloblast-like cells (PA) between solid/multicystic ameloblastoma (SMA) and unicystic (UA) ameloblastoma

Markers	Subtypes	n	Mean Rank	Z	*P* value*
BRAF	SMA	20	22.63	-1.750	.080
	UA	19	17.24		
EGFR	SMA	20	20.53	-.639	.532
	UA	19	19.45		
CD10	SMA	20	20	.000	1.000
	UA	19	20		

* Mann-Whitney U test was performed. Bold values indicate significant difference.

Table 4.7: Comparison of expression of BRAF, EGFR and CD10 within stellate reticulum-like cells (SR) between solid/multicystic ameloblastoma (SMA) and unicystic ameloblastoma (UA)

Markers	Subtypes	n	Mean Rank	Z	*P* Value*
BRAF	SMA	20	23.23	-2.072	**.038**
	UA	19	16.61		
EGFR	SMA	20	20.50	-1.026	.305
	UA	19	19.47		
CD10	SMA	20	23.70	-2.392	**.017**
	UA	19	16.11		

* Mann-Whitney U test was performed. Bold values indicate significant difference.

Table 4.8: Comparison of expression of BRAF, EGFR and CD10 within stromal cells between solid/multicystic ameloblastoma (SMA) and unicystic ameloblastoma (UA)

Markers	Subtypes	n	Mean Rank	Z	*P* Value*
BRAF	SMA	20	23.55	-2.174	**.030**
	UA	19	16.26		
EGFR	SMA	20	20.55	-.669	.504
	UA	19	19.42		
CD10	SMA	20	18.70	-.839	.401
	UA	19	21.37		

* Mann-Whitney U test was performed. Bold values indicate significant difference.

Table 4.9: Association of tumour epithelium versus stromal among BRAF, EGFR and CD10 with site of lesion in solid/multicystic ameloblastoma (SMA) and unicystic ameloblastoma (UA)

Markers / Subtypes	BRAF	EGFR	CD10
SMA (N=20)	100%	100%	100%
UA (N=19)	100%	100%	100%

4.5.3 Association of BRAF, EGFR and CD10 expression in relation to clinical parameters in ameloblastoma subtypes

The Fisher's exact test was used to analyse the association of BRAF, EGFR and CD10 expression with the clinical parameters including age, ethnicity, gender and location of lesion in SMA and UA. Association between expression of EGFR and Malay ethnicity in SMA showed significant difference (P=0.000). There was no significant association observed between BRAF, EGFR and CD10 with other clinical parameters of SMA and UA (Table 4.10-4.13).

Table 4.10: Association between BRAF, EGFR and CD10 expression levels with patients' median age in solid/multicystic ameloblastoma (SMA) and unicystic ameloblastoma (UA)

Ameloblastoma Subset	Variables	N	BRAF				*P* value**	EGFR				*P* value**	CD 10				*P* value**
			-	+	++	+++		-	+	++	+++		-	+	++	+++	
SMA	Age*																
	≤ 38	11	0	1	6	4	.621	0	0	1	10	1.000	0	7	4	0	1.000
	>38	9	0	0	6	3		0	0	0	9		0	6	3	0	
UA	Age*																
	≤ 16	10	0	3	7	0	1.000	0	0	0	10	.211	0	0	7	3	.211
	>16	9	0	2	7	0		0	0	2	7		0	0	9	0	

* Median age of 38 years in SMA and 16 years in UA were used as the cut-off point.
**Fisher's exact test was performed
Level of immunoreactivity is described in Materials and Methods (p.27)

Table 4.11: Association between BRAF, EGFR and CD10 expression levels with patients' ethnicity in solid/multicystic ameloblastoma (SMA) and unicystic ameloblastoma (UA)

Ameloblastoma Subset	Variables	N	BRAF				*P* value*	EGFR				*P* value*	CD 10				*P* value*
			-	+	++	+++		-	+	++	+++		-	+	++	+++	
SMA	Malay	8	0	1	5	2	.806	0	0	0	8	**.000**	0	4	4	0	.640
	Chinese	8	0	0	4	4		0	0	0	8		0	6	2	0	
	Indian	3	0	0	2	1		0	0	0	3		0	2	1	0	
	Others	1	0	0	1	0		0	0	1	0		0	1	0	0	
UA	Malay	7	0	1	6	0	.374	0	0	0	7	.478	0	6	1	0	.550
	Chinese	9	0	4	5	0		0	0	2	7		0	8	1	0	
	Indian	1	0	0	1	0		0	0	0	1		0	1	0	0	
	Others	2	0	0	2	0		0	0	0	2		0	1	1	0	

* Fisher's exact test was performed. Bold values indicate significant difference.

Level of biomarkers immunoreactivity is described in Materials and Methods (p.27)

Table 4.12: Association between BRAF, EGFR and CD10 expression levels with patients' gender in solid/multicystic ameloblastoma (SMA) and unicystic ameloblastoma (UA)

Ameloblastoma Subset	Variables	N	BRAF				*P* value*	EGFR				*P* value*	CD 10				*P* value*
			-	+	++	+++		-	+	++	+++		-	+	++	+++	
SMA	Male	11	0	1	4	6	.056	0	0	1	10	1.000	0	7	4	0	1.000
	Female	9	0	0	8	1		0	0	0	9		0	6	3	0	
UA	Male	10	0	1	9	0	.141	0	0	1	9	1.000	0	9	2	0	.582
	Female	9	0	4	5	0		0	0	1	8		0	7	1	0	

* Fisher's exact test was performed
Level of biomarkers immunoreactivity is described in Materials and Methods (p.27)

Table 4.13: Association between BRAF, EGFR and CD10 expression levels with tumour location in solid/multicystic ameloblastoma (SMA) and unicystic ameloblastoma (UA)

Ameloblastoma Subset	Variables	N	BRAF				*P* value*	EGFR				*P* value*	CD 10				*P* value*
			-	+	++	+++		-	+	++	+++		-	+	++	+++	
SMA	Maxilla	4	0	0	3	1	.748	0	0	1	1	.205	0	1	1	0	.587
	Mandible	16	0	1	9	6		0	0	1	16		0	15	2	0	
UA	Maxilla	2	0	0	2	0	1.000	0	0	0	4	1.000	0	2	2	0	.298
	Mandible	17	0	5	12	0		0	0	1	15		0	11	5	0	

*Fisher Exact Test was performed

Level of biomarkers immunoreactivity is described in Materials and Methods (p.27)

CHAPTER 5: DISCUSSION

Ameloblastoma is a slow growing, locally aggressive odontogenic tumour which occurs mainly in the mandible with high invasion potential and risk of recurrence. The mean patient age in this study was 38 years in SMA and 22 years in UA without any gender predilection which is similar to those reported in the international literatures. Ameloblastoma has been widely studied but little is known regarding the molecular basis of ameloblastoma tumorigenesis, thus preventing the development of non-invasive therapies. Many studies published recently have shown the cellular changes involved which identified a variety of oncogenic mutations and abnormal pathways through immunohistochemical and genomic analysis. This contributed significantly to our contemporary understanding and approach towards the conservative treatment modalities of ameloblastoma.

5.1 Expression of BRAF

BRAF is a potent intermediate protein kinase to activate the downstreaming of mitogen-activated protein kinase (MAPK) pathway. In MAPK pathway, receptor tyrosine kinase (RTK) is triggered by extracellular signals (eg. hormones, cytokines and various types of growth factors) and activates the RAS. Active RAS initiates the phosphorylation of RAF-MEK-ERK which results in the translocation of ERK into the cell nucleus and activates transcription factors involved in the coordination of different cellular processes like cell proliferation, differentiation, migration and survival. More than 90% of the reported BRAF gene mutations were found to involve the substitution of valine by glutamic acid at codon 600 (V600E) subsequent to the transversion of thymine to adenine in exon 15 at nucleotide 1799. BRAF gene mutation has been found exclusively in a wide variety of human benign and malignant neoplasms (eg. ameloblastoma, melanocytic naevi, melanoma, benign ovarian cystadenoma, colon

adenocarcinoma, papillary thyroid carcinoma, pleomorphic xanthoastrocytomas and colorectal carcinoma). Overexpression of BRAF causes aberrant regulation in MAPK pathway which is believed to be involved in tumorigenesis of many tumours. Thus, detection of BRAF mutation has become an important biomarker for the purpose of diagnostic, prognostic, predictive and potential targeted therapy in different types of tumours.

High frequency of gene alterations in different components of MAPK pathway particularly BRAF in ameloblastoma reported in previous studies range from 46% to 82% in their ameloblastoma samples (Brown et al., 2014; Kurppa et al., 2014; Sweeney et al., 2014; Brown & Betz, 2015; Diniz et al., 2015; Fregnani et al., 2016). These results indicate that targeted therapy could be used for ameloblastoma patients either as an adjunct therapy or exclusive therapy. Sweeney et at. (2014) and Brown et al. (2014) have demonstrated the high effectiveness of using FDA-approved BRAF inhibitor (Vemurafenib) in treatment of BRAF-mutation-positive ameloblastomas in vitro. Kaye and coworkers (2015) have shown a remarkably positive clinical and radiographic response of combined BRAF inhibitors (dabrafenib and trametinib) therapy in a case of 40-year-old African American who was diagnosed with stage 4 recurrent ameloblastoma with distant metastasis. Tan and colleagues (2016) have also reported a case of neoadjuvant treatment in BRAF-mutant ameloblastoma using BRAF inhibitor (dabrafenib) which resulted in more than ninety percent of tumour size reduction in 4 months. These two case reports shared the similarity in the efficacy of BRAF-mutant inhibition therapy in ameloblastoma; however, there is a limitation in BRAF inhibitor completely eradicating ameloblastoma tumour cells. We believe that although BRAF-MAPK pathway plays a major role in the tumorigenesis of ameloblastoma, the compensatory activation of MAPK kinase pathway may contribute to the BRAF-inhibitor resistance mechanism (Heikinheimo, Kurppa, & Elenius, 2015).

Similar to Kurppa et al. (2014), that the expression of BRAF mutation did not correlate with the sex, age of patients, ethnics and tumour location (P> 0.05), although Sweeney et al. (2014) and Brown et al. (2014) suggested BRAF mutation exhibited a marked preponderance in mandible compared to maxilla. Although these reports described the BRAF V600E mutation with clinicopathological correlation for the novel conservative treatment of solid/multicystic and recurrent ameloblastoma, it is crucial to further explore the findings.

Currently, the known clinical behaviour of ameloblastoma has been related to their common classification into solid/multicystic or unicystic ameloblastoma subtypes. In this study, we investigated whether the BRAF mutations present in similar proportions in these two variants of ameloblastomas. All ameloblastoma samples (20 SMA; 19 UA) in this current study showed positive immunoexpression of BRAF similar to the study reported by Pereira et al. (2016). This finding indicates SMA and UA might share the same molecular changes, however, the intratumoral heterogeneity cannot be ruled out and further investigations are needed for confirmation. We also investigated the relationship between the BRAF expression and its association with the tumour behaviour in comparing SMA and UA which has never been highlighted in any previous study. Significantly higher expression of BRAF in SMA compared to UA (P<0.05) is consistent with the clinico-behaviour of SMA which are more invasive with higher recurrence rate. This suggests that BRAF mutation can be used as a predictor of clinical aggressiveness of ameloblastoma and as a prognostic marker.

Contrary to findings reported by Brunner et al. (2015), where the BRAF mutations in 19 samples of ameloblastomas were exclusively present at the epithelial component but not at the stromal component; the localisation of BRAF expression in this study was predominantly at SR-like cells. BRAF mutation expression was significantly higher at SMA than UA in epithelial and stromal components especially at the SR-like cells and

stromal cells ($P<0.05$). This observation demonstrated that the presence of high frequency oncogenic mutation might be initiated at SR-like cells. Stronger BRAF staining at cytoplasm with focal nucleus staining in epithelial cells typically strongest in the SR-like cells was consistent with previous studies (Brown et al., 2014; Fregnani et al., 2016). Cytoplasm of SR-like cells might create a microenvironment to promote the growth and proliferation of tumour cells. Higher BRAF stromal positivity in SMA is suggestive of the presence of ameloblastoma-oncogenic mutation in the stromal cells which participate in the invasion mechanism of ameloblastoma. Thus, this observation might indicate its role in evading apoptosis and increasing the survival rate of oncogenic cells in ameloblastoma via paracrine and autocrine manner. This result was suggestive of the eligibility for ameloblastoma patients to use BRAF-targeted therapy as an alternative treatment particularly in SMA cases.

5.2 Expression of EGFR

EGFR is a cell surface transmembrane tyrosine kinase which is also known as ErbB1 or HER1. It consists of an extracellular ligand binding domain and an intracellular tyrosine kinase domain. Binding of the extracellular ligand (epidermal growth factor or transforming growth factor-α) at receptor activates the subsequent phosphorylation of protein kinase cascade. The auto-phosphorylation of tyrosine kinase elicits downstream transduction signalling via MAPK and Akt pathway. Expression of EGFR plays a major role in modulating cellular processes such as cells proliferation, differentiation, repair, cell migration and regulation of cell survival. EGFR is commonly found to be expressed at epithelial, neuronal and mesenchymal origin cells including osteoblasts and chondrocytes. Osteoblasts with negative EGFR expression have been shown to increase in cell differentiation rate and this reflects their inability to proliferate, thereby impaired bone formation (Sibilia et al., 2007). Upregulation of EGFR in human osteoblasts may directly affect bone metastasis and bone carcinoma such as osteosarcomas.

Overexpression of EGFR has also been observed almost hundred percent in different types of head and neck tumours including the common epithelial odontogenic tumour, ameloblastoma (Sibilia et al, 2007). Several studies had reported that expression of EGFR was higher in recurrent ameloblastoma (Vered et al., 2003; Oikawa et al., 2013). Thus, monoclonal antibodies against EGFR (eg. Cetuximab, Gefitinib, Necitumumab) could be one of the treatment modality for advanced ameloblastoma.

All the specimens in the present study showed homogenous positive immunoreactivity of EGFR irrespective of the variant of ameloblastoma (SMA and UA). The results of similar studies were inconsistent and ranged from all positive EGFR expression to completely immunohistochemically negative of EGFR (Shrestha et at., 1992; Ueno et al., 1994; Vered et al., 2003; Payeras et al., 2007). Our findings were consistent with the reports by Vered et al. (2003) and Payeras et al. (2007). The divergence of results might be due to the different scoring criteria used for the ameloblastoma samples and discrepancy in the techniques during immunohistochemical procedure such as choice of fixative solution, storage time of specimens, variety in anti-EGFR antibody, incubation duration and types of antigen retrieval agent used (Vered et al., 2003; Atkins et al.,2004)

Expression of EGFR was significantly higher in SMA compared to UA ($P<0.05$) in the current study, high level of EGFR expression can be associated with increased activity of protein phosphorylation cascade, proto-oncogene expression and proliferation. This evidence suggested that the EGFR may have a key role in the invasiveness of ameloblastoma. Additionally, in our study, EGFR demonstrated the strongest staining at SR-like cells of SMA with maximum score of 3.00 similar to the study by Ueno and colleagues (1994). It seems reasonable to speculate that SR-like cells bound EGFR factors involved in the stimulation of invasion activities of tumour cells in autocrine mode of activation. Interestingly, the localisation of EGFR protein was

detected in both cytoplasm and nucleus. There was no linear membranous staining of EGFR in our samples. The impression was the presence of EGFR in cytoplasm and nucleus being the primary property of ameloblastoma.

Pereira and colleagues (2015) reported experimentally that EGFR nuclear localisation correlated to higher proliferation activities with co-localisation of Cyclin D1 in ameloblastoma samples. Similar studies in breast cancer and oropharyngeal carcinoma also showed nuclear accumulation of EGFR correlated with high proliferation and poorer survival (Lo et al., 2006). Cytoplasmic expression of EGFR has been associated with poorer prognosis in certain tumours including pancreatic adenocarcinoma, squamous cell lung carcinoma and thyroid carcinoma (Ueda et al., 2004). Ueda and colleagues (2004) also suggested that the overexpression of cytoplasmic EGFR was due to the translocation of EGFR from membrane to cytoplasm during tumour cells–stromal interaction similar to the phenomenon described in epithelial-mesenchymal transition. In contrast, some authors postulated that cytoplasmic overexpression of EGFR might be a sign of intensified signalling and EGF ligand saturation with subsequent internalisation of the ligand-receptor complex and degradation within the lysosomes; while pre-lysosomal EGFR can still activate the protein kinase cascade in cell proliferation (Ueda et al., 2004; Braut et al., 2014). Although these internalised receptor cells might react slower to stimuli, yet their role in affecting proliferative activity of tumour cells cannot be overlooked. The cellular localisation pattern of EGFR may be of clinicopathologic significance and its vital role in assessing its biological invasiveness property, proliferative potential and anti-EGFR treatment option for ameloblastoma.

5.3 Expression of CD10

CD10 protein is a 90-110 kd zinc-dependent endopeptidase which is associated with cell differentiation and growth regulation of normal and neoplastic cells through

signalling peptide transduction pathways. CD10 causes cleavage and inactivation of certain peptide hormones which reduces the cellular response toward local peptide hormones. Expression of CD10 was found to be widely distributed at both normal and neoplastic neuronal cells, hematopoietic cells, epithelial cells and lymphoid cells. CD10 expression is associated with apoptosis, differentiation and proliferation of neoplastic cells. The role of CD10 in tumour progression remains controversial. Suzuki and colleagues (2001) had reported a differential expression of CD10; higher in normal endometrium and markedly low in high grade endometrial carcinoma. Similarly Khin et al. (2003) reported positive immunoreactivity of CD10 detected in ovarian surface epithelial tumours and reduced CD10 immunoreactivity in advanced histological grading of the tumours. However, expression of CD10 in gastric carcinoma, hepatocellular carcinoma and squamous cells carcinoma indicates poor prognosis with increased cellular dysplasia, neoplastic invasion and metastatic potential (Maguer-Satta et al., 2011). Maguer-Satta et al. (2011) has identified the role of CD10 in tumorigenesis and metastatic activity of various tumours, including breast, lung, prostate and pancreas cancers. Recent studies by Iezzi et al. (2008), Abdel-Aziz and Amin (2012) and Ahlem et al. (2015) demonstrated intense stromal staining in ameloblastomas correlated to high local tumour recurrence and their important role in tumour invasion. Currently, immunotherapy for CD10 inhibitor (eg.Thiorphan) in tumour still remains at the stage of in-vitro study. Therefore, the CD10 marker could be useful for evaluating the behaviour of ameloblastoma and development of future personalised targeted therapy.

The present study showed significantly higher expression of CD10 in SMA than UA ($P<0.05$). This result is found to be in accordance with the clinico-behaviour of SMA which tends to be more aggressive and higher recurrence after surgery compared to UA. The immunohistochemical result revealed CD10 was particularly expressed in SR-like cells and mainly membranous with focal cytoplasmic staining. These results were

similar with the study by Masloub et al. (2011) where the CD10 expression at SR-like cells especially membranous in intraluminal type of UA; while membranous and cytoplasmic reactivity in mural type of UA. Their study also found that epithelial cells of SMA showed both membranous and cytoplasmic CD10 immunoreactivity. Deschamps and colleagues (2006) had reported that strong membranous CD10 staining pattern associated with poorly differentiated carcinomas and decreased survival. The difference in CD10 immunoreactivity in these two variants of ameloblastoma might explain the higher invasiveness of SMA compared to UA. We also postulated that high expression of CD10 in SR-like cells is associated with tumour invasion; their proliferation playing an important role in invasive growth pattern of ameloblastoma.

Stromal positivity was detected in 9 of 19 UA and 7 of 20 SMA in this study. In contrast to the report by Iezzi et al. (2008), where the mean expression of CD10 at peritumoral stromal cells was higher in solid multicystic ameloblastoma compared to unicystic ameloblastoma and peripheral ameloblastoma. The slightly higher CD10 expression in stromal of UA than SMA in our study might be explained by the fact that most of our UA samples were mural variant in which aggressiveness behaviour is similar to SMA. The exact mechanism of the presence of stromal CD10 increase the invasiveness and infiltrative feature of tumour cells still remained unclear. It has been suggested that the stromal-tumour cells interaction and the presence of CD10 modifies the microenvironment of tumours which stimulates tumour cell migration and invasion by activation of different signalling pathways (Maguer-Satta et al., 2011).

Our data demonstrated negative immunoreactivity of CD10 in all of the PA-like cells. This finding has never been discussed in previous similar studies. It has been suggested that expression of CD10 is not consistently found to be altered in most epithelial transformed cells. The intrinsic deregulation of CD10 could act as an initiating transforming event affecting particularly immature cells. CD10 deregulation in

early common progenitor cells could block the PTEN function of apoptosis and induced cell proliferation by activation of Akt pathway (Maguer-Satta et al., 2011). Alteration of CD10 expression seems to be a consequence of the initial tumour transformation. Thus, this could possibly explain the negative immunoreactivity of CD10 in PA-like cells in our study that the tumorigenesis initiated in PA-like cell of ameloblastoma which modulated the neighbouring cellular environment.

CHAPTER 6: CONCLUSION

Over the decades, a wide variety of different tumour marker proteins in human neoplasm have been explored and studied extensively in the attempt to identify their roles in tumorigenesis and the biological behaviour of tumours. Nevertheless, the roles of the pro-invasive tumour markers in ameloblastoma still remain unknown. Three pro-invasive ameloblastoma tumour markers (BRAF, EGFR and CD10) were investigated in this study for this purpose.

Based on the results of the present study, the expression of BRAF, EGFR and CD10 were consistently higher in SMA than UA variants. It could be concluded that these three pro-invasive markers play an important role in the tumorigenesis of ameloblastoma through their specific signalling pathway to stimulate tumour invasion and progression. These proteins function as a complex network to regulate cellular growth, such as cell proliferation, differentiation, anti-apoptosis and survival. Each of these markers might be a useful tool to identify the local invasive behaviour of ameloblastoma and to predict recurrence risk.

The limitations of this study were small sample size and inadequate clinical information of the samples. The lacking of wide variety of other subtypes of ameloblastoma such as desmoplastic and peripheral variant prevents further comparison of the expression pattern between different ameloblastoma subtypes. The evaluation of correlation between tumour markers expression and recurrence was not feasible due to incomplete clinical information provided.

Further studies are needed to explore the potential role of personalised molecular targeted therapies directed against these pro-invasion markers in preventing tumour relapse and completely tumour eradication in ameloblastoma.

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APPENDICES

APPENDIX A: Protocol for Haematoxylin & Eosin Staining

1. **Tissue sections were deparaffinised and rehydrated as following method:**

Reagent bath	Duration (minutes)
Xylene	5
Xylene	4
Alcohol 100%	3
Alcohol 95%	3
Alcohol 70%	3
Running Water	3

2. **Tissue sections were placed in different solutions for haematoxylin staining as below:**

Reagent bath	Duration (minutes)
Harris Haematoxylin	1
Running water	3
Acid alcohol	3
Running water	3
Potassium acetate	4 dips
Running water	3

3. For Eosin staining and dehydration:

Reagent bath	Duration (minutes)
Alcohol 80%	1
Eosin	4
2 bath of Ethanol 95%	1 (each)
2 bath of Ethanol 100%	1 (each)
3 bath of Xylene	3 (each)

4. Mounting of slides using dibutyl phthalate (DPX)

APPENDIX B: Protocol for Immunohistochemistry

Tissue sections were incubated overnight at 60°C prior to immunohistochemistry processing.

1. Tissue sections were deparaffinised and rehydrated using different reagent bath as following:

Reagent bath	Duration (minutes)
Xylene I	5
Xylene II	4
Alcohol 100%	3
Alcohol 95%	3
Alcohol 70%	3
Running water	3

2. Tissue sections were immersed in the Antigen Retrieval Buffer and pretreated using decloaking chamber at 121°C for 30 seconds for antigen retrieval.
3. Tissue sections were allowed to cool at room temperature for 20 minutes and rinsed under running water for 5 minutes.
4. Tissue sections were fully covered with endogenous peroxidase blocking agent and incubated at room temperature for 10 minutes.
5. Tissue sections were then washed twice with PBS bath. The excess fluid was drained off.
6. Tissue sections were incubated with primary antibody for a specific duration and temperature as recommended by the manufacturer (Table 3.1).
7. After incubation, excess fluid was allowed to drain off and tissue sections were rinsed in three baths of PBS. Any excess fluid was wiped off from the slides.
8. Next, the tissue sections were incubated with secondary antibody for 30 minutes at room temperature, followed by washing in three baths of PBS and draining off

excess fluid.

9. Tissue sections were incubated with 3, 3-diaminobenzidine (DAB) for 5 minute at room temperature and washed under running water for 5 minutes.
10. Tissue sections were counterstained and dehydrate as the following steps,

Reagent bath	**Duration**
Harris haemotoxylin	1 minute
Wash in running water	3 minutes
Acid alcohol 0.5%	10 seconds
Wash in running water	3 minutes
2% potassium acetate	4 dips
Wash in running water	3 minutes
Alcohol 95% - I	4 dips
Alcohol 95% - II	4 dips
Alcohol 100% - I	2 minutes
Alcohol 100% - II	2 minutes
Xylene - I	3 minutes
Xylene – II	3 minutes
Xylene - III	3 minutes

11. The tissues sections were mounted with coverslips using dibutyl phthalate (DPX).
12. Tissues as mentioned in Table 3.1 were used for positive control.
13. Negative control samples used the same tissue sections which were treated without the primary antibody. All of the control sections were negative.