EXPRESSION OF INTERLEUKIN 1-ALPHA, INTERLEUKIN 6 AND CD 10 IN PREDICTING RECURRENCE OF AMELOBLASTOMA

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EXPRESSION OF INTERLEUKIN 1-ALPHA, INTERLEUKIN 6 AND CD 10 IN PREDICTING RECURRENCE OF AMELOBLASTOMA

ABSTRACT

Background: Ameloblastoma (AM) is a benign yet locally aggressive odontogenic tumour with a high post-treatment recurrence rate. Despite advances in surgical techniques, predicting recurrence remains a significant challenge. Recent literature suggests that specific cytokines and markers, such as interleukin 1-alpha (IL-1α), interleukin 6 (IL-6), and CD 10, might play a role in AM's aggressive behaviour and recurrence. **Objectives**: The aim of this study is to evaluate the expression levels of IL-1α, IL-6 and CD 10 and compare their immunoprofile in non-recurrent and recurrent AM. This study also sought to determine the association between expression level of these markers with demographic and clinicopathological parameters in non-recurrent and recurrent AM. **Methods**: A descriptive cross-sectional study comprising of 42 cases were divided into 3 groups; 16 cases of conventional AM with no recurrence (AMNR), 13 cases of primary conventional AM with recurrence (PAMR), and 13 cases of recurrent ameloblastoma from the same patient (RAM) subjected to immunohistochemical staining for IL-1α, IL-6 and CD 10. A semiquantitative scoring, immunoreactive scoring (IRS) was used to evaluate the expression of IL-1α, IL-6 and CD 10 in non-recurrent and recurrent AM. The expression levels were then correlated with demographic and clinicopathological parameters. **Results**: There were no significant differences ($p>0.05$) in IL-1α, IL-6, or CD10 expression across all groups. IL-1α expression was significantly associated with histopathological subtype in AMNR ($p=0.03$) and PR ($p=0.002$). In the AMNR group, IL-6 expression was significantly associated with tumour side localization ($p = 0.01$). CD 10 showed significant correlation with tumour side localization ($p=0.02$) in PAMR, and subsites of tumour localization in PAMR ($p=0.005$) and RAM ($p=0.002$).

Conclusions: We observed an upregulation of IL-6 expression in recurrent AM compared to non-recurrent AM, suggesting the potential of IL-6 in predicting recurrence of AM. Downregulation of IL-1α and CD-10 expression in recurrent AM compared to non-recurrent AM needs further investigation in order to identify their role.

Key words: Ameloblastoma, IL-1α, IL-6, CD10, recurrence.

EKSPRESI INTERLEUKIN 1-ALPHA, INTERLEUKIN 6 DAN CD 10 DALAM MERAMALKAN PENGULANGAN AMELOBLASTOMA

ABSTRAK

Latar Belakang: Ameloblastoma (AM) ialah tumour odontogenik yang jinak namun agresif secara setempat dengan kadar pengulangan yang tinggi selepas rawatan. Walaupun terdapa kemajuan dalam teknik pembedahan, meramalkan pengulangan merupakan cabaran yang ketara. Penemuan terkini mencadangkan bahawa sitokin dan penanda tertentu, seperti interleukin 1-alpha (IL-1α), interleukin-6 (IL-6), dan CD 10, mungkin memainkan peranan dalam tingkah laku agresif AM dan berulang. **Objektif**: Tujuan kajian ini adalah untuk menilai tahap ekspresi IL-1α, IL-6 dan CD 10 dan membandingkan imunoprofil mereka dalam AM tidak berulang dan berulang. Kajian ini juga berusaha untuk menentukan perkaitan antara tahap ekspresi penanda ini dengan parameter demografi dan klinik-patologi dalam AM tidak berulang dan berulang. Kaedah: Matlamat kajian ini adalah untuk menilai tahap ekspresi IL-1α, IL-6 dan CD 10 dan membandingkan imunoprofil mereka dalam kes AM tidak berulang dan berulang. Kajian ini juga berusaha untuk menentukan perkaitan antara tahap ekspresi penanda ini dengan parameter demografi dan klinik-patologi dalam AM tidak berulang dan berulang. **Kaedah**: Kajian keratan rentas deskriptif yang terdiri daripada 42 kes dibahagikan kepada 3 kumpulan; 16 kes daripada konvensional AM tanpa berulang (AMNR), 13 kes daripada primer konvensional AM (PAMR) dan 13 kes daripada ameloblastoma berulang dalam pesakit yang sama (RAM). Semua sampel tertakluk kepada pewarnaan imunohistokimia untuk IL-1α , IL-6 dan CD 10. Pemarkahan separa kuantitatif, pemarkahan imunoreaktif (IRS) digunakan untuk menilai ekspresi IL-1α, IL-6 dan CD 10 dalam AM bukan berulang dan berulang. Data demografi dan klinikal, termasuk umur pesakit, jantina, lokasi tumour, dan subtaip histopatologi AM telah dianalisis. **Keputusan**: Tiada perbezaan ketara ($p>0.05$) dalam ekspresi IL-1α, IL-6 atau CD10 merentas semua

kumpulan. Ekspresi IL-1α dikaitkan dengan ketara dengan subtaip histopatologi plexiform dalam AMNR (p=0.03) dan subtaip folikel dalam PAMR (p=.002). Dalam kumpulan AMNR, IL-6 dikaitkan dengan lokasi tumour, khususnya sebelah kanan (p = 0.01). CD 10 mempunyai penemuan penting pada lokasi kanan tulang mandibel, RAM (p=0.02), subtaip lokasi tumour PAMR (p=0.005), dan RAM (p=0.002). **Kesimpulan:** Kami memerhatikan kenaikan regulasi ekspresi IL-6 dalam AM berulang berbanding AM tidak berulang, mencadangkan potensi IL-6 dalam meramalkan pengulangan AM. Penurunan regulasi ekspresi IL-1α dan CD-10 dalam AM berulang berbanding AM tidak berulang memerlukan siasatan lanjut untuk mengenal pasti peranannya.

Kata kunci: Ameloblastoma, IL-1α, IL-6, CD 10, pengulangan.

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LIST OF SYMBOLS AND ABBREVIATIONS

AM	:	Ameloblastoma
AMNR	:	Conventional ameloblastoma with no recurrence
ANOVA	:	Analysis of variance
BRAF	:	B-Raf proto-oncogene
CALLA	:	Common acute lymphoblastic leukemia antigen
CD 10	:	Cell surface enzyme- neural endopeptidase
ER	:	Estrogen receptor
Fas	:	Fas cell death receptor
FasL	:	Fas cell death receptor ligand
H&E	:	Haematoxylin & Eosin
HPV	:	Human Papilloma Virus
IHC	:	Immunohistochemistry
IL-1α	:	Interleukin 1-alpha
IL-1β	:	Interleukin 1-beta
IL-6	:	Interleukin 6
IRS	:	Immunoreactive scoring
MAPK	:	Mitogen-activated protein kinase
MEK	:	Mitogen-activated protein kinase kinase
MMPs	:	Matrix metalloproteinases
MRI	:	Magnetic resonance imaging
NF- κb	:	Transcriptor factor nuclear factor Kb
NTFs	:	Normal tonsil fibroblasts
OPG	:	Osteoprotegerin

OPN : Osteopontin

OPSCC : Oropharynx squamous cell carcinoma

OSCC : Oral squamous cell carcinoma

PA : Pre-ameloblast

PAMR : Primary conventional ameloblastoma with recurrence

RANK : Receptor activator nuclear kappa

RANKL : Receptor activator nuclear kappa β ligand

RCC : Renal cell carcinoma

TME : Tumour microenvironment

TNF : Tumour necrosis factor

TNF-α : Tumour necrosis factor-α

RAM : Recurrent ameloblastoma of the same patient

SPSS : Statistical Package for Social Sciences

SR : Stellate reticulum

ST : Stromal cell

WHO : World Health Organization

CHAPTER 1: INTRODUCTION

Ameloblastoma (AM) is a common, enigmatic gnathic tumour and constitutes about 1% of the head and neck neoplasm. It is one of the most common benign odontogenic tumours. Its enigmatic nature is manifested by its slow growing, like benign tumours, but having the characteristics of locally aggressive and a high recurrence rate following treatment (Masthan et al., 2015). The 5th edition of World Health Organization (WHO) classification of Head and Neck tumour has classified ameloblastoma into five subtypes, namely conventional, unicystic, extraosseous/peripheral, adenoid and metastasizing ameloblastoma. Conventional AM is the most encountered subtype clinically. There are six histopathological subtypes of conventional AM; follicular, plexiform, acanthomatous; granular cell, basal cell, and desmoplastic. Conventional AM usually recurs if inadequately removed. The standard of care is complete excision with negative margins, irrespective of the histologic subtype. This requires removal of a bone margin of at least 10 mm beyond the radiographic margin to ensure removal of tumour permeating medullary bone, usually a segmental resection. However, several studies have recommended conservative therapy based on the macroscopic appearance of the AM.

High recurrence rate of AM presents a major challenge. The histological subtype also appears to play an essential role in predicting the possibility of recurrence of ameloblastoma. The most reported histopathological subtype to recur is follicular followed by plexiform (Goh et al., 2021; Hendra et al., 2020; Ragunathan et al., 2022; Reichart et al., 1995; Siar et al., 2012). Recurrence of AM has been suggested to be correlated with the type of surgical treatment and the subtyping of AM (Hertog et al., 2011).

In a systematic review and meta-analysis analyzing 942 patients, the recurrence rate following radical treatment was 12%, which was significantly lower than the conservative treatment, with 30% post-treatment recurrence rate (Qiao et al., 2021). They found a similar finding when stratifying individuals based on the histological categorization with multicystic ameloblastoma had greater recurrence rates than unicystic ameloblastoma. In literature, recurrence rates for unicystic AM following conservative treatment are reported to range between 10% and 25%. However, these studies often do not specify the histologic subtypes of the primary lesion (Garcia et al., 2016). Conservative treatment has been recommended for unicystic AM in view off the lower percentage of the post-operative recurrence rate while radical treatment is advised as the mainstay treatment for multi-cystic AM, given its higher recurrence rate (Gardner, 1984; Reichart et al., 1995). A study by Eckardt et al. (2009) rated the cumulative relapses at about 17% after 5 years and 19% after 10 years . Recurrence of ameloblastoma was reported in a European study by Boffano et al. (2021) amounting to 19.3% within 5 years follow up. Strongly emphasize that a period of more than five years is needed for a follow-up and conclude that five years of a disease-free period may not be adequate to cure ameloblastoma (Neville et al., 2023).

Numerous studies in the literature highlight how insufficient treatment leads to recurrence in ameloblastoma. However, because of their extremely varied biological behavior, the mechanism of recurrence may be complex. By identifying many invasive processes of AM through the expression of different molecular markers, biological behavior can be anticipated. Ameloblastomas biological behavior has been investigated using a variety of molecular markers. These markers are involved in a variety of pathophysiological mechanisms, including bone remodeling, cell proliferation related to invasion, angiogenesis-related cytokines, the extracellular matrix (ECM) and tumour stromal cell function, all of which play interconnected roles in ameloblastoma invasion.

Many studies have been done to clarify the invasion phenomenon in ameloblastoma. However, the exact molecular mechanisms of invasion in ameloblastoma have not been well elucidated yet in predicting the recurrence of ameloblastoma. We aim to investigate the correlation between the expression of interleukin 1-alpha (IL-1α), interleukin 6 (IL-6), and CD 10 in predicting the recurrence of AM. These three molecular markers are reportedly associated with increased osteolytic and osteoclastogenesis activity, leading to bone resorption and local invasion by the AM tumour cells. A better knowledge and insight regarding the biological behavior of AM in cellular events may be revealed by the expression of above markers. This study may provide a better knowledge of AM molecular behavior, its relevance to the probability of recurrence, and potential targeted therapeutic therapies in the future.

Research Questions:

1) What is the expression level of IL-1α, IL -6 and CD 10 in recurrent and non-recurrent ameloblastoma?

2) Can the expression profiles of these molecules be used as predictors for recurrence in ameloblastoma?

3) What is the correlation between the expression of these markers and the demographic and clinicopathological parameters of non-recurrent and recurrent ameloblastoma?

Aim: To investigate the expression levels of IL-1α, IL-6 and CD 10 in predicting recurrence of AM using immunohistochemistry.

Specific objectives:

1) To determine the expression levels of IL-1α, IL-6 and CD 10 in recurrent and non-recurrent AM.
2) To compare the immunoprofiles of IL-1α, IL-6 and CD 10 between recurrent and non-recurrent AM.
3) To determine the association between the expression levels of these markers with the demographic and clinicopathological parameters in recurrent and non-recurrent AM.

Null hypothesis:

Ameloblastoma tumour cells do not express IL-1α, IL-6, CD10 and the immunoexpression patterns show no correlation with the recurrence of AM.

Clinical relevance of the study:

IL-1α, IL-6 and CD10 can be used as predictors for recurrence of AM if expression patterns of these markers show correlation with the recurrence.

CHAPTER 2: LITERATURE REVIEW

2.1 Ameloblastoma

2.1.1 Introduction

Ameloblastoma is defined as a benign but locally infiltrative epithelial odontogenic neoplasm of the jawbones characterized by ameloblast-like cells and stellate reticulum in the recent 5TH WHO classification of Head and Neck tumours (Vered et al., 2022). Historically, the tumour was first recognized by Cusack in 1827, detailed by Broca in 1868 and Sir Louis-Charles Malassez was the first to coin the term 'adamantinoma' to this tumour in 1885. He suggested that it originates from the epithelial remnants of developing root sheath. The term 'ameloblastoma' was introduced by Ivy and Churchill in 1930 and has been used up till today (Reichart et al., 1995).

Ameloblastoma is the most prevalent odontogenic tumour in all ethnic groups, constituting around 1% of all head and neck neoplasms in Europe and the USA, likely with the highest frequency in African and Afro-Caribbean populations (Morgan, 2011). The incidence of ameloblastoma per year is 0.92 per million persons in the world (Hendra et al.,2020). The benign designation of this neoplasm does not match with its locally aggressive behavior and high recurrence rate. In São Paulo, Brazil, Fregnani et al. (2010) reported a 22% recurrence rate for ameloblastoma in 27 patients out of 113 ameloblastoma cases. Meanwhile, a study by Bi et al. (2021) in the Eastern Province of China, found that the recurrence rate for AM was observed at 13.29%, an identical finding with Siar et al. (2012) who reported 18 patients out of 340 ameloblastoma cases, accounting for 13.3% of the of the recurrence rate of ameloblastoma in Malaysia.

2.1.2 Epidemiology

In a meta-analysis of biological profile of ameloblastoma by Reichart et al. (1995) found a significant geographical variation in the incidence. They showed that in Asians (Chinese, Indian, Japanese, Malays, Thai) had a higher incidence of 38.47%, slightly higher than the Caucasians with a 24.8%. However, this finding contradicted with Fregnani et al. (2010), which reported that 72% of the patients were Caucasians. Malaysia is a multiracial country consisting of three major ethnicities: Malay (62.5%), Chinese (20.6%), and Indian (6.2%). Interestingly, three local studies reported high occurrences of ameloblastoma in the Malay ethnic group, with rates of 47.6% (Siar et al., 2012), 63.6% (Binti Ismail et al., 2014) and 65.3% (Ismail & Saw, 2018). The reason for this racial predisposition is not fully understood, but it may be related to the fact that Malay makes up the majority of the population in Malaysia. This finding is consistent with Reichart et al. (1995) which noted a higher incidence of ameloblastoma in the Asian population compared to Caucasians.

Most of the literature found the peak incidence of ameloblastoma in the third decade of life (Bi et al., 2021; Fregnani et al., 2010; Hendra et al., 2020; Ragunathan et al., 2022; Reichart et al., 1995). Two local studies documented the incidence peaked in the second decade of life (Ismail & Saw, 2018; Siar et al., 2012). However, in Europe, the occurrence of ameloblastoma showed in the fourth decade of life (Boffano et al., 2021).

Overall, there was a slight male predilection over female globally (Ajila & Hegde, 2022; Hendra et al., 2020; Philipsen et al., 1992; Ragunathan et al., 2022; Reichart et al., 1995; Siar et al., 2012) except in Sao Paulo, Brazil, Fregnani et al.(2010) observed 53% ameloblastoma cases in female. Meanwhile, there were no gender predilections seen in the Sri Lanka (Okada et al., 2007).

The mandible has a high propensity for ameloblastoma compared to maxilla and the tumours predominantly located at the body and posterior part of the lower jaw (Ajila & Hegde, 2022; Bi et al., 2021; Binti Ismail et al., 2014; Hendra et al., 2020; Ismail & Saw, 2018; Siar et al., 2012). A systematic review by Hendra et al. (2020) found that the occurrence of AM was 87.2% in mandible, 8.5% in maxilla, 1.1% in the soft tissue and the rest of 3.1% was not specified.

2.1.3 Aetiopathogenesis

The basal cells of the oral mucosa, the developing enamel organ, remnants of the dental lamina, or the epithelial lining of an odontogenic cyst were thought to be the histological sources of ameloblastoma (Neville et al., 2023). Diverse molecular processes are involved in the growth and development of ameloblastoma including apoptosis, bone remodeling, cell proliferation, adhesion and signaling, tumour suppressor genes, extracellular matrix related proteins and clonality pattern (Jhamb & Kramer, 2014).

Both extracellular and intracellular signals have the ability to activate cell signaling pathways, which are involved in a variety of cellular processes such as proliferation, metabolism, and differentiation (Jhamb & Kramer, 2014). Numerous human cancers have been linked to BRAF, a mitogen-activated protein kinase (Ras/MAPK) pathway intermediate and strong MEK activator (Cantwell-Dorris et al., 2011). The initiation of this signaling cascade, even without external stimuli, is facilitated by a missense mutation in BRAF at residue 600, where valine is substituted with glutamine.

This mutation enables the cells to become self-sufficient in generating growth signals internally. Of note, Kurppa et al. (2014) found two-third of the ameloblastoma epithelial cells expressed the BRAF V600E mutation, which is involved in the growth and spread of tumours.

Matrix metalloproteinases (MMPs) comprise a family of zinc-dependent endopeptidase which have an ability to degrade the components of extracellular matrix thus promoting the invasion and proliferation of neoplastic cells. A strong expression of MMP-1, MMP-2 and MMP-9 was found in the stromal cells of ameloblastoma and mesenchymal components of tooth germs (Kumamoto et al., 2003). These molecules might be the precursor to regulate the tumour progression in ameloblastoma as well as regulation of tooth germ developmental process.

Tumour necrosis factor (TNF) exhibits pro-inflammatory activity through activation of transcription factor nuclear factor κB (NF- κB) which play a vital role in bone remodeling (Kong et al., 1999). Bone resorption is dependent on a cytokine known as receptor activator of nuclear factor kappa B ligand (RANKL), a TNF family member that is essential for osteoclast formation in the normal and pathological state of bone remodeling. RANKL activates the receptor activator of nuclear factor κB (RANK) that presents on the surface of the osteoclast (Kearns et al., 2008). Another molecule that is involved in bone remodeling, osteoprotegerin (OPG) which is a secreted, soluble receptor that binds to RANKL leading to disruption of RANK/RANKL interactions. Interaction between these proteins regulates the activity and maturation of osteoclast in bone remodeling. An elevated level of RANK was found in stromal cells of solid/multicystic ameloblastoma with a high ratio of RANKL to OPG-positive cells, suggesting net bone resorption (da Silva et al., 2008).

Mechanism of bone resorption in ameloblastoma may be represented via the RANKL elevation, as RANKL expressed by tumour cells may interact with RANK on osteoclast progenitors, leading to osteoclastogenesis.

Apoptosis is a programmed cell death initiated intrinsically (within the cell) or extrinsically (activation of pro-apoptotic receptors on cell surface). Apoptosis plays a role in balancing the cell division and tissue homeostasis. Fas and Fas ligand (FasL) are members of the tumour necrosis factor (TNF)-receptor and involved in the regulation of cell death. Ligation of Fas with FasL results in activation of caspase-3 in the caspase cascade that eventually initiates apoptosis (Volpe et al., 2016). A variety expression of Fas, FasL and caspase-3 was found across different histopathological types of ameloblastoma (Kumamoto et al., 2001). This suggests that different variants of ameloblastoma exhibit distinct apoptotic characteristics. However, Fas expression is lower in ameloblastoma compared to normal tooth germ hence it is postulated that this molecule most likely plays a role in the potential survival of the neoplastic cells.

2.1.4 Clinico-radiological findings

Ameloblastoma typically appears clinically as a slow growing, relatively painless tumour. However, because of its locally aggressive nature, it can quickly develop into a large, expanding mass. The tumour often appears asymptomatic and may be detected as an incidental finding during radiographic examination. If left untreated, it may cause tooth mobility, displacement of the tooth, damage to adjacent resulting in numbness and a severely disfigured facial appearance (Effiom et al., 2018; Reichart et al., 1995; Siar et al., 2012). On the other hand, the majority of AM in China (Jing et al., 2007) and Egypt (Tawfik & Zyada, 2010) showed self-limited growth and no clinical signs, while approximately 79% of ameloblastoma in Brazil (Avelar et al., 2008) were asymptomatic.

The radiographic features of ameloblastoma range from unilocular, multilocular radiolucency to mixed radiopaque-radiolucent appearance. Most typical radiographic feature of ameloblastoma is multilocular radiolucent lesion (Binti Ismail et al., 2014; Ismail & Saw, 2018; Siar et al., 2012) . Multilocular lesions described as having a 'soap bubble appearance' with large loculations or 'honeycomb appearance' with a small loculations radiographically.

The classic "soap bubble" appearance is mostly found in the multicystic ameloblastoma (Vered et al., 2022). Meanwhile, the unilocular radiolucency with scalloped margin is often associated with impacted molar tooth (Neville et al., 2023). In desmoplastic ameloblastoma, the radiographic appearance is mixed radiolucent-radiopaque which resembles a fibro-osseous lesion, this might be due to the metaplastic bone formation (Philipsen et al., 1992), or osseous metaplasia within the dense fibrous septa (Neville et al., 2023).

Computed tomography scan is the most effective diagnostic imaging modality to address the limitations of plain x-rays, which lack the sensitivity and specificity to provide a comprehensive assessment of the bone extension and soft tissue involvement in ameloblastoma. Determining the extent of a lesion's involvement in the bone is helpful in surgical planning. In the meantime, magnetic resonance imaging (MRI), especially in cases of maxillary ameloblastoma, provides further details on the soft tissue and marrow extension beyond the bony edge.

2.1.5 Histopathological characteristics

The latest 5th World Health Organization (WHO) classification of Head and Neck tumour has classified ameloblastoma into five histological subtypes namely, conventional, unicystic, extraosseous /peripheral, adenoid and metastasizing ameloblastoma.

2.1.5.1 Unicystic ameloblastoma

Unicystic AM most commonly occurred in younger patients with 50% diagnosed during the second decade of life (Neville et al., 2023). It has three histopathological subtypes which are luminal, intraluminal and mural with the mural type having a greater tendency to recur compared to the other unicystic AM (Goh et al., 2021).

Histopathologically, it is characterized with a single cyst lined by epithelium, with a palisaded columnar basal layer with reverse polarity and stellate reticulum–like upper layers confined to the lining or luminal surface of cyst, constitutes the luminal subtype. The intraluminal type characterized by a plexiform epithelial mass may extend only into the lumen. Additional islands of epithelium extending into the wall constitute the mural subtype (Vered et al., 2022). Recurrence rate of unicystic AM comparatively lower than conventional AM after enucleation and curettage (Goh et al., 2021; Neville et al., 2023).

2.1.5.2 Extraosseous/peripheral ameloblastoma

Peripheral ameloblastoma shows an innocuous clinical behavior most commonly seen in middle-aged patients. Patients respond well to local surgical excision resulting in rare recurrence episodes. Peripheral AM consists of proliferating odontogenic epithelium with histomorphology similar to conventional AM. The histopathological features and growth patterns are the same as in conventional ameloblastoma (Vered et al., 2022).

2.1.5.3 Conventional ameloblastoma

Conventional solid or multicystic intraosseous AM is encountered in patients across the wide age range. It has been reported to have a high recurrence rate among other type of AM (Almeida Rde et al., 2016; Goh et al., 2021; Nwoga, 2022). It shows an infiltrative growth pattern and is more aggressive. Total of 50-90% recurrence rate have been reported after curettage (Neville et al., 2023). The commonest follicular subtype, there are islands of epithelium resembling the epithelial component of the enamel organ in a fibrous stroma. Peripheral cells are columnar to cuboidal (ameloblast-like), with hyperchromatic nuclei arranged in a palisading pattern with reverse polarity, and often subnuclear vacuolation.

Central epithelium is reminiscent of stellate reticulum, with loosely arranged angular cells, and often undergoes cystic change. The second commonest subtype is the plexiform subtype, composed of anastomosing strands of ameloblastomatous epithelium with an inconspicuous stellate reticulum, less prominent ameloblast-like cells, and cyst-like degeneration in the stroma rather than the epithelium. Mitoses are usually scattered but posterior maxillary AM may have high cellularity and frequent mitoses. Follicular subtype has more tendency to recur followed by plexiform subtype (Almeida Rde et al., 2016; Goh et al., 2021).

Several other subtypes are recognized. The acanthomatous subtype has squamous differentiation centrally in islands but maintains the reverse polarization of the nuclei in peripheral columnar cells. In the granular cell subtype the central epithelium develops abundant eosinophilic granular cytoplasm. The basal cell subtype resembles basal cell carcinoma because it consists of islands and strands of basaloid cells with sparse cytoplasm and peripheral palisading.

The desmoplastic subtype has more widely dispersed islands with spiky outlines, cuboidal to flat peripheral cells, and central spindle-shaped cells in a densely collagenous stroma, sometimes with osteoplasia. A recurrence rate of 12.1% was found in desmoplastic AM, which was lower compared to the follicular and plexiform subtypes (Goh et al., 2021).

2.1.5.4 Adenoid ameloblastoma

Recently, 45.4% of adenoid ameloblastoma had at least one recurrence after surgical removal (Jayasooriya et al., 2022). Adenoid ameloblastoma is characterized by a partly cribriform arrangement of basal ameloblast-like cells demonstrating reversed nuclear polarity and a minor component of suprabasal stellate reticulum–like epithelium. Basal cells may be multilayered, with a transition to a round or ovoid morphology. Distinctive features are duct-like structures formed by cuboidal to columnar cells, some of which contain mucin, and focal whorled cellular condensations reminiscent of morules. Two thirds of cases contain varying amounts of dentinoid. Clear cells are often associated with dentinoid, and ghost cell keratinization may be a minor feature (Vered et al., 2022).

2.1.5.5 Metastasizing ameloblastoma

Metastasizing ameloblastoma exhibits identical histopathological features to conventional ameloblastoma, with no specific features predicting metastasis with the plexiform histological pattern being the most common (Vered et al., 2022).

2.1.6 Treatment and recurrence

Ameloblastoma is a benign lesion yet locally aggressive, hence the therapeutic management of the disease is complicated because it must be both sufficiently extensive to avoid recurrence and as minimally destructive as possible.

Treatment controversy is common among the surgeons to weigh the benefit and risk between conservative management and radical therapy. Surgical treatment is the mainstay and primarily structured to ensure recurrence-free tumour resection with satisfying functional and cosmetic benefits to the affected patients. On the other hand, conservative treatment (marsupialization, enucleation, and curettage) preserves the integrity of the bones and allows continuous growth of the mandible (Takahashi et al., 1998).

Most of the studies reported a higher recurrence rate after conservative treatment compared to radical treatment (Laborde et al., 2017; Nakamura et al., 2002; Reichart et al., 1995). Mandibular ameloblastoma had a higher recurrence rate compared to maxillary. However, no significant differences were found in relation to localization of this tumour (Reichart et al., 1995). Generally, unicystic AM has a lower recurrence rate (13-30%) compared to conventional AM (50-90%), and conservative treatments are recommended for unicystic AM. The histological subtypes of ameloblastoma also play a significant role in predicting the post-operative recurrence rate. The follicular subtype, followed by the plexiform subtype, has a greater tendency for local recurrence compared to other histological subtypes of ameloblastoma of conventional type (Ajila & Hegde, 2022; Goh et al., 2021; Hong et al., 2007; Laborde et al., 2017; Reichart et al., 1995).

Interestingly, Goh et al. (2021) found a lower recurrence rate in mixed histopathological type of the follicular and the plexiform variant which postulated that the plexiform histopathological variant behaves less aggressive than follicular subtype. Long-term follow-up of up to ten decades is mandatory for this tumour due to its high recurrence rate despite treatment provided (Neville et al., 2023).

2.2 Cytokines

Cytokines are small, secreted proteins that are released by a cell and have a particular impact on how cells communicate and interact with one another. Cytokine is the general name, whereby interleukin is a cytokine made by one leukocyte and acts on other leukocytes. Their activity is redundant in which similar functions can be stimulated by different cytokines and act synergistically. They are made by a population of cells with helper T cells (Th) and macrophages being the predominant producers (Zhang & An, 2007).

They may initiate signaling cascades, which means that even minute quantities of protein may have disastrous effects (Ibrahim et al., 2017). The pro-inflammatory cytokines lead to activation of immune cells and produce more cytokines.

2.2.1 Interleukin-1 alpha (IL-1α)

The IL-1 family has ten members of receptors and eleven members of cytokines. The interleukin-1 family of cytokines is more intimately associated with harmful inflammation than any other family, yet the same members also serve to promote nonspecific resistance to infection and the maturation of the immune response to foreign antigens (Dinarello, 2018). Primarily, IL-1α is a membrane-anchored molecule and has autocrine signaling mechanisms; however, interleukin-1 beta (IL-1β) is a secreted molecule that acts in a paracrine manner or systemically (Weber et al., 2010).

IL-1α is known for its role as a potent modulator in bone resorption by inducing the activation of osteoclast-like cells and production of matrix metalloproteinase enzymes (Sengüven & Oygür, 2011).

2.2.2 Interleukin 6 (IL-6)

IL-6 is a multifunctional cytokine that can induce an acute phase response, triggered during the early course of an infection. IL-6 expression was demonstrated to be induced by tumour necrosis factor alpha (TNFα) and IL-1β administrations in cell cultures (Gadient & Otten, 1997; Ringheim et al., 1995). IL-6 is a pivotal cytokine in bone microenvironment and formation of osteoclasts (Ishimi et al., 1990).

They bind to IL6-R, a signal transduced via the gp130 coreceptor induced RANKL expression (Palmqvist et al., 2002). Inflammatory cytokines like interleukins 1, 6 and 11 can stimulate osteoclast development and thereby the process of bone resorption occurs. IL-6 has been recognized as the key for antiresorptive factor that stimulates osteoclast differentiation and initiates osteoclasto-genesis (Kudo et al., 2003).

In the HPV-negative oropharynx squamous cell carcinomas (OPSCC), osteopontin (OPN) was produced by OPSCC cells and this was linked to an increase in IL-6 levels in fibroblast. Additionally, treating normal tonsil fibroblasts (NTFs) with recombinant OPN led to changes in their phenotype, including enhanced contraction and IL-6 production (Hendawi et al., 2024).

2.3 Role of IL-1 alpha and IL-6 in osteoclastogenesis in ameloblastoma

RANKL is a membrane-bound factor expressed by osteoclastogenesis-supporting cells and acts as a key player for osteoclastic differentiation (Takayanagi et al., 2000). Cell-cell contact between osteoclast precursor cells and osteoclastogenesis-supporting cells were found to be the inducing agents in osteoclastogenesis (Okamoto et al., 2017).

Cytokines and growth factors act as a soluble agent which upregulate the growth and invasion, are found to be secreted by tumour cells and stromal cells. Cytokine levels, especially osteolytic cytokines found in intracystic fluids of cysts or expressed by the cells may play a significant role in growth of ameloblastoma (Kubota et al., 2001). Upregulation of the production of IL-6 has been implicated in the pathogenesis of disease states characterized by excessive osteoclastic bone resorption.

It is also suggested that IL-1α and IL-6 cytokines play a role in the aggressive behavior of ameloblastoma by inducing the production of matrix metalloproteinase like degradative enzymes and prostaglandins as well as differentiation and activation of osteoclast-like cells (Kudo et al., 2003). The expression of both IL-1α, IL-6 in stroma and epithelial cells suggests that they activate the cells reciprocally in a paracrine and autocrine manner, creating a favorable bone tumoral microenvironment that facilitates ameloblastoma invasion (Goh et al., 2019).

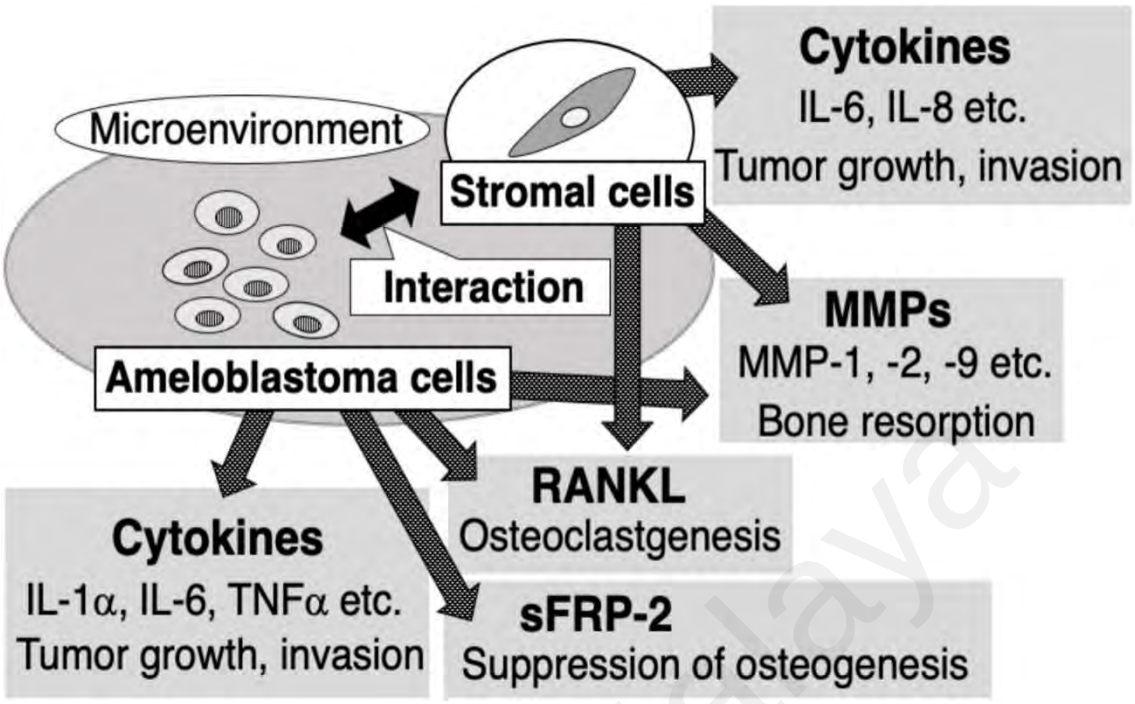

Figure 2.1 Microenvironment constructed by tumor cells and stromal components in ameloblastoma. Source: (Fuchigami et al., 2014)

The ameloblastoma cells or nests are usually separated by a fibrovascular stroma, with a distance from surrounding resorbed bones and osteoclasts for an effective communication through RANKL-RANK interaction (Fuchigami et al., 2014; Sathi et al., 2008). In a recent in vitro and clinicopathologic study, IL-6 was found as a critical factor for RANKL induction in stromal fibroblast leading to osteoclastogenesis in the tumour microenvironment (TME) to further progress the invasion of ameloblastoma (Yoshimoto et al., 2023).

Independent IL-1α or IL-1α dependent production of unidentified chemoattractant by stromal cells may be a key role for local invasiveness of ameloblastoma cells (Ono et al., 2024).

This is supported by the findings of Goh et al. (2019), a higher expression of IL-1α and IL-6 was associated with increasing tumour size in ameloblastoma and concluded that the larger the size of the tumour, more cytokines produced, more destruction to the bone leading to tumour invasion.

2.4 CD 10

CD 10 is a 90 to 100 -kd cell surface zinc dependent metalloprotease glycoprotein with endopeptidase activity presenting on the surface of many cell types, including lymphoid precursor cells, germinal center B lymphocytes, and some epithelial cells. It cleaves and inactivates neuropeptides and peptide hormones at the amino terminus to hydrophobic residues within the peptides sequences, thereby decreasing the cellular response to local peptide hormones (Bahrami et al., 2006). CD10 has also been called neutral endopeptidase, enkephalinase, neprilysin, and common acute lymphoblastic leukemia antigen (CALLA). CD 10 is also known to be useful for the categorization of acute leukemia and the sub-classification of lymphomas (Ogawa et al., 2002).

CD10 was found to be a useful marker for particular malignancies, expression consistently described in renal cell carcinoma (RCC) with 89-100% immunopositivity reported in conventional clear cell RCC. In oral squamous cell carcinoma, stromal cell CD10 positivity was linked with a poor prognosis, and there was also a remarkable association with lymph node metastases, local recurrences, and histologic grade (Piattelli et al., 2006). CD10 expression in BeWo choriocarcinoma cells has been linked to both apoptosis and proliferation (Suzuki et al., 2002). In invasive breast carcinomas, positive CD10 stromal expression is associated with estrogen receptor (ER)-negative (Kamarudin et al., 2021).

In ameloblastoma, CD10 expression was strongly expressed in the membrane of stellate reticulum-like cells in conventional AM and unicystic AM (Tan et al., 2022). A high CD 10 expression in AM might predict the neoplastic potentiality of the epithelial lining of the cyst as well as a tool to identify areas with local invasive behavior and substantial risk of recurrence (Masloub et al., 2011). CD10 could be an indicator for ameloblastoma aggressiveness since its expression was found most in the recurrent AM (Abdel-Aziz & Amin, 2012; Iezzi et al., 2008). CD10 has been identified as a good marker to differentiate between primary and metastasis including gastric, pancreatic, colorectal, melanoma and oral squamous carcinoma which making it as an useful tool for predict tumour invasiveness and recurrence rate (Jhamb & Kramer, 2014).

CHAPTER 3: METHODOLOGY

3.1 Research design

This is a descriptive cross-sectional study to investigate expression of IL-1α, IL-6 and CD10 in recurrent and non-recurrent AM.

3.2 Materials

3.2.1 Samples

The ethics approval for this study was obtained from the Medical Ethics Committee, Faculty of Dentistry, University Malaya [DF0S2119/0069(L)] and University of Peradeniya, Sri Lanka (ERC/FDS/UOP/I/2018/08). A total case of 42 formalin-fixed paraffin-embedded tissue blocks of recurrent and non-recurrent AM were retrieved after reviewing the hematoxylin and eosin-stained sections of these cases. These cases were retrieved from the archives of Diagnostic Oral Pathology Unit, Faculty of Dentistry, Universiti Malaya and Department of Oral Pathology, Faculty of Dental Sciences, University of Peradeniya, Sri Lanka. The selection criteria were based on the definition of ameloblastoma by the 5th edition of WHO Classification of Head and Neck Tumours 2022. The 42 cases are divided into 3 categories:

A. 16 cases : Conventional ameloblastoma with no recurrence (AMNR).

B. 13 cases: Primary conventional ameloblastoma with known recurrence (PAMR).

C. 13 cases: Recurrent ameloblastoma from same patient (RAM).

Case selection for study group was based on the following inclusion and exclusion criteria:

The inclusion criteria:

1. Samples fulfilled the histological criteria established by 5TH edition WHO Classification of Head and Neck Tumours 2022.

2. Samples with sufficient lesional tissue.

3. Samples containing at least 60% tumour tissues.

4. Cases with minimum 5 years of follow up (AMNR).

The exclusion criteria for the selected samples were:

1. Samples with evidence of malignant transformation.

2. Samples with inadequate lesional tissue.

The demographic and clinical data were obtained from the laboratory request form of each case. The sites of tumour were classified into side of mandible, right mandible, left mandible or bilateral; and further classified into subsites of tumour. Subsites of tumour were classified as anterior (midline of mandible to distal surface of canine), middle (medial surface of first premolar to distal surface of first molar), posterior (mesial surface of second molar to most posterior of jaw), mandible (more than one segment of mandible involved in single side), bilateral (involvement of bilateral mandible) and not specified.

3.2.2 Antibodies

Three primary antibodies, anti-IL-1α, anti-IL-6 and anti-CD10 were used in this study. All of these antibodies were sourced from Abcam PLC Cambridge, UK. The immunohistochemical staining process for these antibodies using Dako REAL Envision Kits, Dako REAL Peroxidase Blocking Solution, Dako REAL Antibody diluents (Dako Corporation, Glostrup, Denmark) and target retrieval solutions were sourced from BitaLife Sciences Sdn.Bhd.

3.3 Methods

3.3.1 Specimens preparation

All formalin-fixed and paraffin-embedded block samples were cut into a serial section of 4-micron meter thickness. Each section was mounted on a salinized glass slide which incubated overnight at 60 degree Celsius for deparaffinization.

3.3.2 Immunohistochemistry (IHC)

Before beginning immunohistochemical staining of the research samples, optimization was performed to determine the ideal dilution for each antibody. Positive and negative controls were performed for each marker in accordance with the manufacturer's recommendations to test the marker's specificity. The manufacturer's instructions for the three types of primary antibodies used in this study are summarized in Table 3.1.

Table 3.1: Primary antibodies used in immunohistochemical studies

Primary antibody	Cellular localization	Dilution	Antigen-Retrieval Buffer (Ph)	Incubation period and temperature	Washing buffer	Control tissue
Anti-CD10 [EPR22867-118] Mouse monoclonal to CD 10	Cell membrane	1:25	Citrate buffer (pH6.0)	Overnight at 4°C	Phosphate Buffered Saline (pH7.4)	Tonsil
Anti-IL-1α [EPR25263-3] Rabbit polyclonal to IL-1α	Cytoplasm	1:2000	Citrate buffer (pH6.0)	Overnight at 4°C	Phosphate Buffered Saline (pH7.4)	Human skin tissue
Anti-IL 6 [EPR21710] Mouse monoclonal to IL-6	Cytoplasm	1:5000	Citrate buffer (pH6.0)	Overnight at 4°C	Phosphate Buffered Saline (pH7.4)	Cervical carcinoma

3.3.3 Interpretation of results

3.3.3.1 Descriptive

The stained sections were examined with light microscope (Olympus BX51 Microscope, Olympus Imaging Inc., Tokyo, Japan). Digitalized images were captured with digital slide scanner (Motic Easy Scan Pro 6, Kowloon, Hong Kong).

3.3.3.2 Semi-quantitative

A semi-quantitative method was used to evaluate the expression of IL-1α, IL-6 and CD10 in the tumour cells pre-ameloblast like cell (PA-like cells) and stellate reticulum (SR-like cells). Five hotspots were determined and examined under x200 magnification. To avoid potential misinterpretation, areas with artifacts and intense inflammation were avoided. The level of immunoreactivity of IL-1α, IL-6 and CD10 (Fedchenko & Reifenrath, 2014) were scored as below:

Table 3.2 : Immunoreactive Scoring (IRS)

I (Intensity of staining)	P (Percentage of positive cells)	IRS score (Multiplication of I and P)
0 = No staining reaction	0= No positive cells	0= Negative
1= Mild reaction	1= <10% of positive cells	1-3 =Mild
2= Moderate reaction	2= 10-50% positive cells	4-8=Moderate
3= Intense reaction	3= 51-80% positive cells	9-12 = Strongly positive
	4= >80% positive cells	

Each case was evaluated based on intensity of staining (I) and percentage of positive cells (P) with the total IRS score resulted from multiplication of I and P. As for the intensity of the staining: 0 with no color reaction, 1 with mild reaction, 2 with moderate reaction and 3 when the staining was intense.

Scoring of the percentage positive cells: 0 when there were no positive cells, 1 with number of positive cells were less than 10%, 2 when 10-50% cells were positive, score 3 with 51-80% positive cells and score 4 if there was more than 80% positivity. An example of the intensity of the staining and percentage of positive cells for the AM cases from random markers was demonstrated in Figure 3.1 and Figure 3.2.

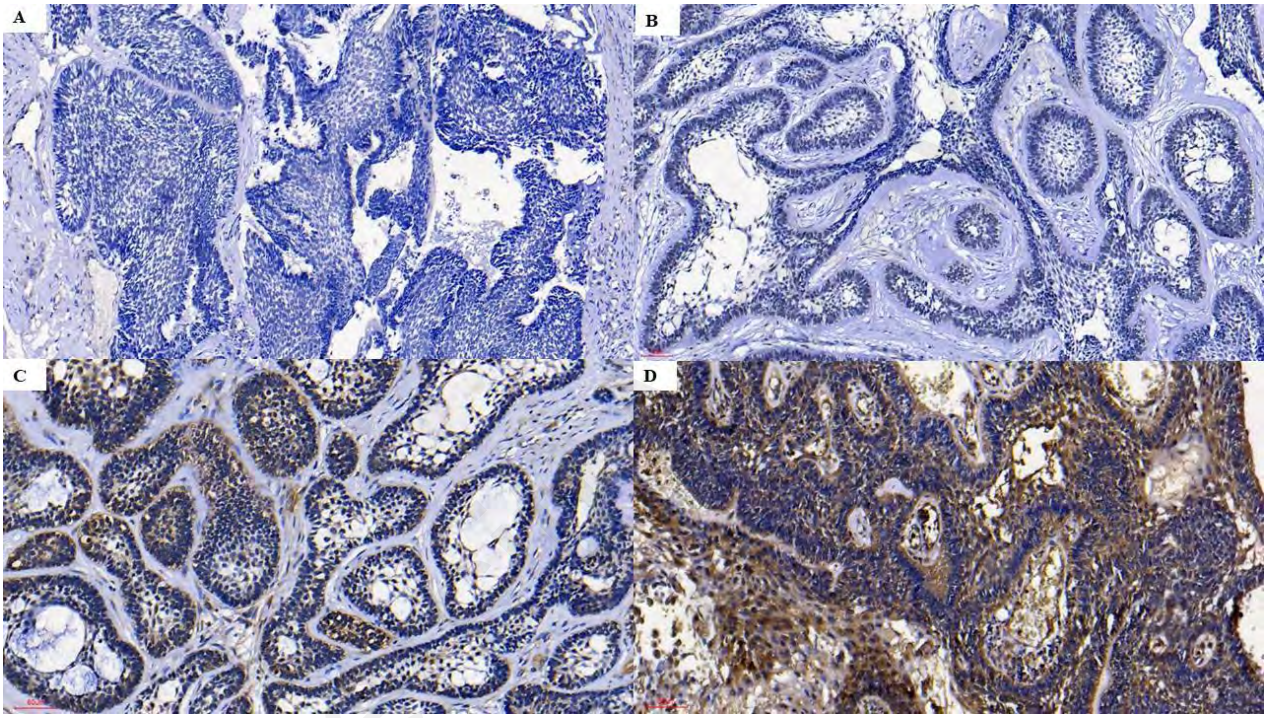

Figure 3.1 : Photomicrograph showing examples of intensity of the staining (I).

A. No color reaction. B. Mild reaction. C. Moderate reaction and D. Intense reaction

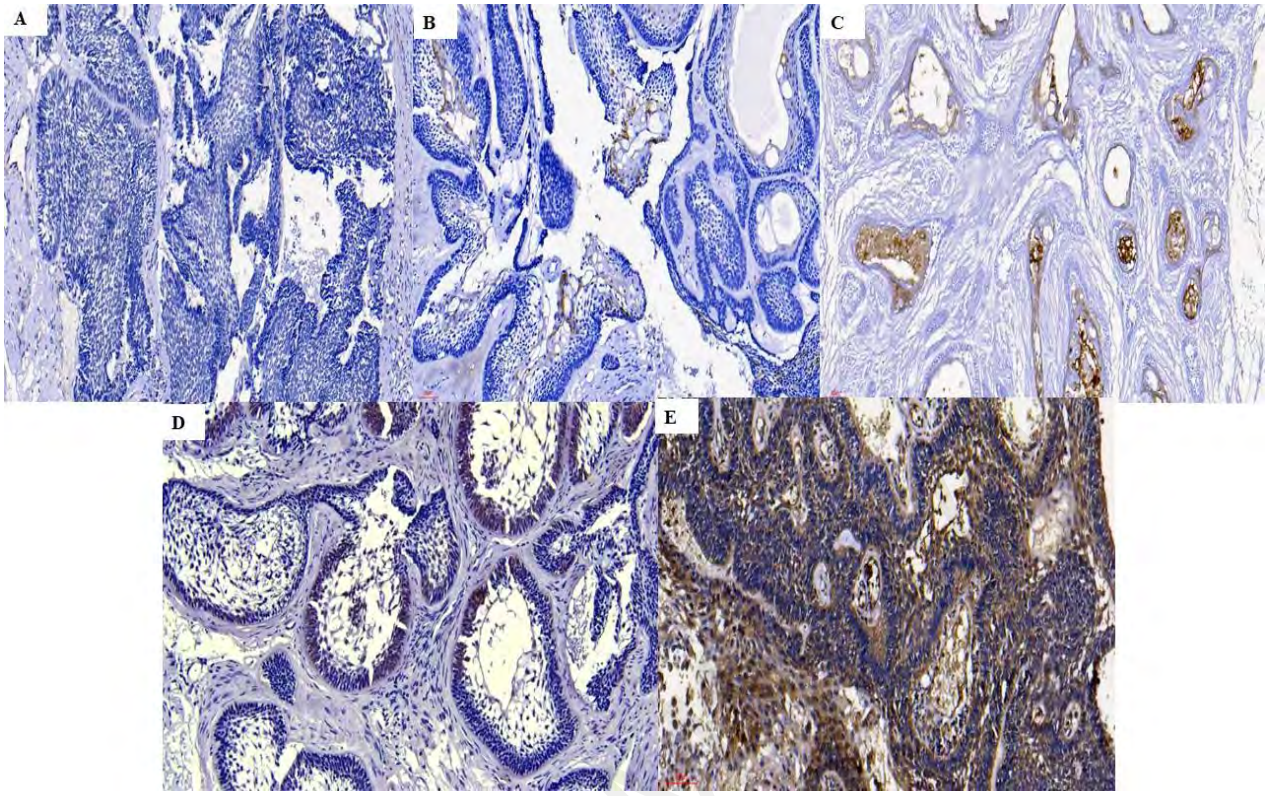

Figure 3.2 : Photomicrograph showing examples of percentages of positive cells (P).

A. No positive cells. B. Less than 10% of positive cells. C. 10-50% of positive cells. D. 51-80% positive cells. E. More than 80% positive of tumour cells.

3.3.4 Calibration

One consultant oral pathologist (WMT), one oral pathologist (YCG) and one oral pathology trainee (HD) were involved in assessing the IHC scoring. Forty-five random hotspots were scored blindly during the inter-examiner calibration. The intraclass correlation coefficient (ICC) test was 0.67 indicating moderate agreement was achieved. Intra-examiner calibration was conducted individually by analyzing ten random protein distributions of different hotspots, at the first and two weeks after data scoring. Cohen's kappa test was 0.80 indicating a strong agreement.

3.3.5 Statistical analysis

The analysis of results was performed using IBM Statistical Package for Social Sciences (SPSS) version 27. The One-Way ANOVA test was used to compare expression of IL-1α, IL-6 and CD10 in tumour cells (PA-like cells and SR-like cells) in both non-recurrent and recurrent AM groups. The association between IL-1α, IL-6 and CD10 with the demographic and clinicopathological parameters in recurrent and non-recurrent AM groups were tested using Pearson Chi square test. For the statistical analysis, *P* value <0.05 was considered to denote statistical significance.

CHAPTER 4: RESULTS

4.1 Demographic characteristics

The samples consisted of 29 patients in total, of which 16 were non-recurrent AM and the other 13 with recurrence AM. Their demographic characteristics are summarized in Table 4.1. Our findings showed that male predominance in AMNR group, with male to female ratio of 3:1 while PAMR and RAM groups showed a female predominance, with a male to female ratio of 1: 3.3. Most AMNR cases were predominantly diagnosed in the second decade and third decade of life whereas PAMR and RAM mostly occur later in the fourth decade of life. The mean age was higher at the recurrence and during the primary diagnosis as compared to non-recurrent group. Similar wide age distribution was observed for the three groups of AM.

Table 4.1 : Demographic characteristics of AMNR, PAMR and RAM

Variables		Category		
		AMNR	**PAMR**	**RAM**
		N (%)	**N (%)**	**N (%)**
Gender	Male	12 (75.0)	3 (23.1)	3 (23.1)
	Female	4 (25.0)	10 (76.9)	10 (76.9)
	M:F ratio	3:1	1:3.3	
Age	10-19 years	5(31.25)	1 (7.7)	0 (0.0)
	20-29 years	5(31.25)	3 (23.1)	2 (15.4)
	30-39 years	1(6.25)	3(23.1)	4(30.8)
	40-49 years	1(6.25)	3(23.1)	3(23.1)
	50-59 years	3(18.75)	2(15.4)	2(15.4)
	60-69 years	1(6.25)	1(7.7)	2(15.4)
	70-79 years	0	0	1(7.7)
	Mean age	31.3	37.2	44
	Age range	12-62	14-64	22-72

4.2 Clinicopathological characteristics

The clinical characteristics of the 42 cases are summarized in Table 4.2. All of our cases occurred in the mandible. For AMNR, tumours were most commonly found on the right side of mandible, often involving multiple segments and exhibiting bilateral involvement. As for the recurrent group, PAMR and RAM were predominantly located at the right posterior of the mandible. The majority of the PAMR patients underwent conservative treatment while most patients with AMNR and RAM received radical surgery. In relation to the histopathologic subtypes, plexiform subtype was the most common among AMNR, while the follicular subtype is more prevalent in PAMR and RAM.

Table 4.2 : Clinicopathological characteristics of AMNR, PAMR and RAM

Variables			Category		
			AMNR	PAMR	RAM
			N (%)	N (%)	N (%)
Known site of occurrence	Side	Right	7 (43.8)	8 (61.5)	9 (69.2)
		Left	4 (25.0)	4 (30.8)	4 (30.8)
		Bilateral	5 (31.2)	1 (7.7)	0 (0.0)
	Subsite(s)	Anterior	1 (6.3)	1 (7.7)	1 (7.7)
		Middle	0 (0.0)	0 (0.0)	0 (0.0)
		Posterior	2 (12.5)	5 (38.5)	6 (46.1)
		Mandible	5 (31.2)	4 (30.8)	3 (23.1)
		Bilateral	5 (31.2)	1 (7.7)	0 (0.0)
		Non specified	3 (18.8)	2 (15.3)	3 (23.1)
Known treatment		Conservative	6 (37.5)	11(84.6)	2 (15.4)
		Radical	9 (56.3)	1 (7.7)	10 (76.9)
		Non specified	1 (6.2)	1 (7.7)	1(7.7)
Known ameloblastoma subtype		Follicular	5 (31.3)	8 (61.5)	6 (46.1)
		Plexiform	9 (56.3)	3 (23.1)	3 (23.1)
		Acanthomatous	1 (6.2)	0 (0.0)	0 (0.0)
		Basal cell	0 (0.0)	0 (0.0)	1(7.7)
		Mixed type	1 (6.2)	1 (7.7)	2 (15.4)
		Non specified	0 (0.0)	1 (7.7)	1 (7.7)

4.3 Immunohistochemical findings

4.3.1 Expression of IL-1α

The expression of IL-1α for AMNR, PAMR and RAM is summarized in Figure 4.1. One case of AMNR showed negative IL-1α immunoreactivity, and most of the other cases expressed mild immunopositivity. For PAMR, there were 7 cases showing mild expression of the IL-1α marker. The majority of the RAM cases expressed moderate immunopositivity to IL-1α. The positive immunoreactivity was detected in PA-like cells and SR-like cells. Figures 4.2 and 4.3 illustrate the distribution pattern of IL-1α in RAM and AMNR group.

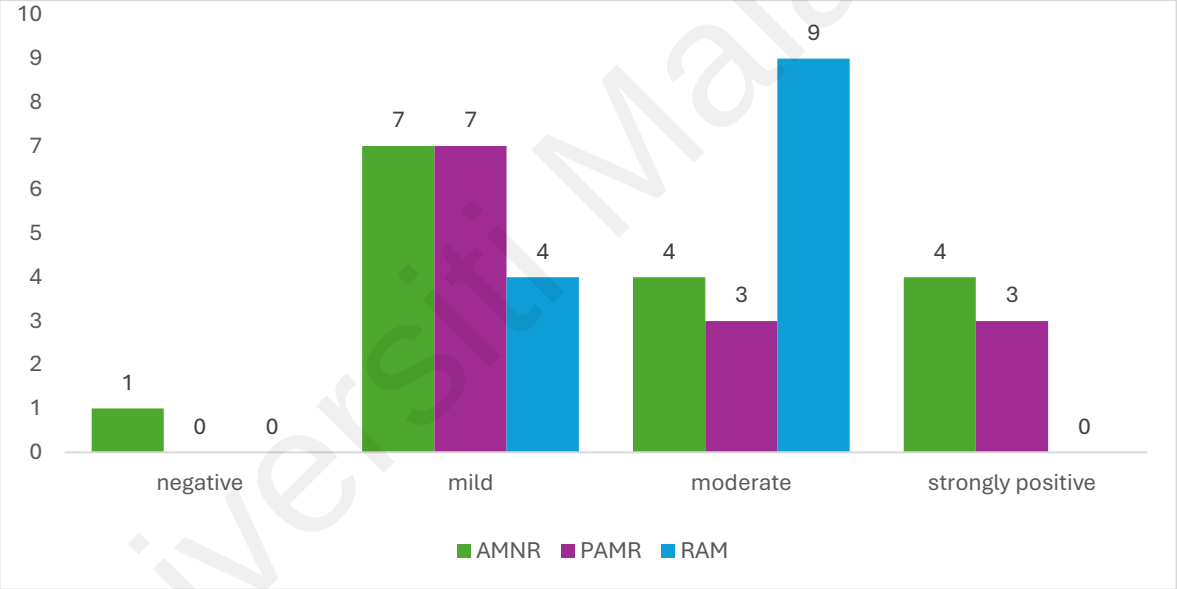

Figure 4.1 : Expression of IL-1α in non-recurrent and recurrent AM

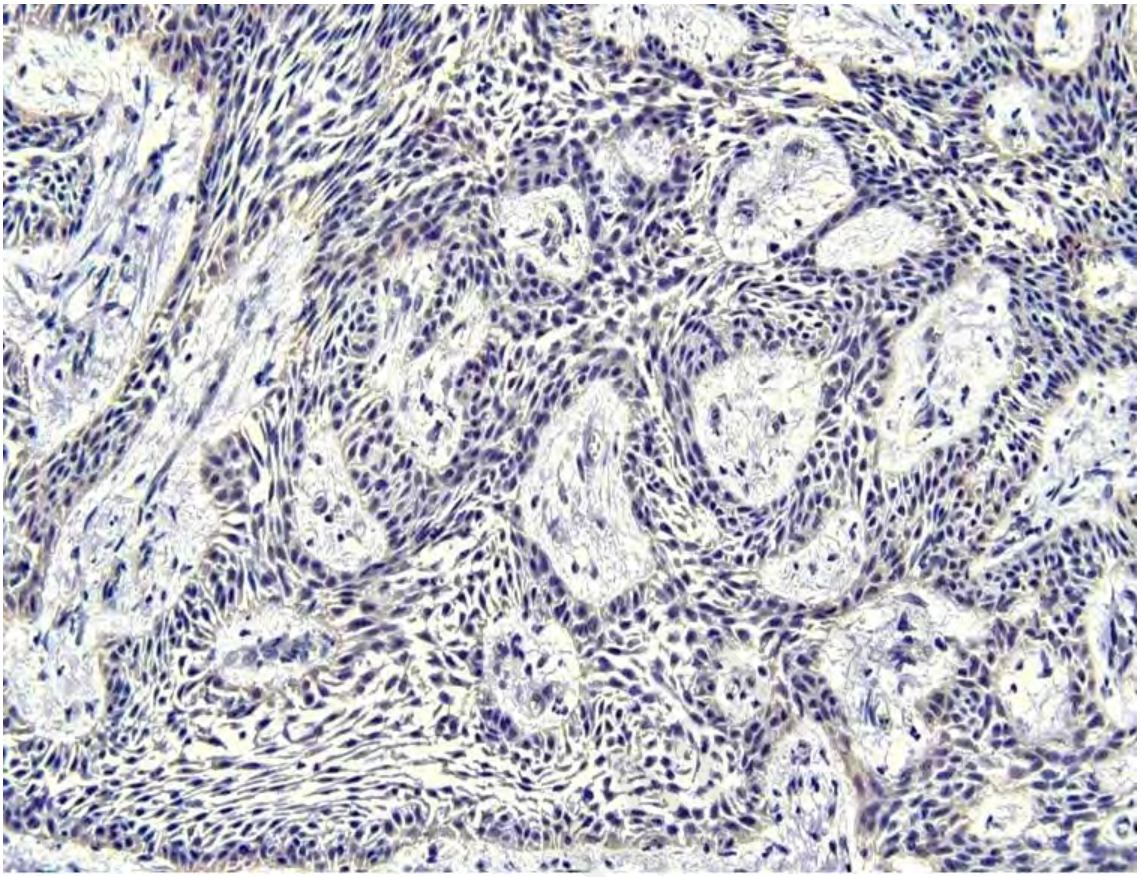

Figure 4.3 : Photomicrograph showing moderate immunopositivity of IL-1 α in RAM group. Original magnification 200x.

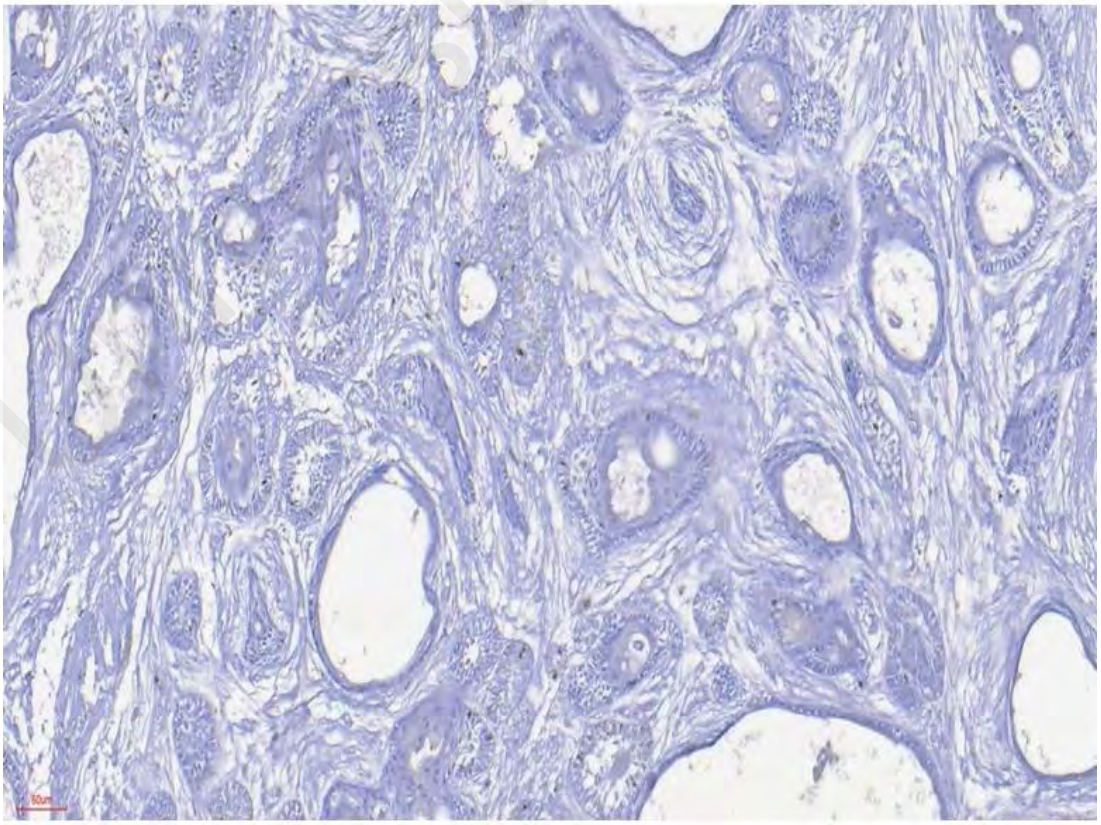

Figure 4.2 : Photomicrograph showing mild immunopositivity of IL-1 α in AMNR group. Original magnification 200x.

4.3.2 Expression of IL-6 in ameloblastoma

Immunoreactivity of IL-6 in AMNR, PAMR and RAM is summarized in Figure 4.4. Strong IL-6 immunoreactivity was observed in more than half of the AMNR and RAM. RAM exhibited almost even cases of moderate and strong immunopositivity. One case from AMNR exhibited negative immunoreactivity to IL-6. The positive immunoreactivity was primarily observed in PA-like cells and SR-like cells. Figures 4.5 and 4.6 illustrate the distribution pattern of IL-6 in RAM group.

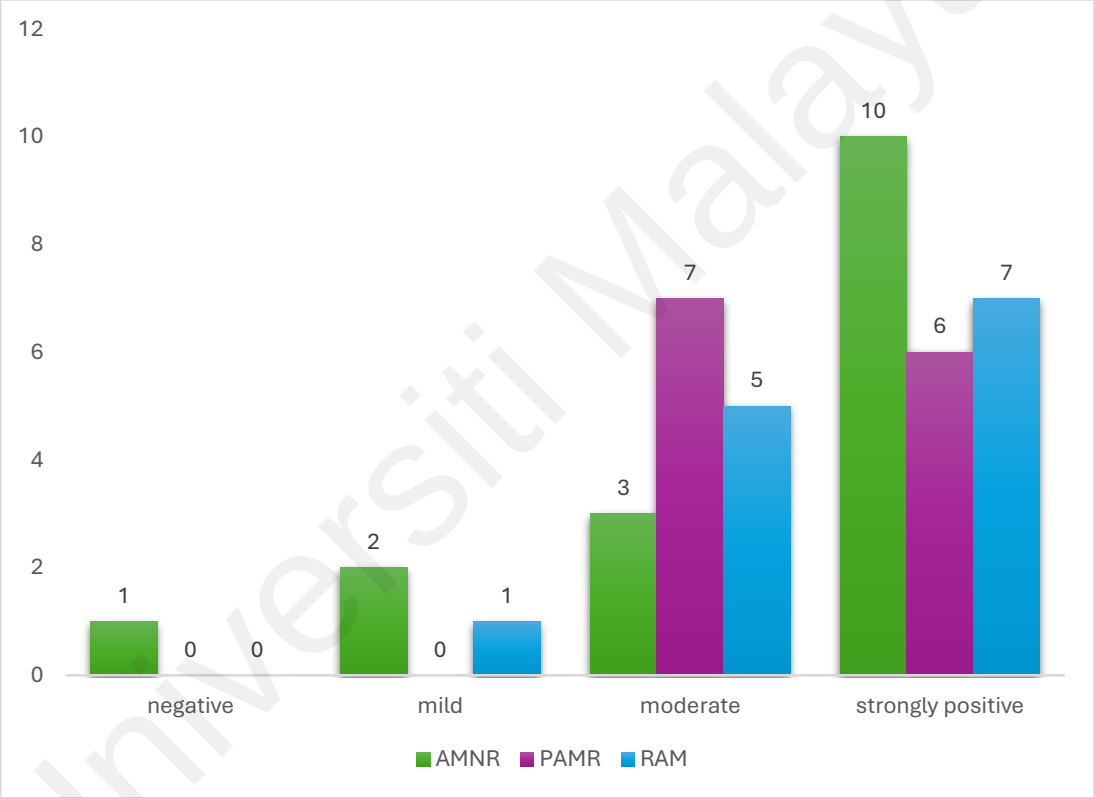

Figure 4.4 : Expression of IL-6 in non-recurrent and recurrent AM

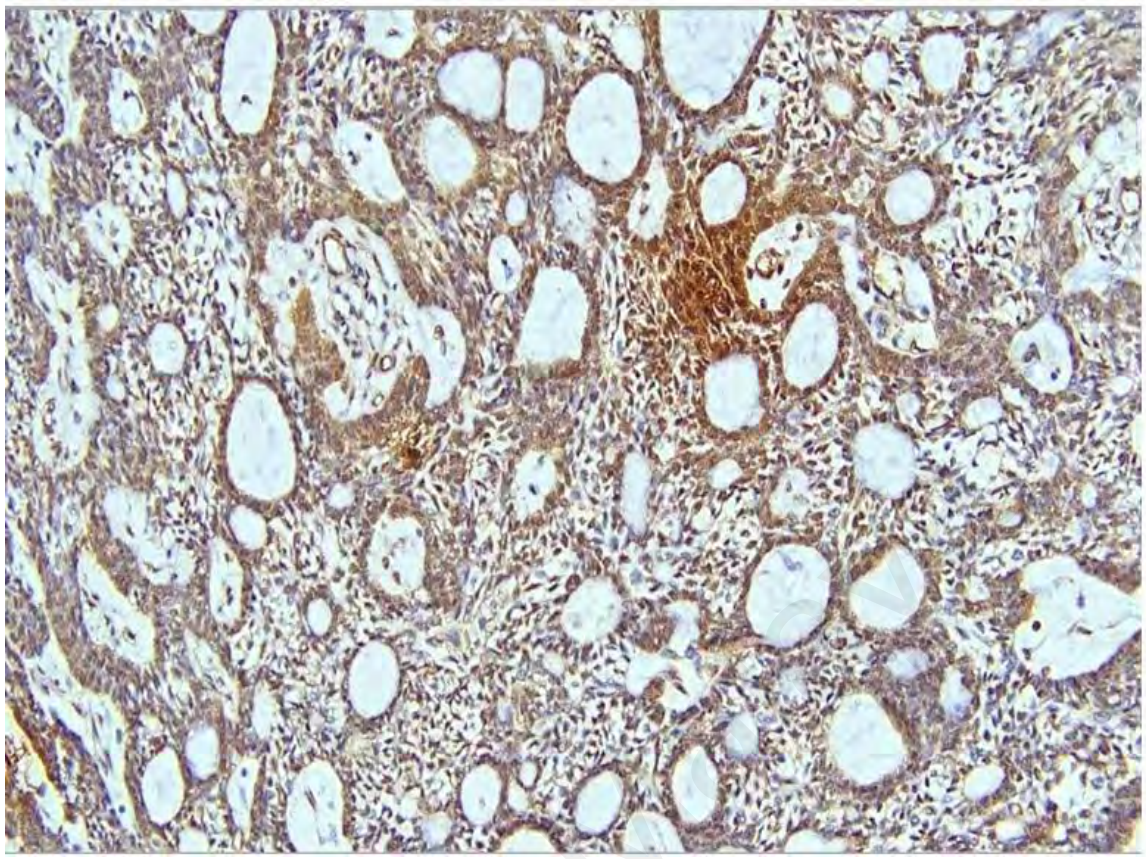

Figure 4.5 : Photomicrograph showing strong immunopositivity of IL-6 in RAM group. Original magnification 200x.

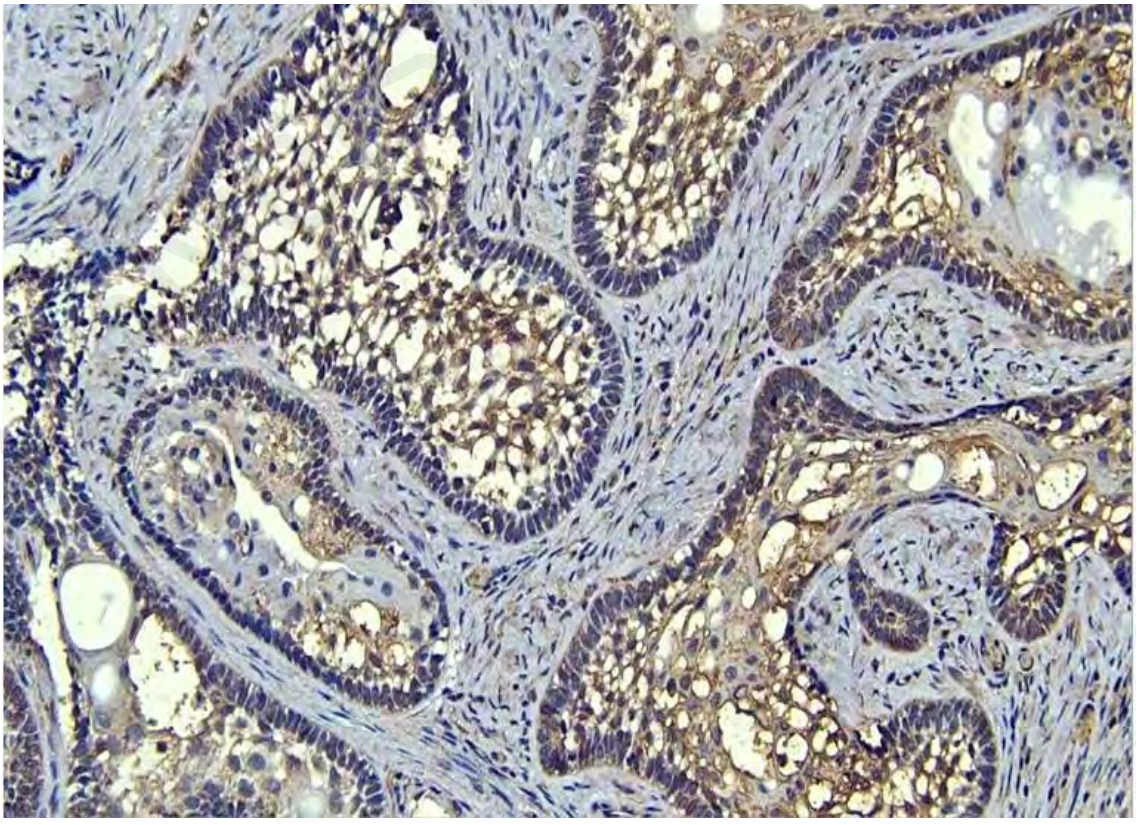

Figure 4.6 : Photomicrograph showing strong immunopositivity of IL-6 in RAM group. Original magnification 200x.

4.3.3 Expression of CD 10 in ameloblastoma

Immunoreactivity of CD10 for AMNR, PAMR and RAM is summarized in Figure 4.7. In AMNR, most of the tumour cells exhibit a strong expression of CD10 marker. Moderate CD 10 immunopositivity was observed in the majority of the PAMR and RAM cases. Only one case in the RAM expressed strong positivity of CD 10. The positive immunoreactivity was observed in PA-like cells and SR-like cells. Figures 4.8 and 4.9 illustrate the distribution pattern of CD10 in PAMR and RAM.

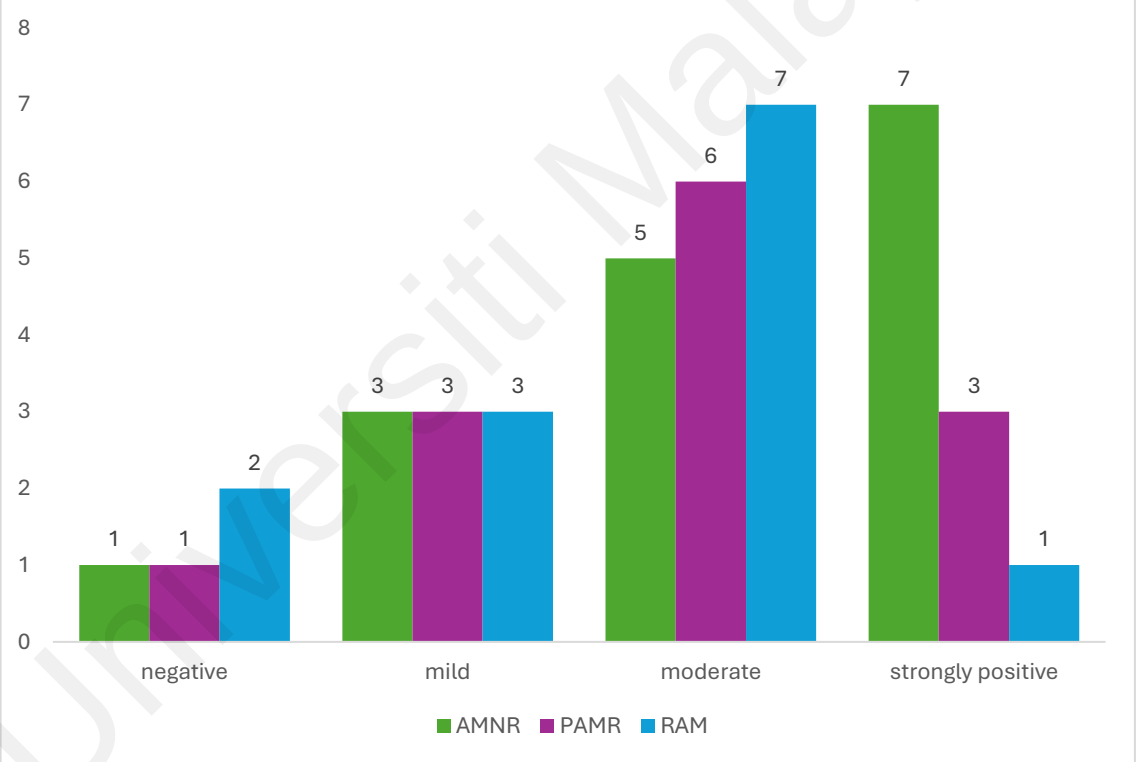

Figure 4.7 : Expression of CD 10 in non-recurrent and recurrent AM

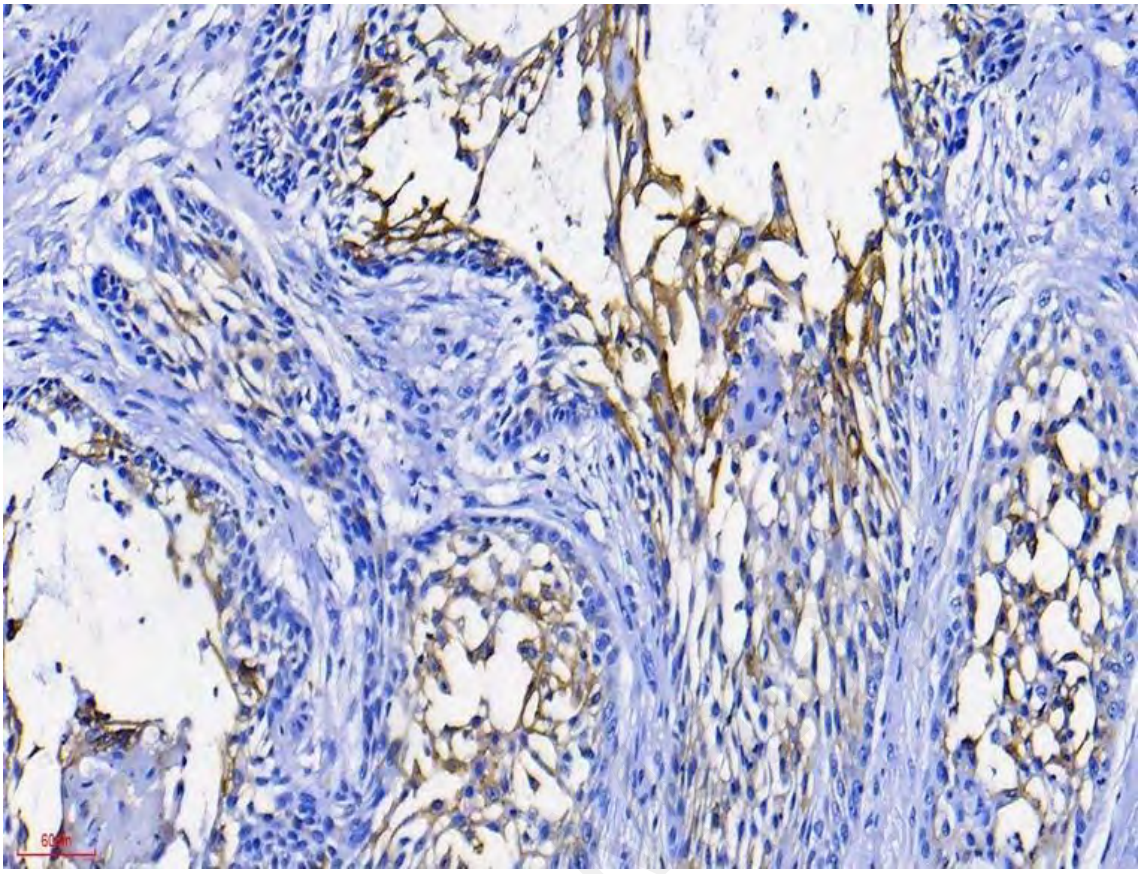

Figure 4.9 :Photomicrograph showing moderate immunopositivity of CD10 in PAMR group. Original magnification 200x.

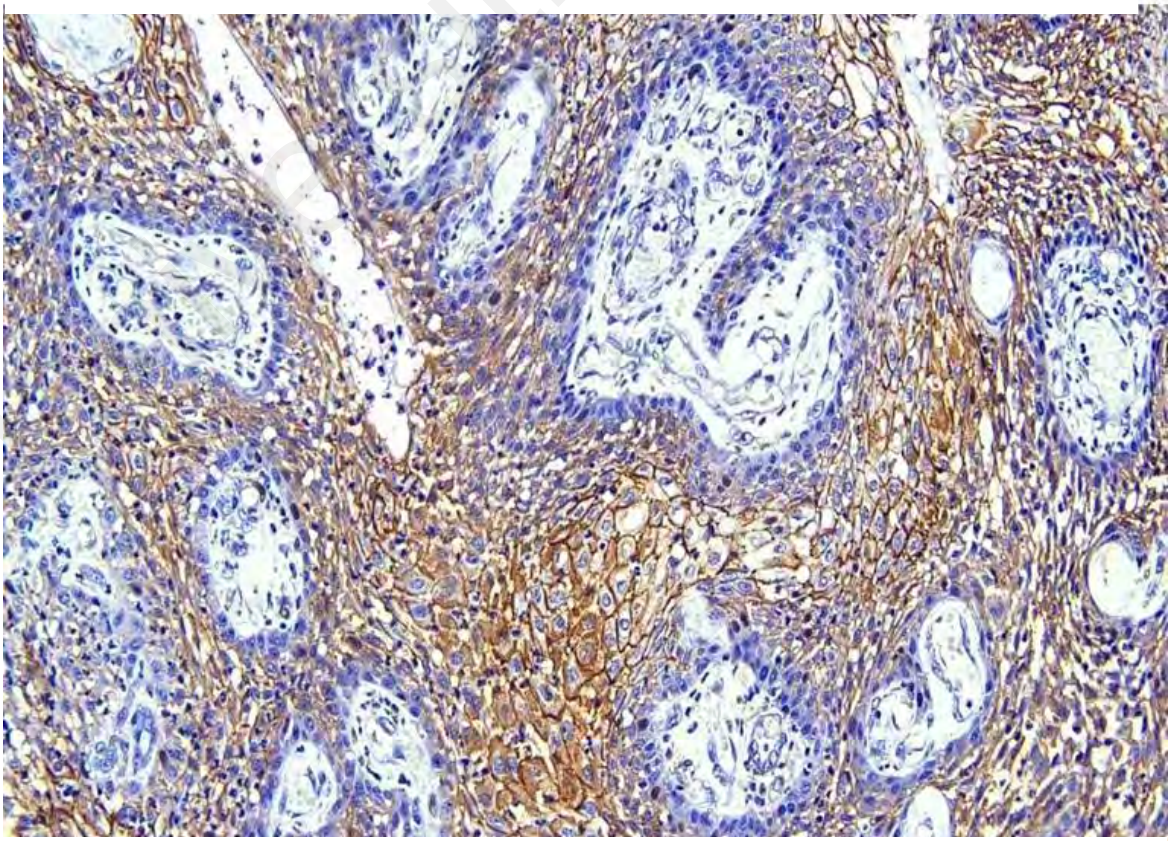

Figure 4.8 : Photomicrograph showing strong immunopositivity of CD 10 in RAM group. Original magnification 200x.

4.4 Statistical analysis

4.4.1 Comparative analysis of immunoreactivity for IL-1α, IL-6 and CD 10 in non-recurrent and recurrent AM

One-way ANOVA test was used to compare the immunoreactivity of three markers (IL-1α, IL-6, and CD10) in recurrent and non-recurrent AM. The AMNR group had the highest mean IL-1α expression score, while the PAMR group had a lower mean score, and the RAM group had the lowest. For the IL-6 immunomarker, the recurrent group had higher levels of IL-6 expression. The RAM group had the highest mean score, followed by PAMR and AMNR. As for the CD10 immunomarker, the highest mean score was found in the AMNR group compared to the recurrent group. However, no statistically significant differences ($p > 0.05$) were found across all categories for the immunoreactivity of IL-1α, IL-6 and CD10.

Table 4.3 : Statistical summary for different immunoreactivity of markers IL-1α, IL-6 and CD 10 between AMNR, PAMR, RAM

Immunomarkers	Group of ameloblastoma	N	Mean	95% Confidence Interval for Mean		F	P value
				Lower Bound	Upper Bound		
IL-1α	AMNR	16	5.81	3.58	8.05	0.364	0.697
	PAMR	13	5.08	2.78	7.37		
	RAM	13	4.69	3.22	6.16		
IL-6	AMNR	16	9.38	7.24	11.51	0.009	0.991
	PAMR	13	9.46	8.14	10.78		
	RAM	13	9.54	7.62	11.46		
CD 10	AMNR	16	6.94	4.86	9.01	1.692	0.197
	PAMR	13	5.46	3.49	7.43		
	RAM	13	4.62	2.78	6.45		

*Test performed: One-way ANOVA

In order to determine which specific group means are different after finding a significant effect in ANOVA test, we performed the Tukey post-hoc test across all of the three markers (IL-1α, IL-6, and CD10), the pairwise comparison indicates that there are no significant differences between the AM groups.

Table 4.4 : Pairwise comparison of immunomarkers expression with AM groups using Tukey post hoc test.

Immunomarkers	Pairwise group			Std. Error	P value
IL-1α	AMNR	vs	PAMR	1.348	0.84
	AMNR	vs	RAM	1.348	0.68
	PAMR	vs	RAM	1.416	0.96
IL-6	AMNR	vs	PAMR	1.223	1.00
	AMNR	vs	RAM	1.223	0.99
	PAMR	vs	RAM	1.284	1.00
CD 10	AMNR	vs	PAMR	1.290	0.49
	AMNR	vs	RAM	1.290	0.18
	PAMR	vs	RAM	1.355	0.81

*Test performed: Tukey post-hoc test.

4.4.2 Association of IL-1α, IL-6 and CD10 expression in relation to demographic and clinicopathological parameters in non-recurrent and recurrent AM

The Pearson Chi-Square test was used to analyze the association of IL-1α, IL-6 and CD 10 expression with the demographic and clinical parameters including age, gender, tumour location, histopathological subtype and treatment modalities of AM in non-recurrent and recurrent group of AM as illustrated in Table 4.5, 4.6, 4.7, 4.8 and 4.9. Correlation analysis revealed a significant association between expression of IL-6 with side of mandible involved in AMNR ($p=0.01$) and between expression of CD 10 with side of mandible involved in PAMR ($p=0.02$) as described in Table 4.7. The subsites of tumour location within the mandible a significant association between CD 10 expression and recurrent groups, PAMR ($p=0.005$) and RAM ($p=0.02$) as described in Table 4.8. A significant association was found between IL-1α expression and histopathological subtypes with AMNR ($p=0.03$) and in PAMR groups ($p=0.002$) as stated in Table 4.9. There was no significant association ($p > 0.05$) observed between expression of IL-1α, IL-6 and CD 10 with patients' age, gender and treatment modalities across non-recurrent and recurrent AM groups.

Table 4.5 : Association between IL-1α, IL-6 and CD 10 expression levels with patients' age of non-recurrent and recurrent AM.

Group AM	Age group	IL-1α				P value	IL-6				P value	CD 10				P value
		negative	mild	moderate	strongly positive		negative	mild	moderate	strongly positive		negative	mild	moderate	strongly positive	
		Count	Count	Count	Count		Count	Count	Count	Count		Count	Count	Count	Count	
AMNR	10-19	0	2	1	2	0.46	0	0	1	4	0.31	0	0	2	3	0.41
	20-29	0	3	2	0		0	0	2	3		0	1	1	3	
	30-39	0	0	1	0		0	1	0	0		0	0	0	1	
	40-49	0	0	0	1		0	0	0	1		0	1	0	0	
	50-59	1	1	0	1		1	1	0	1		1	1	1	0	
	60-69	0	1	0	0		0	0	0	1		0	0	1	0	
	70-79	0	0	0	0		0	0	0	0		0	0	0	0	
PAMR	10-19	0	0	0	1	0.39	0	0	0	1	0.71	0	1	0	0	0.22
	20-29	0	2	1	0		0	0	2	1		1	0	0	2	
	30-39	0	2	0	1		0	0	2	1		0	1	2	0	
	40-49	0	2	0	1		0	0	2	1		0	1	2	0	
	50-59	0	1	1	0		0	0	1	1		0	0	2	0	
	60-69	0	0	1	0		0	0	0	1		0	0	0	1	
	70-79	0	0	0	0		0	0	0	0		0	0	0	0	
RAM	10-19	0	0	0	0	0.54	0	0	0	0	0.76	0	0	0	0	0.38
	20-29	0	0	2	0		0	0	1	1		0	0	1	1	
	30-39	0	1	3	0		0	0	1	3		0	1	3	0	
	40-49	0	2	1	0		0	1	1	1		1	0	2	0	
	50-59	0	1	1	0		0	0	1	1		1	1	0	0	
	60-69	0	0	1	0		0	0	1	0		0	1	0	0	
	70-79	0	0	1	0		0	0	0	1		0	0	1	0	

*Test performed: Chi Square test.

Table 4.6 : Association between IL-1α, IL-6 and CD 10 expression levels with patients' gender of non-recurrent and recurrent AM.

Category	Gender	IL-1α					IL-6					CD 10				
		Negative	Mild	Moderate	Strongly positive	P value	Negative	Mild	Moderate	Strongly positive	P value	Negative	Mild	Moderate	Strongly positive	P value
AMNR	F	0	2	1	1	0.94	0	0	1	3	0.74	0	1	1	2	0.91
	M	1	5	3	3		1	2	2	7		1	2	4	5	
PAMR	F	0	5	3	2	0.55	0	0	5	5	0.61	1	2	4	3	0.63
	M	0	2	0	1		0	0	2	1		0	1	2	0	
RAM	F	0	2	8	0	0.12	0	0	5	5	0.08	1	2	6	1	0.66
	M	0	2	1	0		0	1	0	2		1	1	1	0	

*Test performed: Chi Square test

Table 4.7 : Association between IL-1α, IL-6 and CD 10 expression levels with tumour location (side of mandible) of non-recurrent and recurrent AM groups.

Group	Side of mandible	IL-1α			P value	IL-6				P value	CD 10				P value
		mild	moderate	strongly positive		negative	mild	moderate	strongly positive		negative	mild	moderate	strongly positive	
AMNR	Right	2	2	3	0.56	0	0	0	7	**0.01**	0	2	2	3	0.76
	Left	2	1	1		0	0	2	2		0	1	1	2	
	Bilateral	3	1	0		1	2	1	1		1	0	2	2	
PAMR	Right	5	2	1	0.34	0	0	5	3	0.58	0	1	5	2	**0.02**
	Left	1	1	2		0	0	2	2		0	2	1	1	
	Bilateral	1	0	0		0	0	0	1		1	0	0	0	
RAM	Right	3	6	0	0.45	0	1	3	5	0.85	2	3	4	0	0.13
	Left	1	3	0		0	0	2	2		0	0	3	1	
	Bilateral	0	0	0		0	0	0	0		0	0	0	0	

*Test performed: Chi Square test ; Bold indicates significant

Table 4.8 : Association between IL-1α, IL-6 and CD 10 expression levels with tumour location (subsites) of non-recurrent and recurrent AM groups.

Group	Subsites	IL-1α				P value	IL-6				P value	CD 10				P value
		Negative	Mild	Moderate	Strongly positive		Negative	Mild	Moderate	Strongly positive		Negative	Mild	Moderate	Strongly positive	
AMNR	Anterior	0	1	0	0	0.65	0	0	0	1	0.45	0	0	1	0	0.34
	Middle	0	0	0	0		0	0	0	0		0	0	0	0	
	Posterior	0	2	0	0		0	0	1	1		0	1	0	1	
	Mandible	0	1	3	1		0	0	1	4		0	0	2	3	
	Bilateral	1	3	1	0		1	2	1	1		1	0	2	2	
	Non-specified	0	0	0	3		0	0	0	3		0	2	0	1	
PAMR	Anterior	0	0	1	0	0.78	0	0	0	1	0.59	0	0	0	1	**0.005**
	Middle	0	0	0	0		0	0	0	0		0	0	0	0	
	Posterior	0	2	2	1		0	0	3	2		0	1	2	2	
	Mandible	0	3	0	1		0	0	3	1		0	0	4	0	
	Bilateral	0	1	0	0		0	0	0	1		1	0	0	0	
	Non specified	0	1	0	1		0	0	1	1		0	2	0	0	
RAM	Anterior	0	0	1	0	0.87	0	0	1	0	0.26	0	1	0	0	**0.02**
	Middle	0	0	0	0		0	0	0	0		0	0	0	0	
	Posterior	0	1	5	0		0	0	3	3		0	0	5	1	
	Mandible	0	1	2	0		0	0	0	3		0	2	1	0	
	Bilateral	0	0	0	0		0	0	0	0		0	0	0	0	
	Non specified	0	2	1	0		0	1	1	1		2	0	1	0	

*Test performed: Chi Square test; Bold indicates significant.

Table 4.9 : Association between IL-1α, IL-6 and CD 10 expression levels with histopathological subtypes of conventional AM in non-recurrent and recurrent AM groups.

Category	Histopathologic subtypes	IL-1α				P value	IL-6				P value	CD 10				P value
		Negative	Mild	Moderate	Strongly positive		Negative	Mild	Moderate	Strongly positive		Negative	Mild	Moderate	Strongly positive	
AMNR	Follicular	0	5	0	0	**0.03**	0	1	2	2	0.19	0	1	2	2	0.92
	Plexiform	1	1	3	4		1	0	1	7		1	2	2	4	
	Acanthomatous	0	1	0	0		0	0	0	1		0	0	1	0	
	Basal cells	0	0	0	0		0	0	0	0		0	0	0	0	
	Mixed type	0	0	1	0		0	1	0	0		0	0	0	1	
PAMR	Follicular	0	5	3	0	**0.002**	0	0	4	4	0.66	0	2	3	3	0.58
	Plexiform	0	0	0	3		0	0	2	1		0	1	2	0	
	Acanthomatous	0	0	0	0		0	0	0	0		0	0	0	0	
	Basal cells	0	0	0	0		0	0	0	0		0	0	0	0	
	Mixed type	0	1	0	0		0	0	1	0		0	0	1	0	
RAM	Follicular	0	2	4	0	0.50	0	1	4	1	0.09	1	2	3	0	0.38
	Plexiform	0	1	2	0		0	0	0	3		0	0	2	1	
	Acanthomatous	0	0	0	0		0	0	0	0		0	0	0	0	
	Basal cells	0	1	0	0		0	0	1	0		1	0	0	0	
	Mixed type	0	0	2	0		0	0	0	2		0	1	1	0	

*Test performed: Chi Square test; Bold indicates significant

CHAPTER 5: DISCUSSION

5.1 Demographic and clinicopathological characteristic

Our study found that AMNR cases were common in the second decade and in agreement with Zhang et al. (2010) with the mean age of 14.5 years and 14.7 years in the study of Arotiba et al. (2005) in Nigeria . Our AMNR cases were also found commonly in the third decade of life which in accordance with other studies, where the cases predominantly occurred in the third and fourth decades of life (Dhanuthai et al., 2012; Masthan et al., 2015; Ragunathan et al., 2022). In the recurrent AM, PAMR cases were observed to peak at the mean age of 37.2 ranging from 14 to 64 years and most of the RAM cases occurred at the mean age of 44 between 22 to 72 years. This six-year interval from primary recurrent to secondary recurrent proved that a follow-up period of more than 5 years is crucial in AM cases despite treatment received (Neville et al., 2023).

As for gender analysis, we observed a male predominance in non-recurrent AM group which aligns with the majority of the literature (Ajila & Hegde, 2022; Hendra et al., 2020; Siar et al., 2012). In the present study, we observed that females predominate males in the recurrent AM groups, with a 3.3:1 female-to-male ratio. This female predominance is similar to Goh et al. (2021) where the female-to-male ratio in recurrent AM cases was 1.3:1 and 1.03:1 in a study by Au et al. (2019) in Hong Kong. However, this finding differs from Bi et al. (2021) where recurrences were significantly more in males with male-to-female ratio being reported as 1.92:1. The higher incidence of recurrent occurrences in females may be linked to their tendency to seek treatment later or to the slower onset of the symptoms. Furthermore, this group might have been less inclined for upfront aggressive or radical treatment at the time of primary diagnosis which could lead to a recurrence in an advanced stage.

In relation to the site of occurrence, AMNR was most commonly found in the right mandible involving more than one segment, followed by bilateral involvement. Majority of the recurrent cases were found in the right posterior mandible. These findings are in discordance with Nwoga et al. (2022) where most of the recurrent AM were reported at the anterior region of the mandible.

We hypothesized that the greater frequency of recurrent AM on the right side of the mandible could be attributed to a specific cohort characteristics rather than a generalizable pattern in this study. It is well known that mandible has a high bone mineral density with less vascularity that would favor for recurrence (Devlin et al., 1998). Some research suggests that certain biological or genetic factors may predispose cancers to behave differently based on their location. However, a particular biological explanations for a right-sided preference have yet to be found decisively.

Regarding the AM histopathologic subtypes, all of the markers showed strong expression in the plexiform subtype for AMNR, whereas follicular subtype predominated in recurrent groups. This observation aligns with previous reports indicating that follicular AM is the most prevalent subtype and has the highest recurrence rate, followed by the plexiform subtype (Goh et al., 2019; Siar et al., 2012).

5.2 Expression of IL-α

Members of the IL-1 family play diverse roles across different tissues and cells, influencing functions such as immune response regulation, hematopoietic balance, bone remodeling, neuronal physiology, and synaptic plasticity (Landuzzi et al., 2024).

IL-1α is a pleiotropic potent modulator in bone resorption by inducing the activation of osteoclast-like-cells and production of matrix metalloproteinase enzymes (Pripatnanont et al., 1998; Sengüven & Oygür, 2011). A strong relationship between cytokine expression and matrix metalloproteinase 2 and 9 production in ameloblastoma referring to the role of cytokine and enzymatic activity in tumour spreading within the bone trabeculae was reported by (Kubota et al., 2001). Similarly, in bladder CA, IL-1α was found to regulate MMP-2 and MMP-9 to affect cancer invasiveness and related to short overall survival and progression-free survival (Yao et al., 2023).

We observed that AMNR tumour cells showing moderate to strong immunopositivity for IL-1α, with one case showing negative immunoreactivity. In contrast, majority of RAM exhibited moderate IL-1α expression. No cases showed strong positivity in RAM compared to PAMR and RAM. Tumour cells in PAMR exhibited mild to moderate IL-1α expression. All of the AM tumour cells in the recurrent groups expressed IL-1α, compared to only one case in the non-recurrent group showed negative immunoreactivity for IL-1α. Our findings are supported by previous studies conducted by Pripatnanont et al. (1998) which reported a strong upregulation of IL-1α in stellate-reticulum-like cells. Further confirmation through cytokine mRNA hybridization supported the synthesis of IL-1α cytokine in stellate-reticulum cells (Pripatnanont et al., 1998). A significant expression of IL-1α was secreted by epithelial components, particularly in ameloblastoma epithelial cells (Fuchigami et al., 2014).

IL-1α was observed in the nuclear and/or cytoplasmic compartments in 98% of OSCC (Rajan et al., 2019).

As for the association of the IL-1α marker with the demographic and clinicopathological, our study found a statistically significant difference in IL-1α expression in the histopathological subtype within AMNR group (p=0.03) and PAMR group (p=0.002).

The significant association of IL-1α expression with histopathological subtypes in AMNR and PAMR categories suggests its potential as a biomarker for differentiating between follicular, plexiform, acanthomatous, basal cells and mixed-type histopathological subtypes. This finding warrants further investigation into the role of IL-1α in ameloblastoma pathogenesis and subtype classification. This raises the possibility of therapeutic implications using IL-1α.

5.3 Expression of IL-6

Interestingly, we demonstrated a higher IL-6 upregulation in recurrent group compared to the non-recurrent AM group. This is the first study to investigate the expression level of this marker in the recurrent group of AM; thus, there is limited existing literature to use as a reference for our research. In relation to recurrent AM groups, mild to strong IL-6 immunopositivity was observed in the tumour parenchyma of PAMR while there was a moderate to strong expression of IL-6 in the RAM. Meanwhile, AMNR shows a variety of IL-6 expressions from no immunoreaction to strong reaction. A strong immunoreactivity was detected in tumour cells across seventeen cases of non-recurrent AM (Sathi et al., 2008). Sengüven and Oygür (2011) found majority of the non-recurrent AM cases showed mild to moderate reaction in the stellate reticulum-like cells.

Pripatnanont et al. (1998) demonstrated strong expression of IL-6 in the stellate-reticulum and postulated that AM synthesized two bone-modulating cytokines namely IL-6 and IL-1α.

RAM had the highest mean score for IL-6 expression followed by PAMR and AMNR. Most of the ameloblastoma cells in tumour parenchyma express IL-6, specifically in RAM. This upregulation of IL-6 suggests its potential involvement in advancing bone resorption in AM progression from non-recurrent to local recurrence.

Elevated IL-6 expression has shown a stronger association with recurrent tumours compared to primary tumours (Harmer et al., 2018). Ohsaki et al. (1992) suggested that IL-6 may act in both an autocrine and paracrine ways for human osteoclasts and play a significant role in the bone resorbing capacity of these cells. The expression levels of IL-1α and IL-6 was observed in three cell locations (PA-like cell, SR-like cell, and ST cells) and showed that the tissue and supportive components of AM interact through these cytokines to create a favorable environment for tumour growth (Goh et al., 2019).

Regarding the IL-6 marker's relationship with demographic and clinicopathological factors, our study discovered a statistically significant difference in IL-6 expression in non-recurrent cases ($p = 0.01$) with side of mandible involved. We postulated that this overexpression IL-6 in AMNR cases could explain why the majority of tumours grew on the right side of the mandible and suggest that bone resorption was promoted prior to recurrence of AM.

5.4 Expression of CD 10

CD 10 was found to be strongly expressed in the SR-like cells but showed negative immunoreactivity in the PA-like cells in conventional AM (Tan et al., 2022). Our study's immunohistochemical results revealed that most of the tumour cells in AMNR exhibited a strong expression of CD 10, while PAMR and RAM both expressed CD 10 moderately.

AMNR exhibited a higher mean score for CD 10 immunoreactivity compared to PAMR and RAM. Neoplastic cells showed cytoplasmic and membranous CD 10 immunopositivity in both groups. In relation to non-recurrent AM, these results are in accordance with those reported by Masloub et al. (2011) where high CD 10 expression was noted in neoplastic epithelial cells of the solid/multicystic ameloblastoma.

Most of the literature studied the expression of CD 10 in the stromal cells of a tumour compared to tumour parenchyma. In contrast to our study, we evaluated the CD 10 expression in the tumour parenchyma instead of stromal cells. We hypothesized that this different focus on cell types and their correlation with the CD 10 marker might result in a distinct pattern of CD 10 expression.

This downregulated expression of CD 10 in PAMR and RAM might be explained by the findings of Mishra et al. (2016) who found that decreased CD10 expression could potentially lead to tumour progression by increasing the concentration of active peptides in the tumour milieu, thereby enhancing cell signaling and promoting tumour growth. Our findings support the above conclusion as well. In addition, this downregulation of CD10 expression was corroborated by Toussaint et al. (2010) who found that it was related with a greater risk of relapse in ductal carcinoma in situ.

As for the association of the CD 10 marker with the demographic and clinico-pathological, our study found a statistically significant difference between CD 10 expression with side of mandible involved (p=0.02) in PAMR and subsites of tumour localization in PAMR (p=0.005) and RAM (p=0.02). This suggests that CD10 could potentially be used as a prognostic indicator for recurrence risk and as a predilection for side and subsites of tumour location in the mandible.

CHAPTER 6: CONCLUSION AND RECOMMENDATION

6.1 Conclusion

This is the first study to compare non-recurrent AM, primary recurrent and recurrent AM from the same patient to investigate the expression of IL-1α, IL-6, and CD 10 in predicting recurrence of AM. We observed an upregulation of IL-6 expression in recurrent AM compared to non-recurrent AM, suggesting the potential of IL-6 in predicting recurrence of AM. A downregulation of IL-1α and CD 10 expression in recurrent AM compared to non-recurrent AM may indicate specific tumour behavior, including side and subsites of tumour localization in the mandible as well as the histopathological subtypes of conventional AM. CD 10 expression was notably significantly associated with side of mandible involved and subsites of tumour localization within the PAMR. IL-6 expression was significantly associated with subsite of tumour localization within the AMNR group. IL-1α expression was markedly associated with the histopathological subtype of AM in AMNR and PAMR group.

In summary, we observed an upregulation of IL-6 expression in recurrent AM compared to non-recurrent AM, suggesting the potential of IL-6 in predicting recurrence of AM. Downregulation of IL-1α and CD-10 expression in recurrent AM compared to non-recurrent AM needs further investigation in order to identify their role.

6.2 Limitations

There were several limitations, including small and unequal sample size between the non-recurrent and recurrent ameloblastoma cases. The lack of variety and proportional cases in these histologic subtypes of ameloblastoma denied representable comparison of immunomarker expression across these subtypes.

This study mainly focuses on the expression of the markers in tumour parenchyma thus may limit the findings in association of parenchyma-stromal cell interactions on the tumoral microenvironment in AM.

6.3 Recommendation

Further studies focusing different signaling pathways related to IL-1α, IL-6, and CD 10 using larger samples are recommended to investigate the mechanisms of predicting recurrence of AM.

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