

ORIGINAL ARTICLE

Parenchyma-stromal interleukin-1 alpha and interleukin-6 overexpressions in ameloblastoma correlate with the aggressive phenotype

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Abstract

Introduction: Ameloblastoma is a benign but locally invasive odontogenic epithelial neoplasm with a high recurrence rate after treatment. The two main subsets encountered clinically are unicystic (UA) and solid/multicystic ameloblastoma (SMA). Currently neoplastic progression of many tumour types are believed to be related to parenchyma-stromal cell-cell interactions mediated by cytokines notably interleukins (IL). However their roles in ameloblastoma remain ill-understood. **Materials and Methods:** Thirty-nine formalin-fixed paraffin-embedded ameloblastoma cases comprising unicystic ameloblastoma (n=19) and solid/multicystic ameloblastoma (n=20) were subjected to IHC staining for IL-1 α , IL-1 β , IL-6 and IL-8. A semi-quantitative method was used to evaluate the expression levels of these cytokines according to cell types in the tumoural parenchyma and stroma. **Results:** Major findings were upregulations of IL-1 α and IL-6 in SMA compared to UA. Both cytokines were heterogeneously detected in the tumoural parenchyma and stroma. Within the neoplastic epithelial compartment, IL-1 α expression was more frequently detected in PA-like cells in UA whereas it was more frequently encountered in SR-like cells in SMA. IL-6 demonstrated higher expression levels in the stromal compartment of SMA. IL-1 β and IL-8 were markedly underexpressed in both tumour subsets. **Conclusions:** Overexpression of IL-1 α in SMA suggests that this growth factor might play a role in promoting bone resorption and local invasiveness in this subtype. The expression levels of IL-1 α and IL-6 in three cellular localizations indicate that parenchymal-stromal components of ameloblastoma interact reciprocally via IL-1 α and IL-6 to create a microenvironment conducive for tumour progression.

Keywords: Ameloblastoma, Interleukin-1 alpha, Interleukin-6, Microenvironment

INTRODUCTION

Ameloblastoma is a benign but locally invasive odontogenic epithelial neoplasm which accounts for 13% to 54% of all jaw lesions.¹ It takes its origin from the dental lamina and enamel organ. Microscopically the ameloblastoma tumour epithelium recapitulates the pre-ameloblast and stellate reticulum of the developing tooth.² This tumour shows a distinct predilection for the posterior mandible and usually affects patients between the third to seventh decades

of life.^{1,2} The two major clinicopathological subtypes are unicystic (UA) and solid/multicystic ameloblastoma (SMA). SMA exhibits a more aggressive behaviour because of its local infiltrativeness and high recurrence rate.³ UA, on the other hand, is indolent with a cyst-like characteristic and low recurrence tendency.¹

The most controversial behaviour of ameloblastoma is its invasiveness into the surrounding bone, despite its benign nature. The exact mechanism of its locally aggressive growth

remains unclear. Recent evidence indicates that ameloblastoma could induce osteoclastogenesis which in turn enables ameloblastoma to invade bone.⁴ Interactions between stromal and epithelial cells to create a complex tumoral microenvironment are essential for tumour invasion. The molecular interactions that lead to bone resorption without a proportional osteoblastic reaction in ameloblastoma results in abnormal bone remodelling sequence⁵. Soluble factors such as cytokines and growth factors which upregulate the growth and invasion are found to be secreted by tumour cells and stromal cells. Cytokines particularly IL-1 α , IL-1 β , IL-6 and IL-8 were present at high levels in ameloblastoma cells and cystic aspirate.^{6,7}

The aim of this study was to determine by immunohistochemistry the expression patterns of IL-1 α , IL-1 β , IL-6 and IL-8 in the ameloblastoma parenchyma and/or stroma. Our rationale was to clarify whether the relative distribution of these bone resorbing cytokines in the different tissue compartments/cellular localizations correlate with the biological behaviours of its tumour subsets.

MATERIALS AND METHODS

Tissue sample

This study was approved by the Institutional Medical Ethics Committee [DFOS1517/0054(P)] on 2nd of June, 2015. The study sample consisted of 39 formalin-fixed paraffin-embedded tissue blocks of ameloblastoma cases (20 SMA and 19 UA) retrieved from the archives of Oral Pathology Diagnostic and Research Laboratory, Faculty of Dentistry, University of Malaya. New haematoxylin and eosin stained 4 μ m thick sections of these specimens were reviewed and selected by a qualified pathologist (CHS) according to established criteria.^{1,2} Patients' characteristics were recorded.

Immunohistochemistry

New 4 μ m thick sections were mounted on salinized slides. Optimization for each antibody was performed. The primary antibodies used were rabbit polyclonal anti IL-1 α (AB9614) (Abcam Inc, Cambridge, MA, USA, 1:2000), rabbit polyclonal anti IL-1 β (AB2105) (Abcam, 1:1000), mouse monoclonal anti IL-6 (AB9324) (Abcam 1:5000) and mouse monoclonal anti IL-8 (AB 18672) (Abcam, 1:500). Positive and negative controls according to the manufacturers' recommendations were carried out. Antigen retrieval and endogenous peroxidase blocking

were performed prior to incubation with primary antibodies. Diaminobenzidine (DAB) was used as chromogen and counterstaining with Harris haematoxylin was carried out.

Immunohistochemical analysis

The immunoprofile and antibody localizations of IL-1 α , IL-1 β , IL-6 and IL-8 in pre-ameloblast-like cells (PA-like cells), stellate reticulum-like cells (SR-like cells), and stromal cells (ST cells) were evaluated by descriptive and semi quantitative methods. Five hotspots were selected and examined at x200 magnification. Immunoreactivity was independently scored by two authors (YCG and CHS). Any disagreement was reviewed together to achieve a consensus score. Each case was evaluated based on the percentage of immunoreactive tumoral epithelial cells: (-) negative when there was no immunopositivity present; (+) mild when immunopositivity was observed in focal area (<25%); (++) moderate when significant percentage of tumoral epithelial cells (25-50%) expressed the marker; and (+++) strong when >50% of the tumour epithelial cells expressed the marker. Stromal components were quantified similarly.

Statistical analysis

The result analysis was performed using IBM Statistical Package for Social Sciences (SPSS) version 20. Expression of IL-1 α , IL-1 β , IL-6 and IL-8 in PA-like cells, SR-like cells and ST cells in UA and SMA were subjected to Friedman test. Mann-Whitney U test was performed for comparative analysis of IL-1 α , IL-1 β , IL-6 and IL-8 in relation to ameloblastoma subsets within PA-like cells, SR-like cells and ST cells. For all the statistical analysis, *P* value <0.05 was considered to denote statistical significance.

RESULTS

Patients' characteristics

Details of cohort are summarized in Tables 1. Their outcomes and follow-up records were generally unavailable for analysis.

Immunohistochemical findings

These are summarized in Tables 2 and 3, and illustrated in Figures 1, 2, 3 and 4.

IL-1 α . IL-1 α was the most widely expressed cytokine in both UA and SMA, being identified in all three cell types evaluated (Fig. 1). It was more frequently detected in PA-like cells

TABLE 1: Demographic and clinical characteristics of the 39 ameloblastoma cases

Variables	UA	SMA
No of samples, n (%)	19 (100)	20 (100)
Demographic characteristics		
Gender		
Male, n (%)	9(47.4)	9(45)
Female, n (%)	10(52.6)	11(55)
M:F ratio	1:1.1	1.1:2
Age (Years)		
Mean	22.9	38.8
Age range	8-58	15-67
Clinical characteristics		
Site		
Mandible	18(94.7)	16(80)
Maxilla	1(5.3)	4(20)
Known Clinical Presentations		
Swelling	11 (64.7)	12 (66.7)
Pain + swelling	1 (5.9)	3 (16.7)
Painless swelling	4 (23.5)	2 (11.1)
Asymptomatic	1 (5.9)	1 (5.5)
Unspecified	2 (-)	2 (-)
Known Radiographic finding		
Radiolucency	5(50)	1 (9.1)
Unilocular radiolucency	3(30)	3 (27.3)
Multilocular radiolucency	2(20)	6 (54.5)
Others	-	1 (9.1)
Unspecified	9(-)	9 (-)
Pre-operative diagnosis		
Ameloblastoma	12 (63.2)	18 (90)
Dentigerous cyst	5 (26.3)	1 (5)
Odontogenic keratocyst	1 (5.3)	-
Radicular cyst	1 (5.3)	-
Lateral Periodontal cyst	-	1 (5)

than SR-like cells and ST cells. Intracellular protein localization was predominantly nuclear (Fig. 1). Immunoreactivity of IL-1 α in three different cell types showed no significant difference ($P>0.05$) in UA and SMA (Table 2). There was a significant difference in IL-1 α expression scores between UA and SMA within PA-like, SR-like and ST cells (Table 3).

IL-1 β . IL-1 β was markedly underexpressed in both UA and SMA. Positive expression rate

was higher in UA compared to SMA. Mild immunoreactivity was noted in cytoplasmic and nuclear region of epithelial and ST cells in UA (Fig. 2). Most SMA cases were nonreactive for IL-1 β . Positive tumours demonstrated equal distribution of IL-1 β expression in PA-like and SR-like cells. IL-1 β was weakly expressed in two cases within ST cells. Expression levels of IL-1 β in three different cell types were not significantly different ($p>0.05$) (Table 2). A significant difference in expression of IL-1 β

TABLE 2: Comparison of immunoreactivity for IL-1 α , IL-1 β , IL-6 and IL-8 among pre-ameloblast-like, stellate reticulum-like and stromal cells in UA and SMA

Markers	n		Cells	Mean (SD)		χ^2 (df)		p Value	
	UA	SMA		UA	SMA	UA	SMA	UA	SMA
IL-1 α	19	20	PA-like cells	1.42(.507)	1.85(.366)	1.676(2)	0.200(2)	0.433	0.905
			SR-like cells	1.37(.831)	1.95(.759)				
			ST cells	1.27(.787)	1.90(.718)				
IL-1 β	19	20	PA-like cells	0.37(.496)	0.05(.224)	3.250(2)	0.500(2)	0.197	0.779
			SR-like cells	0.21(.419)	0.05(.224)				
			ST cells	0.16(.375)	0.10(.308)				
IL-6	19	20	PA-like cells	0.95(.229)	1.00(.324)	1.771(2)	0.889(2)	0.412	0.641
			SR-like cells	0.79(.631)	1.00(.324)				
			ST cells	0.84(.765)	1.10(.718)				
IL-8	19	20	PA-like cells	0.21(.419)	0.10(.308)	6.000(2)	3.000(2)	0.050	0.223
			SR-like cells	0.21(.419)	0.05(.224)				
			ST cells	0.05(.229)	0.00(.000)				

Test performed: Friedman test

between PA-like cells of UA and SMA was observed (Table 3).

IL-6. IL-6 was the second most widely expressed cytokine in both UA and SMA. Staining intensity was mild and diffuse. Intracellular protein localization was predominantly cytoplasmic and occasionally nuclear (Fig. 3). No significant differences were observed for IL-6 expression

scores among PA-like, SR-like and ST cells ($P>0.05$) (Table 2). Expression levels for IL-6 between epithelial and stromal cells of UA and SMA were not significantly different ($P>0.05$) (Table 3).

IL-8. IL-8 was markedly underexpressed in UA and SMA (Fig. 4). A weak protein localization in cytoplasmic and nucleus region was generally

TABLE 3: Comparison of expression of IL-1 α , IL-1 β , IL-6 and IL-8 within pre-ameloblast-like, stellate reticulum-like and stromal cells between UA and SMA

Markers	Subtypes	n	Mean Rank			p Value		
			PA-like cells	SR-like cells	ST cells	PA-like cells	SR-like cells	ST cells
IL-1 α	UA	19	15.71	16.18	15.74	0.006	0.030	0.014
	SMA	20	24.08	23.63	24.05			
IL-1 β	UA	19	23.18	21.61	20.58	0.015	0.139	0.594
	SMA	20	16.98	18.48	19.45			
IL-6	UA	19	19.50	18.11	17.92	0.563	0.213	0.201
	SMA	20	20.48	21.80	21.98			
IL-8	UA	19	21.11	21.61	20.53	0.345	0.139	0.305
	SMA	20	18.95	18.48	19.50			

Test performed: Mann-Whitney U Test; Bold values indicate significant difference.

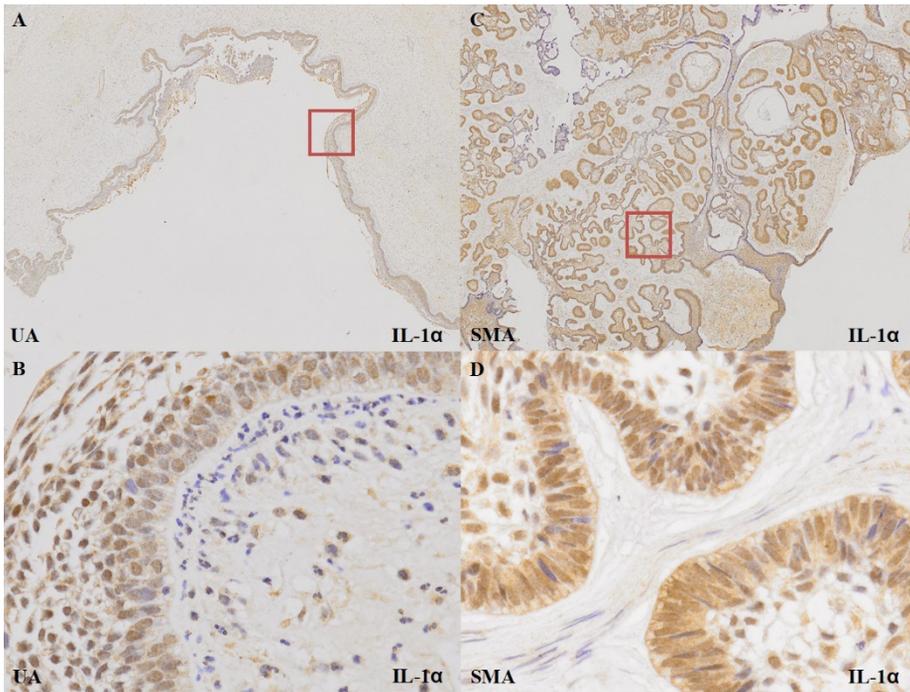


FIG. 1: Representative sections showing expression of IL-1 α in UA and SMA. IL-1 α expression was observed in PA-like, SR-like and ST cells with relatively greater distribution observed in SMA (C and D). (Original magnification: A, C x40; B, D x400; DAB).

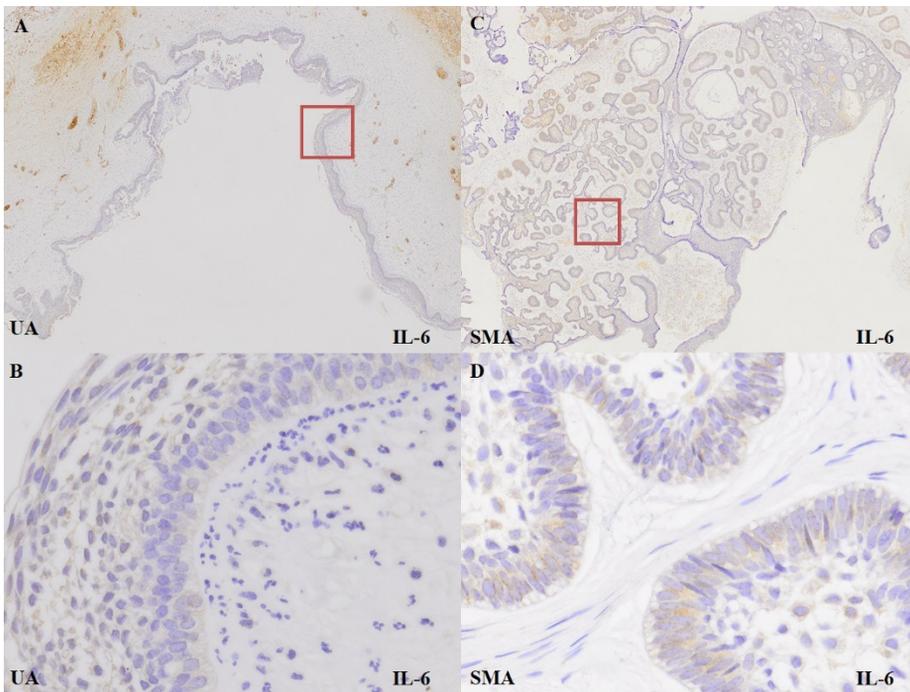


FIG. 2: Representative sections showing expression of IL-1 β in UA and SMA. General immunonegativity was observed among PA-like, SR-like and ST cells. (Original magnification: A, C x40; B, D x400; DAB)

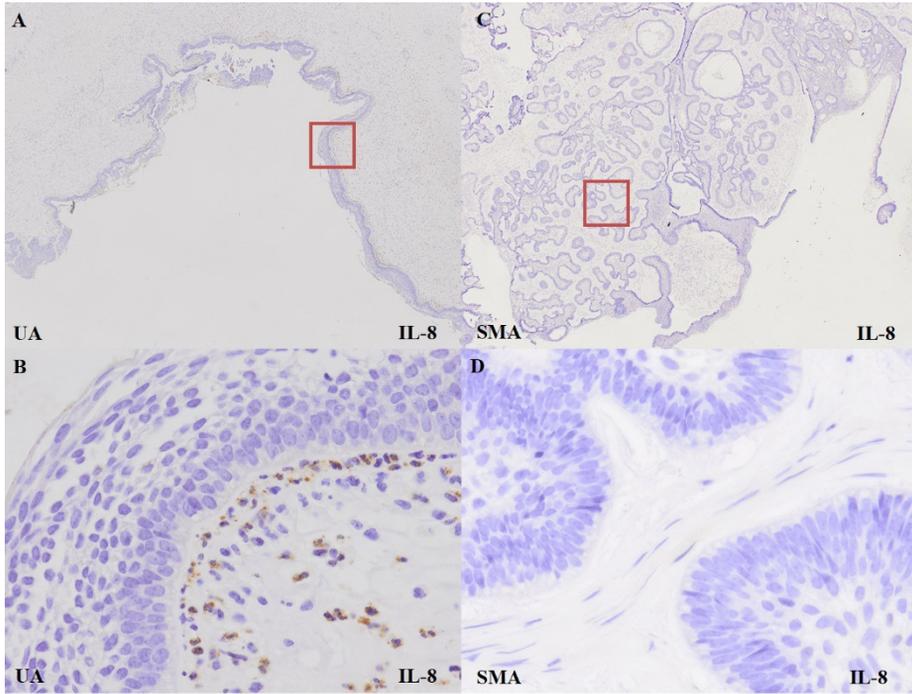


FIG. 3: Representative sections showing expression of IL-6 in UA and SMA. General immunonegativity was observed among PA-like, SR-like and ST cells. (Original magnification: A, C x40; B, D x400; DAB)

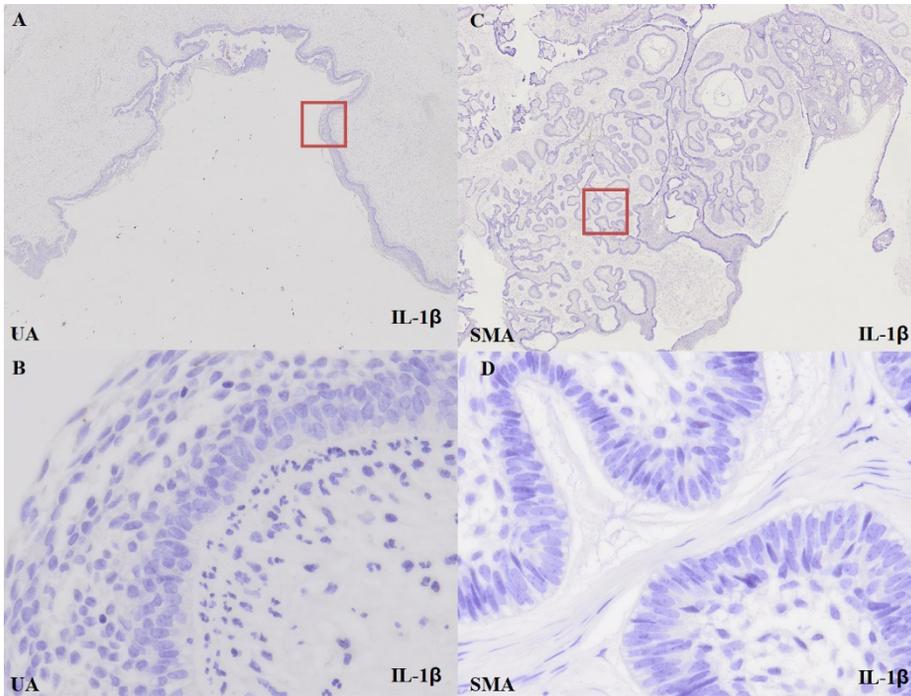


FIG. 4: Representative sections showing expression of IL-8 in UA and SMA. General immunonegativity was observed among PA-like, SR-like and ST cells. (Original magnification: A, C x40; B, D x400; DAB)

observed. Expression levels of IL-8 in three different cell types were not significantly different ($P>0.05$) (Table 2). Expression levels for IL-8 between epithelial and stromal cells of UA and SMA were not significantly different either ($P>0.05$) (Table 3).

DISCUSSION

Cell-to-cell interactions in a complex tumoral microenvironment are believed to be regulated by secreted growth factors and cytokines.^{8,9} Mounting evidence suggests that the bone tumoral microenvironment and ameloblastoma interact reciprocally via cytokines to enhance its aggressiveness.^{5,6,8,9,10,11} The present study was designed to evaluate by immunohistochemistry a set of cytokines, IL-1 α , IL-1 β , IL-6 and IL-8 compartmentalized within the ameloblastoma parenchymatous producing cells and/or in secreted forms within the microenvironment. Our goal was to elucidate whether the relative distribution of these factors in the two tissue sites correlate with the biological behaviours of its tumour subsets.

In a bone tumoral microenvironment, the RANK-RANKL-OPG triad has been shown to be an essential cytokine system to maintain constant bone mass and the balance in bone remodeling.¹² Binding of RANKL to RANK is essential for differentiation and activation of osteoclast precursor cells to active osteoclasts.¹³ Accordingly, RANKL expression in ameloblastoma might reflect the bone dynamic process associated with tumour-induced bone remodeling.^{13,14} Bone resorbing factors such as IL-1, IL-6, IL-8, IL-11 and IL-17 have been proposed as factors influencing RANKL upregulation.¹⁵

IL-1 α is known for its role as potent modulator in bone resorption by inducing the activation of osteoclast-like cells and production of matrix metalloproteinase enzymes.^{10,11} IL-1 α is proposed to stimulate osteoclastogenesis via upregulation of RANKL.¹¹ Steeve *et al.* (2004) and Sathi *et al.* (2008) described stromal cell as an essential element in osteoclastogenesis as OPG and RANKL are synthesised by stromal cells.^{5,16} Meanwhile, da Silva *et al.* (2008) reported elevated RANKL expression in stromal cells and higher ratio of RANKL to OPG in SMA as compared to UA.¹⁷ As such, high expression of IL-1 α in ST cells of ameloblastoma is projected with greater distinction expected in SMA, a finding which was reflected in our results.

In the present study, IL-1 α was identified in the three localizations (PA-like cells, SR-like cells and ST cells) of all the UA and SMA cases. Generally, IL-1 α expression was significantly higher in SMA, distributed more in the SR-like cells, which concurs with previous reports.^{10,11} Within UA however, IL-1 α was more frequently detected in the PA-like cells suggesting that these PA-like cell-bound factors play a key role in stimulating anti-tumour immunity. This could be extrapolated from the biological behaviour of UA which demonstrates lesser aggressiveness compared to SMA. An alternative explanation would be IL-1 α in PA-like cells might be less potent in inducing osteoclastogenesis. On the same note, IL-1 α in SR-like cells of SMA is likely to exert greater potency in inducing osteoclastogenesis. In fact, the overall higher expression level of IL-1 α in SMA compared to UA implies positive association with biological behaviour of SMA. With expression of IL-1 α present in both the epithelial and stromal cells, it is likely that these cells interact reciprocally via IL-1 α to create a microenvironment conducive for growth of ameloblastoma. IL-1 α precursor (pro-IL-1 α) is mainly found intracellularly and fully active as precursor.^{18,19} In our study, most of the IL-1 α expressions were seen localized intracellularly and membrane-bound IL-1 α was less frequently encountered.

Unlike IL-1 α which is constitutive in many cell types, IL-1 β is mainly induced in response to viral and microbial molecules.²⁰ IL-1 β reportedly could promote bone resorption and stimulate IL-6 production in osteoblast cells.²¹ Our study showed marked underexpression of IL-1 β in UA and SMA subsets. Mild staining intensity was similarly observed not only in the SR-like cells, but also PA-like cells and ST cells. Expression of IL-1 β in SMA was noticeably lower than in UA, especially within PA-like cells. It is unclear whether underexpression of IL-1 β is significant due to a lack of previous studies for comparison. However, our results led us to conclude that IL-1 β plays little role in inducing osteoclastogenesis in the tumoral microenvironment of ameloblastoma. This correlates well with the fact that IL-1 β is mainly expressed in response to microbial invasion but ameloblastoma is usually devoid of inflammatory infiltration unless secondarily infected.^{2,20} This explanation is further supplemented by Hoenig *et al.* (1991) who demonstrated high levels of IL-1 β in radicular cysts but absence of this interleukin in healthy control subjects.²²

IL-6 has been recognised as a key osteoresorptive factor that stimulates osteoclast differentiation and initiates osteoclastogenesis.^{23,24,25} Study by Steeve *et al.* (2004) further documented that IL-6 not only leads to stimulation of RANKL production but also acts synergistically with RANKL.¹⁶ Unlike previously reported¹⁰, we observed no intense staining of IL-6 in SR-like cells of UA and SMA. Our results showed positive immunoreactivity of IL-6 more often within PA-like cells in both subsets. IL-6 was more frequently detected in PA-like cells of UA compared to SMA suggesting that PA-like cell bound IL-6 also plays a key role in stimulating anti-tumour immunity in UA. The immunostaining of IL-6 within ST cells in UA seemed similar to findings from Sathi *et al.* (2008) with more than half of the cases for UA showing only mild expression for IL-6.⁵ There is however higher positive staining localized in ST cells of SMA. IL-6 has been acknowledged as one of the most important pro-tumourigenic cytokines by inducing “tumour-elicited inflammation” through IL-6 expression in tumoral microenvironment.²⁶ Hence, strong localization of IL-6 expression in the ST cells of SMA suggests that these secretable factors in the stromal compartment activate inflammation which in turn promote invasion. Besides that, IL-6 expression levels in the epithelial (PA-like and SR-like) cells and ST cells in SMA were notably higher than UA. This corresponds to the more aggressive behaviour of SMA in inducing higher bony resorptive activity. Interestingly, while IL-6 was more frequently detected in epithelial cells, higher immunoreactivity intensity was observed in ST cells. However, presence of IL-6 in both epithelial and stromal components again suggests reciprocal activity in tumoral microenvironment.

IL-8 is one of the α -chemokine family of cytokine produced by a variety of cell types in response to inflammation and injury.²⁷ IL-8 also reportedly, increases the sensitivity of tumour cells to stromal-derived chemokines.²⁸ Fuchigami *et al.* (2014) suggested that ameloblastoma epithelial cells and stromal cells interact reciprocally via IL-8.⁶ However, our study revealed predominantly negative staining for IL-8 in UA cases within PA-like, SR-like and ST cells. A higher immunonegativity was observed in SMA cases with ST cells demonstrating absolute immunonegativity to IL-8. These observations led us to hypothesize that IL-8 plays little role in inducing osteoclastogenesis in the

tumoral microenvironment of ameloblastoma.

In conclusion, the presence of IL-1 α and IL-6 in both epithelial and stromal components suggest that IL-1 α and IL-6 activate these cells reciprocally in both autocrine and paracrine manners to create a bone tumoral microenvironment favourable to growth and invasion of ameloblastoma. IL-1 α in stellate reticulum cells and IL-6 in stromal cells are two key secretable factors inducing osteoclastogenesis in SMA. Further research on the molecular aspect of interleukins would provide the basis for new therapeutic strategies for the treatment of ameloblastoma.

Acknowledgements

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