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ORIGINAL ARTICLE

Immunoexpression of BRAF, EGFR and CD10 in ameloblastoma

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Abstract

The ameloblastoma is the most challenging odontogenic neoplasm to treat because of its locally-invasive behaviour, severe clinical implication, risk of malignant transformation and high recurrence rate. Recent evidence suggests that BRAF, EGFR and CD10 have a role in the local invasiveness of ameloblastoma. However, the spatial distribution characteristics of these pro-invasive factors and their association with clinical parameters in this neoplasm are largely unexplored. We sought to address these issues in ameloblastoma subtypes and to determine their biological relevance. Nineteen unicystic (UA) and 20 conventional ameloblastoma (SMA) were subjected to immunohistochemical staining for BRAF, EGFR and CD10, and semiquantitative analysis was performed. All ameloblastoma cases (n=39/39; 100%) exhibited a $BRAF^+/EGFR^+/CD10^+$immunoprofile. Their expression rates were significantly higher in SMA than UA ($P<0.05$). BRAF, essential for the progression and proliferation of ameloblastoma, was detected mainly in the cytoplasm of stellate reticulum-like>stromal>pre-ameloblast-like cells ($P<0.05$). EGFR, a potent oncogenic protein, showed predominantly nuclear localisation. CD10, an apoptosis-inhibitory factor, was strongly expressed in the membrane of stellate reticulum-like cells. Taken together, present results suggest that the spatial distribution patterns of BRAF, EGFR and CD10 parallel the specific behaviours of SMA and UA. Their cellular and intracellular protein localisations have important targeted therapy implications.

Keywords: BRAF; EGFR; CD10; ameloblastoma; tumour invasiveness.

INTRODUCTION

The ameloblastoma is a slow-growing, benign but locally-invasive odontogenic epithelial neoplasm which accounts for 1% of all oral tumours.[1] Of dental lamina origin, the ameloblastoma is generally asymptomatic until it becomes large. In 2017, ameloblastoma was reclassified by the WHO classification of head and neck tumours, following the updates from the genetic studies. It is broadly grouped into conventional ameloblastoma (SMA), unicystic ameloblastoma (UA), peripheral/extraosseous ameloblastoma and metastasising ameloblastoma.[2] Treatment of ameloblastoma can be conservative treatment or surgical resection, the formal always related to high recurrent while the latter frequently results in facial disfigurement and high morbidity. The reported recurrence rates after resection vary from 0% to 25%, with SMA being higher.[3]

The most controversial behaviour of ameloblastoma is its invasiveness into the surrounding bone, despite its benign nature. Recent evidence suggests that BRAF, EGFR and CD10 have a role in the local invasiveness of ameloblastoma.[4-8] BRAF, a mitogen-activated protein kinase (Ras/MAPK) pathway intermediate and a potent activator of MEK in numerous human cancers,[4] modulates the progression and proliferation of ameloblastoma.[5]

Multiple studies identified mutated BRAF V600E as an important factor in the aetiopathogenesis of ameloblastoma by either genomic or immunohistochemistry analysis. They have found the incidence of BRAF V600E mutation ranged from 33.3% to 82%.[9-12] Interestingly, Brown *et al*. with the 100% agreement on the comparison of efficacy and sensitivity between molecular study (PCR) and immunohistochemistry study in the detection of BRAF mutation. This has demonstrated that using immunohistochemistry analysis alone in this kind of study is valid.[10] The predominant of

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BRAF mutation protein was found to involve mainly in the mandibular ameloblastoma particularly posterior region (from body to condyle of mandible).[13] These findings would proposed a different aetiopathogenesis of maxilla and mandible ameloblastoma which might require different targeted therapy regime.

EGFR, a cell surface transmembrane receptor kinase,[5] may have a role in its locally-invasive behaviour.[7-8] EGFR overexpression in ameloblastoma is related to its phosphorylation increases the matrix metalloproteinase secretion in the mitogen-activated protein kinase/ extracellular signal-regulated kinases pathyway (MAPK/ERK) which induces cell proliferation, differentiation, migration, invasion angiogenesis and apoptosis inhibition. These activities are contributing to the tumour's aggressive behaviour in ameloblastoma. The reported immunopositivity of EGFR in ameloblastoma ranged from 30% to 100% at different localisation of antibody staining, either membrane, cytoplasm, nucleus or in combination.[6,8,14] The different localisation of EGFR expression affect the effectiveness of anti-EGFR therapy as recommended.[15]

CD10, a 90-110kd cell surface zinc-dependent metallo-endoprotease glycoprotein with endopeptidase activities, augments growth in recurrent ameloblastoma.[16] CD10 is not specific to haematopoietic malignancies but it is also expressed by normal cells as in foetal liver, bone marrow, spleen and brain and other solid tumours as detected in renal cell carcinoma, transitional cell carcinoma, prostatic adenocarcinoma, pancreatic adenocarcinoma, malignant melanoma and endometrial stromal sarcoma.[16-17] Controversial results have been reported in bladder carcinomas, which CD10 down-regulation in progressive tumours or inversely upregulation associated with invasion and metastasis.[17] The immunopositivity of CD10 in ameloblastoma has been reported as high as 60-70%.[16,18]

Although BRAF, EGFR and CD10 have been previously evaluated, their relative contributory roles in ameloblastoma local invasiveness remain unclear. As immunohistochemical analysis with anti-BRAF, anti-EGFR and anti-CD10 are useful as surrogate markers to evaluate the respective mutation status of ameloblastomas, we sought to clarify their relationship by investigating immunohistochemically their expressions and localisations in SMA and UA. The rationale was to determine whether the spatial distribution patterns of these pro-invasive factors parallel their biological behaviours.

MATERIALS AND METHODS

Samples

Formalin-fixed paraffin-embedded archival tissues of 39 ameloblastoma cases (20 SMA and 19 UA) were retrieved following the histological criterial established by WHO Histological Classification of Odontogenic Tumour 2017 for SMA and UA which with sufficient tissue representative of the ameloblastoma. Samples with evidence of malignant transformation, insufficient representative tissue and from the same patient were excluded. New haematoxylin and eosin-stained four-μm-thick sections of these specimens were reviewed and selected by a qualified pathologist (CHS) according to established criteria.[19] Patients' characteristics were recorded. The follow-up data on treatment outcome was inadequate, hence, this clinical parameter was excluded from analysis. This study was approved by the Medical Ethics Committee, Faculty of Dentistry, University of Malaya ethics committee [Ethics DF OS1502/0011 (P)].

Immunohistochemistry

Immunohistochemistry was performed on new four-μm-thick sections. Antigen retrieval was accomplished by pressure cooker treatment of deparaffinised sections in 10 nM of citrate buffer (pH 6, 15 min). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide for 20 min, and sections rinsed in 0.05 M phosphate buffered saline (PBS) (5 min, two times) before immersing in blocking solution (Dako Corporation, Carpinteria, CA, USA) for 20 min at room temperature. After rinsing in PBS at pH7.4, tissue sections were incubated with optimally diluted primary antibodies: anti-BRAF (EP152Y, dilution 1:50; Abcam Inc, Cambridge, MA, USA) at room temperature for 2 h, overnight at 4°C for both anti-EGFR (EP774Y, dilution 1:250; Abcam) and anti-CD10 (56C6, dilution 1:25; Abcam). Immunoreactions were performed using the Envision Kit (Dako). Colorimetric development and contrast were achieved using 3,3'-diaminobenzidine-tetrahydrochloride (DAB) (Dako) and Mayer's haematoxylin. Known positive sections of breast carcinoma for BRAF, EGFR and CD10 were used as positive controls. For negative control, sections were treated as above but without the primary antibody. All control sections were negative.

Immunohistochemical assessment

Immunoreactivity was independently scored by two authors (CCT and CHS). Any disagreement

was reviewed together to achieve a consensus score. Strong agreement achieved within intra-observer and inter-observer in immunoreactivity staining scoring with Kappa value 0.85 and 0.90 respectively. The positive staining proportion was scored with modification of criteria given by Barnes *et al*.[20] Four representative fields of interest from the tumour centre and another four from the advancing front of both variants were examined at x400 magnification with a microscope (BX51, Olympus, Japan). Each case was rated according to a score based on the intensity and percentage of immunoreactive cells present: (-) negative when none of these cells were positively stained; (+) mild when staining was present in focal areas (<25%); (++) moderate when staining was evident in significant areas (25-50%); and (+++) strong when staining was present in predominant areas of the tumour (>50%). Immunoreactive cells were categorised as pre-ameloblast-like (PA-like), stellate reticulum-like (SR-like) and stromal (ST-like) cells.

Statistical analysis

All data are presented as mean values ± standard deviations (SD). Chi-square test or Fisher exact was used to analyse expression rates of each marker in ameloblastoma subtypes, and its association with clinicopathologic parameters. Friedman, Wilcoxon Signed-Ranks and Mann-Whitney U tests were used to evaluate expression differences in mean ranks among and between variables. Statistical analyses were performed using IBM SPSS version 20. A *P*-value of <0.05 was considered significant.

RESULTS

Patients' characteristics

Details of cohort are summarised in Tables 1 and 2. Mean onset ages (range) in SMA and UA patients were 38.8±12.74 (15-67) and 22.9±12.75 (8-58) years respectively. The most common presenting complaint was a painless swelling (SMA=65%; UA=78.9%). The histological subtypes for ameloblastoma were plexiform (n=8), follicular (n=4), granular (n=3), basaloid (n=2), acanthomatous (n=1) and mixed (n=2); for unicystic ameloblastoma were mural (n=7), luminal (n=2), intraluminal (n=1) and mixed (n=9). Known treatments were enucleation (SMA=20%; UA=10.5%) and segmental resection (SMA=35%; UA=21.1%). Their outcomes and follow-up records were generally unavailable. Statistical analyses showed significant association between EGFR and ethnicity in SMA, BRAF and radiological features in UA ($P< 0.05$).

Immunohistochemical findings

These are summarised in Tables 1-4 and illustrated in Figs. 1A-I and 2A-F. All ameloblastoma cases (n=39/39; 100%) heterogeneously expressed BRAF, EGFR and CD10. Their protein localisation was predominantly cytoplasmic>membranous, and in addition, nuclear for EGFR (Fig.1D-I). BRAF and CD10 were more frequently detected in SR-like than PA-like cells (Figs. 2D, F). A significantly higher strong intensity of BRAF was found within SR-like cells in SMA with mainly in the cytoplasmic region and some at the nucleus compared to mild-moderate staining in the majority of the PA-like cells and stromal cells in UA cases. Expression of EGFR was markedly strong in both SR-like cells and stromal cells in both SMA and UA. The strong immunoreactivity of EGFR was equally distributed within the cytoplasmic and nucleus regions. EGFR expression in SMA was higher than UA for all three cell types. All PA-like cells show negative immunoreactivity in the expression of CD10 marker in both subsets of ameloblastoma. None of them show strong CD10 immunoreactivity in stromal cells. The majority of the intense immunoreactivity of CD10 is observed at SR-like cells in SMA (80.0%) and UA (42.1%). Exclusive membranous staining was seen at SR-like cells with focal cytoplasmic staining at stromal cells. Stromal cells stained variably positive.

Statistical analyses demonstrated significant differences in mean expression scores according to cellular types within and between SMA and UA (Tables 3 and 4) (Figs.1A, 2A-C) ($P< 0.05$).

DISCUSSION

Although molecular targeted therapy[11, 21-25] has revolutionised treatment for advanced-stage ameloblastoma, the locally-invasive growth and recurrence tendency of this neoplasm are major challenges hindering their effective management. In order to provide a basis for clinical therapeutic decisions, we explored whether there is any potential association between the expression levels of BRAF, EGFR and CD10 with the clinicopathologic variables of patients with ameloblastoma. Two key findings were made. Significant BRAF, EGFR and CD10 upregulation in SMA compared to UA, and significant overexpression of these factors

TABLE 1: Associations between BRAF, EGFR and CD10 expression levels with clinicopathologic parameters in twenty patients with conventional ameloblastoma (SMA)

Variables	n=20	BRAF				*P*-value	EGFR				*P*-value	CD10				*P*-value
		-	+	++	+++		-	+	++	+++		-	+	++	+++	
Age (yr)*						0.621					1.000					1.000
≤38	11		0	1	6	4		0	0	1	10		0	7	4	0	
>38	9		0	0	6	3		0	0	0	9		0	6	3	0	
Gender						0.056					1.000					1.000
Male	11		0	1	4	6		0	0	1	10		0	7	4	0	
Female	9		0	0	8	1		0	0	0	9		0	6	3	0	
Ethnicity						0.806					0.000					0.640
Malays	8		0	1	5	2		0	0	0	8		0	4	4	0	
Chinese	8		0	0	4	4		0	0	0	8		0	6	2	0	
Indian	3		0	0	2	1		0	0	0	3		0	2	1	0	
Others	1		0	0	1	0		0	0	1	0		0	1	0	0	
Site						0.748					0.205					0.587
Maxilla	4		0	0	3	1		0	0	1	1		0	1	1	0	
Mandible	16		0	1	9	6		0	0	1	15		0	15	1	0	
Radiology						0.610					0.532					0.443
ULRL	2		0	0	1	1		0	0	0	2		0	2	0	0	
MLRL	5		0	0	2	3		0	0	1	4		0	2	3	0	
RL NOS	2		0	0	2	0		0	0	0	2		0	2	0	0	
RR	2		0	0	1	1		0	0	0	2		0	1	1	0	
NA	9		0	1	6	2		0	0	0	9		0	6	3	0	

*Median age 38 years in SMA was used as cut-off point; ULRL, unilocular radiolucency; MLRL, multilocular radiolucency; RL NOS, radiolucency not otherwise specified; RR, root resorption; NA, not available.

Chi-square and Fisher Exact tests were performed. Bold values indicate statistical significance (P <0.05).

TABLE 2: Associations between BRAF, EGFR and CD10 expression levels with clinicopathologic parameters in nineteen patients with unicystic ameloblastoma (UA)

Variables	n=20	BRAF				*P*-value	EGFR				*P*-value	CD10				*P*-value
		-	+	++	+++		-	+	++	+++		-	+	++	+++	
Age (yr)*						1.000					0.211					0.211
≤16	10		0	3	7	0		0	0	0	10		0	7	3	0
>16	9		0	2	7	0		0	0	0	9		0	0	9	0
Gender						0.141					1.000					0.582
Male	10		0	1	9	0		0	0	1	9		0	9	2	0
Female	9		0	4	5	0		0	0	1	8		0	7	1	0
Ethnicity						0.374					0.478					0.550
Malays	7		0	1	6	0		0	0	0	7		0	6	1	0
Chinese	9		0	4	5	0		0	0	2	7		0	8	1	0
Indian	1		0	0	1	0		0	0	0	1		0	1	0	0
Others	2		0	0	2	0		0	0	0	2		0	1	1	0
Site						1.000					0.205					0.298
Maxilla	2		0	0	2	0		0	0	1	1		0	1	1	0
Mandible	17		0	5	12	0		0	0	1	16		0	15	2	0
Radiology						0.015					0.637					0.363
ULRL	4		0	0	4	0		0	0	1	3		0	3	1	0
MLRL	2		0	1	1	0		0	0	0	2		0	2	0	0
RL NOS	5		0	4	1	0		0	0	1	4		0	3	2	0
RR	1		0	0	1	0		0	0	0	2		0	1	0	0
NA	7		0	0	7	0		0	0	0	7		0	7	0	0

*Median age 16 years in UA was used as cut-off point; ULRL, unilocular radiolucency; MLRL, multilocular radiolucency; RL NOS, radiolucency not otherwise specified; RR, root resorption; NA, not available.

Chi-square and Fisher Exact tests were performed. Bold values indicate statistical significance (P <0.05).

TABLE 3: Comparison of BRAF, EGFR and CD10 mean expressions in different cellular types

Markers	Cellular types	SMA (n=20)			UA (n=19)		
		Mean (SD)	χ^2 (df)	*P*-values[a]	Mean (SD)	χ^2 (df)	*P*-values[a]
BRAF	PA cells	1.75 (0.44)	21.90 (2)	**0.000**	1.47 (0.51)	20.04 (2)	**0.000**
	SR cells	2.70 (0.57)			2.26 (0.73)		
	ST cells	2.10 (0.78)			1.58 (0.50)		
EGFR	PA cells	1.95 (0.22)	38.10 (2)	**0.000**	1.89 (0.31)	33.65 (2)	**0.000**
	SR cells	3.00 (0.00)			2.95 (0.22)		
	ST cells	2.95 (0.22)			2.84 (0.50)		
CD10	PA cells	0.00 (0.00)	37.28 (2)	**0.000**	0.00 (0.00)	28.67 (2)	0.000
	SR cells	2.70 (0.65)			2.05 (0.97)		
	ST cells	0.40 (0.59)			0.58 (0.69)		

SMA, conventional ameloblastoma; UA, unicystic ameloblastoma; SD, standard deviation; PA, pre-ameloblast; SR, stellate reticulum; ST, stromal. [a]Friedman test was performed. Bold values indicate statistical significance ($P< 0.05$).

within the epithelial compartment compared to their stroma, suggest that these cytokines are fundamentally related to the locally-infiltrative behaviour of ameloblastoma. Of clinical relevance is that identification of these factors as potential biomarkers independently correlated with tumour aggressiveness is invaluable for better therapeutic strategies.[11, 21-25] On the other hand, the significance of BRAF overexpression among male SMA patients, EGFR in Malay SMA patients, and BRAF with radiological presentation in UA patients, remains unclear.

BRAF gene mutation has been found in a variety of human benign and malignant neoplasms. All ameloblastoma samples in this study were BRAF-positive, similar to a previous report[23] while the other studies reported the BRAF gene alteration ranging from 33.3% to 82%.[5,9-12] We found that BRAF mutation has no association with tumour location coincide with Fregnani *et al*. and Diniz *et al*. even though Brown *et al*. and Sweeney *et al*. reported a predilection to mandible ameloblastoma.[10-12, 22] Kurppa *et al*. and Fregnani *et al*. demonstrated discrete predominant of positive BRAF immunoexpression in woman, however, there was no statistical difference found in our study similar to the study of do Canto *et al*.[5,13,22] The BRAF mutation expression was significantly higher in SR-like and stromal cells in SMA ($P<0.05$) suggesting that oncogenic mutation might be initiated in these cells.[10,22] It also indicates its role in evading apoptosis and increasing the survival rate of oncogenic cells within ameloblastoma via paracrine and autocrine manners. González-González R *et al*. and Heikinheimo *et al*. found no proportional difference of BRAF mutation in luminal/intraluminal/mural of UA cases similar in our study.[26-27] This may indicate that BRAF mutation might not entirely related to the tumour behaviour. These findings are important in the eligibility considerations for ameloblastoma patients to undergo BRAF-targeted therapy as an alternative treatment.[11,17,25]

EGFR is an important oncogenic factor in many cancer types including head and neck tumours.[28] In this study, EGFR was widely expressed in ameloblastoma samples, a finding that corroborated with some previous studies but differed from others.[8,29-31] The reasons for these contradictory results are unclear. Differences in sources of antibodies used, methodologies for tissue processing/immunohistochemistry and scoring criteria may contribute to these discrepancies.[8,32] More importantly, EGFR

Table 4: Comparison of BRAF, EGFR and CD10 expressions between conventional ameloblastoma (SMA) and unicystic ameloblastoma (UA)

Variables	N	BRAF		EGFR		CD10	
PA-like cells		Mean rank	*P*-value	Mean rank	*P*-value	Mean rank	*P*- value
SMA	20	22.63	**0.083**	20.53	0.532	20.00	1.000
UA	19	17.24		19.45		20.00	
SR-like cells							
SMA	20	23.23	**0.038**	20.50	0.305	23.70	**0.017**
UA	19	16.61		19.47		16.11	
ST-like cells							
SMA	20	23.55	**0.030**	20.55	0.504	18.70	0.401
UA	19	16.26		19.42		21.37	

n, number of cases; PA, pre-ameloblast; SR, stellate reticulum; ST, stromal.
Mann-Whitney U test was performed. Bold values indicate statistical significance (P<0.05).

was significantly higher in SMA than UA (*P*<0.05). Ameloblastoma with activated EGFR pathway may involve in the epithelial-to-mesenchymal transition (EMT) which causes tumour development and progression. Vered *et al*. reported 100% of ameloblastoma are overexpressed with EGFR.[8] Nevertheless, mutation and gene amplification of EGFR are rare in ameloblastoma.[33] Emerging evidence suggests that high levels of EGFR are associated with

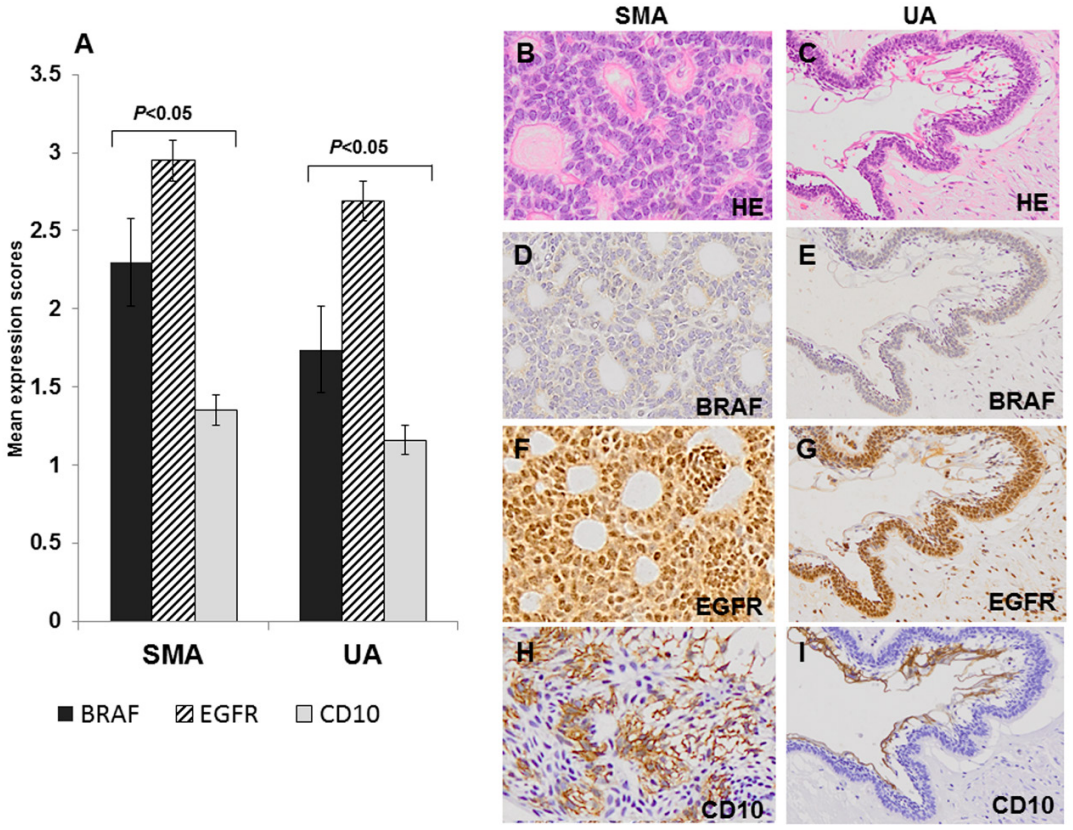

FIG. 1: (A) Comparison of immunoreactivity for BRAF, EGFR and CD10 in conventional ameloblastoma (SMA) and unicystic ameloblastoma (UA) (*P*<0.05). Representative photomicrographs of SMA and UA demonstrating (B, C) haematoxylin and eosin (HE) staining, and differential expression of (D, E) BRAF, (F, G) EGFR and (H, I) CD10 (magnification C,E,G, I x100; B,D,F,H x200).

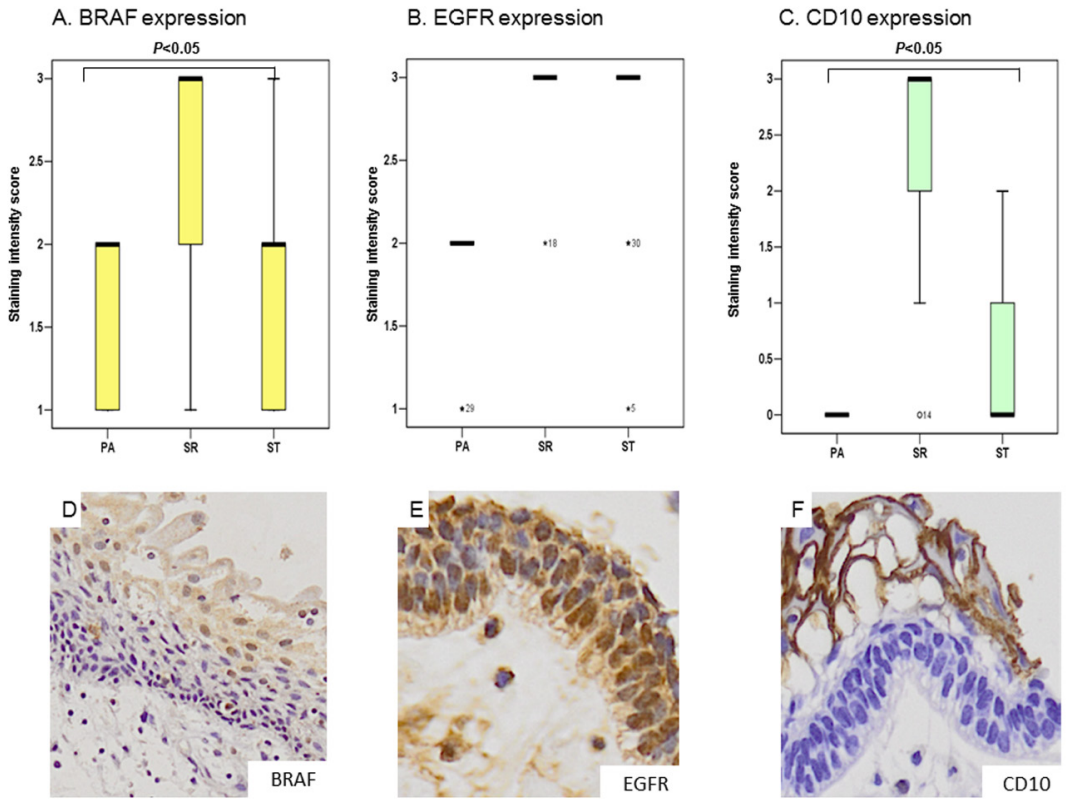

FIG. 2: (A-C) Box plots show data distributed in relation to median values for BRAF, EGFR and CD10 expressions in 39 ameloblastoma stratified by tumoural (PA, pre-ameloblast-like, SR, stellate reticulum-like) and stromal (ST) cell types (P<0.05). Representative photomicrographs demonstrating immunolocalisation of (D) BRAF, (E) EGFR and (F) CD10 in these cellular types (magnification Dx200; E, F x400).

increased activity of protein phosphorylation cascade, proto-oncogene expression and proliferation. The observed preferential EGFR staining in SR-like cells of SMA concurred with the study by Ueno *et al*.[30] The clinical relevance is that SR-like cells bound EGFR factors stimulate tumoral invasion activities in an autocrine mode which participate in tumour proliferation, migration and invasion. In contrast, Li *et al*.[34] found higher expression of EGFR protein in peripheral layer of ameloblastoma which suggestive of the cells' proliferative nature was decreasing from the peripheral to the central layer of ameloblastoma. Interestingly, EGFR protein was detected in both the cytoplasm and nucleus. Previous studies on ameloblastoma, breast and oropharyngeal cancers found that nuclear EGFR correlated with high proliferation and poorer survival.[15,35] Nuclear protein localisation and low membranous immunostaining of EGFR raises a concern about anti-EGFR treatment resistance in ameloblastoma.[15]

CD10 is widely distributed in a variety of normal and abnormal tissues.[17] Aberrant CD10 is associated with apoptosis, differentiation, proliferation and invasion. Recent studies demonstrated intense CD10 stromal staining in ameloblastoma correlated with high local tumour recurrence and invasion.[6,8,36] The slightly higher CD10 expression in stromal of UA than SMA in our study might be explained by the fact that most of our UA samples were mural variant in which aggressiveness behaviour is similar to SMA. In contrast, the present study showed distinct CD10 membranous expression in SR-like cells, similar with an earlier report.[36] Accordingly, membranous CD10 expression in SR-like cells is frequently augmented in intra luminal UA while membranous and cytoplasmic CD10 immunoreactivity is overexpressed in mural UA.[36] Significant higher immunoexpression of CD10 in SMA than UA may explain the aggressive clinic-behaviour and higher recurrence after surgery of SMA in comparison to UA. Mounting evidence suggests that strong membranous CD10 staining pattern is associated with poorly-differentiated carcinomas and decreased survival.[37] The lack

of immunoreactivity for CD10 in PA-like cells was consistently observed in all our tumour samples. CD10 deregulation in early common progenitor cells could block the PTEN function of apoptosis and induced cell proliferation by activation of Akt pathway.[38] Alteration of CD10 expression seems to be a consequence of the initial tumour transformation. Thus, we surmised that the negative immunoreactivity for CD10 in PA-like cells observed in others[36-37] and our study suggested that the tumourigenesis could possibly initiated in PA-like cell of ameloblastoma which modulated the neighbouring cellular environment causing tumour transformation.

In conclusion, although the local aggressiveness and recurrence potential of ameloblastoma are widely-acknowledged, to date the molecular basis for this behaviour remains poorly understood. Present findings suggest that the spatial distribution patterns of BRAF, EGFR and CD10 parallel the specific behaviours of SMA and UA. The cellular and intracellular protein localisation of these factors have important targeted therapy implications. Finally, to assist in predicting the efficacy of the targeted chemotherapeutic agent, all ameloblastoma tumours should be properly evaluated and triaged based on immunophenotypic changes.

The limitation of this study was the inadequacy of the extensive clinicopathological data to determine the prognostic value of these mutations in long-term follow-up. Future works should be extended to determine these characteristics in the histological variants of ameloblastoma to better understand the role of these markers in the tumourigenesis of ameloblastoma. Larger sample size in multicentric studies with prospective follow-up records is required to validate the therapeutic and predictive value in these markers.

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