Original Article

Expression of EGFR and survivin in ameloblastoma, odontogenic keratocyst and calcifying odontogenic cyst – An immunohistochemical study

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Abstract

Context: Odontogenic lesions have diverse biological behaviour which is characterised by local invasiveness, and a high recurrence rate. EGFR and survivin was found to be involved in the aggressiveness, recurrences and metastasis of a variety of epithelial malignancies.

Aims: To assess and compare the expression of EGFR and survivin in Ameloblastoma (AB), Odontogenic keratocyst (OKC) and Calcifying odontogenic cyst (COC).

Settings and Design: The study's goal was to use immunohistochemistry to assess the qualitative and quantitative expression of EGFR and survivin and to correlate their expression patterns in AB, OKC and COC.

Methods and Material: Study included 30 AB, 15 OKC and 10 COC. All the slides were immunohistochemically analysed for qualitative, quantitative and semi-quantitative data. In each group, the presence of EGFR and survivin was assessed in terms of stain localisation, intensity and percentage of positive cells.

Statistical Analysis Used: Data were analysed using Chi-square test and one-way ANOVA, P value < 0.05 was considered statistically significant.

Results: EGFR positivity was found in all cases. Survivin was found to be 96% positive in AB and 100% positive in OKC and COC. Both EGFR and survivin showed predominant cytoplasmic staining. All the slides that are stained with EGFR are also stained with survivin. The intensity varied significantly between the layers. OKC showed higher immunoreactive scores (IRSs).

Conclusions: The current study provides insight into the role of EGFR and survivin in the pathogenesis of AB, OKC and COC. OKC appears to be more aggressive than ameloblastoma and COC, owing to its higher IRS.

Keywords: Ameloblastoma, Odontogenic keratocyst, Calcifying odontogenic cyst, EGFR, Immunohistochemistry (IHC), Immunoreactive score (IRS), Survivin

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INTRODUCTION

Odontogenic cysts and tumours that originate from primordial tooth forming tissue and/or its remnants have diverse clinical, biological, and histological behaviour. Whilst most of the odontogenic lesions are benign, a few exhibit locally invasive behaviour, and a high recurrence rate such as ameloblastoma.[1] However, lesions originally identified as benign odontogenic cysts [odontogenic keratocyst (OKC), especially parakeratinised variant, and calcifying odontogenic cysts (COC)], are considered neoplasms, according to the 2005 World Health Organisation (WHO) classification of odontogenic tumours. Further, these entities were renamed keratocystic odontogenic tumour (KCOT) and calcifying cystic odontogenic tumour (CCOT), respectively, by their aggressive behaviour, and molecular similarity to a wide range of odontogenic neoplasms. However, disagreements about the existence, nomenclature, and diagnosis of various entities persisted. Following much debate, the WHO published the fourth edition of a simplified version of classification in 2017, in which KCOT and CCOT are currently classified as OKC and COC, respectively.[2]

Growth factors and their receptors are important in the development and progression of neoplasms as well as the growth of normal tissues. The Epidermal Growth Factor Receptor (EGFR) is a tyrosine kinase cell surface receptor, which sends signals to regulate many important processes, such as cellular growth, proliferation and differentiation. It is commonly mutated and/or overexpressed in various types of epithelial malignancies.[3,4] Antiapoptotic factors, on the other hand, are important in the regulation of apoptosis and the control of cell division. Survivin is an apoptotic regulator and a unique member of the inhibitor of apoptosis protein (IAP) family. It is required for cell division, and it is normally only expressed in cells that are actively proliferating, but it is increased in the majority, if not all, malignancies. In cancer cells, it is considered that it can extend the life span of the cell, enabling gene mutations to accumulate and allowing growth factor-independent survival.[5-7]

In many cancer cells, the EGFR and survivin pathways are two distinct yet intertwined survival processes. These are two nodal proteins that cross many signalling networks and were separately observed to be involved in the aggressiveness, recurrence and metastasis of several epithelial malignancies.[8] Therefore, assessing these two markers combined in AB, OKC and COC could find similarities and differences between these lesions. OKC and COC are biologically aggressive odontogenic cysts, and the molecular mechanisms underlying their aggressiveness are not fully elucidated. Consequently, comparing the growth factors and apoptotic inhibitors in OKC and COC, to those of ameloblastoma, a well-researched lesion, could provide new insights into their molecular nature. Hence, the purpose of this research was to utilise immunohistochemistry (IHC) to evaluate and correlate the expression of EGFR and survivin in ameloblastoma, OKC and COC.

MATERIALS AND METHODS

The study included 55 retrospective cases (30 AB, 15 OKC and 10 COC) from oral pathology specimen archives. To confirm the diagnosis, archival H&E slides were examined. The slides were then stained. The details of the IHC procedure are shown in Table 1. Two blinded observers carefully examined the slides. Each slide was thoroughly reviewed on a visual monitor screen attached to an olympus BX51 research microscope with a digital camera DP71 until a consensus was reached. To assess the brown-coloured distribution of immunopositive cells, qualitative, quantitative and semi-quantitative approaches were used.

Tissues were examined in a scanner view (4X), and EGFR and survivin staining distribution were classified as

Table 1: Flowchart showing IHC procedure

On a positively charged slide, 3µ sections of tissue were taken.
↓
Dewaxing (2 charges of xylene, 10 min)
↓
Rehydration (alcohol, 100% – 5 min, 80% – 5 min)
↓
Rinse with deionized water for 2-3 min
↓
Antigen retrieval (trisodium citrate buffer for EGFR and Tris EDTA for survivin in microwave oven, 3 cycles, 5 min each at 800W)
↓
Peroxide block (10 min)
↓
Wash (3 times with PBS)
↓
Power block (10 min)
↓
Primary antibody incubation (30 min for EGFR and 1 hour for survivin)
↓
Super enhancer (20 min)
↓
Secondary antibody (poly HRP, 30 min)
↓
Wash (3 times with PBS)
↓
DAB chromogen (5 min)
↓
Wash (distilled water)
↓
H & E staining (Harris haematoxylin)

EDTA – Ethylenediaminetetraacetic acid; DAB – 3,3′- diaminobenzidine; HRP – Horseradish peroxidase; PBS – Phosphate buffer saline; H&E – Haematoxylin and eosin

focal (less than 50% of cells are positive) or diffuse (more than 50% of cells are positive). The staining intensity of immunopositive cells was compared to that of control tissue, which was poorly differentiated oral squamous cell carcinoma (PSCC), which served as an external positive control. Survivin's internal positive control is provided by inflammatory cells within the tissue.[9]

Five representative fields were selected at random for stain quantification and localisation at 40X high power magnification. The presence of EGFR was evaluated in terms of membrane, cytoplasm, or both (membrane and cytoplasmic). The presence of survivin was also classified as nuclear, cytoplasmic, or both (nuclear and cytoplasmic). In each case, the localisation and percentage of positive cells were assessed further between layers, such as peripheral and central cells in AB, basal and parabasal layers in OKC and COC. The semi-quantitative analysis was done using immunoreactive score (IRS).[9] Please see Table 2.

Data were analysed using Chi-square test and one-way ANOVA, P value < 0.05 was considered statistically significant. SPSS software was used to analyse the data.

RESULTS

All the cases are positive except one case of AB did not take up the stain with survivin [Figure 1]. The percentages of immunolabeled cells, the number of positive cases, the predominant cytoplasmic localisation, and the IRSs of EGFR and survivin samples are shown in Table 3. EGFR staining was seen in the membrane, cytoplasm or both, but it was mostly seen in the cytoplasm of all study groups. The intensity of EGFR staining differed significantly between AB (p-0.007), OKC (p-0.005) and COC layers (p-0.006). Survivin staining intensity in AB peripheral and central cells showed a statistically significant difference (p-0.03, Table 4). The IRS scores for EGFR revealed a statistically significant difference between the lesions (p-0.02, Table 3). A separate analysis of the intensely stained subpopulation of cells with survivin clearly showed a statistically significant difference (p-0.001, Table 5). Spearman's correlation coefficient revealed a significant direct correlation between EGFR and survivin.

Table 2: Immunoreactive score

A (% of positive cells)	B (Intensity of staining)	IRS interpretation IRS score=multiplication of A and B
0 - No stain 1 - <10% 2-10–50% 3-50–80% 4 - >80%	0 - No stain 1 - Mild 2 - Moderate 3 - Intense	0=Negative [score ranges between (0 and 1)] 1=Mild [score ranges between (2 and 3)] positive, but weak expression 2=Moderate [score ranges between (4 and 8)] positive but mild expression 3=Strongly positive [score ranges between (9 and 12)] positive and strong expression.

DISCUSSION

In the present study, all cases demonstrated EGFR positivity. Few earlier studies have found similar findings with EGFR in AB and OKC.[10-15] In addition, EGFR positivity was reported in one case of COC in a single study.[1] The immunohistochemical positivity of EGFR in all of these lesions suggests that EGFR plays an important role in the tumorigenesis of these lesions. Since the EGFR gene is frequently amplified and/or mutated in cancer cells, it is assumed to play a role in tumour development.

In this study, the intensity of EGFR staining was found to be higher in the periphery cells of AB than in the central cells, and stronger basal layer staining was seen in OKC and COC than in the parabasal layer. Due to the diverse activities of EGFR, there could be disparities in the strength of staining. The intense staining may possibly suggest a higher protein content, which can help cells to survive, proliferate or differentiate. EGFR expressing neoplasms demonstrate more aggressive pathological features, which may be attributable to the activation of various signalling pathways that control diverse biological processes.[16,17] Thus, it's considered that there's a correlation between EGFR and tumour progression, and that EGFR can enable neoplastic odontogenic epithelium in these lesions to proliferate.

The immunolocalisation of EGFR in these tumours may aid in the detection of proliferative activity, which might subsequently be used to predict biological behaviour.[11,14] Literature suggests that, when EGFR is expressed both on the membrane and in the cytoplasm of cells they proliferate at a normal physiologic rate. When EGFR is only found in the membrane, the cell's response to proliferative stimuli may be accelerated. While cytoplasmic localisation may account for the internalised/inactive receptor, it may also explain the slower response.[14,18,19] In this study, predominant cytoplasmic localisation was observed which reflect inactive cells that can proliferate in the presence of growth factors.

EGFR primarily activates two signalling pathways: the mitogen-activated protein kinase (MAPK) pathway and the phospho-inositol 3 kinase (PI-3 K) pathway. The MAPK is needed for cell proliferation, and the PI-3 K is required for the activation of anti-apoptotic molecules and prevent programmed cell death. Activated forms of EGFR can increase survivin protein levels via the PI-3

Table 3: Positive cases, pattern of staining, localization of stain and final scores of EGFR and survivin. M – Membrane, C – Cytoplasm, M+C – both membrane and cytoplasm

IHC	Study groups	No of positive cases	Pattern of stain	Stain localisation			IRS scores				*P*
			No of cases with diffuse staining	M (%)	C (%)	M+C (%)	Negative	Mild	Moderate	Strongly positive	
EGFR	AB	30/30	28/30	7.52	80.9	8.5	0	0	10	20	0.02
	OKC	15/15	15/15	5.17	84.7	11.38	0	0	0	15	
	COC	10/10	10/10	1.28	96.11	2.63	0	0	4	6	
Survivin	AB	29/30	24/30	73.26	0.30	22.53	1	4	25	0	0.32
	OKC	15/15	15/15	80.35	3.45	17.65	0	1	14	0	
	COC	10/10	10/10	93.14	0.1	6.75	0	4	6	0	

Table 4: Percentage of positive cells and intensity scores of EGFR and survivin

IHC	Study groups	Layers	Percentage of positive cells		Intensity scores			*P*
			Mean % of positive cells	Average %	weak	moderate	strong	
EGFR	AB	Peripheral	94.2±15.7	90.7±19.6	3	18	9	0.007
		Central	87.2±29.1		8	19	3	
	OKC	Basal	98.4±4.12	96.8±6.3	0	3	12	0.005
		Parabasal	95.3±10.01		0	12	3	
	COC	Basal	97.9±3.5	90.0±10.9	0	4	6	0.006
		Parabasal	82.04±22.03		4	6	0	
Survivin	AB	Peripheral	86.3±21.7	87.1±21.4	11	18	0	0.03
		Central	87.1±21.4		21	8	0	
	OKC	Basal	70.5±18.1	86.8±9.17	2	13	0	0.09
		Parabasal	86.86±9.17		6	9	0	
	COC	Basal	91.1±6.4	86.8±12.3	4	6	0	0.06
		Parabasal	86.8±12.3		8	2	0	

Table 5: IRS for intensely stained cells with survivin

Study groups	Negative	Mild	Moderate	Strongly positive
AB	15	15	0	0
OKC	0	5	10	0
COC	1	9	0	0
Chi-square test	Test value=40.57/*P*=0.000			

kinase pathway.[20,21] As a result, in the current study, all cases that tested positive for EGFR expression also tested for survivin positivity. EGFR and survivin positive cells in three groups were compared, and no significant difference was found, implying that OKC and COC have aggressive phenotypic behaviour similar to ameloblastoma. The cells in the superficial parakeratin layer did not express EGFR or survivin, indicating that they are terminally differentiated.[22]

Survivin showed positivity in all the study cases except one case of AB. However, the internal positive control took up the stain; negative staining might be due to the presence of very few proteins detectable by IHC. Previous studies have demonstrated survivin positivity in AB and OKC.[14,23-26] According to the literature, this is the first study to show Survivin expression in COC. Survivin had a predominance of weak to moderate staining in all three lesions. In AB, there was a significant difference in intensity between the central and peripheral cells. This difference in staining intensity might be due to differences in protein functional activity. The overexpression of anti-apoptotic proteins in the odontogenic epithelium is linked to cellular proliferation, differentiation and apoptotic inhibition, all of which influence the clinical behaviour of ameloblastomas.[27] Survivin immunostaining was primarily found in the cytoplasm, and there was a significant difference between the groups such as COC having the highest percentage of cells, followed by AB, and then OKC. Cell survival rates may play a role in the different histomorphogenesis of these tumours, such as ghost cell formations in COC and various histological variants in AB. Further, OKC had significantly lower cell survival rates, which could be seen histologically as thin epithelial lining.

Survivin over-expression has been associated with a more aggressive and invasive phenotype of oral squamous cell carcinoma in recent studies, and the existence of two distinct pools of survivin, nuclear and cytoplasmic, has already been identified.[28,29] The precise mechanisms of nuclear survivin localisation remain unknown but several studies have suggested that nuclear survivin is important in cell proliferation and poor survival rates.[6,30-32] Given the importance of nuclear survivin positivity, cells with intense nuclear staining were counted separately to assess how these positive cells are distributed across all three groups. The intensely stained cells are predominantly found in the parabasal layer of OKC. Significant differences were found between study groups, which might explain the disparity in the behaviour of these lesions.

A reliable immunoreactive scoring (IRS) system was used to compare the final scores of benign aggressive lesions

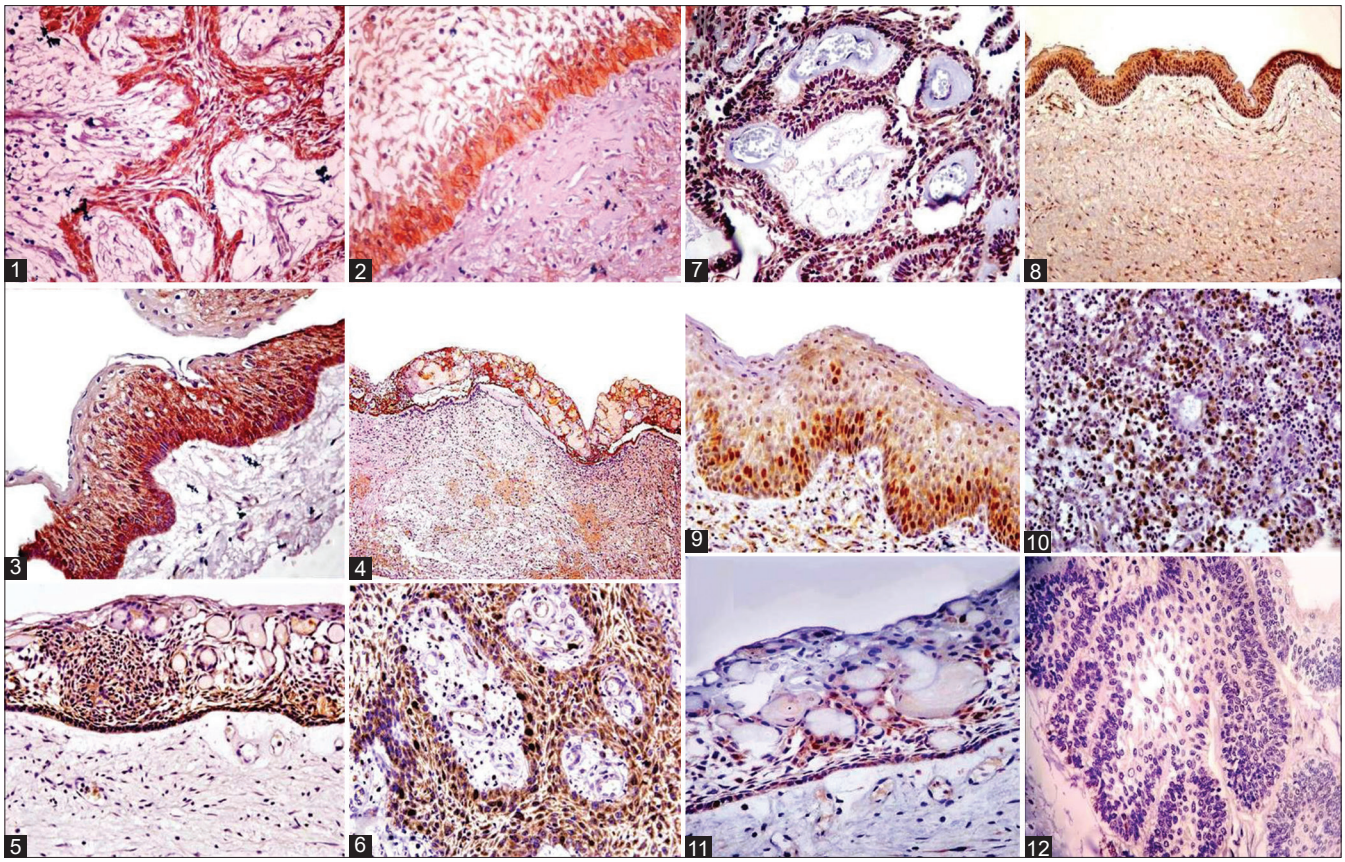

Figure 1: 1) EGFR expression localised in cytoplasm of ameloblastoma - 40X magnification. 2) EGFR expression localised in both cytoplasm and membrane in the basal layer of ameloblastoma (40X). 3) EGFR expression in OKC (40X). 4, 5) Diffuse EGFR expression in COC -10X, 20X magnification, respectively. 6) Nuclear expression of survivin in few cells but predominantly localised in cytoplasm of ameloblastoma (40X). 7) Survivin expression in ameloblastoma (40X). 8) Survivin expression in OKC (10X). 9) Intense survivin expression localised in nucleus of few basal and supra basal layers of OKC (40X). 10) Strong intensity of survivin expression in lymphocytes (internal control) (40X). 11) Survivin expression localised in cytoplasm of COC. 12) Mild of survivin expression in ameloblastoma (40X)

to identify any possible correlations.[9] The EGFR IRS classification scores suggest a significant increase in OKC, as opposed to AB and COC. IRS of survivin positions most of these lesions in the moderately positive category in terms of the aggressiveness of the lesion. Intensely stained subpopulation cells with survivin, on the other hand, revealed a highly significant difference between the study groups and OKC. Thus, only counting these intensely stained cells may provide new insights into determining the aggressive nature of neoplasms. A validated score such as IRS has made an important contribution to understanding the pathogenesis of OKC and has provided evidence that it is an aggressive benign cyst comparable to AB.

Finally, all the areas that are stained with EGFR are also stained with survivin. EGFR targeted therapy is effective in diminishing cellular proliferation in AB cell cultures and recently survivin inhibitors have been developed.[12] Therefore, it is apparent that these locally aggressive tumours are the candidates for anti-EGFR and anti survivin treatment modalities

CONCLUSION

The current study provides insight into the role of EGFR and survivin in the pathogenesis of AB, OKC and COC. According to the findings, OKC appears to be more aggressive than ameloblastoma and COC, due to its higher IRS scores. To the best of our knowledge, this study is the first of its kind, combining EGFR and survivin in oral lesions. As a result of the EGFR-survivin relationship, these proteins can be used to target these lesions. This could also be an option for patients who are not good candidates for surgery. These research findings could be instrumental when newer and more updated classifications are proposed. The limitation of the present study is small sample size and an apparent lack of opportunity to observe the biological behaviour of these lesions. More research is needed to validate our findings and investigate the relationship between EGFR and survivin.

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Conflicts of interest

There are no conflicts of interest.

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