COMPARATIVE IMMUNOHISTOCHEMICAL ANALYSIS OF K-RAS ONCOGEN AND CYCLOOXYGENASE2 ENZYME EXPRESSION IN PLEOMORPHIC ADENOMA AND ADENOID CYSTIC CARCINOMA OF SALIVARY GLANDS

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Abstract

Background: The salivary glands tumors constitute an important area in the field of oral and maxillofacial pathology. Although, such tumors are uncommon, thus they are not rare. Pleomorphic adenoma is the most common salivary benign tumor and from a histological point of view, it contains the epithelial cell, the myo-epithelial cell and mesenchymal ingredient. Adenoid cystic carcinoma is an infiltrative malignant salivary gland tumor with three different histological patterns: cribriform, tubular or solid. RAS is a signal transduction protein for various important cellular processes such as cellular proliferation, differentiation, and survival by mediating the cell's response to extracellular stimulations. K-ras is a protein within the RAS family, and it functions in the same pathway of a Ras –Raf - Mek-Erk-map kinase pathway which plays a role in mediating cellular response to cell growth. K-ras appears to be involved in signal transduction and cell cycle regulation. Cyclooxygenase-2, an inducible enzyme in most cell types including keratinocytes, fibroblast and Tcell, catalyzes the synthesis of prostaglandins. Several processes in cancer may be influenced by Cox-2 including cell proliferation, apoptosis, and angiogenesis. Therefore, cyclooxygense2 may inhibit apoptosis via different pathways like down- regulation of arachidonic, up regulation of proto-oncogene Bcl2 and down-regulation of Bax, thus contributing to an increased survival rate.

Aims of the study: This study was conducted to analyze the immunohistochemical expression of K-ras oncogen and Cox-2 enzyme in pleomorphic adenoma and adenoid cystic carcinoma of salivary glands and to correlate the expression of the studied biomarkers with the clinical parameters and with each other.

Materials and Methods: In this study, 24paraffin embedded tissue blocks of salivary gland tumors were selected retrospectively from the files of the

department of Oral pathology at the College of Dentistry, Baghdad University; 12 cases were pleomorphic adenoma and the other 12 cases were adenoid cystic carcinoma. Immunohistochemistry technique was used to assess K-ras oncogen and Cox-2 enzyme expression in these tumors.

Results: Positive K-ras immunohistochemical expression was found in 11 (91.6%) and 8 (66.6%) of pleomorphic adenoma and adenojd cystic carcinoma studied cases respectively. Regarding Cox-2 expression, the results showed positive expression in 7 (58.3%) and1 (91.6 %) of pleomorphic adenoma and adenoid cystic carcinoma studied cases respectively. Statistically significant correlation was shown regarding K-ras expression with the sex of pleomorphic adenoma (P 0.044), while non-significant correlation was observed with other clinical parameters in pleomorphic adenoma and adenoid cystic carcinoma. Concerning Cox-2 expression, statistically significant correlation revealed with the site of pleomorphic adenoma (P 0.046) and the sex of adenoid cystic carcinoma (P 0.02), while non-significant correlation was found with other clinical parameters of both tumors.

Conclusion: The present study showed a highly expression rate of K-ras in pleomorphic adenoma and highly expression rate of Cox-2 in adenoid cystic carcinoma. The results also showed that there is no significant correlation between the expression rate of both K-ras and Cox-2 among study cases.

Keywords: Pleomorphic adenoma**,** adenoid cystic carcinoma**,** signal transduction, K-ras**,** Cox-2, immunohistochemistry

Introduction

Salivary gland neoplasms are distinguished because of their histological diversity. These neoplasms comprise of benign and malignant tumors of epithelial, mesenchymal, and lymphoid origin. From a pathological point of view, it is not an easy to make a differentiation of benign from malignant tumors; primarily because of the complexity of the classification and the rarity of several entities, which may exhibit a broad spectrum of morphologic diversity in individual lesions (Speight and Barrett, 2002).

Pleomorphic adenoma (PA) is the most common salivary gland tumor and it represents 60% to 73% of the parotid gland tumors, 40% to 60% of the submandibular and minor salivary glands tumors. (Ito et al,2005). PA is comprised of a salivary gland neoplasm with a benign nature. The pathology of PA involves ductal epithelial and myoepithelial cell proliferations in a mesenchymal stroma exhibiting ostensible histomorphologic diversity (Torske, 2006.)

Adenoid cystic carcinoma (ACC) is a malignant tumor with a

deceptively benign histological appearance characterized by indolent and locally invasive growth with high propensity for local recurrence and distant metastasis. The tumor is composed of basaloid cells with small, angulated, and hyperchromatic nuclei and scant cytoplasm arranged prognostically into 3 significant patterns: cribriform, tubular, and solid (Jaso and Malhotra, 2011). It is a rare tumor, which accounts for only 1% of all malignant tumors of the oral and maxillofacial region (Speight and Barrett, 2002), and 22% of all salivary gland malignancies. Also, it is one of the most common malignant tumors of the minor salivary and seromucinous glands (Kokemueller et al., 2004; Dodd and Slevin, 2006).

Numerous studies have attempted to elucidate accurate histological prognostic features but have often yielded conflicting results (Jaso and Malhotra, 2011).

GTPase K-ras, known as V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog and K-ras, is a protein that is encoded by the *K-ras* gene (Popescu,1985) in humans. The protein product of the normal K-ras gene performs an essential function in normal tissue signaling, and the mutation of a K-ras gene is an essential step in the development of many cancers. Like other members of the Ras family, the K-ras protein is a GTPase and is an early player in many signal transduction pathways (Kranenburg, 2005).

Cyclooxygenases (Cox), which are prostaglandin endoperoxide synthase, are the rate-limiting enzymes required for the conversion of arachidonic acid to prostaglandins (Vane et al, 1998). Cox-1 is constitutively expressed in most tissues, and plays a role in regulating normal physiological function and inflammation(Pairet and Engelhardt,1996). Cox-2 is expressed in inflammatory cells, such as in macrophage, fibroblast and vessel endothelial cell after being stimulated by antigens or cytokine (Appleton et al., 1995; Majima et al., 1997). Furthermore, Cox-2 has been reported to be expressed intensively in various malignancies (Sano et al., 1995; Hida et al.,1998; Zimmermann et al.,1999) and it was suggested that Cox-2 overexpression should correlate with tumor aggressiveness and poor prognosis (Tsujii et al., 1997). Thus, the inhibition of Cox-2 activity may have a therapeutic value.

Materials and Methods

In this study, 24 samples of parraffine-embeded blocks of salivary gland tumors were included, of which there were 12 cases of PA and 12 cases of ACC.

The cases involved in this study were retrospectively obtained from the Department of Oral Pathology, College of Dentistry, University of Baghdad; and the clinical information obtained from patients' case sheets

including age, gender, and lesion area. Therefore, the clinico-pathological characteristics of the patients from which the specimens were taken are illustrated in table (1) below:

P A	Age/ Years	Sex	Site	**ACC**	Age/ Years	Sex	Site
1	40	Female	parotid	1	42	Male	Maxilla
2	26	Female	parotid	2	33	Female	Tongue
3	53	Male	Minor s.g.	3	34	Female	Floor of mouth
4	25	Female	parotid	4	30	Female	Floor of mouth
5	56	Male	parotid	5	31	Female	mandible
6	42	Male	parotid	6	44	Male	mandible
7	25	Male	parotid	7	45	Female	Floor of mouth
8	50	Female	parotid	8	34	Male	Soft palate
9	50	Female	parotid	9	42	Male	Hard palate
10	22	Female	Minor s.g.	10	40	Male	Parotid gland
11	28	Male	Minor s.g.	11	55	Female	Hard palate
12	30	Male	Minor s.g	12	43	Male	Floor of mouth

Table 1:Clinico-pathologic data of studied sample

All the 24 paraffin blocks were cut at 4 µM. From each block, one representative section was stained with hematoxylin and eosin for reassessment of histopathological diagnosis and two other sections were prepared on adhesive slides for immunophistochemical staining with anti K-ras and Cox-2 monoclonal antibodies (Abcam).

Positive control is used for indicating the properness of the staining techniques. One positive control was used for each set of test runs. Normal skin and colon cancer were used as positive control for K-ras and Cox-2 respectively.

The negative control slides were prepared from test tissue and all reagents except the primary antibody were applied; thus positive staining indicates a lack of specificity of the antibody.

For immunohistochemistry, the tissue sections on the positively charged slides were baked in hot air oven at 65°C for 1 hr. Sections were sequentially dewaxed through a series of xylene, graded alcohol and water immersion steps. Antigen (Ag) retrieving was done for Cox-2 while this step was omitted for K-ras as recommended by the manufacturer. Then, endogenous peroxidase activity was blocked, and it was followed by blocking the non- specific staining. Primary Abs (100 µl) were applied for each section. A dilution of (1-100) for K-ras and (1:50) for Cox-2 were used. After an overnight incubation and washing with phosphate buffered solution (PBS), secondary Abs were applied, incubated and rinsed with a stream of PBS. Primary Abs were visualized with 3,3-diaminobenzidine (DAB) chromogen, then counterstained with Mayer's hematoxyline, dehydrated and mounted.

The region of staining viewed at 400 magnifications was scored as follows. For K-ras, immunohistochemical expression of three punches per each case were evaluated and regarded as a whole. The immunoreactivity was evaluated on a semi quantitative scale considering the percentage of positive cells (score: 0–4 for respectively, <5, 5–20, 20–40, 40–80, >80%) (DiFlorio et al ,2007). All cases were divided into four expression groups according to their scores which were as follows: score 1=1-20 score 2=20-40 score 3=40-80 score 4=>80 (DiFlorio et al,2007). For Cox-2, expression was evaluated by taking three fields per case. Staining extent was scored as 0 (0%), 1(1-25%), 2 (26-50%), 3(51-75%) and 4 (76-100%) according to the percentage of positively stained cells (Se Min et al., 2010). Therefore, the staining intensity was omitted since it may be subjected to personal variation during examination.

Pearson correlation (χ chi square) test was applied to find the relationship of the studied markers with each other and also with the various clinical parameters; p value $\leq$0.05 was considered significant and the statistical analyses were done using SPSS (Statistical Package for Social Sciences) V17 (2008).

Results

Expression of K-ras and Cox-2

The immunoexpression of K-ras was detected in majority of PA cases 11(91.6 %), of which 5 cases showed strong immunopositivity, while the other 6 cases were moderate. For Cox-2, positive immunoexpression was found in 8 cases (66.6%), 4 of them showed score 3, 2 positive cases showed score 4 and the remaining 2 positive cases showed score 2 of the expression. (Fig.1, 2 and 3)

In ACC, positive immunoexpression of K-ras was observed in 7(58.3%), of which 3cases showed strong immunoexpression score, other 3 cases showed moderate expression score and the remaining one positive case showed weak expression score. Concerning Cox-2 immunoexpression, present findings showed positive expression in 11(91.6%), of which 5 cases showed score 4 of the expression,4 cases showed score 2,and one remaining positive case, showed score 3 and the other one showed score 1of the expression.(Fig.4,5 and 6).

The correlation of the studied markers with each other and with the clinicopathological findings

Regarding the expression of K-ras, in PA, the results of the present study revealed statistically significant correlation of the K-ras expression with the sex(p value =0.044),table 2,whereas non-significant correlations

were observed with the site and the age of the study sample (P value 0.376 and 0.135 respectively).

Concerning ACC, the positive expression of K-ras showed statistically non-significant correlations with age, sex, and site (P value 0.557, 0.519 and 0.315 respectively).

Regarding the expression of Cox-2, in PA, a statistically significant correlation was shown with the site of the study sample (P value =0.046), table 3, whereas non-significant correlation observed with the age and sex of study sample (P value 0.329 and 0.282) respectively.

In ACC, the expression of Cox-2 revealed statistically significant correlation with the sex (p value =0.02), table 4, whereas non-significant correlation were found with age and site of ACC study sample (P value 0.537 and 0.773) respectively.

Correlating K-ras and Cox-2 with each other, a statistically non-significant positive correlation was seen in both PA and ACC (P values 0.225 and 0.727 respectively) (tables 5 and 6).

Therefore, the comparison between ACC and PA regarding the scores of K-ras and Cox-2 revealed a statistically non-significant correlation (P values 0.121 and 0.123 respectively) (Tables 7 and 8).

Table 2: Correlation of K-ras expression with the sex of pleomorphic adenoma

Sex		Scores					Total	Relation			
		0	1	2	3	4		X^2	Likelihood ratio	d.f.	p-value
Female	No.	1	0	0	1	4	6	5.467	6.225	2	0.044
	%	100%	0%	0%	16.7%	80%	50%				
Male	No.	0	0	0	5	1	6				
	%	0%	0%	0%	83.3%	20%	50%				
Total	No.	1	0	0	6	5	12				
	%	100%	100%	100%	100%	100%	100%				

Table 3: Correlation of Cox-2 expression with the site of pleomorphic adenoma

Site		Scores					Total	Relation			
		0	1	2	3	4		X^2	Likelihood ratio	d.f.	p-value
Minor salivary Glands	No.	3	0	0	0	1	4	6.375	8.005	3	0.046
	%	75%	0%	0%	0%	50%	33.3%				
Parotid Gland	No.	1	0	2	4	1	8				
	%	25%	0%	100%	100%	50%	66.7%				
Total	No.	4	0	2	4	2	12				
	%	100%	100%	100%	100%	100%	100%				

Table 4: Correlation of Cox-2 expression with the sex of Adenoid cystic carcinoma

Sex		Scores					Total	Relation			
		0	1	2	3	4		X^2	Likelihood ratio	d.f.	p-value
Female	No.	0	0	4	1	1	6	8.800	11.632	4	0.020
	%	0%	0%	100%	100%	20%	50%				
Male	No.	1	1	0	0	4	6				
	%	100%	100%	0%	0%	80%	50%				
Total	No.	1	1	4	1	5	12				
	%	100%	100%	100%	100%	100%	100%				

Table 5: Correlation of K-ras and Cox-2 with each other in pleomorphic adenoma

K-ras		Cox-2					Total	Relation			
		0	1	2	3	4		X^2	Likelihood ratio	d.f.	p-value
0	No.	0	0	0	1	0	1	6.800	8.179	6	0.225
	%	0%	0%	0%	25%	0%	8.3%				
1	No.	0	0	0	0	0	0				
	%	0%	0%	0%	0%	0%	0%				
2	No.	0	0	0	0	0	0				
	%	0%	0%	0%	0%	0%	0%				
3	No.	2	0	0	2	2	6				
	%	50%	0%	0%	50%	100%	50%				
4	No.	2	0	2	1	0	5				
	%	50%	0%	100%	25%	0%	41.7%				
Total	No.	4	0	2	4	2	12				
	%	100%	100%	100%	100%	100%	100%				

Table 6: Correlation of K-ras and Cox-2 with each other in adenoid cystic carcinoma

K-ras		Cox-2					Total	Relation			
		0	1	2	3	4		X^2	Likelihood ratio	d.f.	p-value
0	No.	0	1	2	1	1	5	8.080	8.720	12	0.727
	%	0%	100%	50%	100%	20%	41.7%				
1	No.	0	0	0	0	0	0				
	%	0%	0%	0%	0%	0%	0%				
2	No.	0	0	0	0	1	1				
	%	0%	0%	0%	0%	20%	8.3%				
3	No.	1	0	1	0	1	3				
	%	100%	0%	25%	0%	20%	25%				
4	No.	0	0	1	0	2	3				
	%	0%	0%	25%	0%	40%	25%				
Total	No.	1	1	4	1	5	12				
	%	100%	100%	100%	100%	100%	100%				

Table 7: Comparison between ACC and PA regarding the scores of K-ras

Lesion		Scores					Total	Comparison			
		0	1	2	3	4		X^2	Likelihood ratio	d.f.	p-value
ACC	No.	5	0	1	3	3	12	5.167	5.822	3	0.121
	%	83.3%	0%	100%	33.3%	37.5%	50%				
PA	No.	1	0	0	6	5	12				
	%	16.7%	0%	0%	66.7%	62.5%	50%				
Total	No.	6	0	1	9	8	24				
	%	100%	0%	100%	100%	100%	100%				

Table 8: Comparison between ACC and PA regarding the scores of Cox 2

Lesion		Scores					Total	Comparison			
		0	1	2	3	4		X^2	Likelihood ratio	d.f.	p-value
ACC	No.	1	1	4	1	5	12	6.552	7.249	4	0.123
	%	20%	100%	66.7%	20%	71.4%	50%				
PA	No.	4	0	2	4	2	12				
	%	80%	0%	33.3%	80%	28.6%	50%				
Total	No.	5	1	6	5	7	24				
	%	100%	100%	100%	100%	100%	100%				

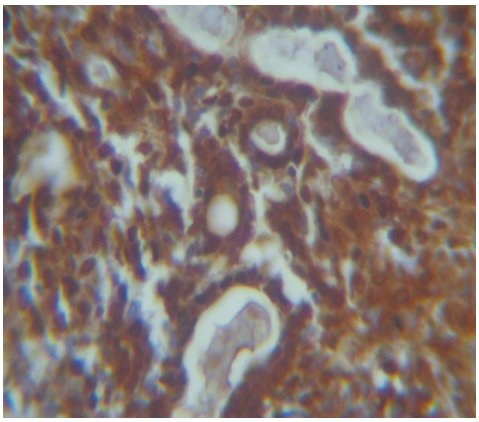

Figure1: Positive expression of K-ras in PA(X40)

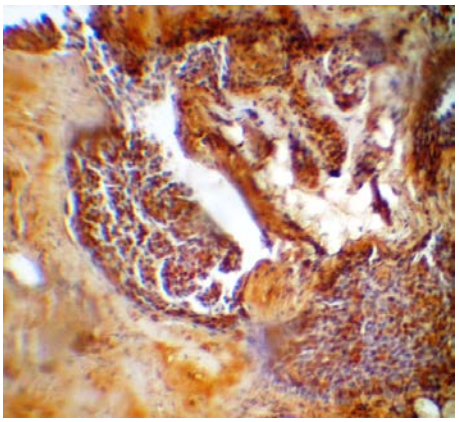

Figure2: Positive expression of K-ras in PA(X20)

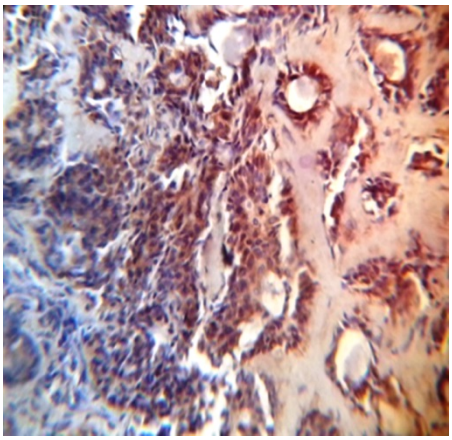

Figure 3: Positive expression of Cox-2 in PA(X40)

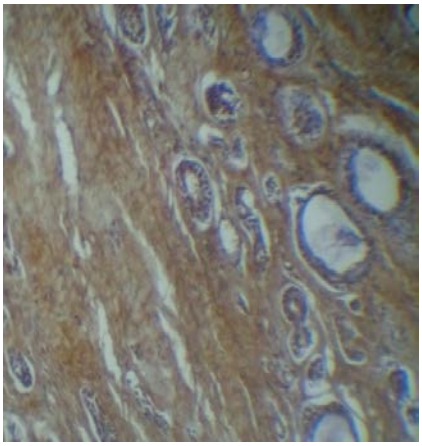

Figure 4: Positive expression of K-ras in ACC(X20)

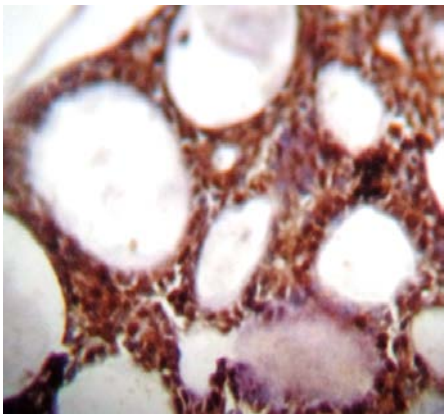

Figure 5: Positive expression of K-ras in ACC (X40)

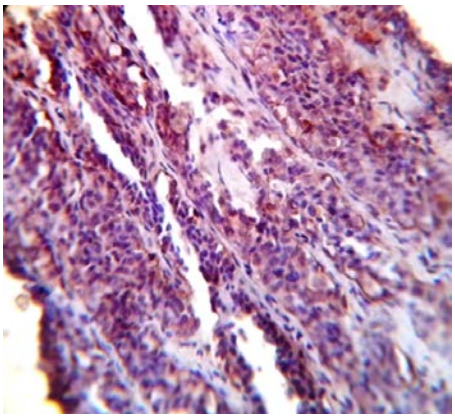

Figure 6: Positive expression of Cox-2 in ACC (X40)

Discussion

The results of the current study overemphasized the important role played by K-ras in PA. About 92% of PA cases exhibited immunoreactivity for K-ras. These findings are consistent with other studies in which the KRAS protein was reported to be an early player in many signal transduction pathways (Kranenburg, 2005). Other studies pointed to detection of *ras* genes in several malignant neoplasms of diverse origin (Yoo and Robinson, 2000). K-*ras* mutations are more likely to occur in tumors from glandular epithelial tissues. K-*ras* mutation occurs mainly in mucin-producing adenocarcinomas (Bos, 1998). However, our data do not agree with studies reported by Yoo and Robinson (2000) who found only 8% mutations of the K-*ras* gene in salivary gland tumors. The expression of COX-2 in PA in the present study was about 67%. Our data did not agree with other studies as the study of Sakurai (2001) who demonstrated an

increased expression of COX-2 in 90% of cases with salivary gland tumors.

The expression of K-ras in ACC was shown in about 58% of cases, while the expression of COX-2 was shown in about 92% of ACC cases. These findings suggested that the role of COX-2 in ACC is more pronounced compared with K-ras, while this relation was inversed in PA. It is worth to mention that COX-2 is down-regulated in estrogen deficient animals when they have been treated with high concentrations of estrogen (Radeva, 1977).

Regarding correlation of studied biomarkers with each other, the data of the present study pointed to independent pathways between K-ras and Cox-2 in both PA and ACC.This finding disagree with other studies that investigated the relationship between the expression of K-ras and Cox-2 in other types of tumors. Li et al (2006) conducted a study in the light of the fact that K-ras plays an important role in the induction of COX-2 expression in tumor cells. They reported a significant correlation between the expression rate of COX-2 and K-ras in gastric cancer.

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