Immunohistochemical expression of basic fibroblast growth factor-2 and Heparanase in salivary pleomorphic adenoma

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ABSTRACT

Background: The aim of this study was to evaluate the expression of fibroblast growth factor-2 and Heparanase in salivary pleomorphic adenoma, and to correlate the two studied markers with each other and with clinicopathological parameters including: age, sex, tumor site and histopathological presentation.

Methods: Sections of twenty five formalin-fixed paraffin embedded tissue blocks specimens of salivary pleomorphic adenoma were immunostained using monoclonal antibodies (Fibroblast growth factor-2 and Heparanase) to assess their expression in this tumor.

Results: The expression of fibroblast growth factor-2 and Heparanase were positive in all pleomorphic adenoma cases (100%). The positive expression of fibroblast growth factor-2 was significantly correlated with histopathological presentation (p-value=0.032), but it was non-significantly correlated with FGF-2 and other clinicopathological parameters (age, sex, tumor site). The positiveexpression of Heparanse was non-significantly correlated with the histopathological presentation (p-value=0.088) as well as with other clinicopathological parameters (age, sex, tumor site). Statistically significant correlation was found between the expressions of both studied markers (p-value= 0.0005).

Conclusion: The fibroblast growth factor-2 and Heparanase positive expression was noted in all cases of salivary pleomorphic adenoma signifying that both fibroblast growth factor-2 and Heparanase might contribute in the biological behavior of pleomorphic adenoma. The highly significant correlation found in the expression of both markers suggests their synergistic and cooperative role in the tumorigenesis of pleomorphic adenoma.

Keywords: Pleomorphic adenoma, FGF-2, Heparanases. **(J Bagh Coll Dentistry 2014; 26(1):121-127).**

INTRODUCTION

Pleomorphic adenoma (PA) is a benign neoplastic tumor of the salivary glands. It is the most common type of salivary gland tumors and the most common tumor of the parotid gland. It derives its name from the architectural pleomorphism (variable appearance) seen by light microscopy, it is also known as "Mixed tumor, salivary gland type", which describes its pleomorphic appearance as opposed to its dual origin from epithelial and myoepithelial elements [(1)]. Clinically, the tumor is usually solitary and presents as a slow growing, painless, firm single nodular mass. Isolated nodules are generally outgrowths of the main nodule rather than a multinodular presentation. It is usually mobile unless found in the palate and can cause atrophy of the mandibular ramus when located in the parotid gland. Though it is classified as a benign tumor, pleomorphic adenoma have the capacity to grow to large proportions and may undergo malignant transformation to form carcinoma ex pleomorphic adenoma, a risk that increase with time. Although it is benign the tumor is aneuploid, it can recur after resection, it invades normal adjacent tissue and distant metastases have been reported after long time intervals [(2)]. Histologically, it is highly variable in appearance, even within individual tumors. Classically it is biphasic and is characterized by an admixture of polygonal epithelial and spindle-shaped myoepithelial elements in a variable background stroma that may be mucoid, myxoid, cartilaginous or hyaline. Epithelial elements may be arranged in duct-like structures, sheets, clumps and/or interlacing strands and consist of polygonal, spindle or stellate-shaped cells,areas of squamous metaplasia and epithelial pearls may be present.The tumors are not enveloped but it is surrounded.by a fibrous capsule of varying thickness. The tumors extend through the normal glandular parenchyma in the form of finger-like pseudopodia [(1)]. Little is known about specific transcription and growth factors involved in human salivary gland tissue morphogenesis and cytodifferentiation. Identification of such molecules through basic research is likely to furnish potential new tools for tumors [(3)]. Fibroblast growth factors (FGFs) are one of the largest growth factor families, comprising 22 members with 13%–71% sequence similarity in mammals [(4)]. FGF type 2 (FGF2), or basic FGF, is a prototype member of the family. It interacts with high-affinity receptors (FGF receptors [FGFRs]), which are transmembrane tyrosine kinases; the FGFR1 isoform being its prime target. The

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binding of FGF2 to FGFR1 induces receptor autophosphorylation on several tyrosine residues, which in turn activates downstream effector molecules, leading to the activation of the Ras-mitogen-activated protein kinase (MAPK) cascade [5]. This cascade promotes translocation of MAPKs to the nucleus, where they phosphorylate and directly activate specific target proteins, including transcription factors. FGF2 is also highly expressed in various somatic cell types where it has an intrinsic function in the regulation of cell proliferation, differentiation, and survival. It also regulates self-renewal and immaturity of many tissue-specific stem cells, including cells from the mouse striatum [6], bone marrow mesenchymal stem cells and adipose tissue-derived stem cells [7,8]. Heparanase is an endoglycosidase that degrades heparan sulfate in the extracellular matrix and cell surfaces, and fulfills a significant role in tumor angiogenesis and plays a critical role in fibroblast growth factor-2 bioactivation by facilitating the releases of immobilized fibroblast growth factor-2 from the extracellular matrix [9,10]. Similarly, Heparanase has been shown to facilitate cell invasion associated with autoimmunity, inflammation and angiogenesis [11,12].Traditionally, heparanase activity was correlated with the metastatic potential of tumor-derived cells, attributed to enhanced cell dissemination as a consequence of heparan sulfate cleavage and remodeling of the extracellular matrix barrier [13,14].

MATERIALS AND METHODS

The sample of this study included twenty five formalin-fixed, paraffin-embedded tissue blocks, diagnosed as salivary pleomorphic adenoma which was dated from (2000 till 2012). The blocks were obtained from Al-Shaheed Ghazi Hospital/ Medical City /Baghdad (12 cases); Al-Kadhimiya teaching hospital (10 cases); the archives of the department of Oral and Maxillofacial Pathology/ College of Dentistry/ University of Baghdad (3cases). Demographic and clinical data provided by the surgeon were obtained from the surgical and pathological reports available with the tissue specimens, including patient's age, sex, clinical presentation, site of the tumor and histopathological description The diagnosis of each case was confirmed by the examining of Hematoxylin and Eosin (H&E) sections by two specialized pathologists to determine the most predominant histopathological appearances whether it is mostly cellular, stromal or mixed type.Two other 4µm thick sections for each case were cut and mounted on positively charged slides (Fisher scientific and Eschosuperfrost plus (USA) for immunohistochemical staining with monoclonal antibodies: fibroblast growth factor-2 (US. Biological) and Heparanase (US. Biological). Positive tissue controls was obtained according antibodies manufacturer's datasheet and added to each test run.

Evaluation of Immunohistochemical results

Immunohistochemical signal specificity was demonstrated by the absence of immunostaining in the negative control slides and its presence in recommending positive controls. For FGF-2 tumor cells with clear brown cytoplasmic staining pattern were considered positive, and membranous or membranous and cytoplasmic immunoreactivities were considered positive for Heparanase. Immunohistochemically stained pleomorphic adenoma sections were studied by light microscope under (10X) objective. In each tissue section, five representative fields (areas showed well preserved tumor islands in which the reaction was clearly positive) were selected for FGF-2 and Heparanse monoclonal antibodies immunostaining evaluation, with an average of 1000 tumor cell per case and 200 tumor cells per field. Only the number of cells that were positive for FGF-2 and positive for Heparanse were quantified by counting at least one thousand cells in representative five fields at(40X) objective in each case. Membranous or membranous and cytoplasmic immunoreactivities were scored for Heparanase, while cytoplasmic expression was the parameter scored for FGF-2 expression. The extent of staining was scored using the following scale: 0 = no staining (negative), I = staining of 0–5% of tumor cells (very weak positive), II =staining of 6–25% of cells (weak positive), III = staining of 26–75% of tumor cells (moderate positive), IV = staining of 76–100% of tumor cells (strong positive) [15].

Statistical Analysis

The studied parameters were scored and considered as categorical data thus they presented as count and percentage. The relationship between categories was tested by Chi-square test.

Spearman's rho correlation was applied to assess the linear association between FGF-2 and Heparanse. The level of significance was 0.0005 (two-sided) in all statistical testing.

RESULTS

PositiveFGF-2 immunostaining was detected as brown cytoplasmic staining of the tumor cells, fig. (1,2) Positive IHC expression was found in all pleomorphic adenoma casesasillustrated intable (1) whichreveals that only (1) case (4.0%) showed

very weakpositiveexpression,(8)cases(32.0%) showed weakpositiveexpression, (9) cases(36.0%) showed moderatepositive expression and (7) cases (28.0%) showed strong expression. Positive Heparanase Immunostaining was found in all pleomorphic adenoma casesas brownmembranous ormembranousand cytoplasmic expression. Fig (3,4)Heparanase immunostaining of the pleomorphic adenoma cases was summarized in table (2) which reveals that only (1)case(4.0%) showed very weak positive expression, (2) cases (8.0%)showedweakpositive expression while (12) cases(48.0%) showedmoderatepositiveexpression and (10) cases (40%) showed strong positive expression.

The positive expression of fibroblast growth factor-2 was non-significantly correlated with age(p=0.737),sex(p =0.456) and tumor site(p =0.765),while there was significant correlation with histopathological presentation(p =0.088) as shown in table (3).

The positive expression of Heparanase was non significantly correlated with all clinicopathological parameters including age((p=0.737), sex(p =0.456),tumor site(p =0.765) and histopathological presentation(p =0.088);table(4).

Regarding the correlation between FGF-2 and Heparanase IHC expressions, the results of the present study revealed a highly significant correlation between both markers (p= 0.0005) as clarified in table (5).

DISCUSSION

The selection of pleomorphic adenoma in this study is attributed to its consideration as the most common benign salivary gland neoplasm which characterised by neoplastic proliferation of parenchymatous glandular cells along with myoepithelial components, and having a malignant potentiality. Also it is the most common type of salivary gland tumor and the most common tumor of the parotid gland[1].Regardless of the great variety of the histopathological aspects, the main diagnostic feature was the presence of both epithelial and mesenchymal- like tissue (mixed) which was corresponded to (60%) of the cases, cellular (32%) and stromal (8%), this is in agreement with [16]who stated that the mixed histopathological type was the most predominan. Growth factors mediate a wide variety of biological processes such as development, tissue repair and tumorigenesis, and also contribute to cellular proliferation and transformation in neoplastic cells[3].

Of the growth factors that may play important roles in tumor progression, FGF-2 was unique, since it was epithelial, mesodermal and neuroectodermal mitogens as well as being a potent angiogenic factor [17].

A variety of studies in vitro and in vivo suggest that alterations in the expression of FGF-2 and its receptor are associated with growth deregulation in neoplastic cells and are thought to contribute to cellular transformation and continued proliferation[18,19].

The results of this study showed a positive expression of FGF-2 in all cases of PA with a moderate positivity in (36%) of cases and strong positivity in (28%) of cases, these findings are compatible with previous studies[17,19]who identified FGF-2 in tumor cells and stated that FGF-2 are expressed in neoplastic cells of PA, particullarymyoepithelial cells which may be related to the differentiation of neoplastic myoepithelial cells and mesenchymal-like tissue formation including fibrous, hyaline, myxoid and chondroid tissues.

Since there is an evidence which suggest that FGF-2 is released from cells through a novel pathway [20], the result of this study and other observations suggest that FGF-2 may be released from tumor cells inducing autocrine tumor cell proliferation in the tubular and solid areas, while in the myxoid and chondroid areas, tumor cells showed to be differentiated and produce an extacellular containing collagen, laminin and tenascin[21,22]. FGF-2 was intensely localized in the basement membrane of tubular cells, this storage of FGF-2 in the basement membrane is thought to be stable and temporarily inactive due to its high affinity for heparin -like molecules[23], So immunohistochemical result of this study is consistent with the hypothesis that the expression of FGF-2 may be involved with the regulation of the growth and differentiation of tumor cells[24].

Heparanase also cleaves perlecan HS in the basement membrane and relases FGF-2, making it available for growth factor-dependent signalling during angiogenesis, wound healing and tumor formation.

To the best of our knowledge, the present study is the first of its kind in assessing HP expression immunohistochemically in PA of salivary gland.

The expression of Heparanase was detected as brown granular membranous\ cytoplasmic localized in tumor cells.The result of this study showed a positive expression of Heparanase in all studied cases of PA with a moderate positivity in (48%) of cases and strong positivity in (40%) of cases.

Since this is a pioneer study in assessing HP in PA, it's difficult to establish a comparison with other studies however conclusive remarks can be

withdrawn from other studies on the salivary gland malignancies [25] assumed that heparanase induction contributes to tumor progression through enhanced angiogenesis,release of ECM-sequestersd growth factors, generation of bioactive HS fragments and creation of a growth-supportive microenvironment

Concerning to histopahological presentation ,positive expression was observed in mixed type of tumor (60%) more than cellular type (32%) of cases followed by stromal type(8%) ,there is no study related to be compared with but this may be suitable with the hypothesis that Heparanase cleaves perlecan HS in the basement membrane and releases FGF-2 making it available for growth factor-dependent signaling during angiogenesis and tumor formation[25,26], also the bioactivity of FGF-2 may be modulated by its release from ECM as a complex with a fragment of Heparanase[25,27]and the high –affinity activation of FGF receptors (FGFRs) and FGFs requires the formation of a ternary complex with HS[28,29], and as mentioned that FGF-2 positivity is more in the mixed type of tumor than cellular and stromal ,so the more positive expression of FGF-2 is a land mark of more positive expression of Heparanase.

In agreement with the role of the Heparanase in releasing FGF-2 from the ECM, the results of the present study revealed that both FGF-2 and Heparanase showed similar pattern of expression, they were highly correlated by Pearson chi square with significant correlation between either proteins expression was found (p-value=.0005).

The correlation between the two markers in PA is firstly done in Iraq and no other study correlate between them regarding salivary gland tumor so, the comparison could be withdrawn from other studies regarding other tumor affecting oral cavity.

The positive correlation between the two markers agree with Chen et al. [30] that showed Heparanase mRNA and FGF-2 mRNA are associated with higher tumor MVD in OSCC and Shareef et al. [31] also showed, there was significant correlation between the immunohistochemical expression of Heparanase and FGF-2 regarding oral squamous cell carcinoma.

Heparanase degradation of HS could change the availability of FGF-2 in the tumor microenvironment by releasing FGF-2 from the matrix and the cell surface.Besides to modifying FGF-2 action, Heparanase could alter signaling initiated by multiple heparin binding growth factors [32].

It has been revealed that Heparanase degradation of cell surface HS can augment the FGF-2 activity, depending on the Heparanase concentrations used to alter cell surface HS. FGF-2 binding and signaling require HS sequence-specific interactions .Depending on the extent of HS degradation, HS sequences, which bind to either FGF-2 or FGFR, could be removed or cryptic sites could be revealed, angiogenesis is dependent multiple components that can be affected by Heparanase in the ECM provide binding sites for angiogenic factors such as FGF-2 and vascular endothelial growth factor. Cell surface HSPG acts as growth factor and adhesion receptors on tumor cells and vascular endothelial cells. Modifying the HS may affect tumorigenicity by modifying the responsiveness of multiple receptors to the extracellular environment [33,34].

Finally, the statistically significant correlation between FGF-2and HP expression revealed in this study suggest their close and synergistic cooperation and co activation in PA. Therefore, they could be considered important biomarkers acting together in the angiogenesis, proliferation and aggressiveness of PA. In conclusion: both FGF-2 and HP might contribute in biological behaviour of PA.

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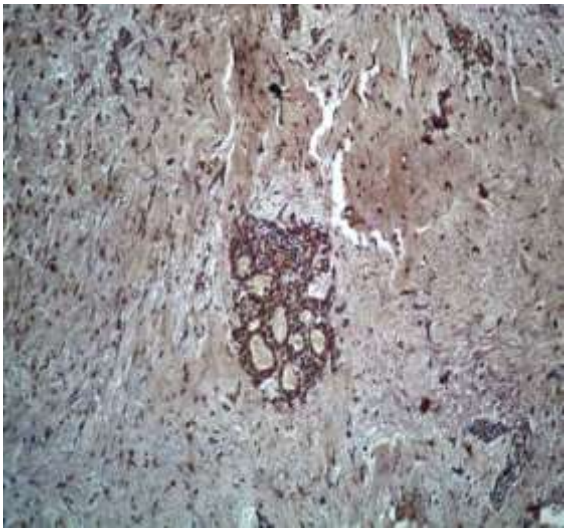

Figure 1: Positive brown cytoplasmic expression of FGF-2 in cellular, ductal and myxoid components (20X).

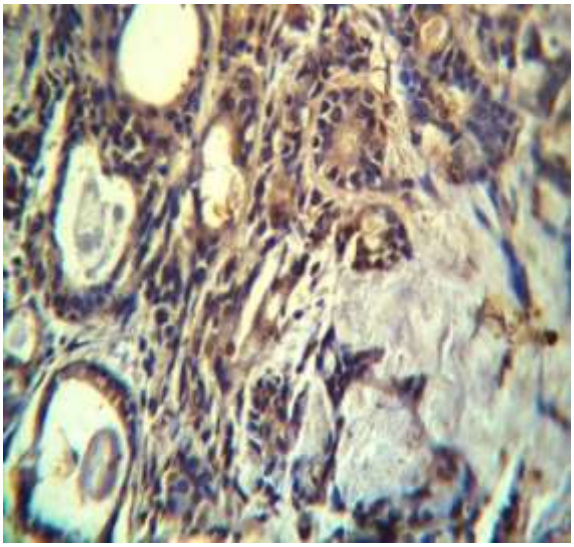

Figure 2: Positive brown cytoplasmic immunostaining of FGF-2 in cellular andductal componenets of pleomorphic adenoma (40X).

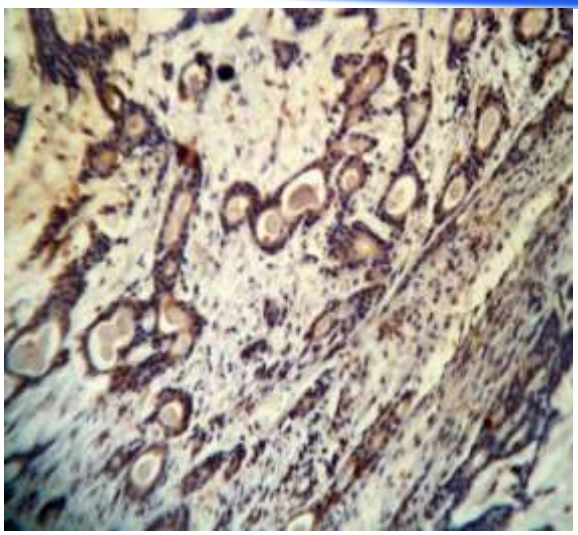

Figure 3: Positive brown membranous/cytoplasmic immunostaining of Heparanase in ductal and cellular components of PA (20X).

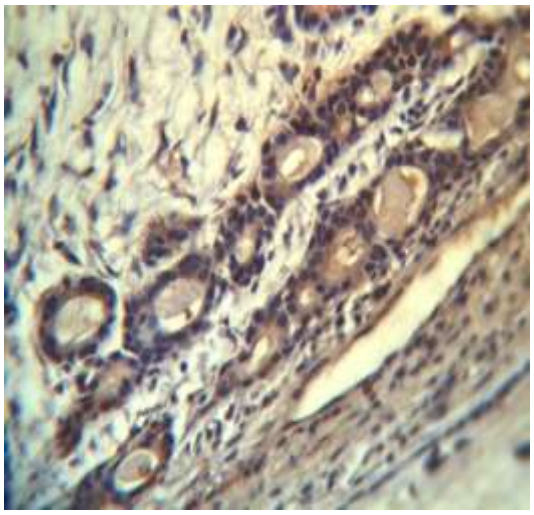

Figure 4: Positive brown membranous/cytoplasmic immunostaining of Heparanase in ductal and myxoid components of PA (40X).

Table 1: FGF-2 IHC expression in pleomorphic adenoma cases

FGF-2FGF-2 score*	No.	%
I	1	4.0%
II	8	32.0%
III	9	36.0%
IV	7	28.0%
Total	25	100%

***I (very weakexpression), II(weakexpression), III (moderateexpression),. IV (strong expression)**

Table 2: Heparanase IHC expression in pleomorphic adenoma cases

Heparanasescore*	No.	%
I	1	4.0%
II	2	8.0%
III	12	48.0%
IV	10	40%
Total	25	100%

I (very weakexpression), II (weakexpression), III (moderate expression), IV(strong expression)

Table 3: Correlation of FGF-2 with histopathological presentation

Crosstab	FGF				Total
Histopathology	I	II	III	IV	
Cellular	0	0	3	5	8
	.0%	.0%	37.5%	62.5%	100.0%
	.0%	.0%	33.3%	71.4%	32.0%
Stromal	0	0	2	0	2
	.0%	.0%	100.0%	.0%	100.0%
	.0%	.0%	22.2%	.0%	8.0%
Mixed	1	8	4	2	15
	6.7%	53.3%	26.7%	13.3%	100.0%
	100.0%	100.0%	44.4%	28.6%	60.0%
Total	1	8	9	7	25
	4.0%	32.0%	36.0%	28.0%	100.0%
	100.0%	100.0%	100.0%	100.0%	100.0%
Pearson Chi-Square	Value		df	Asymp. Sig. (2-sided)	
	13.757		6	.032	

Table 4: Correlation of HP expression with histopathological presentation

Crosstab	Hp				Total
Histopathology	I	II	III	IV	
Cellular	0	0	2	6	8
	.0%	.0%	25.0%	75.0%	100.0%
	.0%	.0%	16.7%	60.0%	32.0%
Stromal	0	1	1	0	2
	.0%	50.0%	50.0%	.0%	100.0%
	.0%	50.0%	8.3%	.0%	8.0%
Mixed	1	1	9	4	15
	6.7%	6.7%	60.0%	26.7%	100.0%
	100.0%	50.0%	75.0%	40.0%	60.0%
Total	1	2	12	10	25
	4.0%	8.0%	48.0%	40.0%	100.0%
	100.0%	100.0%	100.0%	100.0%	100.0%
Pearson Chi-Square	Value	df	Asymp. Sig. (2-sided)		
	11.000	6	0.088		

Table 5: Hp * FGF Crosstabulation

	FGF				Total
Hp	I	II	III	IV	
I	1	0	0	0	1
	100.0%	.0%	.0%	.0%	100.0%
	100.0%	.0%	.0%	.0%	4.0%
II	0	1	1	0	2
	.0%	50.0%	50.0%	.0%	100.0%
	.0%	12.5%	11.1%	.0%	8.0%
III	0	6	4	2	12
	.0%	50.0%	33.3%	16.7%	100.0%
	.0%	75.0%	44.4%	28.6%	48.0%
IV	0	1	4	5	10
	.0%	10.0%	40.0%	50.0%	100.0%
	.0%	12.5%	44.4%	71.4%	40.0%
Total	1	8	9	7	25
	4.0%	32.0%	36.0%	28.0%	100.0%
	100.0%	100.0%	100.0%	100.0%	100.0%
Pearson Chi-Square	Value		df	Asymp. Sig. (2-sided)	
	30.906		9	.0005	