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Immunohistochemical Expression of P_{53} and bcl-2 in benign and malignant salivary glands tumors

Dr. Natheer H. AL-Rawi M.Sc, Ph.D [*]
Dr. Jamal N. Ahmed M.Sc[*]
Dr. Husam Omer Mohammed M.Sc [**]

Abstract

Objectives: Salivary glands tumors are relatively uncommon neoplasm with widely variable histopathologic and biologic characteristics. Alteration in some proto-oncogenes and tumor suppressor genes may lead to the development and progression of these tumors. The purpose of the present study was to analyze the immunohistochemical expression of P_{53} and bcl-2 in some salivary gland tumors in relation to tumor size, histological grade and extent of invasion.

Study Design: The series consisted of 22 formalin-fixed paraffin embedded blocks of salivary glands tumors collected from the files of Oral pathology Department./College of Dentistry/ Baghdad University. The specimens consisted of (10) benign salivary gland tumors (Pleomorphic Adenomas) and (12) malignant salivary gland tumors [(2) Carcinoma ex Pleomorphic Adenoma, (3) Muco-Epidermoid Carcinoma, (2) Adenocarcinoma (5) Adenoid cystic carcinoma]. These were analyzed for immunohistochemical detection of P_{53} and bcl-2 in their cell types.

Results: Pleomorphic Adenoma showed negative expression of P_{53} and bcl-2 in 70% of cases, whereas all malignant salivary glands tumors were positive for P_{53} and bcl-2. P_{53} and bcl-2 immune-reactivity were associated with larger tumor size, higher tumor grade and greater extent of invasion without reaching statistical significance level.

Conclusion: P_{53} expression could be used as a useful tumor marker for malignant transformation of Pleomorphic adenoma and bcl-2 expression could be used as a useful monitor for measuring the aggressiveness of these neoplasms.

Keywords: P_{53}; bcl-2; salivary gland tumors; immunohistochemistry

Introduction

Salivary glands tumors comprise a significant proportion of oral tumors and are the next common neoplasm of the mouth after squamous cell carcinoma [(1)]. They are relatively uncommon neoplasm with widely variable histopathologic and biologic characteristic which makes it difficult to determine the pathogenesis and selection of therapeutic modalities [(2)].Recent advances in molecular genetic techniques have allowed for extensive analysis of a wide variety of human tumors, such information are important not only to the understanding the tumor biology, but also for the potential development of novel diagnostic and biologic markers

* Assistant Professor, Dept. Oral Diagnosis, College of Dentistry, Baghdad University, Baghdad, Iraq.
** Dept. Oral Medicine, College of Dentistry, University of Anbar, Anbar, Iraq.

for their management [3]. Apoptosis (programmed cell death) is a specific form of cell death that constitutes an important mechanism of maintaining homeostasis. Apoptosis occurs physiologically as well as in the course of many diseases. Alterations of apoptosis are always coupled with pathological conditions and/or oncogenesis [4].Apoptosis / anti-apoptosis markers like P_{53} and bcl-2 may potentially be able to predict the tumor behaviors [5].P_{53} is a tumor suppressor gene that controls the induction of apoptosis and growth arrest at cell cycle check points. The function of P_{53} leads to elimination of damaged cells from a population and is believed to be a basis of its tumor suppressor function. Mutation of P_{53} gene diminishes cells ability to repair damaged DNA before entry into S-phase leading to greater chance of mutational fixation into the genome and passed onto successive generations of cells [6]. Bcl-2 encoded by the proto-oncogene bcl-2 and expressed in many types of malignant tumors, protect cells from apoptosis- induced DNA damaging agents. This anti-apoptotic effect is hypothesized to be caused, at least in part, by retardation of cell proliferation due to cell's accumulation in the G_o and G_1 phases of the cell cycle [7].Loss of function of P_{53} protein combined with bcl-2 has been suggested to be a major cause of carcinogenesis. Loss of function of P_{53} protein combined with bcl-2 up regulation might give the tumor cells double growth advantage, because uncontrolled proliferation is combined with a reduced cell death rate [8]. Due to the scanty information available on salivary glands tumorigenesis the present study tried to investigate the immunohistochemical expression of P_{53} and bcl-2 in benign & malignant salivary glands tumor sample and to clarify the clinico-pathological significance of P_{53} and bcl-2 abnormalities.

Materials and method

Twenty two routinely fixed paraffin embedded blocks were retrieved from the files of the department of oral pathology/ College of Dentistry/ Baghdad University from the period of January 2001 till March 2006. The sample consisted of 10 benign tumors (Pleomorphic Adenomas) and 12 malignant tumors (2 carcinoma ex pleomorphic adenoma, 3 Muco-epidermoid carcinoma, 2 polymorphous low-grade adenocarcinoma and 5 Adenoid cystic carcinoma). All the 22 blocks were retreated as (Deblocking) and (Re-embedding) to achieve a regular and homogenous shape. A thin 3 µM thickness sections were cut from the tissue blocks, collected and prepared for immunohistochemical procedure at the Department. of Pathology /King Hussein's Cancer center/ Jordan. Forty four silanized slides represented different salivary glands tumors divided into 22 slides for P_{53} immunoassaying and other 22 slides for bcl-2 immune- staining. One specimen of adenocarcinoma of Colon and other specimen of follicular Lymphoma were immuno stained to represent the positive control slides for P_{53} and bcl-2 respectively. After drying and dewaxing, all slides were incubated in automated slide preparation stainer system (Bench Mark®XT-

Ventana ™-France) for 32 minutes with one drop of monoclonal anti-human P_{53} (Ventana ® Confirm ™P_{53} mouse IgG-ready to use, clone D07) and monoclonal anti-human bcl-2 (Ventana ® Confirm ™bcl-2 mouse IgG-ready to use, clone 100/D5). Negative control slides were incubated

with normal phosphate buffer solution instead of primary monoclonal antibodies. All sections were washed by reaction TRIS buffer for 2 minutes to remove excess unconjugated antibodies, then sections were incubated for 8 minutes at 37ºC with secondary monoclonal antibodies (Ventana ® -i- VIEW™ Biotin Ig®-ready to use). Slides were then washed again in TRIS buffer for 1 minute. The bound antibodies were detected using Labeled Strept- Avidin -Biotin Complex (LSAB) following the manufacturer's instructions. Slides were incubated for 8 minutes with (Ventana ®-i-VIEW SA-HRP ™-ready to use) then washed with TRIS buffer for 2 minutes, 3',3' diaminobenzidine (DAB) chromogen was then applied on all slides using (Ventana ®-i- VIEW DAB ®-ready to use & i- VIEW H_2O_2® ready to use as substrate for 8 minutes. Slides were rewashed with TRIS-buffer for 2 minutes, then a drop of (Ventana ® i-VIEW Copper ™) was applied for 4 minutes and washed again with TRIS buffer for 2 minutes. Hemotoxylin counter stain was then applied to all slides for 2 minutes and washed with tap water for other 2 minutes, dried in graduated alcohol concentrations, then mounted with DPX and slide cover. Examination of the slides was done by Biocular Microscope at 10X, 40X & 100 X magnifications.

Scoring:

The P_{53} and bcl-2 immune-reactivity and intensity was semi quantitatively evaluated in at least 1000 cells examined at 40X magnification and recorded as the percentage of (P_{53} or bcl-2) positive tumor cells over the total number of neoplastic cells present in the same area.

P_{53} immuno reactivity was divided into three groups according to the classification of KARAJA et al [9], as follow:

Score (0): negative [no staining],

Score (1): + weak positive [staining in 10% or less of neoplastic cells],

Score (2): ++ intensely positive [staining of more than 10X of neoplastic cells.

Bcl-2 immuno reactivity was divided into four groups according to the classification of Aoki et al [10] , as follows:

Score (0): negative [no staining],

Score (1): + weak positive [staining in 5-20% of neoplastic cells],

Score (2): ++ moderate positive [staining in 20-50% of neoplastic cells]

Score (3): +++ strong positive [staining in more than 50% of neoplastic cells].

Statistical analysis had been made using Chi-square test & Fischer exact test. $P<0.05$ was considered significant.

Results

Ten cases of Pleomorphic adenoma were identified in this study as benign salivary glands tumors. They present as epithelial sheets and ductal structures with a myxoid, chondroid, hyaline and osteoid materials. Seven out of ten cases (70%) of these tumors were negative for P_{53} expression and three out of ten (30%) of them expressed weak to moderate P_{53} immuno staining.

Bcl-2, on the other hand, has negative staining reaction in 70% of cases. Two cases demonstrated weak staining and one case demonstrated strong staining reaction (Table 1).

Table (2) illustrates the combined P_{53}/bcl-2 staining pattern in relation to tumor stage of Pleomorphic adenomas. The (P_{53} negative/ bcl-2 negative) patterns appeared with relatively high frequency in Pleomorphic adenomas with stage I-II (62.5%) , whereas (P_{53} positive/ bcl-2 negative) and /or (P_{53} negative/ bcl-2 positive) pattern have a

low frequency with stage I-II (12.5%) and relatively moderate frequency with stage III-IV (50%).

P_{53} positive/ bcl-2 positive pattern exhibit itself with low frequency in Pleomorphic adenomas with stage I-II (25%) and completely absent in stage III-IV (0%).However, a non significant association between the combined P_{53}/bcl-2 immuno expression patterns with tumor stage was seen (Table 2).

All malignant salivary glands tumors were diagnosed as P_{53} positive, the immuno staining intensity ranged from 0-76% with mean of 25± 6.5%. The P_{53} immuno reactive cells were evenly distributed in all positive cases. The highest mean of P_{53} expression was belonged to Polymorphous low grade adenocarcinoma (63%), followed by Muco epidermoid carcinoma (35%), then Adenoid cystic carcinoma (14%) & lastly was the Carcinoma ex Pleomorphic adenoma.

For bcl-2 expression, ten cases (83.33%) of malignant tumors were bcl-2 positive and 2 cases (16.66%) were negative. The immunostaining intensity ranged from (0-90%) with a mean of 32±6.7%. The highest bcl-2 expression was detected in Adenoid cystic carcinoma, followed by Polymorphous low grade adenocarcinoma and the lowest expression was detected on Carcinoma ex pleomorphic adenoma and Muco epidermoid carcinoma (Table3).

Table (4) demonstrates the combined P_{53}/bcl-2 pattern in relation to tumor grade, nodal status and TNM staging of malignant salivary gland tumor samples. P_{53} positive/ bcl-2 positive pattern appeared in 6 cases (50%) with high tumor grade and negative N status. The TNM staging was at stage III-IV. However, a non significant association between the combined P_{53}/bcl-2 immuno expression patterns with tumor grade, nodal stage and TNM staging was seen.

Discussion

Pleomorphic adenoma is the most common benign salivary glands tumors with well known clinical and microscopic characteristics [11]. The present investigation indicates that P_{53} was negative for about 70% of cases. Most studies that done on studying P_{53} expression among Pleomorphic adenoma proved negative P53 expression in the tumor cells [10,12,13&14].

Pleomorphic adenoma tends to have negative or low P_{53} expression when compared with malignant counterpart, indicating a low frequency of mutational events of P_{53} gene. The positively of P_{53} in three cases may be related to the accumulated mutations that affect this gene due to prolonged time of onset of this tumor in salivary glands. It is interesting that "*high P_{53} immuno expression in some Pleomorhic adenoma cases may harbor for malignant transformation to carcinoma ex pleomorphic adenoma especially when the benign lesion stay for a long period of time with no treatment*".

The negative bcl-2 expression in about 2/3 of Pleomorphic adenoma cases was disagreed with other studies [10, 14] they demonstrated a high bcl-2 expression in these tumors.

Ten cases of malignant salivary glands tumors (83.3%) had a late stage of disease, seven cases were at tumor stage III & three cases were ate stage IV. The reason that makes these tumors presented in late stage may be due to patient delay in seeking treatment due to painless nature of these tumors at their early stages. Immunohistochemical detection of the P_{53} in Carcinoma ex Pleomorphic adenoma was seen in all cases with a

mean of 8.5% expression (figure 1). In larger series reported in the literatures, the positive P_{53} rating from 50-75% for this tumor [15, 16]. Generally, P_{53} immuno reactivity was associated with larger tumor size, higher grade and greater extent of invasion. The presence of P_{53} protein over expression in malignant mixed tumor and the absence or very low expression in Pleomorphic adenoma has led to hypothesize that P_{53} mutation is an early event in the malignant transformation of Pleomorphic adenoma.

Immunohistochemical expression of the bcl-2 in Carcinoma ex Pleomorphic adenoma was present in half of cases with a mean of 24 ± 6.7% (figure 2) which was in accordance of Andreadis et al [17] study. This finding supports the idea of multi potential capacity of cell proliferation of this type of tumor especially when associated with P_{53} abnormalities. The accumulation of bcl-2 may be one of the sequences of P_{53} mutation and expression.

P_{53} expression was obvious in all Mucoepidermoid carcinomas that lie in stage III and in one case with positive regional lymph node (figure 3); all have had the disease with a mean of five months duration. . The expression of bcl-2 in Mucoepidermoid carcinoma has an average of 24% in 67% of cases(figure4) .This result may accuse P_{53} and bcl-2 to be responsible for progression and aggressiveness of Mucoepiermoid carcinoma, since all the three cases of Mucoepidermoid carcinomas were in stage III and having an intermediate to high histological grade.

Adenoid cystic carcinoma expresses moderate P_{53} activity (figure 5). This immune reactivity was associated with large tumor, high tumor grade and great extent of invasion as suggested by Kiyoshima et al [5] and Perez et al [14]. All Adenoid cystic carcinoma were positive for bcl-2 also (figure 6), the mean bcl-2 immuno expression was generally higher than that of Carcinoma ex Pleomorphic adenoma, Mucoepidermoid carcinoma and adenocarcinomas . This finding is in accordance with Garlifante et al [8] findings, they suggested that bcl-2 alteration may be responsible for recurrence and metastasis especially when associated with P_{53} abnormalities. The interaction between these genes could trigger a mechanism that is able to promote tumor progression.

Similar to that of Carcinoma ex Pleomorphic adenomas, P_{53} and bcl-2 immuno reactivity

was associated with large Polymorphous low grade adenocarcinoma with great extent of invasion, this finding also support the role of these two genes in development of this type of malignant tumor.

Conclusion

1-P_{53} could be used as a marker to differentiate between benign and malignant salivary glands tumors.

2-P_{53} and bcl-2 immune- reactivity were associated with the larger tumors, high histological grades & greater extension of invasion.

3-P_{53} and bcl-2 altered expression may be responsible for the poor prognosis of Adenocystic carcinoma.

4-The association of combined P_{53}/bcl-2 patterns with salivary glands tumors may co operatively provides clinician with exact information to evaluate tumor aggressiveness.

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Table.1. Immuno expression of p53 and Bcl-2 in Benign Salivary Gland Tumors

Tumor type	Immunoexpression				Site	TNM	stage
	P53 (%)		Bcl-2(%)				
PA(myxoid)	(-)	2%	(-)	0%	Hard palate	$T_2N_0M_0$	II
PA(myxoid& adipose)	(-)	2%	(-)	1%	Parotid	$T_2N_0M_0$	II
PA(myxoid)	(-)	0%	(-)	0%	Upper lip	$T_1N_0M_0$	I
PA(myxoid)	(+)	5%	(+)	5%	Parotid	$T_2N_0M_0$	II
PA(myxoid)	(-)	1%	(-)	0%	Hard palate	$T_1N_0M_0$	I
PA(myxoid)	(-)	0%	(-)	1%	Upper lip	$T_1N_0M_0$	I
PA(myxoid)	(++)	25%	(-)	1%	Floor of mouth	$T_1N_0M_0$	I
PA(myxoid)	(-)	0%	(-)	0%	parapharyngeal	$T_3N_0M_0$	III
PA(myxoid)	(-)	4%	(++)	30%	Parotid	$T_3N_0M_0$	III
PA(myxoid)	(+)	6%	(++)	40%	Hard palate	$T_1N_0M_0$	I

Table.2. Relation of combined bcl-2/p53 staining pattern to Tumor stage of benign Salivary Gland Tumors.

Variable	No. of Cases	P53 -ve/ bcl-2 –ve (%)	P53 +ve/ bcl-2 –ve P53 -ve/ bcl-2 +ve (%)	P53 +ve/ bcl-2 +ve (%)
T category				
I-II	8	5 (62.5%)	1 (12.5%)	2 (25%)
III-IV	2	1 (50%)	1 (50%)	0 (0.0%)
x^2 =2.04				

Sex	Age	Type of Tumor	Histologic Grade	Site	duration	Mean size	TNM	TNM stage	P_{53} (%)	Bcl-2 (%)
M	44	CXPA (AD)	Low	Palate	17 yrs	3	$T_3N_0M_0V_1$	III	7% +	3% -
F	70	CXPA (AC)	High	Para – pharyngeal	10 months	4.5	$T_3N_1M_0$	III	10% mean 8.5% ±	45% ++ Mean 24% ±
F	60	MEC (cystic)	Intermediate	Floor of the mouth	1 month	4.5	$T_3N_0M_0$	III	15% ++	3% -
F	70	MEC (solid)	High	Palate	2 month	4.5	$T_3N_0M_0$	III	30% ++	30% ++
F	70	MEC (solid)	High	Palate	1 year	4	$T_3N_1M_0$	III	60% mean 35%±	40% ++ mean 24%±
M	28	ACC (cribriform)	Moderate	Alveolar ridge	1 year	3	$T_2N_0M_0$	II	10% +	25%++
M	31	ACC (cribriform)	High	Palate	3 yrs	5	$T_3N_0M_0$	III	7% +	30% ++
M	31	ACC (cribriform)	High	Palate	2 yrs	4.5	$T_3N_0M_0$	III	8% +	90%+++
M	35	ACC	High	Palate	2 yrs	7	$T_{4a}N_0M_0$	IV	25% ++	50%++
M	38	ACC	Moderate	Cheek	1 year	3	$T_1N_0M_0$	I	25% ++ mean 14%±	35%
F	15	AC	Low	Alveolar ridge	1 year	3.5	$T_2N_0M_0$	II	50% ++	20%+

M	68	AC	Low	Parotid	2 yrs	3	$T_{4a}N_1M_0$	IV	76% ++ mean 63%±	50% mean 35%±
				Overall mean			25%±6.5		32%±6.7	

Table.3.The clinicopathological data and immunohistochemical findings of malignant tumors of salivary gland of 12 patients

Table.4. Relation of Combined Bcl-2/P53 Staining Patterns to Tumor Grade, TNM staging and N status of Malignant Salivary Gland Tumors.

Variable	**No. of Cases**	**P53+/Bcl-2- P53-/Bcl-2+**	**P53+/Bcl-2+**
High	6	0 (0.0%)	6 (50 %)
Moderate	3	1 (8.33%)	2 (16.66%)
Low	3	1 (8.33%)	2 (16.66%)
x^2 =2.4			
Positive	3	0 (0.0%)	3 (25 %)
Negative	9	2 (16.66 %)	7 (58.33%)
x^2 = 0.99			
I + II	3	0 (0.0%)	3 (25%)
III + IV	9	2 (16.66%)	7 (58.33%)
x^2 = 0.99			

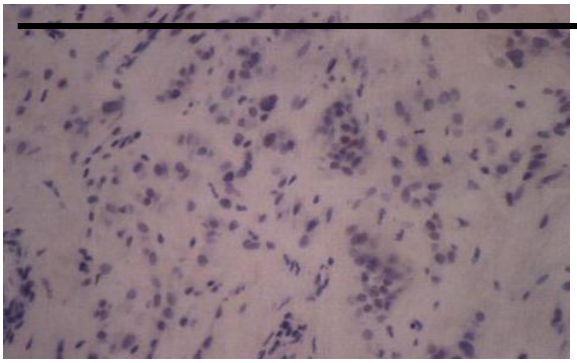


Figure ١: The expression of p53 in Carcinoma ex pleomorphic adenoma (positive expression; score 2) X40 magnification

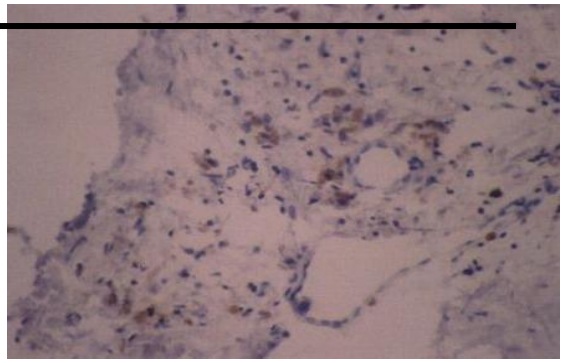


Figure ٢: The expression of bcl-2 in Carcinoma ex pleomorphic adenoma (positive expression; score 2) X40 magnification

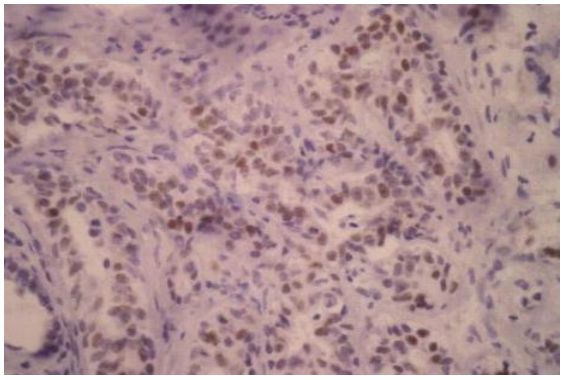


Figure ٣: The expression of p53 in Mucoepidermoid carcinoma (positive expression; score 3) X40 magnification

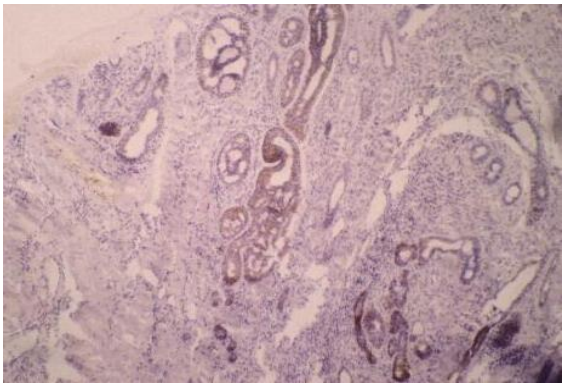
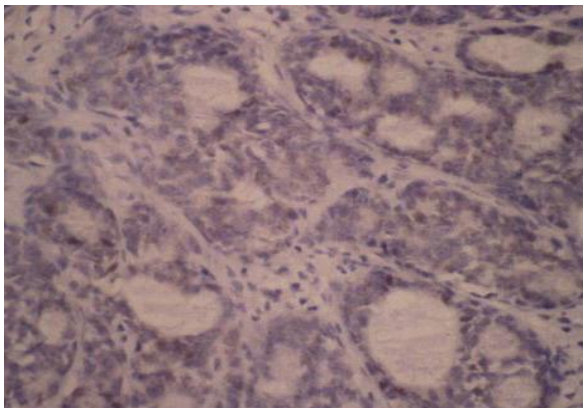


Figure ٤ : The expression of bcl-2 in Mucoepidermoid carcinoma (positive expression; score 3) X10 magnification



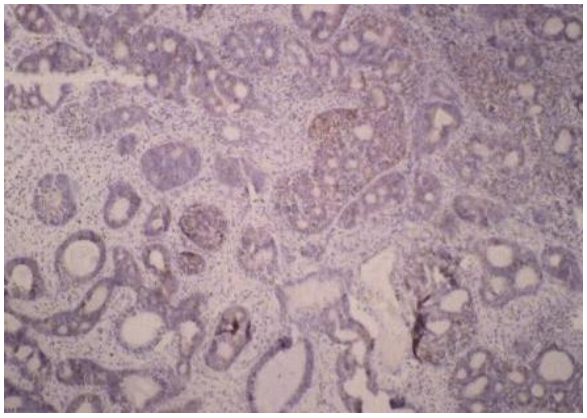


Figure ٥: The expression of p53 in Adenoid cystic carcinoma (positive expression; score 2) X40 magnification

Figure ٦: The expression of bcl-2 in Adenoid cystic carcinoma (positive expression; score 3) X10 magnification