Evaluation of Bax *In situ* hybridization and PCNA Immunohistochemical Expression in Adenoid Cystic Carcinoma

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Abstract

Background: Adenoid cystic carcinoma constitutes the second common malignant epithelial salivary gland tumour.

Objective: To evaluate the expression of Bax mRNA and PCNA and to correlate between the clinicopathological features of Adenoid cystic carcinoma with expressions of Bax mRNA and PCNA protein.

Materials and Methods: Nineteen formalin fixed paraffin embedded tissue blocks of Adenoid cystic carcinoma were used in this study. Bax mRNA gene was analysed by in situ hybridization techniques and PCNA was examined by immunohistochemistry.

Results: *In situ* hybridization detection show low expression of Bax and was noted in 10 cases in Adenoid cystic carcinoma cases (52.63%), Immunohistochemical analysis show high expression of PCNA and was noted in 15 of 19 Adenoid cystic carcinoma cases (78.95 %). No significant relationship was demonstrated between Bax mRNA or the immunostaining PCNA and the morphological growth pattern or patient clinical profile. However, lymph node involvement showed statistically significant differences. Positive significant correlation was found between Bax mRNA and PCNA in Adenoid cystic carcinoma.

Conclusion: The decreased expression of bax in Adenoid cystic carcinoma and high proliferative rate could explain the natural course of these tumours indicate that loss of bax expression might give the tumour cells a double growth advantage because uncontrolled proliferation is combined with reduce cell death rate.

Key words: adenoid cystic carcinoma, Bax, epithelial salivary gland tumour.

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Introduction

Adenoid cystic carcinoma (ACC) is a common salivary gland cancer subtype accounting for 22% of salivary gland malignancies and 1% of all head and neck cancers [1, 2]. Slow growth with frequent local recurrences, perineural invasion and delayed onset of hematogenous are characteristics of this cancer [3, 4]. Adenoid cystic carcinoma is histologically characterized by cribriform pattern, tubular and solid patterns are also recognized, and is composed of epithelial and myoepithelial cells [5]. Adenoid cystic carcinoma was originally described by Lorain and Laboulbene in 1853 [6, 7].

The Bcl-2 gene family seems to act as a regulator of the apoptotic pathway. The two most important apoptosis regulating proteins of this family are most likely Bcl-2 and Bax (Bax is a member of the proapototic family; proapoptotic Bcl- 2 -

associated X protein). Loss of function mutations have been identified in the Bax gene of human tumours. The expression of mutated Bax protein may fail to release cytochrome c and increase the Bax-Bcl-2 ratio resulting in the escape from programmed cell death [8 - 10]. Because the programmed cell death function of Bax is important to counteract tumour growth, they hypothesized that changed Bax expression from normal tissue to primary tumours and metastases may provide additional information rather than only considering Bax protein expression in primary tumours [11]. Thus, they addressed the biologic and clinical significance of changed expression from normal mucosa to primary tumours and metastases related to patients' gender, age, tumour location, histologic stage, growth pattern, differentiation, and prognosis [8, 12-15].

The complexity of PCNA functions are reflected by the history of its discovery and subsequent investigation. This protein was identified over 30 years ago as an antigen for an autoimmune disease in the serum of patients with systemic lupus erythematosus. Two years later, another group found a 36-kDa protein that was differentially expressed during the cell cycle and named it 'cyclin'. Later, it was shown that expression levels of PCNA are associated with proliferation or neoplastic transformation [16-19]. The control of DNA replication is a key element in the proper functioning of a cell, and it may influence genome stability [19-22]. PCNA a potential anticancer target forms a homotrimer and is required for DNA replication and numerous other cellular processes. PCNA is synthesized in all stages of the cell cycle with a half-life of approximately 20 h and is elevated in early S phase to support cell cycle progression [20, 23]. Tumour cells, regardless of their origins, express higher levels of PCNA. A consistent feature in many studies is the positive correlation or association between apoptosis and proliferation [23, 24].

This study aimed to determine the expression of the Bax mRNA using in situ hybridization technique and expression of PCNA immunohistochemically and to correlate between the clinicopathological features of ACC with the expressions of Bax mRNA and PCNA protein.

Materials and Methods

Sample collection

Archival formalin-fixed, paraffin-embedded tissue blocks of 19 cases of salivary gland tumours diagnosed as ACC were obtained from the department of oral and maxillofacial Pathology, College of Dentistry – University of Baghdad and other centres in Baghdad dated from (1998- 2006).

Sample processing

Sections of 5 µm thick of the paraffin embedded tissue were cut and stained with haematoxylin and eosin for histopathological examination. The histological types of ACC diagnosed and an estimate was made of the percentage of each tumour depend on growth pattern into three types: cribriform, tubular and solid patterns (the case categorized by their predominant growth patterns, although two or more patterns can coexist in a single tumour) according to scheme of Szanto et al. (1984) [25]. Another 5 µm paraffin sections were cut and mounted on coated glass slides for in situ hybridization and immunohistochemical analysis.

In situ hybridization staining method

The section were deparaffinised in xylene and rehydrated in descending alcohol series then the tissue slides were pre-treatment solution (citric buffer) was heated in a beaker on a hot plate at 98°C. to prevent buffer from evaporating, the beaker was covered with a glass cover. Slides then transferred immediately to deionized water at room temperature and wash three times, 2 minutes

each. To each tissue section, 2-3 drops of freshly diluted 1x protinase K solution were applied. Then slides were incubated at 37°C for 10-15 minutes. Slides were dehydrated and dried by incubating them at 37°C for 5 minutes then hybridization and detection by one drop of the working DNA probe/hybridization solution was added on the tissue section. Place a cover slip over each slide. The slides were placed in an oven at 70°C for 10 minutes to denature the secondary structure of RNA. The slides were removed in a humid chamber and incubated at 37°C for 24 hours, then soaked in 1x detergent wash at 37°C until the cover slips fall off. One to two drops of RNAase (15 ng/ml) were placed on tissue section. Then slides were incubated at 37°C for 30 minutes. Slides were washed with protein block (pre-warmed) at 37°C for 3 minutes; three times. Then 1-2 drops of conjugate were added to tissue section and slides were kept in a humid chamber at 37°C for 20 minutes final slides were incubated at room temperature (25-37Cº) for about 10 minutes, or until colour development. Colour development was monitored by viewing the slides under the microscope. A blue colored precipitate formed at the site of the probe in positive cells. Colour appeared after 3-5 minutes, usually reached sufficient development after 10 minutes. Slides were counterstained using nuclear fast red. Slides were washed well in running tap water for 5 minutes, and then dehydrated by serial ethanol and xylene and mounted with (DPX) finally the examination and scoring were done.

Blue coloured precipitate at the site of the probe in positive cells and the back ground of red stain belong to the counter stain (nuclear fast red) were noticed. Five high power fields (HPF) 400X were evaluated for localization percentage of positive cells by counting 1000 cells (0% to 100% in 5% steps), the percentage of positively stained cells distributed as follows; 0 (-), <25% (+), 25% - 75% (++), and >75% (+++), by using double blind scoring method [12, 14, 15].

Immunohistochemical method

Antigen retrieval was done using citrate buffer (pH 6.0) by microwave digestion. Endogenous peroxidase was blocked with 0.05% hydrogen peroxide for 30 min. After incubation with a 1:20 dilution of normal horse serum to reduce non-specific binding, the slides were incubated overnight at 48C with primary antibodies against PCNA (Dako-patts, PC-10, 1: 50). Secondary antibodies associated with a streptavidin–biotin-peroxidase method was used (Dako A/S, Strept AB Complex Duet, mouse/rabbit), complemented with diaminobenzidine as the chromogen. All slides were counterstained with hematoxylin. After each step the sections were washed with phosphate buffered saline. Negative controls sections were obtained using non-immune serum instead of the primary antibody. Samples of squamous cell carcinoma were used as positive control. Immunoreactivity was classified as: (-) negative ≤5%, (+) low 6–25%, (++) moderate 26–50% and (+++) high >50% of positive tumour cells, counting at least 1000 cells at high magnification (40x objective and 10x eyepiece). The quantitative analysis of PCNA positive cells were counted by two independent examiners. The PCNA labelling index, (LI) value (number of positive tumour cells/total number of tumour cells expressed as a percentage) was calculated in every case [26].

Statistical Analysis

The results were analyzed using SPSS (16) statistical software. Frequency and percentage was calculated for each parameter. The relationship among PCNA, Bax expression and clinicopathological types and histologic grade were assessed using Chi – square test. Mean ± S.D was calculated for

age and labelling index of PCNA protein. Statistical significance of differences was analyzed by using one - way analysis of variance (ANOVA). The Spearman correlation coefficient (r) was used between PCNA protein and Bax mRNA gene in ACC cases. The $p \leq 0.05$ was considered statistically significant.

Results

The mean age of ACC was mainly occurring in age above 40 years (47 ± 16.34) and gender distributions were nearly equal ratio males/ females. Site distribution of the cases occur mainly in minor salivary gland, hard palate was the site involved with tumour (8 cases; 42.11 %). The clinical staging showed that in ACC equal number of cases were noticed in tumour size (2 – 4) cm T2 and T3 (7 cases 36.84%). Lymph node involvement was located in two in cases of adenoid cystic carcinoma. The histological grading categorised according to the growth pattern into 9 cases (47.4 %) of cribriform, 6 cases (31.6 %) of tubular and 4 cases (21%) of solid growth. Nerve involvement with adenoid cystic carcinoma was histologically diagnosed in two cases (table 1).

The analysis of Bax mRNA in 19 case with ACC revealed 10 cases (52.63%) were positive (4 ++, 6 +) and 9 cases were negative. The staining intensity result was 4 shown strong intensity and 6 cases of weak staining intensity of Bax mRNA expression (figure-1). The positive Bax mRNA expression within cases of the cribriform histological pattern were expressed in 4 cases in parotid gland, the 3 cases of tubular histological pattern were present in minor salivary gland, and finally single case of solid histological pattern of ACC was present in the submandibular gland. Nodal involvement was detected in cribriform positively for Bax mRNA (table 1).

Table (1): Frequency and percentage of positively PCNA protein IHC and Bax m RNA ISH analysis according to clinicopathological finding of ACC

Variables	ACC Cases			
	No. (%)	PCNA	Bax mRNA	P- value
Age M ± S.D.	19 47 ± 16.34	15 45.66 ± 18.45	10 44. 33± 12.58	
Gender Male Female	 11 57.89 8 42.11	 8 53.33 7 46. 67	 5 50 5 50	NS
Site - Major Parotid Submandibular Minor Palate Hard Soft Buccal mucosa	 4 21.05 1 5.263 8 42.11 4 21.05 2 10.53	 4 26.67 1 6.67 7 46.66 2 13.33 1 6.67	 4 40 1 10 2 20 2 20 1 10	NS
Tumour size T_1 T_2 T_3 T_4	 3 15.79 7 36.84 7 36.84 2 10.53	 2 13.33 5 33.34 6 40 2 13.33	 3 30 3 30 3 30 1 10	NS
Lymph node - ve + ve	 17 89.47 2 10.53	 13 86.67 2 13.33	 9 90 1 10	S (0.03)
Clinical stage I II III IV	 3 15.79 7 36.84 7 36.84 2 10.53	 2 13.33 5 33. 34 6 40 2 13.33	 3 30 3 30 3 30 1 10	NS
Histological grading cribriform tubular solid	 10 52.6 6 31.6 3 15.8	 6 40 6 40 3 20	 6 60 3 30 1 10	NS

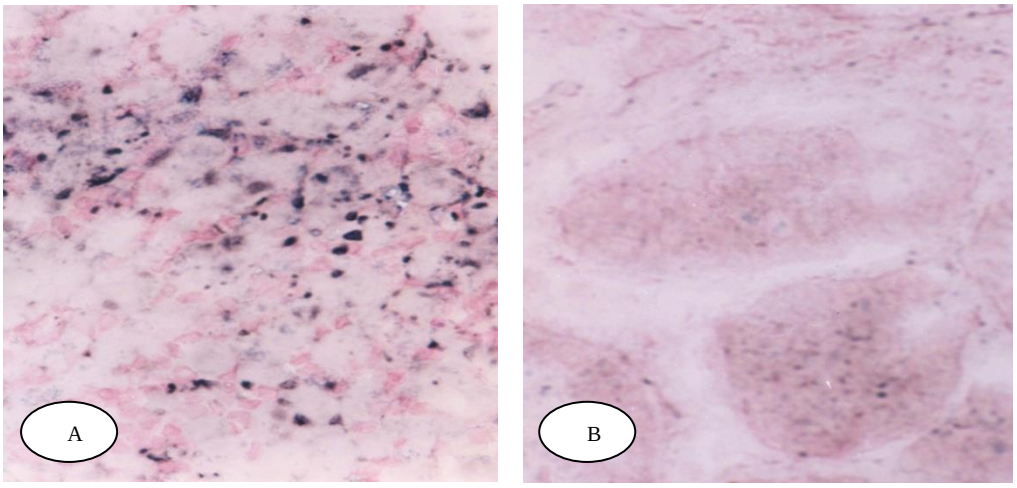
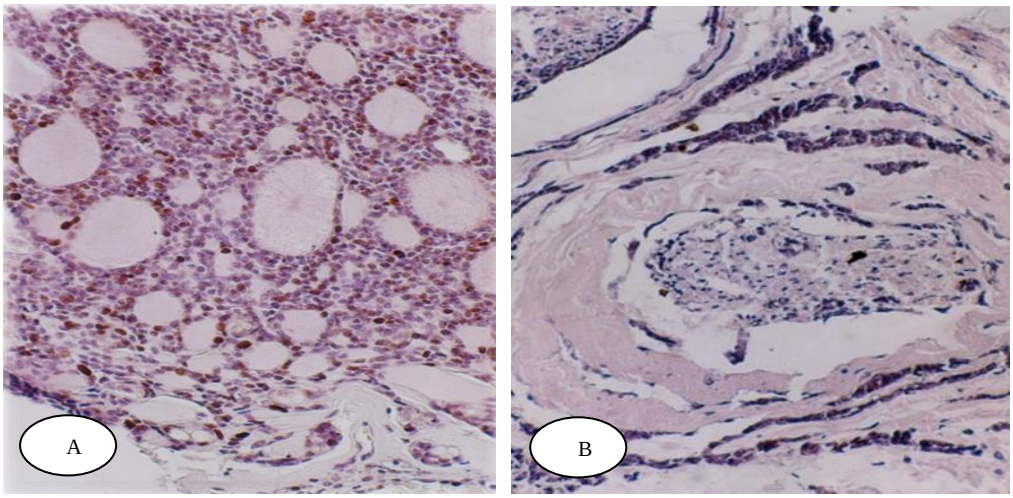


Figure (1): Expression of Bax mRNA gene by *in situ* hybridization in ACC, A- Cribriform growth pattern, B. Solid growth pattern (at 40x magnification), black - blue spots the localization of Bax mRNA gene.

Cells labeled by the PCNA antibody display a staining almost entirely confined to the nucleus with a diffuse or granular pattern or both (figure - 2). The analysis of 19 cases with ACC revealed 15 cases (78.95 %) (4 +++, 7 ++, 4 +) and 4 cases were negative. The staining intensity result were 3 shown strong intensity, 5 cases of moderate and 7 cases of weak staining intensity of PCNA protein expression. The positive PCNA protein expression cases of the cribriform histological types of adenoid cystic carcinoma were frequent in 4 cases in parotid gland and two cases in hard palate. However the hard palate was the frequent site of the primary tumour location in the tubular and solid histological types of adenoid cystic carcinoma, only one case for both cribriform and solid with lymph node involvement was positive for PCNA expression. The results of this study showed that PCNA expression increased as tissues progressed from cribriform to tubular and solid growth pattern of ACC. No statistically significant differences were found in ACC cases with regard to clinical features, morphological growth patterns and ISH of Bax mRNA, immunohistochemical reactivity of PCNA (table 1). However, a significant difference was observed for lymph node ($p = 0.03$).



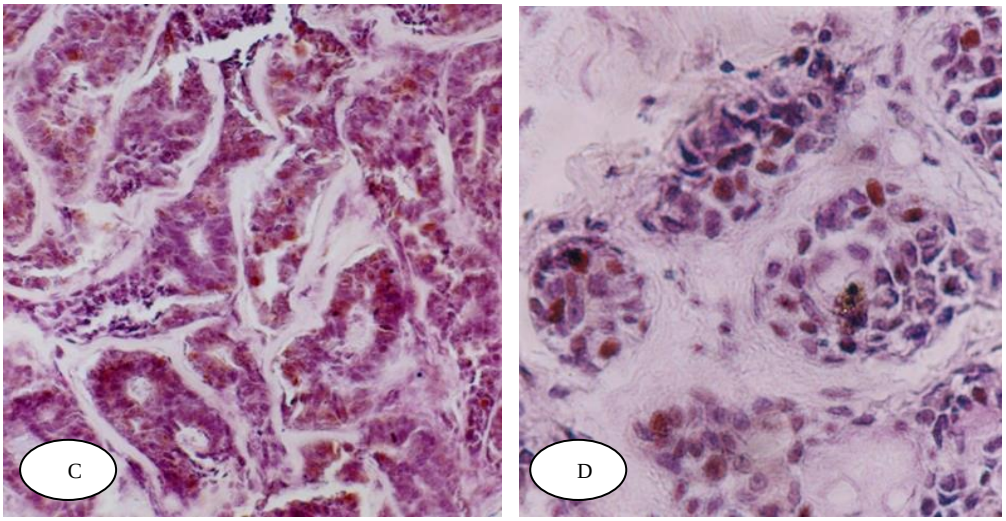


Figure (2): Expression of PCNA in ACC, A. Cribriform growth pattern, B. Neural infiltration (at 20x magnification), C and D. Tubular and solid growth pattern (at 40x magnification).

Figure (3) summerized the cases of ACC that showed the positive and negative cases of both PCNA protein and Bax mRNA expression. A positive correlation was found between PCNA protein and Bax mRNA of ACC cases (r = 0.444). The relation of PCNA LI (The labelling index "mean value ± SD." of positive expression of PCNA) and Bax staining rate to site showed a weak positive correlation (table 2). There is a tendency to increase the proliferative LI of positive expression of PCNA was observed in cases of ACC with positive cases of Bax expression (table 3).

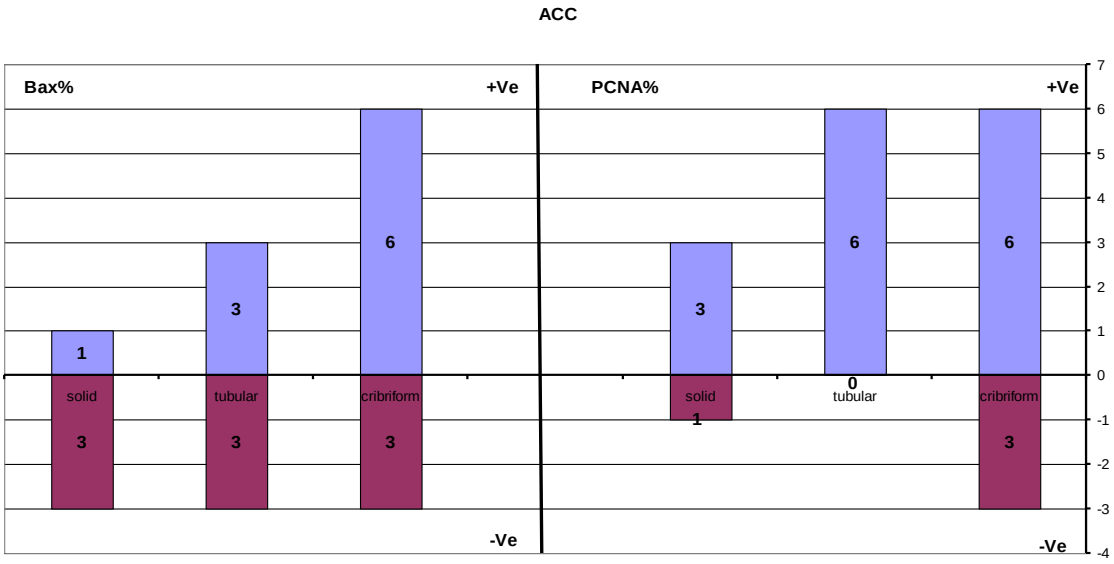


Figure (3): Histogram showed number of adenoid cystic carcinoma cases according to histologic grading in relation to immunostaining of PCNA and Bax mRNA expression (positive and negative cases)

Table (2): Coefficient of association correlation (*r*) between positive PCNA protein and positive Bax mRNA cases of ACC in relation to site

Variables	Site	Mean of + ve PCNA cases	Mean of + ve Bax cases	*r = coefficient of association*
ACC	*Major* *Minor*	45.2 34.61	37.66 17	0.258

Table (3): Labelling index of positive PCNA protein versus positive Bax mRNA cases of ACC

Variables No. of cases	Bax mRNA No. of cases	PCNA Labelling index (Mean ± SD.)
ACC 19	- ve (9) + ve (10)	32.48 ± 19.9 41.9 ± 18.2

Discussion

Adenoid cystic carcinoma is a form of malignant neoplasm that arises most commonly in the major and minor salivary glands of the head and neck. It is often slow to metastasize, but has a persistent and relentless growth with a poor long term prognosis. Major and minor salivary glands including seromucinous glands are the frequent sites of occurrence [2, 3, 6, 7]. The histological picture is variable which determine the prognosis with cribriform being the most common and easily recognized pattern and solid the least common histopathologic subtype, the results of this study as well as others reports showed that the cribriform growth pattern variant is the most common type representing 52.6% of the cases [1, 3, 5].

The cell proliferation / death balance is of the important in the development of salivary gland tumour. The bcl-2 families has been shown to play an important role in the regulation of apoptosis and modulation of cell cycle regulating proteins illustrating the crosstalk in mechanisms controlling cell death and proliferation [10]. The expression of Bax mRNA in ACC has, to our knowledge, not been studied, and the authors could not find comparable studies in the literatures. The expression of this study was observed in ACC irrespective of morphological growth pattern and suggests that loss of Bax expression may play a role in the development of epithelial malignant salivary gland tumours. It has been shown that reduced Bax mRNA and protein expression correlates with tumour progression in head and neck cancer [8, 28 - 30], esophageal and basaloid squamous cell carcinomas [12, 16], salivary gland tumours [14, 15]. The finding of this study is in accordance with results from previous findings [8, 12, 14-16, 27-29] and contradict report suggesting that there is no correlation [30] , the different results may be explained by different methods regarding evaluation of Bax expression using immunohistochemical technique in ACC while this study conducted on using Bax mRNA ISH. The studied method is based on both the percentages of positive tumour cells and staining intensity and allows a more detailed in evaluation and its reproducibility is acceptable.

In PCNA - positive long - lived cells, the probability of accumulation of genetic abnormalities necessary for malignant transformation is increased and plays an important role in epithelial tumour development. The IHC demonstration of PCNA allows an estimation of the growth

fraction in tumour tissue and PCNA can be detected in cells that have recently left the cell cycle which is caused by the prolonged half – life of this protein [20, 21]. The results of this study showed that PCNA expression increased as tissues progressed from cribriform to tubular and solid growth pattern of ACC indicate that PCNA immunostaining can help to estimate the histological grade of malignant morphological growth pattern. In addition, the two cases of ACC with neural invasion were positive to PCNA expression. PCNA has been used as a proliferation marker in different neoplasms in relation to clinical behaviour and the result of this study is similar to previous reports [4, 15, 26, 31 - 33].

The enhancement of cell proliferation and promotion of cell survival via the inhibition of apoptosis is thought to be the key to the initiation and progression of cancers. Either inactivation of pro – apoptotic pathway or activation of antiapoptotic pathway results in failure of apoptosis. This study found a positive strong association between PCNA protein and Bax expression in positive cases, scoring and intensity of expression in ACC and a low – grade malignancy of epithelial malignant salivary gland tumours that conducted in this study. PCNA positivity rate was high in strongly Bax positive group, in ACC, indicating a possible positive correlation between proliferation and death balance of the cell and suggest that proliferative rate increase in absence of apoptotic pathway activation.

In conclusion: The high proliferative rate could explain the natural course of ACC and the decreased expression of Bax in salivary gland tumours indicate that loss of Bax expression might give the tumour cells a double growth advantage because uncontrolled proliferation is combined with reduce cell death rate. The interaction may trigger a multistep process which is able to promote and may play a role in salivary gland tumourgenesis, possibly by inhibiting the apoptosis mediated by Bax.

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References

[1] Jaso J, Malhotra R. Adenoid cystic carcinoma. Arch Pathol Lab Med 2011; 135: 511-515.

[2] Malhotra KB, Agrawal V, PandeyR. High Grade Transformation in Adenoid Cystic Carcinoma of the Parotid: Report of a Case with Cytologic, Histologic and Immunohistochemical Study. Head and Neck Path 2009; 3: 310-314.

[3] Ashkavandi Z, Ashraf M, Moshaverinia M. Salivary Gland Tumors: A Clinico-pathologic Study of 366 Cases in Southern Iran. Asian Pacific J Cancer Prev, 2013; 14: 27-30.

[4] Terada T:.Adenoid cystic carcinoma of the oral cavity: immunohistochemical study of four cases Int J Clin Exp Pathol 2013; 6: 932-938.

[5] El-Naggar AK, Huvos AG. Adenoid cystic carcinoma. In: Barnes L, Eveson JW, Reichart P, Sidransky D (eds). World Health Organization Classification of tumours. Pathology and genetics of head and neck tumours. Lyon: IARC Press, 2005. pp: 221-222.

[6] Ajila V, Hegde S, Gopakumar N, Babu S. Adenoid cystic carcinoma of the buccal mucosa: A case report and review of the literature. Dent Res J 2012; 9: 642- 6.

[7] Mehta DN, ParikhSJ: Adenoid cystic carcinoma of palate. J Nat Sci Biol Med. 2013; 4: 249-252.

[8] Tano T, Okamoto M, Kan S, Nakashiro S, Shimodaira S, Koido S *et al.* Prognostic Impact of Expression of Bcl-2 and Bax Genes in Circulating Immune Cells Derived from Patients with Head and Neck Carcinoma. Neoplasia 2013; 15: 305-315.

[9] Adams JM and Cory S. The Bcl-2 protein family: arbiters of cell survival. Science 1998; 281: 1322 - 6.
[10] Cotter TG. Apoptosis and cancer: the genesis of a research field. Nat Rev Cancer. 2009; 9: 501-507.
[11] Castillo K, Rojas-Rivero D, Lisbona F, Caballero B, Nassif M, Court S. BAX inhibitor-1 regulates autophagy by controlling the IRE1a branch of the unfolded protein response. The EMBO J 2011; 30: 4465-4478.
[12] Isrid S, Henrik P, Roland V, Matthias L, Silke R, Timo H, *etal.* Analysis of p53/Bax/p16ink4a/ CDKN2 in esophageal squamous cell carcinoma: high Bax and p16ink4a/CDKN2 identifies patients with good prognosis. J Clin Oncol 2001; 19: 2272 - 2281.
[13] Gomes C, Bernardes V, Diniz M, Marco L, Gomez R. Anti-apoptotic gene transcription signature of salivary gland neoplasms. BMC Cancer 2012, 12: 61 -6.
[14] Xie X, Nordgård S, Clausen OP, Boysen M. Prognostic Significance of Bax and Bcl-2 Expressions in Adenoid Cystic Carcinoma of Major and Minor Salivary Glands of Nasal and Oral Epithelium. The Open Otorhinolaryngology Journal, 2010, 4: 20-26.
[15] Hussain QA and Al - Azzawi LM. Bax *in situ* hybridization and proliferating cell nuclearantigen immunohistochemical expressions in salivary gland tumours. J Bagh Coll Dentistry 2014; 26: 112-120.
[16] Miyachi K, Fritzler MJ, Tan EM. Autoantibody to a nuclear antigen in proliferating cells The Journal of Immunology 1978; 121: 2228–2234.
[17] Bravo R and Celis JE. A search for differential polypeptide synthesis throughout the cell cycle of HeLa cells. The Journal of Cell Biology. 1980; 84: 795 -802.
[18] Bravo R, Fey SJ, Bellatin J, Larsen PM and Celis JE. Identification of nuclear polypeptide ('cyclin') whose relative proportion is sensitive to changes in the rate of cell proliferation and to transformation. Progress in Clinical and Biological Research 1982; 85 (part A): 235–248.
[19] Strzalka W and Ziemienowicz A. Proliferating cell nuclear antigen (PCNA): a key factor in DNA replication and cell cycle regulation. Annals of Botany, 2011, 107: 1127-1140.
[20] Tan Z, Wortman M, Dillehay K, Seibel W, Evelyn C and Smith S. Small-Molecule targeting of proliferating cell nuclear antigen chromatin association inhibits tumor cell growth. Mol Pharmacol 2012; 81: 811–819.
[21] Waga S and Stillman B. The DNA replication fork in eukaryotic cells. Annual Review of Biochemistry 1998; 67: 721–751.
[22] Al-Azzawi L, Al-Talabani N and Al-Salihi A. DNA histochemical analysis in oral squamous cell carcinoma. Archives of oral Biology 2006; 51: 64 - 68.
[23] Bravo R and Macdonald-Bravo H. Existence of two populations of cyclin / proliferating cell nuclear antigen during the cell cycle: association with DNA replication sites. J Cell Biol 1987; 105: 1549-1554.
[24] Hetz C and Glimcher L. The daily job of night killers: alternative roles of the BCL-2 family in organelle physiology. Trends Cell Biol 2008; 18: 38–44.
[25] Szanto PA, Luna MA, Tortoledo E and White RA. Histologic grading of adenoid cystic carcinoma of the salivary glands. Cancer 1984; 54: 1062 – 1069.
[26] Ogawa I, Miyachi M, Takata T, Vuhahula E, I juhin N and Nikal H. Proliferative activity of salivary gland pleomorphic adenoma and myoepithelioma as evaluation by the proliferating cell nuclear antigen (PCNA) labeling index (LI): Oral Pathol Med 1993; 22: 447-50.
[27] Sampaio-Go´ es FC, Oliveira DT, Dorta RG, Nonogaki S, Landman G, Nishimoto IN, *et al*. Expression of pcna, p53, bax, and bcl-x in oral poorly differentiated and basaloid

squamous cell carcinoma: relationships with prognosis. Head Neck 2005; 27: 982– 989.
[28] Xie X, Clausen OPF, De Angelis P and Boysen M. Prognostic value of spontaneous apoptosis, Bax, Bcl-2, and p53 in tongue squamous cell carcinoma. Cancer 1999; 86: 913-20.
[29] Bandoh N, Hayashi T, Kishibe K, *et al* Prognostic value of p53 mutations, bax, and spontaneous apoptosis in maxillary sinus squamous cell carcinoma. Cancer 2002; 94: 1968-80.
[30] Jia L, Esguerra R, L, Tang X, *et al*. Prognostic value of apoptosis and apoptosis-associated proteins in salivary gland adenoid cystic carcinoma. Pathol Int 2004; 54(4): 217-23.
[31] Alves FA, Pires FR, De Almeida OP, Lopes MA and Kowalski LP .PCNA, Ki-67 and p53 expressions in submandibular salivary gland tumors. Int J Oral Maxillofa Surg 2004; 33: 593-597.
[32] Nagao T, Sato E, Inoue R, Oshiro H, Takahashi RH, Nagai T, *et al*. Immunohistochemical Analysis of salivary gland tumors: Application for Surgical Pathology Practice. Acta Histo chem. Cytochem. 2012; 45: 269-282.
[33] Lee S, Kwon M, Lee Y, Choi S, Kim SY, Cho K, *et al*. Prognostic value of expression of molecular markers in adenoid cystic cancer of the salivary glands compared with lymph node metastasis: a retrospective study. World Journal of Surgical Oncology 2012, 10: 266 -274.