BAX In situ hybridization and proliferating cell nuclear antigen immunohistochemical expressions in salivary gland tumours

Qutaiba A. Hussain, B.D.S. [1]
Lehadh M. Al- Azzawi, B.D.S., M.Sc., Ph.D. [2]

ABSTRACT

Background: Epithelial salivary gland tumours are relatively uncommon and constitute a wide spectrum of variable morphologic and biologic entities. The cell proliferation / death balance is most important in the development of salivary gland tumours. The aim of this study was to examine the expression of PCNA protein immunohistochemically and Bax mRNA gene using in situ hybridization techniques and to correlate between the clinicopathological features of salivary gland tumours with the expressions of PCNA protein and Bax mRNA.

Materials and Methods: Forty nine formalin fixed paraffin embedded tissue blocks of epithelial salivary gland tumours were used in this study. Haematoxylin and Eosin stain was used for reassessment of the histopathologic diagnosis. The cell proliferation activity was examined by proliferating cell nuclear antigen (PCNA) immunohistochemistry and proapoptotic cell death Bax mRNA gene was analysed by in situ hybridization techniques.

Results: Immunohistochemical analysis show high expression of PCNA and was noted in 8 of 12 pleomorphic adenoma cases (66.67%), 15 of 19 adenoid cystic carcinoma cases (78.95 %), 6 of 7 mucoepidermoid carcinoma cases (85.71%), and 3 of 5 adenocarcinoma case (60 %). Significant difference was found between labeling index of benign and malignant salivary gland tumours, while no significant relationship was noted in labeling index between adenoid cystic carcinoma and mucoepidermoid carcinoma neither between mucoepidermoid carcinoma and adenocarcinoma. In situ hybridization detection show low expression of Bax and was noted in only 3 cases of pleomorphic adenoma cases (25%), 10 cases in adenoid cystic carcinoma cases (52.63 %), however, mucoepidermoid carcinoma showed high expression of these markers than other salivary gland tumours, whereas adenocarcinoma show equal number of cases expressed both PCNA protein and Bax mRNA. No significant relationship was demonstrated between the immunostaining PCNA or Bax and the morphological growth pattern or patient clinical profile. Positive significant correlation was found between PCNA and Bax mRNA in pleomorphic adenoma, adenoid cystic carcinoma, mucoepidermoid carcinoma and adenocarcinoma cases.

Conclusion: The high proliferative rate could explain the natural course of these tumours 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 tumour genesis, possibly by inhibiting the apoptosis mediated by Bax.

Keywords: Mucoepidermoid carcinoma, adenoid cystic carcinoma, pleomorphic adenoma, PCNA, Bax mRNA. **(J Bagh Coll Dentistry 2014; 26(1):112-120).**

INTRODUCTION

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 [1-4].

The control of DNA replication is a key element in the proper functioning of a cell, and it may influence genome stability. DNA replication is regulated mainly at the initiation step as a result of cooperation between different signalling pathways controlling the cell cycle [5,6]. 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 [5, 7]. Tumour cells, regardless of their origins, express higher levels of PCNA. Expression levels of PCNA correlate positively and can serve as an independent prognosis marker [8].

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). 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 [9 - 11]

Because the programmed cell death function of Bax is important to counteract tumour growth, they hypothesized that changed Bax expression

(1) M.Sc. Student, Department of Oral Maxillofacial Pathology, Collage of Dentistry, University of Baghdad.
(2) Assistant professor, Department of Oral Maxillofacial Pathology, Collage of Dentistry, University of Baghdad.

from normal tissue to primary tumours and metastases may provide additional information rather than only considering Bax expression in primary tumours [12]. Thus, they addressed the biologic and clinical significance of changed expression from normal mucosa to primary tumours and metastases related to patients' sex, age, tumour location, histologic stage, growth pattern, differentiation, and prognosis [13,14].

A consistent feature in many studies is the positive correlation or association between apoptosis and proliferation, suggesting that they are mechanistically linked. One link relates to the fact that although apoptosis may be initiated in any phase of the cell cycle, the majority of cells undergo apoptosis primarily in the G1 phase of cycling cells [15,16].

To our knowledge the present study is the first the first describing the expression of bax mRNA in situ hybridization in epithelial salivary gland tumours. PCNA protein and Bax mRNA gene expressions in relation to the clinicopathological profile in epithelial benign and malignant salivary gland tumours have not been reported previously. This study aimed to determine expression of proliferating cell nuclear antigen protein (PCNA) immunohistochemically and the expression of the proapoptotic Bcl – 2 – associated X protein (Bax mRNA) using in situ hybridization technique and to correlate between the clinicopathological features of salivary gland tumours with the expressions of PCNA protein and Bax mRNA.

MATERIALS AND METHODS

Archival formalin – fixed, paraffin – embedded tissue blocks of 49 cases of salivary gland tumors diagnosed as (12) pleomorphic adenoma (PA), (7) mucoepidermoid carcinoma (MEC) and (19) adenoid cystic carcinoma (ACC), (5) cases of adenocarcinoma and two cases of each of acinic cell carcinoma, carcinoma ex pleomorphic adenoma (CXPA) and squamous cell carcinoma (SCC) were obtained from the department of oral and maxillofacial Pathology, Oollege of Dentistry – University of Baghdad and other centers in Baghdad dated from (1998 – 2006).

Sections of 5 µm thick of the paraffin embedded tissue were cut and stained with hematoxylin and eosin for histopathological examination. The MECs were graded according to Auclair et al., 1992 [17]. 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. [18]. Adenocarcinomas were graded to scheme of Auclair et al. [19]. The other malignant types of salivary gland were excluded their grading because of minimal number of cases available during the time of collect the study sample. 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 tissue slides were pretreatment 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. Be careful to avoid trapping any air bubbles and 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 to allow hybridization of the probe with the target nucleic acid, and 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 placed in a humid chamber and incubated at 37°C for 30 minutes.

Slides were washed with protein block (pre-warmed) at 37°C for 3 minutes; three times. Excess buffer from around the tissue section was wiped off carefully. 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 color development was complete. Color development was monitored by viewing the slides under the microscope. A blue colored precipitate formed at the site of the probe in positive cells. Color appeared after 3-5 minutes, usually reached sufficient development after 10 minutes. Slides were counterstained using nuclear fast red.

ISH staining analysis of Bax mRNA

Color and distribution of stain: blue colored 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.

Four 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 [13].

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 cromogen. 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) [20]. Intensity of staining was not considered for evaluation. The quantitative analysis of PCNA positive cells were counted by two independent examiners. The PCNA labeling index, value was reported as the percentage of the tumour cells staining per 1000 cells counted. The incidence of positive cells against the antibody was expressed as the labeling index which is a percentage of the positive tumour cells in the total number of tumour cells counted [21].

Statistical Analysis

Data were analyzed by SPSS software for window 10. 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 X^2 test.

Mean ± S.D. was calculated for age and labeling 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 among salivary gland tumours. The $p \leq 0.05$ was considered statistically significant.

RESULTS

The positive reaction of PCNA protein expression was observed in 8 cases (66.67%) of benign epithelial salivary gland tumours (PA) and 29 cases (78.38%) of malignant tumours. twelve cases of benign PA, the mean labeling index of PCNA protein expression was 26.46±10.76 and range (15−48%) and 37 cases of malignant salivary gland tumour was 37.82 ± 20.12 and range (6.2−80). There was a significant difference between LI of benign and malignant tumours.

Bax mRNA expression give an intense blue – black color at the specific sites of the hybridized probe in positive test tissues and positive control and the background of red stain belong to the counter stain (nuclear fast red) was noticed. The specificity of the ISH signal is reflected by the absence of positive ISH signal in the negative control slides. The positive reaction of Bax mRNA expression was observed in 3 cases (25%) of benign salivary gland and 21 cases (56.76%) of malignant salivary gland. There is a significant difference statistically between benign and malignant cases for positive and negative expression of Bax mRNA. Table (1 and 2) shows the positive cases of PCNA protein and Bax mRNA expression in malignant salivary gland tumours in relation to their histological grading with the clinical findings of these cases.

Figures 1-3 show positive nuclear staining of PCNA protein and figures 4-6 show localization of Bax mRNA gene.

The positive case of acinic cell carcinoma with PCNA protein was located in the palate, and the 2 cases of acinic cell carcinoma were positive (++ for each case) of Bax mRNA expression. The two positive cases of squamous cell carcinoma with PCNA protein located in the tongue and submandibular gland, and one case of the two of squamous cell carcinoma showed positive expression of Bax mRNA. The 2 cases of carcinoma ex pleomorphic adenoma were positive for PCNA protein expression and negative expression of Bax mRNA and located in the soft palate.

The strong positive correlation was found between the PCNA protein and Bax mRNA of PA (r = 0.999), where a positive correlation (r = 0.693) regarding the scoring of positively PCNA protein and Bax mRNA expressed cases.

The relation of tumour location (the major and minor salivary gland sites) between immunopositivity of PCNA and Bax mRNA expression in the epithelial salivary gland tumours were shown in table 3. The LI (mean value ± SD.) of positive expression of PCNA versus Bax staining rate in the epithelial salivary gland

tumour were demonstrated in table 4. A tendency to increase the proliferative LI was observed in cases of PA and ACC with positive cases of Bax expression whereas in MEC the negative cases of Bax expression showed high rate of LI than those with positive expression and the same result was found in adenocarcinoma cases.

DISCUSSION

The epithelial salivary gland tumours are relatively uncommon lesions and salivary gland carcinomas are a heterogeneous group of lesions; represent a great number of diverse entities with histological patterns overlapping between different tumour pathologies complicating their diagnosis.

PCNA protein and Bax mRNA gene expressions in relation to the clinicopathological profile in epithelial benign and malignant salivary gland tumours have not been reported previously. The large number of histological types of salivary gland tumours makes them a very heterogeneous group of neoplasias, most of them rare. The relative frequency of malignant tumours has changed over years, due to the changes in diagnostic criteria and classification. The results of this study had been cited the palate were the most common site for minor salivary gland tumours, of which (20 "40.8 %" from 49 cases) occur in the palatal mucosa, through the figure was lower than that in the literature [22 -26]. Some anatomic sites were prone to the development of certain types of salivary gland tumours. The results of this study showed the parotid gland was the commonest site for benign salivary gland tumours, while the malignant tumours the commonest site were both the parotid and submandibular for major gland and palate for the minor glands.

Histological classification of malignant salivary gland tumours is well established as an important prognostic factor for both ACC and MEC. The three recognized histopathologic patterns of ACC are cribriform, tubular and solid 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 80% of the cases [25, 27]. MEC is considered the most common malignant salivary gland tumours accounting for approximately one third of all salivary malignancies [28,29]. In this study MEC of submandibular glands were cited and being associated more frequently with lymph node. This though is not a constant finding but was shown in this study and this is in accordance with others [20, 22,26,27]. Most cases of MEC conducted in this study were low grade lesions; in accordance with the results of literatures were high percentage (85 %) with low grade MEC, favoring a good prognosis [28].

The cell proliferation / death balance is of the important in the development of salivary gland tumour. 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 [5, 6]. PCNA has been used as a proliferation marker in different neoplasms in relation to clinical behaviour [8].

LI of benign epithelial salivary gland tumours had (26.46 ± 10.76) and increase in the malignant epithelial salivary gland tumours had (37.82 ± 20.1) and reach the level of significant statistically. Indeed, LI of the heterogeneous group of malignant epithelial salivary gland tumours had nearly the same value indicate the consistent aggressive behaviour of all epithelial salivary gland tumours, but MEC showed highest PCNA LI (48.3 ± 26.5). Interestingly, the results of this study showed that PCNA expression increased as tissues progressed from cribriform to tubular and solid growth pattern of ACC and from moderate to poorly differentiated adenocarcinoma indicate that PCNA immunostaining can help to estimate the histological grade of malignant morphological growth pattern. The two cases of ACC with neural invasion were positive to PCNA expression and this result similar to others [29-33].

The bcl – 2 family 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 [11]. It has been shown that reduced Bax mRNA expression correlates with tumour progression in head and neck cancer [9]. The expression of this study was observed in malignant salivary gland tumours irrespective of histological subtypes or morphological growth pattern and suggests that loss of Bax expression may play a role in the development of epithelial salivary gland tumours.

The overexpression of Bax protein as a marker of induce apoptosis had been reported in limited studies to initiate tumour cell to apoptosis in salivary gland tumours using immunohistochemical methods for expression of

Bax protein in MEC of salivary gland, therefore, in this study MEC had a higher frequency of strong Bax expression than ACC and adenocarcinoma of salivary glands, but there was no significant difference and this is accordance with Yin [34] and this colleagues that found Bax protein expression in MEC (97 %) were positive and this is not relate to that Bax expression associate with better survival in MEC because the negative cases were too few to be compared with positive group.

In acinic cell carcinoma, the two cases expressed Bax positive while the cases of CXPA expressed negative Bax indicate that the former is a low – grade of malignancy behaviour and the latter is high – grade and aggressiveness behaviour with high proliferative rate [33].

SCC of salivary gland expressed one negative and another positive Bax indicate this tumour had metaplasia of glandular cell to squamous cell, aggressive behaviour and differ from oral mucosal squamous cell carcinoma which was used as housekeeping, expressed Bax positive, this is due to similarity in histological appearance and differentiation of squamous cell to form cell nest and produce keratin but differ in the etiology and pathogenesis of salivary gland that is prove a heterogeneous group of the salivary gland lesions.

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 both benign and malignant salivary gland tumours and this is due to slowly growing of PA benign tumour [31] and a low – grade malignancy of epithelial malignant salivary gland tumours that conducted in this study.

MEC showed higher expression of these two markers than those of other salivary glands. This could be in part explained by the fact that most of MEC were high grade tumours. While in acinic cell carcinoma had a minimal proliferative activity and active inhibitor activity of Bax was detected in both cases and is convincing evidence with its low grade – malignancy of salivary glands [27] and in CXPA, the immunoreactivity of PCNA protein with negative expression of Bax among tumour cells of CX PA indicated that tumour cells are under their active division cycles.

The PCNA positivity rate was high in strongly Bax positive group, in PA and ACC, indicating a possible positive correlation between proliferation and death balance of the cell and the high PCNA positive rate in the two Bax negative cases of MEC and adenocarcinoma may suggest that proliferative rate increase in absence of apoptotic pathway activation. .

In conclusion: The high proliferative rate could explain the natural course of these tumours 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.

REFERENCES

1. Miyachi K, Fritzler MJ, Tan EM. Autoantibody to a nuclear antigen in proliferating cells The Journal of Immunology 1978; 121: 2228–34.
2. Bravo R, Celis JE. A search for differential polypeptide synthesis throughout the cell cycle of HeLa cells. The Journal of Cell Biology 1980; 84: 795 -802.
3. Bravo R, Fey SJ, Bellatin J, Larsen PM, 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): 235–48.
4. Strzalka W, Ziemienowicz A- Proliferating cell nuclear antigen (PCNA): a key factor in DNA replication and cell cycle regulation. Annals of Botany 2011; 107: 1127–40.
5. Tan Z, Wortman M, Dillehay K, Seibel W, Evelyn C, Smith S. Small-Molecule targeting of proliferating cell nuclear antigen chromatin association inhibits tumor cell growth. Mol Pharmacol 2012; 81: 811–9.
6. Waga S, Stillman B. The DNA replication fork in eukaryotic cells. Annual Review of Biochemistry 1998; 67: 721–51.
7. 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; 105:1549–54.
8. Miyamoto S, Ito K, Kurokawa K, Suzuki K, Suzuki K. Clinical validity of proliferating cell nuclear antigen as an objective marker for evaluating biologic features in patients with untreated prostate cancer. Int J Urol 2006; 13**:**767–72.
9. Tano T, Okamoto M, Kan S, Nakashiro S, Shimodaira S, Koido S, Kawakami Y and Hamakawa H. 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: 305-15.
10. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. Science 1998; 281: 1322 - 6.
11. Cotter TG. Apoptosis and cancer: the genesis of a research field. Nat Rev Cancer 2009; 9: 501-7.
12. Castillo K, Rojas-Rivero D, Lisbona F, Caballero B, Nassif M, Court. BAX inhibitor-1 regulates autophagy by controlling the IRE1a branch of the unfolded protein response. The EMBO J 2011; 30: 4465–78.

13. Isrid S, Henrik P, Roland V, Matthias L, Silke R, Timo H, Gerhard W, Steffen H, Bernd D, Peter TD. 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 – 81.
14. Gomes C, Bernardes V, Diniz M, Marco L and Gomez R Anti-apoptotic gene transcription signature of salivary gland neoplasms. BMC Cancer 2012; 12: 61.
15. Hetz C, 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.
16. Xu X, Zhang TL, Jin S, Wang R, Xiao X, Dong Zhang W, Wang PY. Wang XJ. Ardipusilloside I induces apoptosis by regulating Bcl-2 family proteins in human mucoepidermoid carcinoma Mc3 cells. BMC Complementary and Alternative Medicine 2013; 13:322.
17. Auclair PL, Goode RK, Ellis GL.Mucoepidermoid Carcinoma of intra-oral salivary glands. Evaluation and application of grading criteria in 143 cases. Cancer 1992; 69: L2021-30.
18. Szanto PA, Luna MA, Tortoledo E, White RA. Histologic grading of adenoid cystic carcinoma of the salivary glands. Cancer 1984; 54: 1062-9.
19. Auclair P, Ellis G. Adenocarcimona, not otherwise specified. In: Ellis G., Auclair P, Gnepp D (eds). Surgical pathology of the salivary gland. Philadelphia: WB Saunders; 1992. P. 318 – 23.
20. Alves FA, Pires FR, De Almeida OP, Lopes MA, Kowalski LP .PCNA, Ki-67 and p53 expressions in submandibular salivary gland tumors. Int J Oral Maxillofa Surg 2004; 33: 593-7.
21. Ogawa I, Miyachi M, Takata T, Vuhahula E , I juhin N, 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.
22. Pires FR, Pringle GA, de Almeida OP, Chen SY. Intra-oral minor salivary gland tumors: A clinicopathological study of 546 cases. Oral Oncology 2006; 42: 68 -73.
23. Ungari C, Paparo F, Colangeli W, and Innetti G. Parptid glands tumor: over view of a 10-years' experience with 282 patients, focusing on 231 benign epithelial neoplasms. European Review for Medical and pharmacological Sciences 2008; 12: 321-325.
24. Eveson JW, Auclair P, Gnepp DR, El-Naggar AK. Tumors of the salivary glands. In: Barnes L, Eveson JW, Reichart P, Sidransky D (eds). World Health organization classification of tumors. Pathology and Genetic of Head and Neck Tumors. Lyon: IARC Press; 2005. P. 209-81.
25. Ashkavandi Z, Ashraf M, Moshaverinia M. salivary gland tumors: a clinicopathologic study of 366 cases in southern Iran. Asian Pacific J Cancer Prev 2013; 14: 27-30.
26. Choudhary K, Beena VT, Rajeev R, Manju S, Sivakumar R, Pad makaumar SK. Adenoid cystic carcinoma of the hard palate: A case report with a review of the literature on therapeutic and prognostic aspect. J Oral Sci 2012; 3: 113-116.(IVSL)
27. Nagao T. Dedifferentiation and high-grade transformation in salivary gland carcinomas. Head and Neck Pathol 2013; 7:S37–S47. (IVSL).
28. Rapidis AD, Givalos N, Gakiopoulou H, Stavrianos SD, Faratzis G, Lagogiannis GA, Katsilieris L, Patsouris E. Mucoepidermoid carcinoma of the salivary glands. Review of the literature and clinicopathological analysis of 18 patients. Oral Oncology 2007; 43: 130-6.(IVSL)
29. Wolfish E, Nelson B, Thompson L. Sinonasal Tract mucoepidermoid carcinoma: a clinicopathologic and immunophenotypic study of 19 cases combined with a comprehensive review of the literature. Head and Neck Pathol 2012; 6:191–207.
30. Nagao T, Sato E , Inoue R, Oshiro H, Takahashi RH, Nagai T, Yoshida M, Suzuki F, Obikane H, Yamashina M and Matsubayashi J. Immunohistochemical Analysis of salivary gland tumors: Application for Surgical Pathology Practice. Acta Histo Chem Cytochem 2012; 45: 269-82.
31. Gorden-Nunez MA, Godoy GP, Soares RC, Souza LB, Freitaz RA, Queiroz LMG. Immunohistochemical expression of PCNA, p53 and bcl-2 in pleomorphic adenoma. Int J Morphol 2008; 26: 567-72.
32. Lee S, Kwon M, Lee Y, Choi S, Kim SY, Cho K and Nam SY. Prognostic value of expression of molecular markers in adenoid cystic cancer of the salivary glands compared with lymph node metastasis: a retrospective study. World J Surgical Oncology 2012; 10: 266 -74.
33. Saghravania N, Nooshin M, Jafarzadeh H. Comparison of immunohistochemical markers between adenoid cystic carcinoma and polymorphous low grade adenoma. J Oral Sci 2009; 51: 509-14. **(IVSL).**
34. Yin HF, Okada N, Takagi M. Apoptosis and apoptotic-related factors in mucoepidermoid carcinoma of the oral minor salivary glands. Pathology international 2000; 50: 603 – 9.

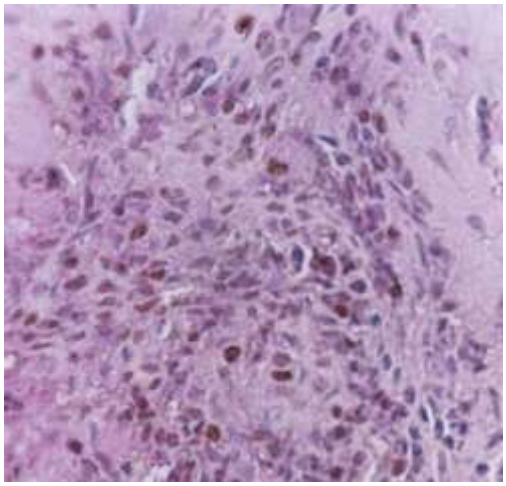

Figure 1: Pleomorphic Adenoma demonstrating nuclear immunostaining for PCNA. Original magnification 400x.

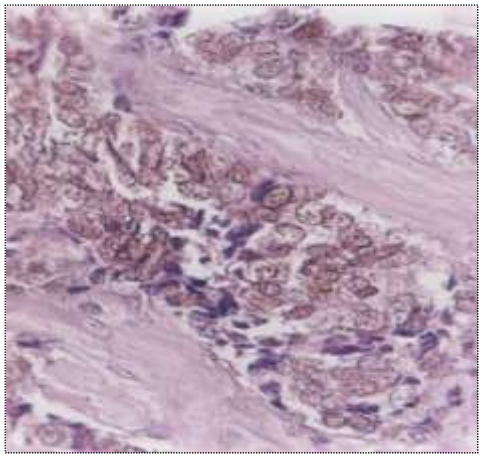

Figure 2: Mucoepidermoid Carcinoma. High grade the positive PCNA staining in the tumour cell. Original magnification 400x.

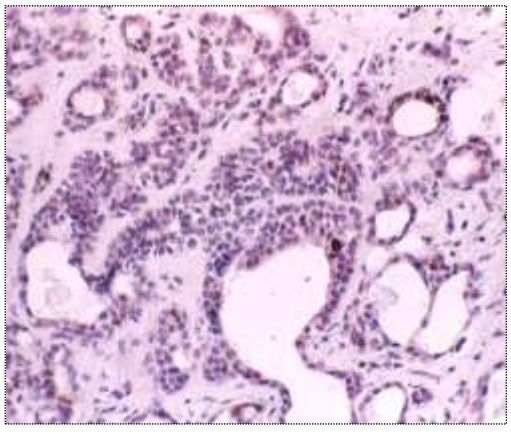

Figure 3: Carcinoma ex Pleomorphic Adenoma demonstrating nuclear brown immunostaining for PCNA. Original magnification 400x

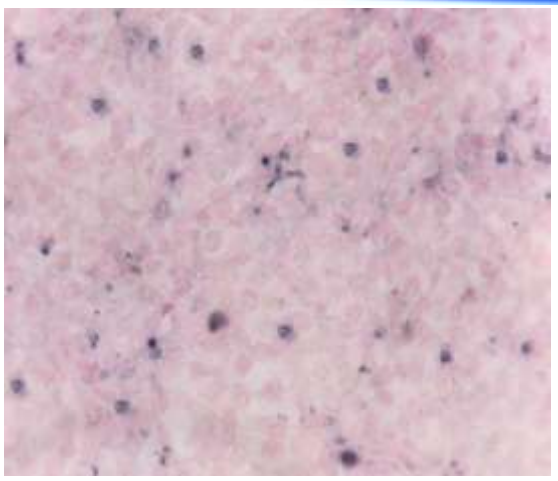

Figure 4: Acinic cell carcinoma, black - blue spots the localization of Bax mRNA gene. Original magnification 400x.

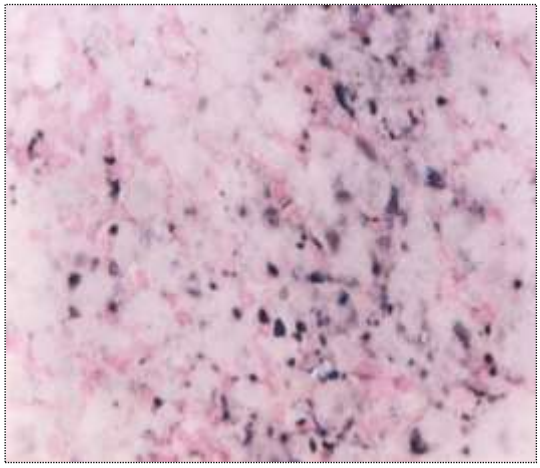

Figure 5: Adeniod cystic carcinoma - cribriform growth pattern, black - blue spots the localization of Bax mRNA gene. Original magnification 400x.

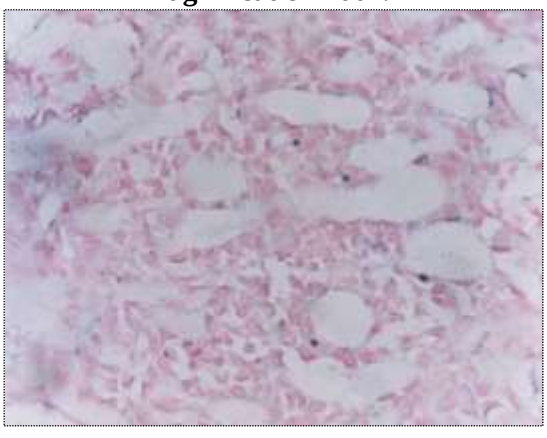

Figure 6: Adenocarcinoma, black - blue spots the localization of Bax mRNA gene. Original magnification 400x.

Table 1: The frequency and percentage of positively PCNA protein IHC analysis according to histologic grading of malignant Epithelial Salivary Gland tumours

Variable	ACC No. (%) 15	MEC No. (%) 6	Adenocarcinoma No. (%) 3
	Cribriform (6) Tubular (6) Solid (3)	Low (4) Intermediate (1) High (1)	Well Moderate(1) Poor diff.* (2)
Age M ± S.D.	45.66± 18.45 41.0±11.4 58 ±13.11	42±20.2 51±0.00 65±0.00	- 36 ± 0.0 41 ± 4.242
Sex			
Male	1(16.7) 5(83.3) 2(66.7)	1(25) 1(100) 1(100)	- 1(100) 1(50)
Female	5(83.3) 1(16.7) 1(33.3)	3(75) - -	- - 1(50)
Site			
Major			
Parotid	4(66.67) - -	1(25) - -	- 1(100) -
Submandibular	- - 1(33.3)	- - 1(100)	- - 1(50)
Sublingual	- - -	- - -	- - -
Minor			
Palate	- -	- - -1(25)	- - -
Hard	2(33.3) 3(50) 2(66.7)	- -	- - -
Soft	- 2(33.3) -	- 1(100) -	- - -
Tongue	- - -	1(25) - -	- - -
Upper lip	- - -	1(25) - -	- - 1(50)
Buccal mucosa	- 1(16.67) -	- - -	- - -
Tumour size			
T_1	- 2(33.3) -	1(25) 1(100) -	- - -
T_2	2(33.3) 1(16.7) 2(66.7)	2(50) - -	- 1(100) 2(100)
T_3	4(66.7) 2(33.3) -	1(25) - 1(100)	- - -
T_4	- 1(16.7) 1(33.3)	- - -	- - -
Lymph node			
- ve	5(83.3) 6(100) 2(75)	4(80) 1(100) -	- 1(100) 2(100)
+ ve	1(16.7) - 1(25)	1(20) - 1(100)	- - -
Clinical stage			
I	- 2(33.3) -	1(25) 1(100) -	- - -
II	2(33.3) 1(16.7) 2(66.7)	2(50) - -	- 1(100) 2(100)
III	4(66.7) 2(33.3) -	1(25) - 1(100)	- - -
IV	- 1(16.7) 1(33.3)	- - -	- - -

Table 2: Bax mRNA gene ISH analysis of positive cases according to histologic grading of malignant epithelial salivary gland tumours

Variable	ACC No. (%) 10	MEC No. (%) 5	Adenocarcinoma No. (%) 3
	Cribriform (6) Tubular (3) Solid (1)	Low (3) Intermediate (1) High (1)	Well Moderate (2) Poor diff.*(1)
Age M ± S.D.	42+12.07 48.33+12.58 44+0.0	51+16.52 51+0.0 65+0.0	- 52+22.63 28+0.0
Sex			
Male	2(33.3) 2(66.7) 1(100)	1(33.3) 1(100) 1(100)	- 2(100) -
Female	4(66.7) 1(33.3) -	2(66.7) - -	- - 1(100)
Site Major			
Parotid	4(66.7) - -	- - -	- 2(100) -
Submandibular	- - 1(33.3)	1(33.3) - 1(100)	- - -
Sublingual	- - -	- - -	- - -
Minor			
Palate	- - -	- - -	- - -
Hard	1(16.7) 1(33.3) -	- - -	- - -
Soft	1(16.7) 1(33.3) -	- 1(100) -	- - -
Tongue	- - -	1(33.3) - -	- - -
Upper lip	- - -	- - -	- - 1(100)
Buccal mucosa	- 1(33.3) -	1(33.3) - -	- - -

Tumour size								
T_1	1(16.7)	2(66.7)	-	1(33.3)	1(100)	-	-	-	1(100)
T_2	2(33.3)	-	1(100)	2(66.7)	-	-	-	1(50)	-
T_3	3(50)	-	-	-	-	1(100)	-	1(50)	-
T_4	-	1(33.3)	-	-	-	-	-	-	-
Lymph node								
- ve	5(83.3)	3(100)	1(100)	3(100)	1(100)	-	-	2(100)	1(100)
+ ve	1(16.7)	-	-	-	-	1(100)	-	-	-
Clinical stage								
I	1(16.7)	2(66.7)	-	1(33.3)	1(100)	-	-	-	1(100)
II	2(33.3)	-	1(100)	2(66.7)	-	-	-	1(50)	-
III	3(50)	-	-	-	-	1(100)	-	1(50)	-
IV	-	1(33.3)	-	-	-	-	-	-	-

Table 3: coefficient of association correlation (*r*) between positive PCNA protein and positive Bax mRNA cases of epithelial salivary gland tumours in relation to site.

Variables	Site	Mean of + ve PCNA cases	Mean of + ve Bax cases	*r*
PA	***Major***	23.625	16	
	Minor	29.3	28	0.368
ACC	***Major***	45.2	37.66	
	Minor	34.61	17	0.258
MEC	***Major***	80	71	
	Minor	32.5	51.333	0.28
Adenocarcinoma	***Major***	23.6	9.3	
	Minor	57	3	0.76
Acinic cell ca	***Major***	6.2	73	-
	Minor	-	70	
Cx PA	***Major***	-	-	-
	Minor	37.5	-	
Squamous cell ca	***Major***	24.3	20	-
	Minor	25	-	

Table 4: Labeling index of positive PCNA protein versus positive Bax mRNA cases of epithelial salivary gland tumours

Variables No. of cases	Bax mRNA No. of cases	PCNA Labeling index (Mean ± SD.)	
PA 12	- ve (9) + ve (3)	22.28 ± 6.8	39 ± 12.7
ACC 19	- ve (9) + ve (10)	32.48 ± 19.9	41.9 ± 18.2
MEC 7	- ve (2) + ve (5)	57 ± 32. 6	44 ± 27.2
Adenocarcinoma 5	- ve (2) + ve (3)	43 ± 19. 8	18.2
Acinic cell ca 2	- ve – + ve(2)	-	6.2
Cx PA 2	- ve(2) + ve -	37.5 ± 3.535	-
Squamous cell ca 2	- ve(1) + ve(1)	24.3	25