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EXPRESSION OF IL-10 AND KI-67 IN SOME BENIGN AND MALIGNANT SALIVARY GLAND TUMORS "AN IMMUNOHISTOCHEMICAL STUDY"

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ABSTRACT

INTRODUCTION: Salivary gland tumors (SGTs) are infrequent tumors representing 2.5-7 % of all head and neck tumors. It's very difficult to reach proper diagnosis and prognosis depending on histomorphologic criteria alone due to overlapping clinicopathologic features. IL-10 is a homodimeric protein that has various anti-inflammatory and immune functions. In cancer, IL-10 has both immunosuppressive properties and anti-angiogenic properties. Several studies proved the prognostic role of IL-10 in different human cancers. However, its role in SGTs still needs to be confirmed. Ki-67 is a nuclear protein that is encoded by the gene MKi-67. Its expression as a proliferative cell and a prognostic marker has been largely investigated in many types of human tumors including those of salivary gland origin, such as acinic cell and adenoid cystic carcinomas.

OBJECTIVES: To evaluate IL-10 and Ki-67 immunoexpression in benign and malignant SGTs.

MATERIAL AND METHODS: IL-10 and Ki-67 expression was measured in 30 SGT cases (15 benign and 15 malignant) and 15 normal salivary gland tissue (NSGT). Immunohistochemical (IHC) staining was carried out by the Labeled Strept-Avidin Biotin complex method (LSAB).

RESULTS: IL-10 and Ki-67 expression was detected in normal salivary gland tissues (NSGT) as well as benign and malignant salivary gland tumors (SGTs) with different intensities. The Lowest expression levels were detected in normal salivary gland tissues, while the highest were detected in malignant salivary gland tumors.

CONCLUSION: IL-10 and Ki-67 could be used as prognostic markers in salivary gland tumors.

KEYWORDS: Salivary gland tumors, Interleukin 10 (IL-10), immunohistochemical marker, Ki-67, prognosis

RUNNING TITLE: Expression of IL-10 and Ki-67 in benign and malignant Salivary gland tumors

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INTRODUCTION

Salivary gland tumors (SGT's) account for 2.5-7% of all head and neck tumors (1). They mostly occur in adults and are less common in children where their occurrence encompasses a higher proportion of malignancy (usually low-grade mucoepidermoid carcinomas) (2).

Salivary gland tumors are classified into benign and malignant, where the benign neoplasms show higher percentage (65-70%) than malignant and mainly affect the parotid gland (3). The most common benign salivary gland tumor (BSGT) is pleomorphic adenoma, followed by Warthin's tumour (4). Benign salivary gland tumours (BSGTs) have better prognosis and lower rate of recurrence than malignant salivary gland tumours (MSGTs) (1).

The etiology of salivary gland cancer is not definite. However, radiation therapy involving the head and neck region may be a risk factor.(5).

Salivary gland tumors show variations in their clinical and histological appearance making their diagnosis difficult (2). Immunohistochemical staining could improve the accuracy of diagnosis. The utilization of these biomarkers helps in enlightening the biologic behavior of the tumor and thus guiding to the proper diagnosis (6).

Inflammation has an important role in tumorigenesis. Cytokines are cell-signaling proteins which affect the communication between cells. They are produced in the tumor microenvironment, which indicates their role in cancer pathogenesis (7).

Interleukin-10 is a cytokine with a homodimeric structure, its role is suppressing the immune activity by blocking the pro-inflammatory cytokines synthesis, it also has anti-cancer actions (8). T-cells, monocytes, B cells are sources of IL-10. Macrophages are the major source of IL-10 (9). IL-10 was noted to be highly expressed in serum, saliva and tumor tissues in different cancer types. (10-12).

Several studies proved the prognostic role of IL-10 in different human cancers (9,12-14). High

concentration of IL-10 indicates poor prognosis (7,9). However, IL-10 role in salivary gland tumors still needs to be confirmed.

Ki-67 is a nuclear protein encoded by the gene MKi-67, that plays a role in cell proliferation (15).

It's found to be regulating the cell cycle, synthesis of ribosomes and help in finding proper prognosis of different neoplasms. Ki67 antibody can be considered an easy way to rely on to estimate growth fraction in normal and malignant neoplasia. Ki67 expression as a proliferative factor has been largely studied in different types of human tumors specially those of salivary gland origin, such as acinic cell and adenoid cystic carcinomas. Ki-67 is related to increased tumor aggressiveness and metastases (15,16).

The aim of this work is to evaluate the expression of IL-10 and Ki-67 in different salivary gland tumors.

MATERIAL AND METHODS

Sample

The current study was carried out at the Faculty of Dentistry, Alexandria University after obtaining the approval of the Research Ethics Committee. It included thirty SGT paraffin-embedded blocks (15 paraffin-embedded blocks of benign salivary gland tumors and 15 paraffin-embedded blocks of malignant salivary gland tumors). Most paraffin-embedded blocks were collected from the archives of the Oral Pathology Department between the periods of 2015 to 2018. Few fresh tissue specimens were taken from the Cranio-Maxillofacial and Plastic Surgery Department. Normal salivary gland tissues are considered control, they were taken from a safety margin of the same patient's resected specimens, away from the tumor tissue. Patients participating in the study were told to fill up a consent.

Histological and immunohistochemical examination

10% neutral buffered formalin was used to fix the studied specimens. They are then processed and soaked in paraffin wax using the common procedures. 3-4 µm thickness sections were placed on glass slides and stained by Hematoxylin and Eosin (H&E). The histopathological diagnosis was confirmed at the Oral Pathology Department at the Faculty of Dentistry, Alexandria University.

Immunohistochemical staining was done using a Labelled Strept-Avidin Biotin complex method (LSAB), following the instruction of the manufacturer's kit manual. 4 µm thick sections were taken from the same tissue blocks and mounted on polyl-L-lysine coated glass slides. The tissue sections were deparaffinised in Xylene for 10 minutes dehydrated in graded series of ethanol and washed twice in phosphate buffered saline for 5 minutes. All sections were micro-waved in 0.01 citrate buffer for 15 minutes. This is done to unmask the antigenic sites in formalin-fixed tissues and to increase the staining intensity of the primary antibody, the slides were then left to cool at room temperature for 20 minutes. Blocking the endogenous peroxidase was achieved by treating sections for 20 minutes at room temperature with 0.3% hydrogen peroxide (H2O2) and then blocking with 1% bovine serum albumin (Sigma) in phosphate-buffered saline (PBs) for one hour to evaluate IL10 and ki-67 antigen expression, E.coli-derived human IL-10 recombinant protein Cat. #144728 (100ug) (US Biological, USA) was used on 30 sections and rabbit monoclonal antibodies (Biocare Medical, USA). For ki-67 was used on 30 sections.

Sections were then incubated with the primary antibody for 1 hour at room temperature with 1:50 dilution according to the manufacturer's specifications. After washing in PBS three times for two minutes, sections were incubated in biotinylated secondary antibody in PBS for 30 minutes at room temperature and subsequently with streptavidin-peroxidase conjugate. Then sections were washed in PBS in the same manner. The 0.02% diaminobenzidinehydrochloride (DAB) containing 0.03% hydrogen peroxidase used as chromogen to visualize the peroxidase activity. Then they were washed in PBS in the same manner. The tissue sections were washed in water, counterstained by Mayer's hematoxylin (Sigma) and covered with glass slip.

The sections were then examined and the intensity of the immunostaining was Quantified in terms of mean area percentage (MA%) and mean optical density (MOD) by the computer image analyzer Image J software (NIH, USA). Unpaired student's t test was used to compare the intensity of immunostaining of IL-10 between the two groups.

Statistical analysis

IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp) was used to analyze our data. To ensure that our data is normally distributed we use the Kolmogorov-Smirnov test. We described the quantity of our data by using range (minimum and maximum), mean, standard deviation and median. While, we used the number and percentage to describe the Qualitative data. A p value < 0.05 was considered statistically significant. The values were recorded as a mean value ± SD. F-test (ANOVA) was used for comparison between normal salivary gland tissue (NSGT), benign salivary gland tumors (BSGTs) and malignant salivary gland tumors (MSGTs) according to mean area percentage (MA%) and mean optical density (MOD).

RESULTS

Clinical evaluation

In this study, the samples were collected from 30 patients, (13 males and 17 females). The diagnosis of the samples as salivary gland tumors was done at Oral Pathology Department, Faculty of Dentistry, Alexandria University. Demographic data of all patients is summarized in (Table 1).

Histopathological results

Out of the 15 BSGTs, 13 were pleomorphic adenoma (PA), Warthin's tumor (WT) and myoepithelioma (ME) were represented by 1 case each, Among the 15 MSGTs, 8 were carcinoma ex pleomorphic adenoma (Ca ex PA), 3 were adenoid cystic carcinoma (AdCC), mucoepidermoid carcinoma (MEC) and

adenocarcinoma were represented by 2 cases each. The histologic features of the studied cases are summarized in (Table 2).

Immunohistochemical results

Normal salivary gland tissue showed negative immunoreactivity for both Ki-67 and IL-10 in epithelial and myoepithelial cells.

Results of Ki67 marker in Benign and malignant tumors:

All the fifteen benign salivary gland tumors showed positive immunoreaction for Ki-67. Pleomorphic adenoma cases showed high nuclear and cytoplasmic immunoreaction in myoepithelial cells for Ki-67 (figure 1a).

The myoepithelioma case showed positive expression in the cytoplasm of myoepithelial cells for Ki-67 (figure 1b).

Papillary cystadenoma lymphomatosum (warthin's tumor) showed high positive immunoreaction for in the oncocytic epithelial lining, while, there was no reaction in the lymphoid stroma (figure 1c).

All the fifteen malignant salivary gland tumors exhibited strong positive immunoxpression for Ki-67. The mucoepidermoid carcinoma with low-grade showed positive expression in the epidermoid cells although, Ki-67 showed positive membranous reactions in the clear and mucous secreting cells (figure 2a). Moreover, mucoepidermoid carcinoma with high grade expressed strong positive immune reaction for Ki-67 highly noted in the epidermoid cell (figure 2b).

Carcinoma ex pleomorphic adenoma exhibited positive nuclear and cytoplasmic immunoreaction for Ki-67 (figure 2c).

Adenocarcinoma, showed immunoreactivity in the nucleus and cytoplasm for Ki-67 (figure 2d)

Adenoid cystic carcinoma showed positive immunoreaction for Ki-67 in the nucleus and cytoplasm shown in basaloid myoepithelial and clear cells (figure 2e).

Results of IL-10 in benign and malignant salivary gland tumor

All the fifteen benign salivary gland tumors showed positive immunoreaction for Ki-67. Pleomorphic adenoma revealed positivity cytoplasmic IL-10 immuno expression. (Figure 3a).

The myoepithelioma showed total cell positive immunoreaction for IL-10. Also, warthin's tumor showed high positive immunoreaction in the oncocytic epithelial lining while there was no reaction in the lymphoid stroma.

All the cases of malignant tumors exhibited strong +ve immunoexpression for IL-10 showing varied intensities.

IL-10 immunoreactivity was predominatly cytoplasmic, membranous reaction was detected in mucus secreting cells of low grade MEC.

Carcinoma ex pleomorphic adenoma exhibited positive immunoreaction for IL-10 and membranous reaction was also detected in the ductal cells, in addition cytoplasmic reaction and clear myoepithelial cells were detected. (Figure 3b) Adenocarcinoma, showed positive immunoreactivity for IL-10 (Figure 3c). Also, Adenoid cystic carcinoma showed positive immunoexpression of IL-10, varying expression intensity in the nucleus as well as clear cells were noticed. (Figure 3d)

Statistical results

The intensity of immunoreactivity of Ki-67 and IL-10 in normal salivary gland tissues (NSGTs) (control), benign salivary gland tumors (BSGTs) and malignant salivary gland tumors (MSGTs) was measured in terms of mean area percent (MA %) and mean optical density (MOD).

As regard to IL-10 the lowest MA% was recorded in normal salivary gland tissues (26.88 ± 6.47), while the highest value was recorded in malignant salivary gland tumors (127.7 ± 4.18). Regarding MOD, the lowest value was recorded in NSGT (26.88 ± 6.47), while the highest value was recorded in MSGTs (201.7 ± 10.45).

As regards to Ki-67 the lowest MA% was recorded in NSGT (24.63±5.80) while the highest value was recorded in MSGTs (129.7±9.95). Regarding MOD, the lowest value was recorded in NSGT (24.63±5.80), while the highest value was recorded in MSGTs (203.0±10.77).

Comparing BSGTs with MSGTs

It revealed significantly greater MA % and MOD of immunoexpression in MSGTs compared to BSGTs, the same test was used to compare the intensity of immunostaining Ki-67 between the two groups, revealing the same results (Figure 4).

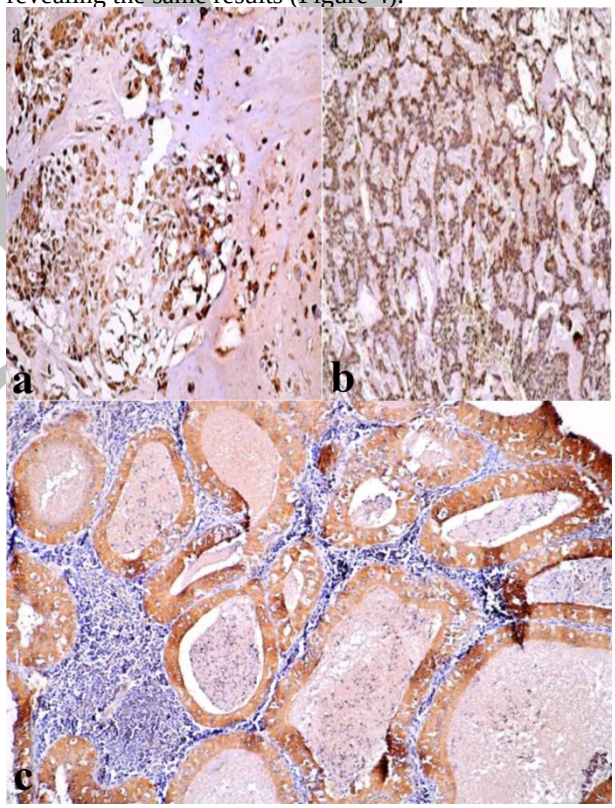

Figure (1): a) Pleomorphic adenoma showing intense immunopositivity to ki67 in glandular epithelial and myoepithlial cells (x200). b) Myoepithelioma showing positive immunoreaction at myoepithelial cells for ki67 (x200). c) Warthin's tumor showing immunoreactivity to Ki-67 in the epithelial, while no reaction noticed in the stroma (x200).

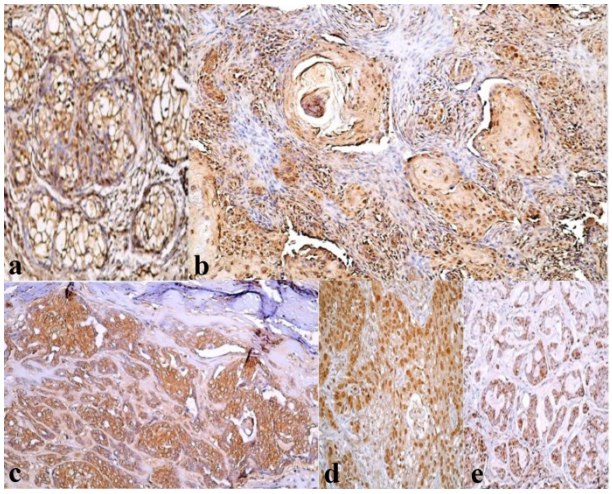

Figure (2): a) MEC (Low-grade) exhibiting +ve immunoreactivity to Ki-67 (x200), b) High grade mucoepidermoid carcinoma revealing diffuse positive immunoreaction to Ki-67 in malignant epidermoid cells (x200). c) Carcinoma ex-pleomorphic adenoma exhibiting strong +ve reactive in the nucleus and cytoplasm of all malignant cells (x200). d) Adenocarcinoma exhibiting total cell positive immune signals to Ki-67 (x200), e) adenoid cystic carcinoma showed positive immune signals to Ki-67 in the nucleus and cytoplasm of basaloid myoepithelial cells (x200).

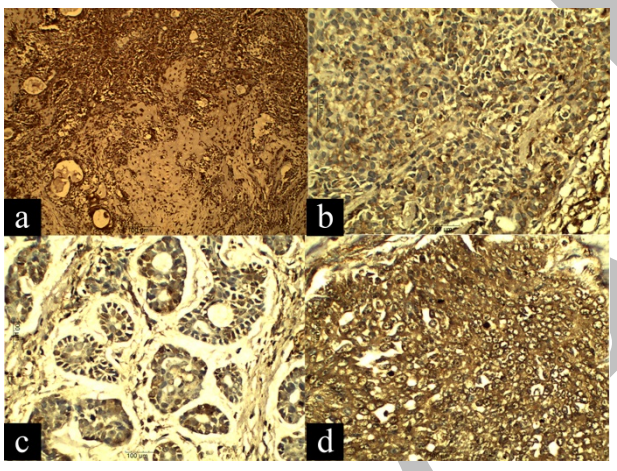

Figure (3):a) Pleomorphic adenoma showed positive immunoreactivity to IL-10 (x200). b) Carcinoma ex-pleomorphic adenoma showing strong positive expression IL-10 in the nucleus and cytoplasm of all cell showing malignancy (x200). c) Adenocarcinoma exhibiting positive immunoreactivity to IL-10 (x200). d) Adenoid cystic carcinoma exhibiting diffuse total immune positivity to IL-10 in almost all malignant to basaloid cells (x200).

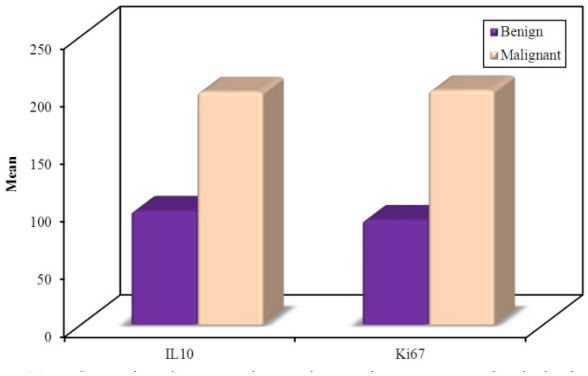

Figure (4): Comparison between Ki-67 and IL-10 immuno expression in benign and malignant

Table (1): The clinical data of SGT patients (N=30)

Variable	No.	%
Type		
• Benign	15	50.0
• Malignant	15	50.0
Age (years)		
Mean ± SD.	42.87 ± 11.07	
Gender		
• Male	13	43.3
• Female	17	56.7
Site		
• Palate	13	43.3
• Parotid gland	9	30.0
• Retro molar area	2	6.7
• Maxilla	1	3.3
• Tongue	2	6.7
• Buccal mucosa	2	6.7
• Labial mucosa	1	3.3

Table (2): Distribution of the studied cases according to types, numbers and percentages of the studied salivary gland tumor cases (n =30)

Type of salivary gland tumor	No.	%
Benign salivary gland tumors	**15**	**50.0**
Pleomorphic adenoma	13	43.3
Worthin's tumor.	1	3.3
Myoepitheliomma	1	3.3
Malignant salivary gland tumors	**15**	**50.0**
Carcinoma ex.P.A	8	26.7
Adenoid cystic C.	1	3.3
Mucoepidermoid carcinoma MEC (low grade)	1	3.3
MEC (high grade)	2	6.7
Adenocarcinoma	3	10.0

DISCUSSION

SGTs are the most heterogenous tumors in the human body. They are characterized by their complex biological behavior and variable morphology. They show variations in their clinical and histological appearance which make their diagnosis difficult. They have annual incidence of 0.4-13.5 per 100,000 individuals worldwide (17).

One of the most important ways to early diagnose different diseases is biomarkers. They show ability to early diagnose diseases and to reach proper prognosis (18). Tumor markers are biochemical substances that is formed and produced by cancerous cells or the host due to the presence of cancerous substances. They are used to monitor or identify the presence of a cancerous growth (19).

There is more than one assay to measure and identify tumor biomarker. These assays could only be approved if they have analytical validation, they have to be with high degree of accuracy, reproducibility, can be relied on , and have clinical utility (19).

In the present study, IL-10 expression was measured in NSGT, BSGTs and MSGTs aiming at evaluating the role of IL-10 in the progression of salivary gland pathology.

Interleukin10 plays an important role in immunosuppression. It can also help in treatment of various diseases such as, chronic inflammation, autoimmune diseases, transplant rejection, graft versus – host disease and sepsis (20).

Different researches stated that various malignant tumor cells produce high levels of Interleukin 10. Sakamoto et al (10) stated that during cancer, gastric cells produce IL-10 and related this to patients low survival rate. Also Ali et al reported IL-10 expression in oral squamous cell carcinoma (7). In addition, in a study conducted by Heckel et al (11), immunohistochemistry showed that IL-10 was found localized in the tumor of primary breast adenocarcinomas. Moreover, interleukin 10 was found to be expressed in the cells of specific type of lung cancer (12), in addition to hepatocellular carcinoma (14).

To our knowledge, no previous studies has investigated the role of IL-10 in SGTs, In this study IL-10 immunoexpression was detected in normal salivary gland tissues, benign salivary gland tumors and malignant salivary gland tumors, the expression was higher in (MSGTs) than in (BSGTs).

In this study, interleukin 10 expression was detected in NSGT at very low level particularly in the ductal cells and myoepithelial cells. In a study of IL-10 expression in primary ovarian carcinoma, similar results were found by Zhou et al who observed IL-10 expression in normal controls at low levels (21). In contrast with our results, Chavey et al in their study done on breast cancer failed to detected any IL-10 expression in normal healthy breast tissue samples (22). The results of the present work are supported by many previous investigations on different glandular tumors. Lianes –Fernandez et al stated that Interleukin 10 was expressed strongly in breast cancer tissues (23). Another study by Ali et al reported strong IL-10 expression in oral squamous cell carcinoma (7).

In this study, IL-10 expression was higher in MSGTs than in BSGTs with a statistically significant difference between them supporting its role in tumor aggressiveness. This was similar to the finding of Zhou et al in their study on primary ovarian carcinoma where they showed higher expression of IL-10 in malignant tumors than benign and normal control (21).

The significance of IL-10 detection in cancer patient's serum was an interesting field of research that revealed many contradictory results. Interestingly, Tavares-Murta et al., found that high serum levels of IL-10 was detected in various malignant cases, this suggests that interleukins can be synthesized by that malignant cells ,this helps in promoting and developing ovarian carcinoma (24).

In contrast, patients having squamous cell carcinoma and head and neck adenoid cystic carcinoma didn't show Interleukin-10 in their serum, this was stated by Hoffmann et al (25). On the contrary, high interleukin 10 serum levels was found by De Vita et al (26) to be higher in patients with gastrointestinal carcinoma than normal controls.

Therefore, more researches to study IL-10 expression on salivary gland tumors is mandatory to discover it's diagnostic and prognostic role.

Almost all neoplasms have the ability to proliferate very fast. Knowing the fraction of the proliferation in tumor cells helps to diagnose different cancerous diseases and to reach proper diagnosis. One of the effective ways to calculate the proliferation fraction is using biological markers (27).Therefore investigation of a proliferative marker as Ki-67 is useful in diagnosis of SGTs.

In this study, the examination of Ki-67 was done in normal salivary gland tissues, BSGTs and MSGTs. The sections of normal tissues which are considered as control showed negative immunoreaction to Ki-67, MSGTs showed higher expression to Ki67

than BSGTs showing statistically significant difference in between.

Ki-67 is a protein of non-histone nuclear type that is encoded by the gene MKi-67, and it is considered a proliferative marker, during the cell cycle interphase (28).

In this study, Pleomorphic adenoma cases exhibited Ki67 immunosignals in both the nucleas and cytoplasm of myoepithelial cells. Carcinoma-ex Pleomorphic adenoma showed positive immunoreaction for Ki67 in all the cells showing malignancy. This was also supported by the study of Abdallah et al (27), Tadbir et al (29), they stated that Ki67 marker plays a role in the proliferation in pleomorphic adenoma, giving an indication on the risk of malignancy. The high Ki-67 proliferative index could definitely be considered in distinguishing carcinoma ex-pleomorphic adenoma from benign pleomorphic adenoma, (27,30,31), despite Vergas et al (32) who unusually detected very low Ki-67 immunoreactivity in pleomoephic adenoma.

In this study, myoepithelioma showed positive immunosignals for Ki67 in the myoepithelial cells.

Supporting the results of Abdallah et al., (27) and contradicting Ferri et al. (30) and Alves et al., (34) who mentioned low expression and negative expression of Ki-67 in myoepithelioma respectively.

Papillary cystadenoma lymphomatosum exhibited positive immunoreactivity in the oncocytic linning for Ki-67, although the lymphoid stroma showed no reaction, in agreement with Sangeetha et al (35) and Abdallah et al (27) in their study.

In this study, mucoepidermoid carcinoma showed positive immunoreactivity which goes along with Abdallah et al (27), Nguyen et al (36) and Triantafillidou et al. (37) who stated that patients having high grade mucoepidermoid carcinoma showed high staining for Ki67, while patients with low grade carcinoma showed week stain for Ki67 which indicates that Ki67 proliferative index can help in determining the grade of mucoepidermoid carcinoma (38).

Adenoid cystic carcinoma showed strong positive immunoexpression of Ki67 in the nucleus and cytoplasm, in accordance with Abdallah et al (27) and Lazzaro (39) but in contrast with Fonseka et al (40) who noticed low Ki67 expression in Adenoid cystic carcinoma, it was accounted for using immunohistochemical staining technique, which is affected by tissue aging, staining technique, enzyme antibody used and single observer bias.

CONCLUSION

IL-10 and Ki-67 immunoexpression was detected in NSGT, BSGTs and MSGTs. The expression was higher in MSGTs than in BSGTs, thus IL-10 and Ki-67 can be used as potential prognostic markers.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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