EXPRESSION OF MCM3 AND KI-67 AS DIAGNOSTIC MARKERS IN BENIGN AND MALIGNANT SALIVARY GLAND TUMORS

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ABSTRACT

Introduction: Salivary gland tumors (SGTs) may represent a considerable diagnostic challenge, primarily because of the complexity of the classification and the rarity of several entities. Since proliferative activity is a reliable method to assess tumor biology. There has been continuous research to find such biological markers, Ki-67 is a widely accepted proliferation marker, with its expression tightly associated with the cell cycle. It is implicated in many of human cancers as a prognostic factor, MCM-3, member of minichromosome maintenance proteins family, is up-regulated in proliferating cells. MCM-3 overexpression in almost all human cancers implicates that it might facilitate the tumorigenesis by playing a role in the malignant transformation of cells.

Objectives: to evaluate the MCM-3 protein expression in benign and malignant salivary gland tumors and compare the obtained results with the expression of Ki-67 proliferation antigen.

Materials and methods: Immunohistochemical analysis of 20 cases of SGTs with 2 sections from each specimen (20 sections for antiKi-67antibody and 20 sections for antiMCM3antibody) and 5 control cases. Immunohistochemical staining was performed using a Labeled Strept-Avidin Biotin method (LSAB).

Results: Normal salivary gland tissue showed negative immunoreactivity for both Ki-67 and MCM-3 in epithelial and myoepithelial cells. All the examined cases showed positive expression for both proliferative markers in benign and malignant SGTs, with different intensities.

Conclusions: The proliferative markers Ki-67 and MCM-3 proteins are overexpressed in malignant salivary gland tumors, than benign ones. Both Ki-67 and MCM-3 may be reliably applied as diagnostic markers to distinguish benign from malignant salivary gland tumors.

Keywords: Salivary gland tumor, Immunohistochemistry, MCM-3, Ki-67.

INTRODUCTION

Since proliferative activity is a reliable method to assess tumor biology, there has been continuous research to find such biological markers (4). Ki-67 is a widely accepted proliferating marker, with its expression tightly associated with the cell cycle (5). The absence of Ki-67 in quiescent cells and its universal expression in proliferating tissues created great interest on its potential role as a marker of cell proliferation, and its expression in resting cells has rarely been reported (6).

Although the Ki-67 is not associated with any type of human cancer as a causative factor, it is implicated in many of them as a prognostic factor based on the expression profile of tumor cells (7).

Studies have proven that the minichromosome maintenance (MCMs) protein expression has a relationship with diagnosis and prognosis (8). MCM-2 and MCM-3 seem to have the most important role in several types of neoplasia (9).

The mini chromosome maintenance (MCM) proteins are a family of essential eukaryotic replication proteins with six distinct members (MCM-2–MCM-7) (10). The six proteins are highly similar to each other and form a hexameric complex (11).

MCM-3, member of minichromosome maintenance proteins family, expression is up-regulated in proliferating cells, whereas intracellular levels decrease significantly in differentiated and growth-arrested cells (12,13,14). MCM-3 protein enters the nucleus at the end of mitosis, remains in the nucleus during most of G1 phase, and becomes predominantly cytoplasmic at the G1/S transition, and disappears at the beginning of S phase (15,16).

Although genetic characterization of tumor tissues shows that mutation of the p53 is the most common genetic alteration in human cancers, the mutation rate of the p53 in cervical cancer is relatively low (17,18). It implies that there might be other factors involved in cervical carcinogenesis. MCM-3 is overexpressed in almost all human cancers; therefore, it might facilitate the tumorigenesis by playing a role in the malignant transformation of cells (19).

Studies on Ki-67 expression in salivary malignancies indicate a biological role in salivary gland cancer pathogenesis (20). Although the expression of MCM-3 has not yet been widely studied in salivary gland neoplasm, MCM-3 might be a useful proliferation marker for differential diagnosis and recognition of clinical behavior of salivary gland tumors (21). This study aimed to evaluate the MCM-3 protein expression in benign and malignant salivary gland tumors and comparing the obtained results with expression of Ki-67 antigen.

MATERIALS AND METHODS

The present study was conducted on 20 specimens diagnosed as salivary gland tumor. Two sections from each specimen (40 sections) were used. The cases were collected from the...
Maxillofacial and Plastic Surgery Department, Faculty of Dentistry, Alexandria University. Five specimens of normal salivary gland tissue served as control group, taken from a safety margin of the same patient’s specimens, away from the tumor tissue.

A written informed consent was obtained from all the patients. The research protocol was approved by the Ethical Committee of the Faculty of Dentistry.

Patients’ clinical data were collected from their files, including the patient’s age and gender as well as the site of the tumor. The specimens were fixed in 10% neutral buffered formalin, processed and embedded in paraffin wax using the conventional procedures.

Serial sections of 4 µm thick were placed on glass slides and stained by (H&E) for routine histopathological examination. A total of 20 cases were examined, 5 cases were benign salivary gland tumors (3 pleomorphic adenoma, myoepithelioma and Warthin’s tumor one case each), and 15 cases were malignant salivary gland tumors (4 mucoepidermoid carcinoma (MEC), 3 myoepithelial carcinoma, oncocytic carcinoma, adenocarcinoma (NOS) and sebaceous adenocarcinoma 2 cases each, carcinoma ex-pleomorphic adenoma (CXPA) and adenoid cystic carcinoma (AdCC), one case each.

Immunohistochemical staining was performed using a Labeled Strept-Avidin Biotin complex method (LSAB), following manufacturer’s kit manual instructions.

Serial sections of 4 µm thick were taken from the same tissue blocks and mounted on poly-L-lysine coated glass slides. The tissue sections were deparaffinized in xylene for 10 minutes, dehydrated in graded series of ethanol and washed twice in phosphate buffered saline (PBS) 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 (H$_2$O$_2$) and then blocking with 1% bovine serum albumin (Sigma) in phosphate-buffered saline (PBS) for one hour. To evaluate the MCM3 and Ki-67 antigen expression, rabbit polyclonal antibody (US Biologicay, USA) for MCM-3 was used on 20 sections, and rabbit polyclonal antibody (Thermo Fisher Scientific, USA) for Ki-67 was used on 20 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 number of Ki-67 and MCM-3 positive nuclei was counted using Leica Quin 500 analyzer computer system, (Leica Microsystems, Switzerland). The cursor was used to point to the positive nuclei, which was then automatically counted by the computer system. The number of positive nuclei was calculated in 10 different fields using magnification (x400).

**Statistical analysis**

Mean values and standard deviation (SD) were calculated for each group. Analysis of data was performed using SPSS 17 (Statistical Package for Scientific Studies) for Windows. Description of quantitative variables (ex. Age, number of positive nuclei) was in the form of mean, standard deviation (SD). Mean values were recorded in control, benign and malignant neoplasms were compared using analysis of variance (ANOVA) test.. Comparison between the groups (ex. Benign and malignant) was performed using unpaired t test.

**RESULTS**

**Clinical evaluation**

The present study comprised tissue samples obtained from 20 patients (12 males and 8 females) which were diagnosed as salivary gland tumor at Oral Pathology Department, Faculty of Dentistry, Alexandria University. Base line data of all patients is illustrated in (Table 1).

**Table (1):** The clinical data of SGT patients(N=20).

<table>
<thead>
<tr>
<th>Variable</th>
<th>%</th>
<th>N</th>
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<tbody>
<tr>
<td><strong>Type</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benign</td>
<td>25%</td>
<td>5</td>
</tr>
<tr>
<td>Malignant</td>
<td>75%</td>
<td>15</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Mean ± SD)</td>
<td>55.7±13.5</td>
<td></td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>60%</td>
<td>12</td>
</tr>
<tr>
<td>Female</td>
<td>40%</td>
<td>8</td>
</tr>
<tr>
<td><strong>Site</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parotid</td>
<td>35%</td>
<td>7</td>
</tr>
<tr>
<td>Submandibular</td>
<td>5%</td>
<td>1</td>
</tr>
<tr>
<td>Sublingual</td>
<td>5%</td>
<td>1</td>
</tr>
<tr>
<td>Minor salivary gland</td>
<td>55%</td>
<td>11</td>
</tr>
</tbody>
</table>
Immunohistochemical results

• Patterns of Ki-67 and MCM-3 immunostaining in normal salivary gland tissue:
  Normal salivary gland tissue showed negative immunoreactivity for both Ki-67 and MCM-3 in epithelial and myoepithelial cells.

• Patterns of Ki-67 and MCM-3 immunostaining in benign salivary gland tumors:
  All the five benign salivary gland tumors showed positive expression for Ki-67 and MCM-3.
  Pleomorphic adenoma cases showed intense nuclear and cytoplasmic immunosignals in myoepithelial cells for Ki-67. In MCM-3, the examined cases of PA showed diffuse total cell reactivity with different nuclear intensity (Fig.1).

Fig. (1): Pleomorphic adenoma showing intense diffuse positive immunosignals to Ki-67(a) and to MCM-3 (b) in glandular epithelial and myoepithelial cells (x200).

Myoepithelioma case exhibited positive cytoplasmic immunoreaction of myoepithelial cells for Ki-67, while intense total cell reactivity in myoepithelial cells for MCM-3 was noted (Fig.2).

Fig. (2): Myoepithelioma exhibiting positive immunoreactivity Ki-67 (a,) and to MCM-3(b) (x200).

Papillary cystadenoma lymphomatosum (Warthin's tumor) exhibited intense immunoreactivity for both markers in the oncocytic epithelial lining. However, the lymphoid stroma was free from any reaction.

• Patterns of Ki-67 and MCM-3 immunostaining in malignant salivary gland tumors:
  All malignant tumors showed positive immunorexpression for both Ki-67 and MCM-3 with different intensities.
  Low-grade MEC expressed positive immunoreactivity in the epidermoid cells, whereas membranous immunoreactions were noted in the clear and mucous secreting cells for both Ki-67and MCM-3 (Fig.3), more over the high-grade MEC showed intense immunopositivity particularly in the epidermoid cells.

Fig. (3): Low-grade mucoepidermoid carcinoma showing positive to Ki-67(a), and to MCM-3(b) immunore-activity in the epidermoid and clear cells (x200).

Myoepithelial carcinoma showed total cell positivity for both markers (Ki-67, and MCM-3) in the malignant epitheloid and plasmacytoid myoepithelial cells.
  Non otherwise specified adenocarcinoma (NOS), showed intense diffuse positive immunostaining for both Ki-67 and MCM-3 (Fig.4).

Fig. (4): Adenocarcinoma exhibiting total cell positive immunosignals to Ki-67 (a) and to MCM-3 (b) (x200).
Oncocytic carcinoma, showed also total cell reactivity in the malignant oncocytes. It was more intense to Ki-67 than to MCM-3 (Fig.5).

**Fig. (5):** Oncocytic carcinoma exhibiting diffuse cytoplasmic immunoreaction of Ki-67(a), and to MCM-3 (b) in the malignant oncocytes (x200).

Sebaceous adenocarcinoma, exhibited positive nuclear and cytoplasmic immunorepression of Ki-67 and total cell immunoreaction of MCM-3. In one case, squamous metaplasia with keratin formation was seen with positive immunoreactions to MCM-3.

In Carcinoma ex-pleomorphic adenoma intense diffuse positive nuclear and cytoplasmic immunoreactivity were detected in almost all malignant cells for both ki67 and MCM-3.

Adenoid cystic carcinoma exhibited positive nuclear and cytoplasmic immunorepression of Ki-67 in basoloid myoepithelial and clear cells. Some nuclei were free from any reaction. Diffuse total immunopositivity to MCM-3 in almost all malignant basoloid and clear cells with different nuclear intensities was detected as well (Fig.6).

**Fig. (6):** Adenoid cystic carcinoma showing positive reaction in the basoloid myoepithelial cells to Ki-67(a), and MCM-3 (b) (x200)

### Statistical results:

**Ki-67 immunorepression**

The mean number of Ki-67 positive nuclei in benign and malignant tumors was (93.6±11.99), (155.4±20.73) respectively. The greatest mean number of positive nuclei was recorded in the malignant tumors. One way analysis of variance (ANOVA) test revealed a statistically significant difference (p<0.0001). Tukey’s post hoc test revealed a significant difference between the two groups.

**MCM-3 immunorepression**

The mean number of MCM-3 positive nuclei in benign and malignant tumors was (87.64±9.51), (123.73± 7.4) respectively. The greatest mean number of positive nuclei was recorded in the malignant tumors. One way analysis of variance (ANOVA) test revealed a statistically significant difference (p<0.0001). Tukey’s post hoc test revealed a significant difference between the two groups.

**Comparison between Ki-67 and MCM-3 immunorepression**

The mean number of Ki-67 positive nuclei in benign and malignant tumor were greater than the mean number of MCM-3 positive nuclei. Unpaired t test revealed that the difference was not statistically significant (p=0.234) in benign tumor, and the difference was statistically significant in malignant tumors (p=0.0002).

### DISCUSSION

Salivary gland tumors (SGTs) are relatively uncommon lesions accounting for 3-6% of all head and neck neoplasms. World Health Organization (WHO) has given the global incidence of salivary gland tumors as 0.4-13.5 cases per 100,000 population annually (22). The rapid proliferation rate is one of the features common to most neoplasms. Identification of a proliferating fraction within the tumor cell population has been useful in diagnosis and/or prognosis in a range of human cancers. Biological markers indicative of cell cycle state has proven useful for determining growth fractions(23).

In the present study, Ki-67 protein was examined in normal salivary gland tissues as well as benign and malignant salivary gland tumors. All normal control sections of salivary gland tissues revealed negative immunoreactivity to Ki-67.

Ki-67 was expressed in all the examined salivary gland tumors with different intensities. The malignant salivary gland tumors showed intense and abundant staining of Ki-67 compared to benign tumor.

In this work, the pleomorphic cases adenoma showed positive nuclear and cytoplasmic Ki-67 immunosignals in myoepithelial cells, while carcinoma ex- pleomorphic adenoma (CXPA ) exhibited intense diffuse total cell immunopositivity in the almost all malignant cells, These findings are in agreement with Tadbir et al (24), and Trandafirescu et al (25), who mentioned that, Ki-67 is useful in assessing the intensity of proliferation in pleomorphic
Adenoid cystic carcinoma adenoma and it gives indications on the risk of malignancy. Also Freitas et al (26) suggested that CXPA having a higher Ki-67 proliferative index than pleomorphic adenoma, therefore it may be useful in distinguishing CXPA from benign pleomorphic adenoma (26).

Myoepithelioma, in the present study, exhibited positive immunoreaction to Ki-67 in myoepithelial cells, whereas myoepithelial carcinoma showed diffuse total cell positivity to Ki-67 in the malignant epitheloid and plasmacytoid myoepithelial cells. This is in accordance with Nagao et al (1), they stated that Ki-67 labeling index (LI) is helpful in the differential diagnosis between myoepithelioma and myoepithelial carcinoma.

The only studied case of Warthin's tumor showed intense immunoreactivity to Ki-67 in the oncocytic epithelial cells. However, the lymphoid stroma was free from any reaction, this is in accordance with, Sangeetha et al (27) in their work on salivary glands.

The cases of mucoepidermoid carcinoma included in this study were of the high and low grade types. Both types were positively immunoreactive to Ki-67, being obviously more intense in the high grade type especially in the epidermoid cells. Accordingly Nguyen et al (28), found that, strong Ki-67 staining occurred in patients with high-grade MEC, whereas low-grade MEC revealed negative or weak staining. This indicated that the proliferating index of Ki-67 may be used as an important parameter for grading mucoepidermoid careinoma (29).

In the present work, AdCC exhibited intense positive nuclear and cytoplasmic immunoexpression to Ki-67. This is in agreement with Lazzaro et al (30), while Fonseka et al (31), reported low expression of this marker in adenoid cystic carcinoma. This difference may be due to measuring the expression by using immunohistochemical staining, which is affected by tissue aging, staining technique, enzyme antibody used, and single observer bias.

Previous studies have proposed that MCM proteins may be sensitive proliferation markers and may serve as novel biomarkers for prognostication and diagnosis of various premalignant and malignant lesions (32, 33). As suggested by Ashkavandi et al (21), MCM-3 is useful to determine the prognosis and in the differential diagnosis of salivary gland tumors.

In the present work, MCM-3 was expressed in all the examined salivary gland tumors with different intensities. In general all salivary gland malignancies, compared to benign lesions, were highly expressive to MCM-3, whereas the normal control section of salivary gland tissues revealed negative immunoreactivity to MCM-3.

In this research, both pleomorphic adenoma and carcinoma ex-pleomorphic adenoma, showed positive total cell immunoreaction to MCM-3, with increased intensity of staining in CXPA. This agrees with Vargas et al (32). They found MCM-2 LI was higher in CXPA than pleomorphic adenoma.

The case of adenoid cystic carcinoma included in this research revealed diffuse total cell immunopositivity to MCM-3. This is in accordance with Ashkavandi et al (21) and Ghazy et al (34), although the latter found that adenoid cystic carcinoma revealed only cytoplasmic MCM-2 staining. The heterogeneity in staining is cell cycle dependent as suggested by Yan et al (16). Who found that, un budded cells showed clear nuclear staining, whereas premiotic budded cells (single nucleus) showed cytoplasmic staining. In contrast, postmitotic large budded cells (two divided nuclei) showed either nuclear or cytoplasmic staining.

This study showed that mucoepidermoid carcinoma expressed positive immunoreactivity in epidermoid and mucus secreting cells, with the high grade MEC cases showing a marked increase of MCM-3 expression when compared to the low grade cases. This is in agreement with Ashkavandi et al (21), Vargas et al (32), and Ghazy et al (34). This might be explained by the fact that in cancer, differentiated neoplastic cells tend to grow and spread at a slower rate than undifferentiated or poorly differentiated cells, which lack the structure and function of normal cells and grow uncontrollably (35).

In the present study, the myoepithelioma and myoepithelial carcinoma exhibited total positive cell reactivity to MCM-3. The malignant epitheloid and plasmacytoid myoepithelial cells were more intensely immunostained. This was in conform with Ghazy et al (34), who reported that myoepithelial carcinoma showed higher value of MCM-2 expression, which might indicate the aggressive behavior of this neoplasm.

MCM-3 is part of the replication licensing complex and thus tightly associated with cell proliferation (36). Therefore, it was important to compare the expression levels of MCM-3 and the established proliferation marker (Ki-67) in SGTs.

According to this work the expression of Ki-67 protein in malignant SGTs was significantly higher than expression of MCM-3. In contrast, most studies have demonstrated that, LI of MCM-3 was significantly higher than Ki-67 LI, in the different human tumor including SGTs (19, 21, 36). The difference may be related to the type of antibody used (monoclonal and polyclonal), and the differences in how the cells are counted.

The fact that the MCM-3 LI was considerably higher than the Ki-67 LI suggests that Ki-67 may be expressed during a shorter interval of the cell cycle than MCM-3. The mRNA levels of MCM-3 are dramatically induced at the G1–S boundary, whereas Ki-67 is predominantly expressed during the S, G2, and M phases, MCM-3 is expressed even in normal and neoplastic cells at the early G1 phase, Ki-67 expression cannot be detected at this stage (37).

In the present work, positive correlation was documented between MCM-3 expression and Ki-67 expression in malignant SGTs (the correlation coefficient r=0.2309). This is in agreement with a study conducted by Shetty et al (38), who reported that, on mammary carcinomas, a positive correlation.
was disclosed between the expression intensities of MCM-2 and Ki-67. In contrast, Lee et al (39), in papillary thyroid carcinoma (PTC) demonstrated that, there was no significant correlation between labeling indices of MCM-3 and Ki-67.

Since only one previous study has assessed MCM-3 expression in salivary gland tumors Ashkavandi et al (21), more work is required to add to the growing body of knowledge on the application of this novel proliferation marker in the study of salivary gland tumours.

CONCLUSION
This study concludes that
• Ki-67 and MCM-3 proteins are overexpressed in malignant salivary gland tumors than benign ones.
• Both Ki-67 and MCM-3 may be reliably applied as diagnostic markers to distinguish benign from malignant salivary gland tumors.

CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest.

REFERENCES


