Original Article
ISSN 2228-5032

# Efficiency of Silver Staining in Differential Diagnosis of Adenoid Cystic Carcinoma from Polymorphous Low-Grade Adenocarcinoma

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(*Submitted: 21 June 2017– Revised version received: 5 May 2018– Accepted: 7 March 2018– Published online: Winter 2018*)

**Objectives** The aim of this study is find a practical and easy way to histologically differentiate between two malignant tumors namely Adenoid Cystic Carcinoma (ACC) and Polymorphous Low-grade Adeno Carcinoma (PLGA) silver nitrate staining.

**Methods** In this cross-sectional study, 30 parrafin-embedded blocks of ACC and nine paraffin-embedded blocks of PLGA with the most acceptable standards were selected and stained with silver nitrate. Then the number and quality (size and pattern) of the stained spots in five random microscopic fields at 100× magnification (at least 100 cells) were evaluated. T-test was used to compare the number of dots between the two tumors while the quality of dots was compared with the Mann-Whitney U test.

**Results** The mean silver stainable nucleolus organizer region (AgNOR) count was 3/45 for ACC and 2/45 for PLGA. Significant differences were observed in the number of dots between ACC and PLGA (p=0.004), but there was no statistically significant difference between the two tumors in terms of quality of dots.

**Conclusion** AgNOR count can be useful as an available method in confirm the diagnosis of ACC and differentiate it from PLGA.

**Keywords** Carcinoma Adenoid Cystic, Adeno Carcinoma, Silver Nitrate, Cell Proliferation

## Introduction

Salivary gland tumors are less pervasive compared to other tumors and account for less than 3% of the head and neck neoplasms, but they comprise a significant percentage of tumors of the mouth, jaw, and face. [1] These tumors have diverse morphologies that make their diagnosis difficult or even impossible in small biopsy samples. In such cases, precise clinical and pathological features are important and helpful, but there are cases requiring specific techniques for diagnosis.[2,3] Adenoid cystic carcinoma (ACC) and Polymorphous low-grade adenocarcinoma (PLGA) are examples of these cases. ACC and PLGA are two adenocarcinoma with different prognosis that overlap in histological features.[4, 5]

ACC is one of the most pervasive and most recognized salivary gland malignancies described for the first time in 1853.[6] It is a fatal tumor with infiltrative nature and slow growth[1], which is prone to local recurrence. It eventually leads to distant metastases and has a poor prognosis. The preferred treatment is usually surgical removal of the tumor, but in some cases, radiation can help improve the patient survival.[7, 8]

PLGA is a malignant, invasive and persistent tumor, with slow metastasis which is almost exclusive to salivary glands.[1] This tumor was first identified in the early 1980s prior to its recognition as a distinct tumor. Its samples used to be classified as ACC and sometimes pleomorphic adenoma. However, it was found that the tumor has distinct clinical and pathological features and was recently emerged as a distinct malignant tumor of the salivary glands.[9, 10]

The best treatment for PLGA is wide excisional surgery sometimes necessitating bone resection. Local recurrence is not rare and 9% to 17% of the patients show recurrence within 5 years after surgery, but it is often successfully controlled with wide excisional surgery in more than 50% of the cases.[1]

Similarity of histological features may cause confusion in the diagnosis of this tumor, particularly if the biopsy sample is small and from the minor salivary glands.[1, 11] The use of advanced diagnostic methods can help in histopathological diagnosis of these two tumors, but most of these techniques are not used in pathology laboratories due to the need for training of experts, expensive kits, and lack of access to the kits. Silver staining is a rapid, simple, cheap, available and one-stage method used in oral pathology with hematoxylin and eosin staining.[12, 13] Several studies have introduced this type of staining as a useful prognostic indicator and a method for diagnosis of premalignant and malignant lesions, and as an indicator for staging of malignant tumors.[14-17]

To the best of authors' knowledge, no previous study has examined silver staining to differentiate between malignant ACC and PLGA.[18] Therefore, the present study was conducted to introduce a simple and accessible method to differentiate between ACC and PLGA using silver staining.

## Materials and Methods

Forty paraffin-embedded blocks, including 30 ACC and 9 PLGA samples, with proper fixation and texture and no edema, hemorrhage, or necrosis were selected and 4µm-thick sections were made and stained by silver nitrate (Merck, Darmstadt, Germany) according to the method described by Ploton et al.[19] Breast cancer sample was considered as the positive control. Qualitative and quantitative assessments were made by light microscopy (CHS model; Olympus, Tokyo, Japan) in a double-blind manner by two observers in areas with the lowest cell overlapping and stain deposition. Slides stained poorly were excluded and the remaining slides that contained 39 samples (30 ACC and 9 PLGA) were evaluated.

Quantitative assessment took place using standard methods as explained by Crocker [(9)], such that on each slide, 100 cells per smear in five random microscopic fields at ×100 magnification optical microscope were selected and the number of Nucleolus Organizer Regions (NORs) was counted. Cells at the core, which had one or more dots were selected and cells lacking these points were not counted. Accumulate points, which were not distinguishable and the cases where the structure of the nucleus was stained as rings, were considered as one point. Then, the mean NORs in 100 cells were calculated per slide.

Qualitative assessment included the size and distribution of points.[20]

The ranking of the size of dots size was as follows:

0: same size dots, +1: two different sizes, +2: three different sizes, +3: all sizes.

Scale of distribution of dots: 0: Limited to the nucleolus, +1: rarely out of the nucleolus, +2: medium dispersion out of the nucleolus, +3: wide dispersion outside the nucleolus.[20]

The t-test was used to compare the number of stained points between the two tumors, and non-parametric Mann-Whitney U test was used to analyze and compare the size and pattern.

## Results

Analysis of the data was performed by two observers using 30 ACC samples and 9 PLGA samples. The intraclass correlation coefficient was 0.998 for the number of AgNORs and 1 for the size and distribution of AgNORs.

As shown in table 1, the men number of AgNORs dots, was 3.45±1.26 in ACC (Fig 1) and 2.45±1.25 in PLGA (Fig 2); this difference was statistically significant (P=0.004).

**Table 1-** Number of AgNORs in ACC and PLGA

| Lesion | Minimum Num spot | Maximum Num spot | Mean Num spot | P-value |
|---|---|---|---|---|
| ACC | 1.00 | 7.00 | 3.45±1.26 | 0.004 |
| PLGA | 1.00 | 5.00 | 2.45±1.25 | |

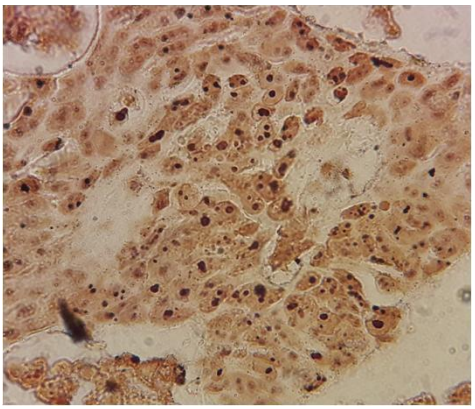

**Figure 1- AgNORs dots in ACC, X100 magnification**

**Figure 2- AgNORs dots in PLGA, X100 magnification**

As shown in tables 2 and 3, the size and distribution pattern of the points did not show statistically significant differences between ACC and PLGA (P=0.30 and P=0.42, respectively).

The sensitivity and specificity of this test were 0.967 and 0.667 respectively.

**Table 2**-The size of nuclear organization region in ACC and PLGA

| Lesion | Same Size | Two Different Size | Three Different Size | All Size |
|---|---|---|---|---|
| ACC | 1.7% | 61.7% | 36.7% | 0.0% |
| PLGA | 0.0% | 77.8% | 22.2% | 0.0% |
| P-value | | 0.30 | | |

**Table 3**- Distribution pattern of nuclear organization region in ACC and PLGA

| Lesion | Limited to the nucleolus | Rarely out of the nucleolus | Medium out of the nucleolus | Wide dispersion out of the nucleolus |
|---|---|---|---|---|
| ACC | 0.0% | 56.7% | 43.3% | 0.0% |
| PLGA | 0.0% | 66.7% | 33.3% | 0.0% |
| P-value | | 0.42 | | |

## Discussion

Histological similarity of ACC and PLGA tumors despite their different prognoses and biological processes calls for a method to help their differential diagnosis.[18] Although the current known methods have high sensitivity, they require high cost and time. The results showed that the rate of cell proliferation in ACC was significantly higher than that in PLGA. Therefore, AgNOR staining technique can be suggested as a simple and available method to differentiate between suspected tumor cases before any sophisticated technique. According to our results, the number of points stained is a reliable parameter while the quality of the stained points (size and distribution of points) has little diagnostic value.

Previous studies in this regard on a variety of tumors suggest that NORs in the nucleus of hyperplasic and malignant cell express their proliferative activity; thus, NORs are a reflection of synthetic activity of hyperplastic cells and increase the speed of cell cycle and malignancy.[21-24] Thus, it seems that presences of these cells in high number in a tumor are associated with more aggressive behavior of tumor. In other words, higher cell proliferation indicates higher growth rate, recurrence, and metastasis of the tumor. Since ACC has more aggressive biological behavior, compared to PLGA, greater presence of NOR's in PLGA is justified.

Silver staining has been used as an indicator to determine cellular proliferation and its relationship with the diagnosis of benign, premalignant and malignant lesions and grade and prognosis of tumors in other parts of the body has been previously studied.[25-30]

One limitation of this study was unstable staining of samples by AgNORs, which resulted from factors such as fixing solution, temperature, reaction time and concentration of silver and formic acid.[19] To fix this problem, the Ploton's modified method was used. [6, 31]

*Yamamoto et al.*[32] stated that the number of AgNOR points in solid pattern ACC was higher than that in tubular and trabecular patterns. They attributed the poorer prognosis of solid pattern ACC to higher cell proliferation in this pattern. They concluded that AgNOR staining is a proper method to assess cell proliferation in different patterns of ACC.[32]

Matsumura et al.[12] Proposed AgNOR staining as an appropriate staining method in differential diagnosis of benign and malignant salivary tumors. Other researchers found no relationship between AgNOR counts with prognosis or differentiation of ACC tumor, which could be due to the difference in the nature of tumors examined, insufficient sample size, differences in methodology or the counting method. [31,33] A previous study found no statistically significant association between the average number of AgNOR points and stage of malignant neoplasm or the clinical course of malignant or benign masses in salivary glands.[15] According to some previous studies, AgNOR staining is useful in distinguishing benign from malignant lesions, but not for distinguishing between histology types or stages of malignant neoplasm of slivery glands and predicting prognosis.[14, 15] Future studies are required to assess AgNORs in ACCs according to their histological pattern.

## Conclusion

The results showed that silver staining techniques could be used as an aid in the differential diagnosis of ACC and PLGA. Since the technique used in this study is low cost, fast, easy and accessible, it could be an alternative to complex and expensive methods.

## Conflict of Interests

None Declared ■

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**How to cite:**
Mahbube Valipour Tahamtan, Shahrzad Shahbeik, Alireza Abdollahi, Gita Rezvani. Silver Staining Efficiency in Differential Diagnosis of Adenoid Cystic Carcinoma (ACC) from Polymorpheus Low-Grade Adenocarcinoma (PLGA). J Dent Sch 2018; 36(1):23–26.