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How Useful is the AgNOR Staining Method for the Diagnosis of Salivary Gland Tumors?

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Abstract

Objectives: The purpose of this study was to determine the numbers and mean areas of the AgNOR dots in the 3 most common salivary gland tumors.
Materials and Methods: One hundred and seventy paraffin blocks histologically diagnosed as pleomorphic adenoma, adenoid cystic carcinoma, and mucoepidermoid carcinoma were selected from the archives of the pathology department of Loghman hospital, Tehran, Iran and then cut and stained using the standard AgNOR method. The number and area of AgNOR dots were evaluated. Chi-square and Pearson correlation (2-tailed) tests were used for statistical analysis. The significance level was $P < 0.05$.
Results: The chi-square test showed significant differences between tumor types and the numbers of AgNORs ($P = 0.000$) and between tumor types and areas of AgNORs ($P = 0.000$).
Conclusions: The AgNOR technique can be used to diagnose salivary gland tumors.

Keywords: AgNOR Method, Salivary Gland Tumors, Diagnosis

1. Background

Salivary gland tumors are rare and comprise 3% - 6 % of the neoplasms in the head and neck area (1). They comprise diverse lesions of uncertain histogenesis, phenotypically and biologically (2). Distinguishing between low-grade malignant and benign tumors and differentiating between different histological grades, particularly in small biopsy specimens, are particularly difficult issues for pathologists faced with these types of lesions (3). In addition, differentiating between types of salivary gland tumors is a diagnostic challenge for pathologists, as they have diverse histomorphological features in individual lesions, as well as a number of types and variants, and even similar patterns (4). On the other hand, pleomorphic adenoma (PA) is the most common benign salivary gland tumor with several variations of cell types. Additionally, mucoepidermoid carcinoma (MEC) and adenoid cystic carcinoma (ACC) are the most common malignant salivary gland tumors with different grades and patterns (5). All these factors make these tumors the best candidates for this study.

Nucleolar organizer regions (NORS) are chromosal segments that contain ribosomal genes (6). The NORs stained silver and the argyrophilic NOR-associated proteins are called AgNORs (7). The amount of AgNOR protein in malignant cells is frequently greater than in benign lesions or normal tissue. Therefore, the amount of AgNOR proteins is believed to be related to cell proliferation activity and the degree of malignant transformation (8). AgNOR expression can be used to differentiate benign from malignant lesions and also be used to grade tumors (3, 9). Additionally, the AgNOR surface area (size) that each dot occupies in each nucleous is another factor to predict the risk of tumor recurrence and also provides useful information for treatment planning (10).

To achieve reliable results, in 1993 the committee on the AgNOR Quantitation within the European society of pathology recommended the utilization of a standardized AgNOR technique to assess the AgNOR quantity in fixed and paraffin-embedded pathological tissues (11).

2. Objectives

We carried out the present study to determine the diagnostic value of AgNOR in diagnosing three salivary gland tumors: PA, ACC, and MEC with different grades (low, intermediate, and high).

3. Materials and Methods

All cases histologically diagnosed as PA, ACC, and MEC were selected from the Archives of the pathology department of Loghman hospital, Tehran, Iran. First, paraffin blocks were cut and stained using the hematoxylin and eosin (H and E) method to confirm the previous diagnosis by two pathologists independently. For better review, all cell and tissue types of PA were reviewed. ACC cases were histologically categorized as tubular, solid, and cribriform patterns. MEC cases were graded as low, intermediate, and high grades. For MEC cases, mucicarmine staining was performed as well to detect mucous cells (1).

A total of 170 blocks from well characterized salivary gland tumors were selected, 80 blocks for PA (consisted all cell and tissue types), 45 blocks for ACC (15 for each histologic pattern), 45 for MEC (15 for each histological grade), and 20 blocks for normal salivary gland tissue (containing mucosal and serous type acini).

AgNOR staining was done according to the standard protocol recommended by the Committee on the AgNOR Quantitation within the European society of pathology (7). Briefly, from each tissue block, two consecutive 3 μm-thick sections were cut, one for H and E staining and one for the AgNOR staining method. For the AgNOR staining method, the sections were immersed in sodium citrate buffer (a pH of 6), incubated in a wet autoclave at 120°C for 20 minutes, and then allowed to cool down to 37°C. The slides were then immersed in a freshly prepared silver-staining solution containing one part of 2% gelatin in 1% formic acid (by volume) and two parts of 25% aqueous silver nitrate solution for 11 minutes. After washing the slides with double-distilled deionized water, the sections were dehydrated and mounted. The sections were stored in a dark cool place. In each case, approximately 100 nuclei were randomly examined from different areas and tissue types. The parameters examined and calculated for each nucleus were as follows:

1. The number of AgNORs per nucleus (nNOR) was calculated at an oil-immersion magnification (x1000) done by two different pathologists.

2. The AgNOR profile surface area (size) per nucleus (μm^2) (aNOR) was measured with the aid of the Zeiss AxioVision 4.5 image capture procedure.

All data were entered into the statistical analysis software, statistical package for social sciences, SPSS version 21.0 (SPSS Inc., New York, USA). Chi-square and Pearson correlation (2-tailed) tests were used for statistical analysis. The significance level was $P < 0.05$.

4. Results

Table 1 shows a summary of the means of nNOR and aNOR in different tumor types. The chi-square test showed significant differences between tumor types and nNOR (P = 0.000) and between tumor types and aNOR (P = 0.000). In addition, there were significant differences between the numbers (Figure 1) and areas of AgNOR in different cell types of PA (P = 0.000, P = 0.000, respectively), between the numbers (Figure 2) and areas of AgNOR in different histological types of ACC (P = 0.000, P = 0.000), between the numbers (Figure 3) and areas of AgNOR in different cell types of low-grade MEC (P = 0.000, P = 0.000), between the numbers and areas of AgNOR in different cell types of intermediate-grade MEC (P = 0.0000, P = 0.000), and between the numbers and areas of AgNOR in different cell types of high-grade MEC (P = 0.000, P = 0.000).

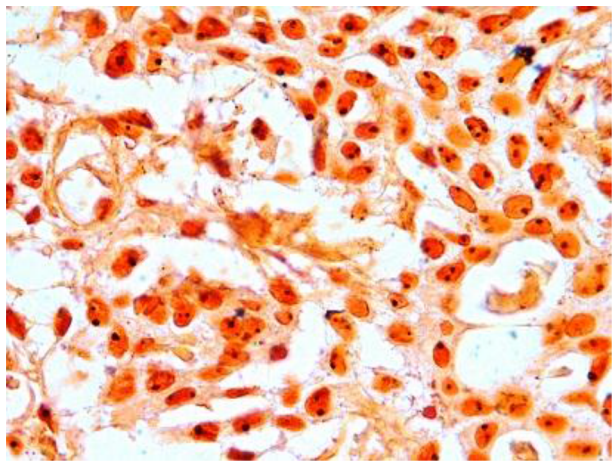

Figure 1. Several small AgNOR Dots Within the Ductal Cells Surrounded by Myoepithelial Cells in PA (x 1000)

Pearson correlation testing showed a significant positive correlation between the tumor type and nNOR (r = 0.159, P = 0.001). In addition, there was a positive and significant correlation between the cell type and nNOR (r = 0.164, P = 0.001). The Pearson test also showed a negative and significant correlation between nNOR and aNOR (r = -0.239, P = 0.001) in general.

5. Discussion

The AgNOR staining technique has been employed for the diagnosis and prognosis of many types of lesions. In the past few years, many studies have attempted to concern the potential diagnostic and prognostic applications of AgNOR staining (12). In the current study, the differences

Table 1. Summary of the Means of nAgNOR and AgNOR in the Nuclei of Different Salivary Gland Tumors and Cell types

Type of Tumor	Mean nAgNOR (± S.D.)	Mean AgNOR (± S.D.)
Normal Salivary Gland		
Mucous acini	1.63 ± 0.49	2.35 ± 0.31
Mucous ductal cells	2.04 ± 0.51	2.06 ± 0.30
Serous acini	1.47 ± 0.50	1.94 ± 0.30
Serous ductal cells	1.84 ± 0.37	1.86 ± 0.31
Overall mean	1.72 ± 0.50	2.05 ± 0.36
PA		
Myoepithelial cells	2 ± 0.74	2.81 ± 0.53
Ductal cells	2.20 ± 0.88	2.53 ± 0.51
Chondroid area cells	1.96 ± 0.71	2.26 ± 0.40
Osteoid area cells	1.91 ± 0.69	2.29 ± 0.45
Myxomatous area cells	1.84 ± 0.78	2.55 ± 0.59
Keratinized area cells	1.42 ± 0.61	2.24 ± 0.34
Atypical cells	2.42 ± 1.05	2.31 ± 0.46
Plasmacytoid cells	2.14 ± 1.01	2.44 ± 0.50
Overall mean	2.03 ± 0.84	2.48 ± 0.53
ACC		
Cribriform pattern	2.07 ± 0.78	2.89 ± 0.51
Tubular pattern	1.59 ± 0.67	3.08 ± 0.36
Solid pattern	3.01 ± 0.74	2.92 ± 0.41
Overall mean	2.13 ± 0.93	2.98 ± 0.43
MEC (low grade)		
Mucous cells	1.49 ± 0.63	3.37 ± 0.29
Intermediate cells	1.83 ± 1.01	3.29 ± 0.35
Epidermoid cells	2.38 ± 0.90	2.98 ± 0.45
Overall mean	1.8 ± 0.934	3.23 ± 0.40
MEC (intermediate grade)		
Mucous cells	2.61 ± 0.86	2.67 ± 0.42
Intermediate cells	2.78 ± 0.83	2.81 ± 0.57
Epidermoid cells	3.13 ± 0.74	3.06 ± 0.40
Overall mean	2.85 ± 0.84	2.85 ± 0.51
MEC (high grade)		
Intermediate cells	3.27 ± 0.86	3.11 ± 0.43
Epidermoid cells	3.09 ± 1.20	3.37 ± 0.31
Overall mean	3.18 ± 1.05	3.24 ± 0.39

between the means of AgNOR counts and areas and the types of tumors were significant.

It is worthy to note that the standard staining method was not employed in any of the previous studies on salivary gland tumors. Table 2 shows a summary of the results of previous studies.

Table 2. Comparing the Mean Number and Areas of AgNOR Dots in the Nuclei of Salivary Gland Tumors in Previous Studies

Type of Tumor	Mean of nAgNOR	Reference
Normal salivary gland		
Overall mean	1.21 ± 1.4	Fonesca
PA		
Overall mean	1.52 ± 0.32	Van Heerden
Overall mean	1.7	Freitas
Overall mean	3.02 ± 1.03	Ogawa
Overall mean	1.47 ± 0.074	Morgan
ACC		
Overall mean	4.20 ± 0.99	Fonesca
Overall mean	3.92 ± 0.18	Morgan
Overall mean	3.65 ± 0.52	Adeyemi
Overall mean	2.83 ± 0.89	Van Heerden
MEC (low grade)		
Overall mean	1.35 ± 0.20	Satyajitraji
Overall mean	0.64 ± 0.15	Alaeddini
Overall mean	1.93 ± 0.55	Van Heerden
Overall mean	3.09 ± 0.86	Adeyemi
Overall mean	4.25 ± 0.15	Morgan
Overall mean	1.62	Epivatianos
MEC (intermediate grade)		
Overall mean	1.04 ± 0.21	Alaeddini
Overall mean	2.62 ± 0.30	Satyajitraje
MEC (high grade)		
Overall mean	1.42 ± 0.23	Alaeddini
Overall mean	2.91	Epivatianos
Overall mean	3.81 ± 0.65	Satyajitraje
Overall mean	3.68	Adeyemi

Auclair and Ellis found atypical cells in 2% of benign cases of PA, which were associated with a higher potential of malignant changes, particularly in those with hyalinization, necrosis, and a high mitotic rate (13).

In our study, the highest mean of the nNOR for PA was due to atypical cells with 2.42 ± 1.05 and the lowest was due to keratinized area cells with 1.42 ± 0.61. Regarding the high risk of the malignant transformation rate in PA, it could be said that the possibility of malignant changes would be higher in tumors consisting of large areas of atypical cells and the lowest in PA tumors consisting mainly of keratinized areas. Landini, based on AgNOR staining findings, reported that the cells in solid/ductal areas have higher metabolic activity than those in chondroid areas. They also suggested that the metabolic activity may have a direct relationship with malignant transformation in PA (14). In Fujita et al.'s study, the number of AgNOR dots in the solid nests was the highest. The authors indicated that tumor cells within stroma, consisting of myxoid, chondroid, or hyalinized matrices, have lower proliferative activity (15).

In our series, the lowest AgNOR number was found in the cribriform pattern of ACC. These findings are in agreement with those of Yamamoto et al., which demonstrated the lowest proliferative activity in the cribriform pattern (16), but inconsistent with Freitas et al.'s findings indicating the lowest proliferative rate in the tubular pattern (17).

Prior studies have indicated that the AgNOR count

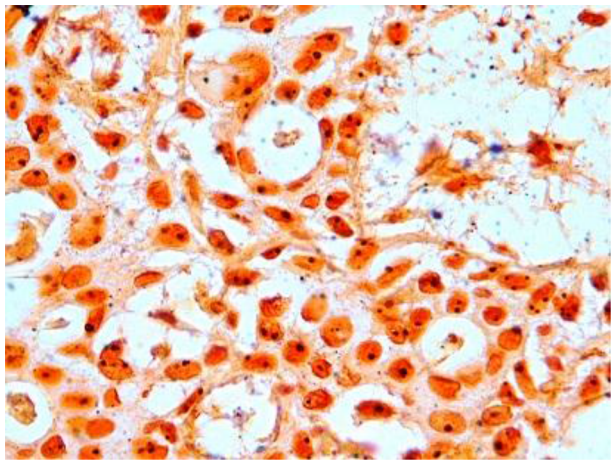

Figure 2. AgNOR Dots Within the Glandular Epithelium in ACC (x 1000)

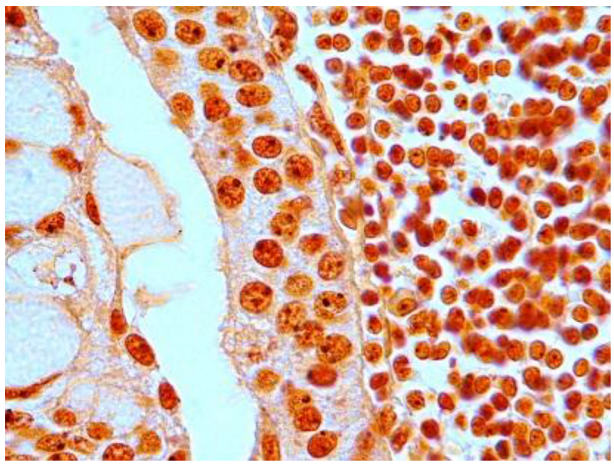

Figure 3. AgNOR Dots Within the Cystic Lining and Epidermoid Cells in MEC (x 1000)

corresponds with the clinical behavior (18). Our results demonstrated that the proliferative rate depends on the histological pattern of ACC. Therefore, it might be possible to predict the clinical behavior of ACC. In the present study, consistent with previous studies, the highest AgNOR number was seen in the solid type, which might explain the worst prognosis in ACC mainly consisting of a solid pattern (5).

In the present study, the mean number of AgNOR dots in the mucous cells of MEC was lower than those of intermediate and epidermoid cells. This result is in agreement with that of Fujita et al. (15). In addition, our results are consistent with the histological grading of MEC (5, 15). Furthermore, the nNOR means of intermediate and epidermoid cells were lower in low-grade tumors than the intermediate-grade tumors, which might explain the lower tendency for malignancy in low-grade tumors. On the other hand, the mean of number of AgNOR dots in intermediate cells increased by increasing the tumor grade, which might prove the intermediate cells as precursors for other cell types. The AgNOR dot numbers also increased with progressing MEC grades. The mean of AgNOR dots in epidermoid cells also increased as the tumor grade increased, which confirms the higher tendency for malignancy. A similar increase in the AgNOR dot numbers has also been reported in other high-grade tumors (19, 20). These findings also seem to be in agreement with the overall indolent biological behavior of MEC. In this study, the overall nNOR mean for low-grade tumors was the lowest, it was highest for the high-grade MEC, and intermediate for the intermediate grade. Alaeddini et al. also found the same results (21). These findings demonstrate that the mean number of AgNOR dots increases as the tumor grade increases. This may serve as an explanation for the different grades of MEC. Chomette et al. reported that the AgNOR count is related to the prognosis and histological grading of MEC (22).

Feritas et al. measured the mean diameter of AgNORs in their study. Overall means were as follows: PA (1.5 μm), solid ACC (1.4 μm), cribriform (1.2 μm), and tubular ACC (1.9 μm). They found a significant difference between the mean diameters of the AgNORs in different histological types of ACC (17).

In the present study, high levels of AgNOR were found in more aggressive tumors and low levels in less aggressive and well-differentiated tumors. Therefore, there is a correlation between the AgNOR count and the histologic differentiation and also between the AgNOR count and the histologic differentiation. In our study, the mean of the AgNOR number was correlated with the tumor grade in ACC and MEC, and there was also a reverse relationship between the AgNOR number and the tumor size, which is in agreement with other studies indicating that a small size, a large number, and a scattered distribution of AgNORs are characteristics of malignant tumor cells whereas a large size, a small number, and a clustered distribution of AgNORs are characteristics of benign tumor cells (9).

In our study, the means of the AgNOR number of ductal cells (both mucous and serous) in the normal salivary gland tissues were higher than those of acini cells (both mucous and serous). These findings indicate the potential role of ductal cells as a main source of salivary gland tumors' histogenesis, which was suggested in previous articles (23).

In conclusion, it is suggested that the change in AgNOR parameters reflects the changes in the cellular metabolic activity and can help in the differentiation of various sali-

vary gland tumors. In addition, the mean of the AgNOR number correlates with the degree of malignancy. Therefore, the AgNOR technique can be used to assess the biologic behaviors of the examined salivary gland tumors.

Footnotes

Authors' Contribution: Study concept and design: Soussan Irani; acquisition of data, Soussan Irani; analysis and interpretation of data, Soussan Irani, Farahnaz Bidari Zerehpoush, Shahram Sabeti; drafting of the manuscript, Soussan Irani; critical revision of the manuscript for important intellectual content, Soussan Irani; statistical analysis, Soussan Irani; administrative, technical, and material support, Soussan Irani; study supervision, Soussan Irani.

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