# RESEARCH ARTICLE

# Could Argyrophilic Nucleolar Organizer Regions count mirror DNA Ploidy in Malignant Salivary Gland Tumors?

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## Abstract

   **Objective:** Nucleolar organizer regions (NORs) are DNA coils that transcribe to ribosomal RNA. The NOR-associated protein, termed argyrophilic NOR (AgNOR), was visible within the nucleus by staining with silver nitrate examination via the light microscope. AgNOR counting is a proliferation marker and may help in the diagnosis and prognosis of various neoplastic lesions. Aneuploidy (abnormal DNA content) can predict the progression, survival and prognosis of the tumors. The aim of this study was to evaluate the role of AgNORs, DNA ploidy status, and total S-phase fraction (TSPF) as prognostic parameters in malignant salivary gland tumors (MSGTs). **Methods:** The current study is a retrospective study on a cohort of MSGTs (N=47), to assess AgNORs using Silver Nitrate stain, DNA index (DI), and TSPF using flow cytometry (FCM). Data including tumor size and site, lymphovascular invasion (LVI), lymph node metastasis (LNM) were collected. **Results:** The AgNORs count was statistically significant with MSGT type. DI was found to have a significant association with tumor site, tumor size and MSGT type. In addition, TSPF was found to be significantly associated with LVI. A moderate positive correlation was noted between AgNORs count and TSPF. LNM, tumor site, high AgNORs and low DI were all associated with short disease-free survival (DFS) and poor overall survival (OS). **Conclusion:** The present study revealed that high AgNORs count, DNA aneuploidy and TSPF had a poor influence on MSGTs prognosis.

   **Keywords:** AgNORs- DNA ploidy- malignant salivary gland tumors- survival

## Introduction

   Salivary gland tumors account for 5% of all head and neck tumors. Most of these tumors are benign and only 20% are malignant, with an estimated global annual incidence varying from 0.4 to 13.5 cases per 100,000 (Dos Santos et al., 2020).

   Malignant salivary gland tumors (MSGTs) are still being studied in terms of pathogenesis and behavior. To date, only a few studies on MSGTs have been conducted to investigate their invasive potentials, proliferative activity, and the ability to predict tumor therapy response or prognosis. Among these studies, Argyrophilic Nucleolar Organizer Regions (AgNORs) evaluation has been considered a simple, reliable, easily applicable and cost-effective approach for assessing cellular proliferation (Mohamed Mahmoud et al., 2017). The number and distribution of AgNORs number within the nucleus reflect the nucleolar and proliferative activity of tumor cells. Nucleolar organizer regions (NORs) are DNA coils from which r-RNA genes were transcribed, which can be found on the short arms of on the chromosomes 13, 14, 15, 21 and 22. Since the NORs are linked with acidic proteins in the form of (C23, B23 and possibly RNA Polymerase I), it is considered to be argyrophilic. These proteins are rich in carboxyl and sulfhydryl groups which precipitate the silver ions (Gupta et al., 2018).

   The AgNORs technique is a good indicator of cell activity, nevertheless, it should not be applied to differentiate between benign and malignant epithelial tumors (Mohamed Mahmoud et al., 2017). In oral squamous cell carcinoma (OSCC), AgNOR is a supportive guide for the clinician and the histopathologist predicting the proliferation rate, in addition to understanding the biological behavior of OSCC (Jagtap MM et al., 2020). Moreover, a previous study suggested that AgNOR mirrors the metabolic activity changes in the cells, which can applied aid in the differentiation between various salivary gland tumors (SGTs) (Gupta et al., 2018). Furthermore, it has been proposed that AgNOR in SGTs reflects the

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changes in the cellular metabolic activity and can help in distinguish various salivary gland tumors (Zahran AM et al., 2018).

In malignant neoplasms, DNA cell content is an efficient prognostic marker. DNA cell content and rapid cell turnover have been reflected in the ploidy status and SPF of the neoplasm (Khanna et al., 2010). Previous research has demonstrated that the DNA content alteration are evident in advanced stages of SGTs, which are attributed to specific genetic and epigenetic alterations that accumulate in the neoplastic cells. These changes caused chromosomal instability, resulting in structural or numerical aberrations known as as a`1neuploidy (Monteiro et al., 2009). DNA ploidy assessment has been established as a prognostic factor in many solid cancers such as ovarian, endometrial (Matias-Guiu and Davidson, 2014), breast, prostate and urinary bladder carcinomas (Danielsen et al., 2016).

Despite wide variety of tumors were analyzed by FCM, few studies were carried out on MSGTs. Although DNA content analysis using FCM is conducted in a wide range of tumors, few studies have been carried out on MSGTs and the results have been contradictory. According to some studies, the overall proportion of MSGTs with DNA aneuploidy is low (Monteiro et al., 2009). On the contrary, other studies have reported that DNA aneuploidy occurs in a large number of MSGTs. Furthermore, aggressive behavior has been associated with DNA content alterations in mucoepidermoid carcinomas (MEC), adenoid cystic carcinomas (ACC), and acinic cell carcinomas (AciCC) (Vargas et al., 2007).

This study aimed to evaluate AgNORs in MSGTs using Silver Nitrate and DNA ploidy status, and S-phase fraction (SPF) using flow cytometry (FCM) in malignant salivary gland tumors . In addition, their association with clinicopathologic parameters was investigated.

## Materials and Methods

The research was approved by Institutional Research Board (IRB) of the Faculty of Dentistry, Minia University Esthical Committee (IRB. 86 NO. 380/2020). A written informed consent for participation and publication was obtained from each participant after receiving information about the details of the study. Confidentiality of patient's records was assured and maintained throughout the study.. All H&E slides was re-evaluated to ensure the diagnosis and retrieved other histopathologic parameters including lymphovascular invasion (LVI), perineural invasion (PNI), lymph node metastasis (LNM).

Forty-seven formalin fixed paraffin embedded (FFPE) tissue blocks of MSGTs had been collected from the Oral and Maxillofacial Pathology Department, Faculty of Dentistry, Minia University and Oncological Pathology Department, South Egypt Cancer Institute (SECI), Assiut University, covering a period of time from 2014-2018 years. The sample size was estimated by a statistician consultation. Ten formalin fixed blocks of normal salivary gland tissue were taken as data standard.

Clinicopathological data were collected from Faculty of Dentistry, Minia University and SECI. Data included

patient's age, gender, site, tumor size, overall survival (OS) and disease free survival (DFS).

The research was approved by Institutional Research Board (IRB) of the Faculty of Dentistry, Minia University Ethical Committee (IRB. 86 NO. 380/2020). A written informed consent forms were obtained from any patient admitted to both Faculty of Dentistry, Minia University and South Egypt Cancer Institute (SECI), Assiut University for seeking their medical treatment. At data presentation, each patient had a private file with a non-disclosure policy; none of the presented data contained any personal information revealing the patient's identity. The informed consent included all the required information about this study for the patients. Only those who signed the consent were enrolled in the study. Participants had the right to withdraw from this study anytime without indicating any reason. Only cases with available tissue blocks and patient records were included in this study.

This study was conducted on 47 formalin-fixed paraffin-embedded (FFPE) tissue blocks of MSGTs collected from the Oral and Maxillofacial Pathology Department, Faculty of Dentistry, Minia University, and Oncological Pathology Department, SECI, Assiut University, in the interval between November 2014 and May 2018. Ten FFPE blocks of normal salivary gland tissue served as controls. These blocks of normal salivary glands were obtained from cases that were not included in the current study and were removed surgically during neck dissection for other causes.

Clinicopathological data were collected from patients' records of both Faculty of Dentistry, Minia University, and SECI, Assiut University. Data included patient's age, gender, site, tumor size, overall survival (OS), and disease-free survival (DFS). OS was calculated from the start of treatment to the date of death due to any cause or loss of follow up, whereas DFS was calculated from the start of treatment till the date of disease recurrence or death. The last follow-up data were retrieved in May 2021.

All H&E slides were re-evaluated to ensure the diagnosis and retrieved other histopathologic parameters, including lymphovascular invasion (LVI), perineural invasion (PNI), lymph node metastasis (LNM).

### AgNORs Method
*Technique*

Sections were cut from FFPEs at a thickness of 4 μm, then deparaffinized in xylene and dried using an alcohol gradient. Then, two solutions were prepared and mixed with a ratio of 2:1. The first solution was a 50% silver nitrate puriss solution (SIGMA-ALDRICH, USA), according to Peloton protocol (Ploton et al., 1986), while the second solution was a gelatin solution. The latter was 2gm of gelatin, 1ml of formic acid, and 100 ml of distilled water 100 ml mixture prepared immediately before use. Afterward, the slides were incubated for 2 hours at room temperature. Finally, samples were washed in distilled water for one minute, followed by dehydration, and mounted in Di-n-butyl Phthalate in Xylene (DPX).

### Evaluation of AgNORs count using quantitative method

Slides were examined under Olympus 22X light

microscopy at ×1,000 magnification with oil immersion and manually counted.

AgNORs dots included in counting must be intensely black stained, and sharply defined, while dots located within the nuclei at the periphery of the microscopic field or overlapped nuclei were excluded. The number of AgNOR dots/100 cells at five consecutive fields was calculated for each case.

*DNA measurement by Flow Cytometry Technique*

DNA analysis was done in the FCM unit, Clinical Pathology Department, SECI, using a DNA cycle test kit [Cycle Test ™ Plus DNA Reagent Kit (BD Bioscience)]. Single-cell suspension was prepared for flow-cytometric analysis following the Modified method described by Leers et al. (Leers et al., 1999). Five sections, each of 20 μm thick, were cut from each FFPE and placed in an Eppendorf tube. Deparaffinization of sections in 3ml of xylene was done three times for 5 min, followed by rehydration in ethanol in descending manner, and then washed in 3 ml Phosphate Buffer Saline (PBS) and immersion in 3 ml of cold citrate solution. Subsequently, they were incubated in a water bath for 80 min at 80 ˚C. The samples were allowed to cool at room temperature before being centrifuged for 5 min. Afterward, the samples were exposed to 3 ml Tris-EDTA for 10 min, for digestion, in a water bath at 37 ˚C. The digestion was stopped by adding 3 ml of PBS and 1% Bovine serum albumin. The sample was filtered through a 50 m nylon mesh filter and then washed in 3 ml PBS for 5 minutes. DNA staining Procedure for DNA ploidy analysis was done using Cycle Test ™ Plus DNA Reagent Kit (BD Bioscience). FACSCalibur flow cytometry (Becton Dickinson, Bioscience, San Jose, USA) was used to analyze the stained nuclei samples, whereas ModFit software was used to acquire and analyze the gated cells. Analysis of DNA histogram was done as following: We applied an FL2-area vs FL2-width pulse processing gating strategy during the analysis to discriminate between single cells and debris (nuclear fragments) and cell aggregates, which strongly enhance the enrichment for single intact nuclei. The Y-axis represents the number of events (cells or nuclei), while the X-axis denotes the fluorescence intensity of propidium iodide bound to DNA. The quality of the DNA histogram was controlled by the coefficient of variation (CV) of less than 8% for FFPE MSGTs.

*Assessment of flow cytometric analysis*

Diploid tumors were defined as one G0/G1 peak compared to the reference sample, while DNA aneuploidy referred to the presence of two discrete peaks including an abnormal G0/G1 peak containing a minimum of 15% of total events and having a corresponding G2/M peak. (Mahmood et al., 1995). DNA index (DI) was calculated as the ratio of abnormal (aneuploid) G0/G1 peak to the mean channel number of normal (diploid) G0/G1 peak. In the current study, the DI for diploid tumors ranged from 0.95 to 1.05. Therefore, samples were considered aneuploid if their DI was less than 0.95 (hypodiploid). DI was statistically calculated and recorded by the machine.

SPF was calculated as the percentage of cells located in the region between the mean channel number for G0/G1 and G2/M, measured by the DNA analysis calculation program. Total S-Phase Fraction (TSPF) cutoff was applied as the mean and set as low or high.

*Statistical Analysis*

Data analysis was conducted utilizing the Statistical Package for Social Sciences (SPSS version 21). The numerical data has abnormal distribution. For two and more than two mean variables, the Mann- Whitney U and Kruskal- Wallis tests were used to assess the association between AgNORs count, DI, TSPF, and clinicopathological parameters of the studied cases. Spearman correlation coefficient tests were used to correlate between AgNORs count, DI, and TSPF of the studied cases. OS and disease DFS were analyzed using the Kaplan-Meier Survival test. Log-rank test was used to evaluate the significance of the difference between the survival curves. Multivariate analysis using Cox proportional hazards model was utilized. All statistical analysis was two-sided, and the level of significance was defined as p < 0.05.

## Results

*Clinicopathological characteristics of patients*

There were 26 cases of MEC, 15 cases of ACC, 4 cases of myoepithelial carcinoma (MECA), and 2 cases of AciCC included in the study. LVI was present in 36 cases. PNI was evident in 38 cases, whereas LNM occurred in 29 cases.

The mean OS was 27.28±7.156 months, with a mean DFS of 26.04 ± 8.196 months. The total number of deaths at the end of the study was 22 (46.8%), while 14 (32.6%) cases had local recurrence. All clinicopathological features are summarized in (Table 1).

*Evaluation of AgNORs staining and DI in studied cases*

ACC had the highest median AgNORs count/cell, while MECA had the lowest, followed by MEC then AciCC. The ACC cases had a mean value of 2.30 dot/cell. Among the ACC variants, the tubular pattern had the highest AgNOR number. AgNORs were numerous and less uniform in shape in ACC. In MECA cases, AgNORs count/cell was 1.50 dot/cell, and AgNORs were small black dots with a less uniform shape.

In MEC, on the contrary, the high number of AgNORs was densely aggregated and irregular in shape. MEC cases had a median value of 1.73 dot/cell, and AgNORs were numerous, aggregated, and less uniform in shape (pleomorphic). AgNORs count/cell of AciCC was 1.86 dot/cell, and AgNORs were was densely aggregated and irregular in shape (Figure 1).

Of the 47 cases studied, 28 were DNA diploid, and 19 were DNA aneuploid. All aneuploidy cases were hypoploidy (Figure 2). The median DI was 1.0 (range 0.30-1.0), and the median TSPF was 16.46 (range 0.00-100) (Table 1).

*Association of AgNORs count, DNA index, and TSPF*

Table 1. Clinicopathological Parameters and DNA Index of the Studied Cases

| Parameters | NO. (%) |
|---|---|
| **Age** | |
| < 50 Years | 20 (42.6) |
| ≥ 50 Years | 27 (57.4) |
| **Gender** | |
| Male | 27 (57.4) |
| Female | 20 (42.6) |
| **Type of MSGT** | |
| MEC | 26 (55.3) |
| ACC | 15 (31.9) |
| AciCC | 2 (4.3) |
| MECA | 4 (8.5) |
| **Site** | |
| Parotid | 28 (59.6%) |
| Submandibular | 14 (29.8%) |
| Nasal | 2 (4.3%) |
| Palatal | 2 (4.3%) |
| Tongue | 1 (2.1%) |
| **Size** | |
| Median (Range) | 5.00 (2.00-11.00) |
| **Size** | |
| >5 cm | 18 (38.3) |
| <5 cm | 29 (61.7) |
| **LVI** | |
| Absent | 11 (23.4) |
| Present | 36 (76.6) |
| **PNI** | |
| Absent | 9 (19.1) |
| Present | 38 (80.9) |
| **LNM** | |
| Absent | 18 (38.3) |
| Present | 29 (61.7) |
| **DNA Index** | |
| Median (Range) | 1.0 (0.30-1.0) |
| **DNA Index** | |
| Aneuploid | 19 (40.4) |
| Diploid | 28 (59.6) |
| **TSPF** | |
| Median (Range) | 16.46 (0.00-100) |
| **Recurrence free interval in months** | |
| Mean ± SD | 27.05±7.778 |
| **Recurrence** | |
| Present | 14 (32.6) |
| Absent | 29 (67.4) |
| **Survival time in months** | |
| Mean ± SD | 27.28±7.156 |
| **Status** | |
| Alive | 25 (53.2) |
| Dead | 22 (46.8) |

Data was expressed as (Mean ± SD), number and percentage; SD, Standard Deviation; MEC, Mucoepidermoid carcinoma; ACC, Adenoid cystic carcinoma; AciCC, Acinic cell carcinoma; MECA, Myoepithelial carcinoma; LVI, Lymphovascular invasion; PNI, Perineural invasion; LNM, Lymph node metastasis.

with clinicopathological parameters of the studied cases

AgNORs count was statistically significant with histopathologic MSGT type (P=0.033). A high AgNORs count was statistically significant with the presence of LVI (P=0.001) and LNM (P>0.001).

A significant association was present between DI and tumor site (P=0.001), with the median DI being aneuploidy in submandibular, palatal, and tongue sites (0.50, 0.65, and 0.43, respectively), while median DI was diploid in parotid and nasal sites. DI was found to be significantly associated with tumor size (P=0.011).

There was also a significant association between DI and histopathologic MSGT type (P=0.047). All AciCC cases in this study were aneuploid with an average of (0.3) while all MECA cases were diploid.

TSPF was found to be substantially correlated with LVI (P=0.012). The median TSPF in the cases of LVI was (27.00).

There was no significant correlation between DI, TSPF, and either patient's age, PNI, or LNM. Association between AgNORs count, DI, TSPF, and clinicopathological features was assessed and summarized in Table 2.

*Correlation between AgNORs count, DI, and TSPF of the studied cases*

A moderate positive correlation was present between AgNORs count and TSPF (P=0.010, r= 0.373). A moderate negative correlation was present between DI and TSPF (P=0.001, r= -0.465). (Table 3).

*Survival analysis of the AgNORs count, DI, and clinicopathological variables in the cases studied*

The Kaplan-Meier survival and log-rank tests were used to examine the relationship between DI, AgNORs count, different clinicopathological features, and patients' outcomes (OS and DFS) (Table 4).

In terms of DFS, tumor site (P<0.001), the presence of LVI (P=0.021), high AgNORs count (P=0.009), and low DI (P=0.001) have been found to be associated with short DFS (Fig 3 A, B). Regarding OS, a significant association was observed between tumor site (P<0.001) LVI (P =0.001), LNM (P=0.003), increased AgNORs count (P<0.001), decreased DI (P=0.011), and poor OS (Fig 3 C, D).

*Multivariate analysis*

Multivariate analysis for OS and DFS was applied, including all the significant parameters in Kaplan Miere analysis for adjusting the confounders. The tumor site was the only independent predictor for the OS in the current study (P=0.017, HR= 2.435, 95% CI: 1.172 - 5.058). Moreover, the tumor site showed borderline significance as a predictor for tumor recurrence (P=0.069, HR= 3.338, 95% CI: 0.908 - 2.265). DI displayed borderline significance as predictors for OS and DFS (P=0.083, HR=0.284, 95% CI: 0.068 - 1.179) and (P=0.099, HR=0.417, 95% CI: 0.148 - 1.179), respectively (Table 5).

Table 2. Association between AgNOR Count, DI, TSPF, and Clinicopathological Parameters of the Studied Cases

|  | P | Median (Range) | P | Median (Range) | p | Median (Range) |
|---|---|---|---|---|---|---|
| **Age** | | | | | | |
| < 50 Years | 0.36 | 1.73 (1.15-2.75) | 0.343 | 1.0 (0.30-1.0) | 0.414 | 14.78 (0.00-80.10) |
| ≥ 50 Years | | 2.02 (1.15-2.75) | | 1.0 (0.30-1.0) | | 27.0 (0.00-100.00) |
| **Gender** | | | | | | |
| Male | 0.738 | 1.73 (1.15-2.75) | 0.258 | 1.0 (0.30-1.0) | 0.094 | 1.40 (0.00-80.40) |
| Female | | 1.86 (1.15-2.70) | | 0.825(0.30-1.0) | | 28.20 (0.00-100.00) |
| **Type of MSGT** | | | | | | |
| MEC | | 1.73 (1.25-2.75) | | 1.0 (0.30-1.0) | | 26.17(0.00-80.40) |
| ACC | 0.033* | 2.30 (1.15-2.65) | 0.047* | 1.0 (0.34-1.0) | 0.07 | 0.00 (0.00-60.00) |
| AciCC | | 1.86# | | 0.3# | | 80.18# |
| MECA | | 1.50 (1.50-2.70) | | 1.0# | | 0.00 (0.00-100.00) |
| **Site** | | | | | | |
| Parotid | | 1.73 (1.15-2.75) | | 1.0 (0.30-1.0) | | 11.98 (0.00-100.00) |
| Submandibular | 0.223 | 2.225 (1.29-2.45) | 0.001* | 0.50 (0.30-1.0) | 0.179 | 48.70 (0.00-67.50) |
| Nasal | | 2.65# | | 1.0# | | 0.00# |
| Palatal | | 1.65# | | 0.65# | | 13.10# |
| Tongue | | 2.17# | | 0.43# | | 0.00# |
| **LVI** | | | | | | |
| Absent | 0.001* | 1.50 (1.25-1.65) | 0.356 | 1.0 (0.34-1.0) | 0.012* | 0.00 (0.00-37.40) |
| Present | | 2.15 (1.15-2.75) | | 1.0 (0.30-1.0) | | 27.00 (0.00-100.00) |
| **PNI** | | | | | | |
| Absent | 0.304 | 1.65 (1.29-2.65) | 0.68 | 1.0 (0.34-1.0) | 0.063 | 1.40 (0.00-37.40) |
| Present | | 1.94 (1.15-2.75) | | 1.0 (0.30-1.0) | | 26.585 (0.00-100.00) |
| **LNM** | | | | | | |
| Absent | >0.001* | 1.495 (1.15-1.86) | 0.595 | 0.85 (0.30-1.0) | 0.335 | 1.40 (0.00-80.10) |
| Present | | 2.270 (1.45-2.75) | | 1 (0.30-1.0) | | 26.17 (0.00-100.00) |
| **Size** | | | | | | |
| ≥5 cm | 0.228 | 2.02 (1.15-2.75) | 0.011* | 1.0 (0.30-1.0) | 0.654 | 16.46 (0.00-100.00) |
| <5 cm | | 1.725 (1.30-2.30) | | 0.50 (0.30-1.0) | | 36.55(0.00-80.10) |

P, P value; MEC, Mucoepidermoid carcinoma; ACC, Adenoid cystic carcinoma; AciCC, Acinic cell carcinoma; MECA, Myoepithelial carcinoma; LVI, Lymphovascular invasion; PNI, Perineural invasion; LNM, Lymph node metastasis; DI, DNA index; TSPF, Total S-Phase Fraction; *, Significant; AgNOR, DNA index and TSPF were constant

## Discussion

MSGTs are a heterogeneous group with varying histologic shape and biological behavior; their clinical presentation can vary depending on the lesions. MSGTs were investigated in order to better understand and study their invasive potentials and proliferative activity (El-Naggar et al., 2017). The current study attempted

Table 3. Correlation between AgNORs Count, DI, and TSPF of the Studied Cases Correlations

| | | Correlations | | | |
|---|---|---|---|---|---|
| | | | AgNOR count | DI | TSPF |
| Spearman's rho | AgNORcount | Correlation Coefficient | 1 | -0.123 | 0.373* |
| | | Sig. (2-tailed) | | 0.409 | 0.01 |
| | | N | 47 | 47 | 47 |
| | DI | Correlation Coefficient | -0.123 | 1 | -0.465* |
| | | Sig. (2-tailed) | 0.409 | | 0.001 |
| | | N | 47 | 47 | 47 |
| | TSPF | Correlation Coefficient | 0.373* | -0.465* | 1 |
| | | Sig. (2-tailed) | 0.01 | 0.001 | |
| | | N | 47 | 47 | 47 |

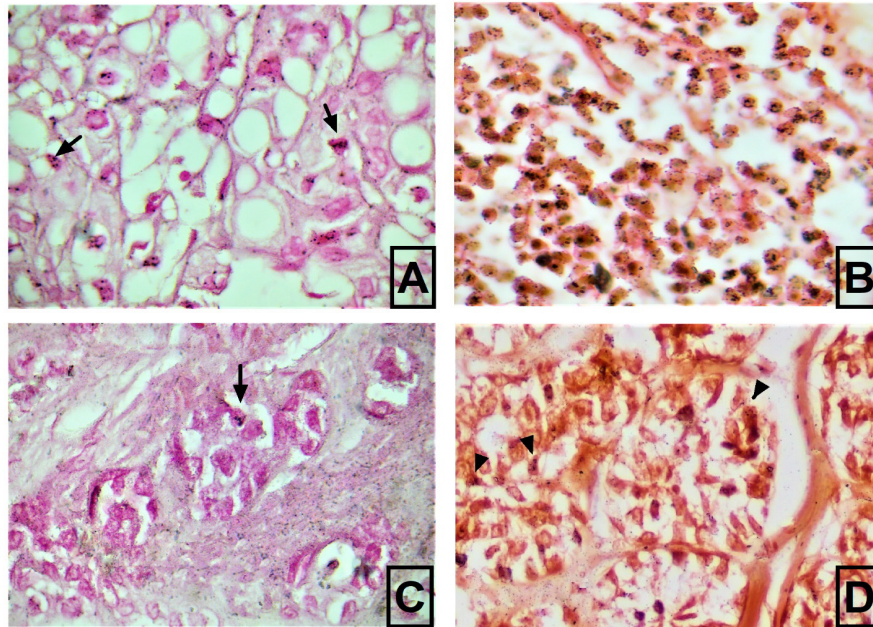DI, DNA index; TSPF, Total S-Phase Fraction; *, Significant

Figure 1. Silver Nitrate Histochemical Staining for AgNOR Evaluation in MSGTs. (A), Photomicrograph of mucoepidermoid carcinoma shows 8-10 AgNORs per nucleus (arrow) (oil immersion view: ×1,000); (B), Photomicrograph shows a high number of AgNORs as black dots (arrow) within the nucleus of adenoid cystic carcinoma (oil immersion view: ×1,000); (C), Photomicrograph of acinic cell carcinoma shows AgNORs having irregular size and clustered shape (arrow), AgNORs staining, x1,000 oil immersion lens (arrow); (D), Photomicrograph shows AgNOR as black dots (arrowhead) within the nucleus of myoepithelial carcinoma (oil immersion view: ×1,000).

to evaluate the correlation between AgNORs count and DNA ploidy in MSGTs. The clinical significance of the current study stems from the fact that proliferative markers such as AgNORs and ploidy status coupled with the clinicopathological parameters can aid the surgeon and the histopathologist to predict the biological behavior of the tumor and therefore facilitate effective therapeutic standards.

In the current study, the data analysis revealed that 57.4% of the cases were males and 42.6% were females, which aligns with a study done by Monteiro et al., (2009), in which the male gender represented as 67.7%. In contrast, more than half of the patients were 50 years or older. This finding is consistent with those of an Egyptian study performed by Zahran et al., (2018). The mean age of incidence of MSGTs in Asian countries is 53.39 years (Lee et al., 2020).

The number of AgNORs and their pleomorphism varied with tumor types (Mohanty et al., 2020). The present work demonstrated that ACC had the highest median AgNORs count/cell, whereas MECA had the lowest, followed by MEC then AciCC. The AgNORs were numerous and pleomorphic in ACC. This finding is compatible with the study done by Irani et al., (2016), who detected significant differences between the type of tumor and AgNOR, with ACC having the highest AgNOR number (Irani et al., 2016). These findings contradict the findings of Adeyemi et al., study (2006), which showed no significant difference in their mean AgNOR value and different histological types. This discrepancy can be attributed to the inclusion of both malignant and benign tumors in Adeyemi study and inflammatory non-tumorous lesions.]

We demonstrated that AgNORs count was statistically significant with the presence of LVI and LNM. This finding is in agreement with the study of Cortegoso et al. ,
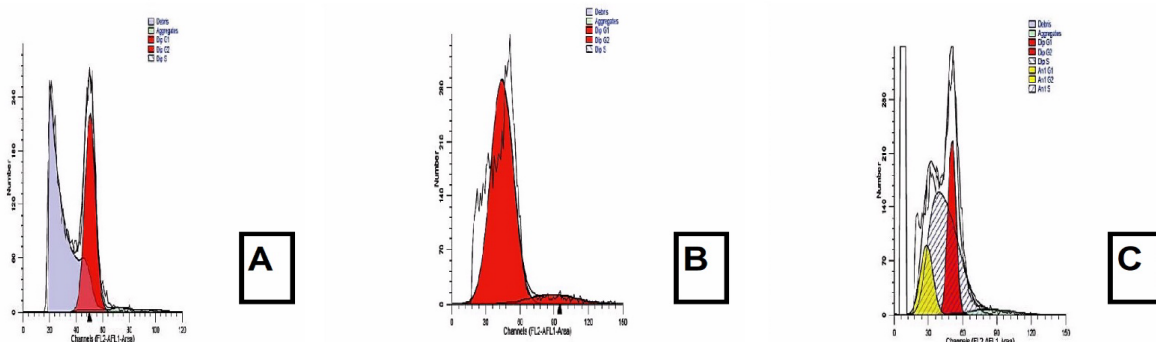


Figure 2. DNA Frequency Histogram. (A),Frequency histogram of diploid standard (normal salivary gland tissue), shows single G0/G1 peak and no SPF cells; (B), DNA frequency histogram of diploid MSGTs, shows single G0/G1 peak; (C), DNA frequency histogram of MSGTs shows hypodiploid (DI= 0.56) and SPF (60.49 %).

Table 4.Kaplan Meier Analysis of Clinicopathological Features, AgNORs Count and DI for DFS and OS

| Parameters | DFS | | | OS | | |
|---|---|---|---|---|---|---|
| | E±SE (%) | Log Rank (χ2) | P | E±SE (%) | Log Rank (χ2) | P |
| Age | | 0.583 | 0.445 | | 1.833 | 0.176 |
| < 50 Years | 73.7±10.1 | | | 70.0±10.2 | | |
| ≥ 50 Years | 62.5±9.9 | | | 55.6±9.6 | | |
| Gender | | | | | | |
| Male | 70.8±9.3 | 0.14 | 0.708 | 63.0±9.3 | 0.007 | 0.934 |
| Female | 63.2±11.1 | | | 60.0±11.0 | | |
| Type of MSGT | | 1.396 | 1.002 | | 2.194 | 0.533 |
| MEC | 64.0±9.6 | | | 61.5±9.5 | | |
| ACC | 66.7±13.6 | | | 53.3±12.9 | | |
| AciCC | 66.7±13.6 | | | 40.0±12.6 | | |
| MECA | 75.0±21.7 | | | 75.0±21.7 | | |
| Site | | 23.119 | <0.001* | | 57.317 | <0.001* |
| Parotid | # | | | # | | |
| Submandibular | | | | | | |
| Nasal | | | | | | |
| Palatal | | | | | | |
| Tongue | | | | | | |
| LVI | | 6.305 | 0.012* | | 10.129 | 0.001* |
| Absent | 100.0±0.0 | | | 100.0±0.0 | | |
| Present | 56.3±8.8 | | | 50.0±8.3 | | |
| PNI | | 1.42 | 0.233 | | 2.171 | 0.141 |
| Absent | 87.5±11.7 | | | 77.8±13.9 | | |
| Present | 62.9±8.2 | | | 57.9±8.0 | | |
| LNM | | 3.213 | 0.073 | | 9.08 | 0.003* |
| Absent | 83.3±8.8 | | | 83.3±8.8 | | |
| Present | 56.0±9.9 | | | 48.3±9.3 | | |
| Size | | 0.091 | 0.762 | | 0.098 | 0.755 |
| >5 cm | 62.5±12.1 | | | 55.6±11.7 | | |
| <5 cm | 70.4±8.8 | | | 65.5±8.8 | | |
| AgNORs count | | 6.906 | 0.009* | | 16.874 | <0.001* |
| <1.8 | 86.4±7.3 | | | 86.4±7.3 | | |
| ≥1.8 | 47.7±10.9 | | | 40.0±9.8 | | |
| DI | | 11.171 | 0.001* | | 6.489 | 0.011* |
| Aneuploid | 35.3±11.6 | | | 31.6±10.7 | | |
| Diploid | 88.5±6.3 | | | 82.1±7.2 | | |

P, P value; E, Estimate; SE, Standard error; MEC, Mucoepidermoid carcinoma; ACC, Adenoid cystic carcinoma; AciCC, Acinic cell carcinoma; MECA, Myoepithelial carcinoma; LVI, Lymphovascular invasion; PNI, Perineural invasion; LNM, Lymph node metastasis; TSPF, Total S-Phase Fraction*, Significant ; #, Regarding tumor site, two cases has nasal site of tumor and died before 24 months during follow up, also, only one case has tongue tumor site which died at 13 months; So, we could not extrapolate behind the period to calculate 2 years DFS and OS for tumor site.

(2017) on OSCC (Cortegoso et al., 2017). Nonetheless, it is inconsistent with the findings of Kapila et al., (2017), who found no significant association between LVI, LNM, and AgNORs count in OSCC (Kapila et al., 2017). This discrepancy could be attributed to the difference in aggressive behavior of OSCC and MSGT.

Tyagi et al., (2020) reported that actively proliferating cells have impaired nucleolar association thus having a higher AgNORs count, regardless of the ploidy state of the cell. Evaluation of the AgNORs count aids in investigating the rate of cellular proliferation and predicting tumor

aggressiveness. The black dots of AgNORs were more significant in number and more irregular in shape in malignant lesions (Jagtap et al., 2020). The AgNORs are generally considered to be proliferation markers. Proliferating cells exhibit increased biosynthesis, leading to increased rRNA, increased nucleolar activity, and a higher number of AgNOR dots. Recent histopathologic studies of NORs have resulted in more accurate diagnosis, classification, and prognosis of different lesions (Tyagi et al., 2020). Since it is simple and reproducible, counting is the most commonly used method for evaluating AgNORs.
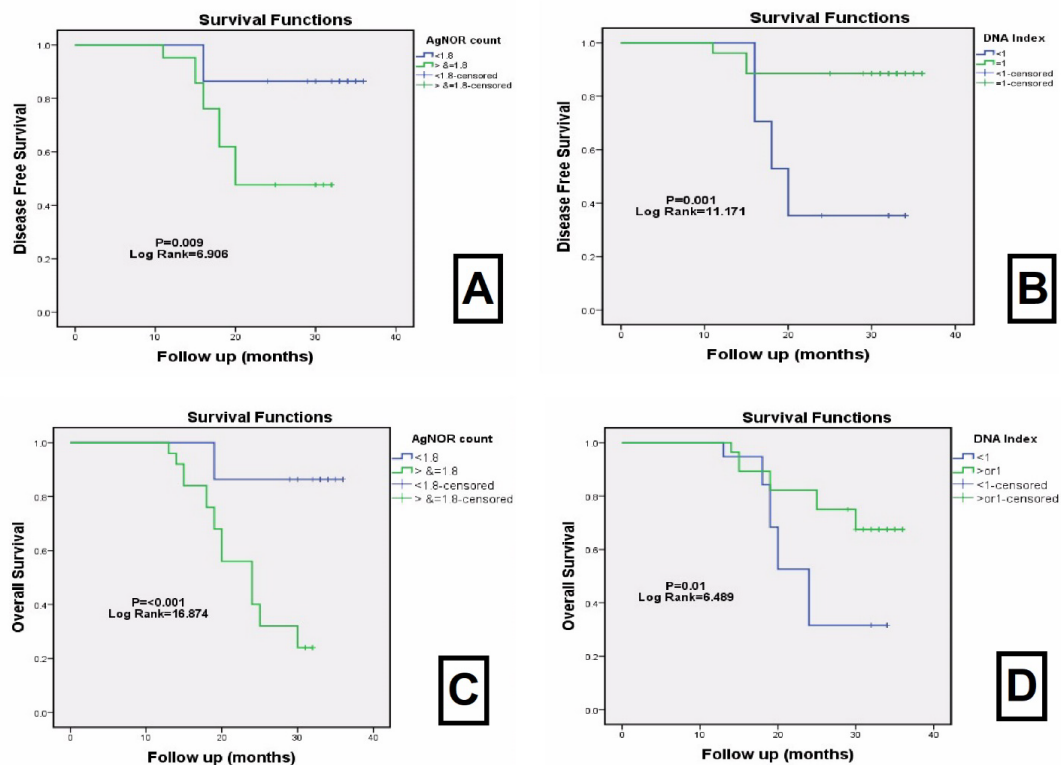
Figure 3. Kaplan Meier Survival Plots for OS and DFS with AgNORs Count and DNA Index. (A), High AgNORs count in MSGTs is significantly associated with decreased DFS; (B), Low DI (aneuploidy) in MSGTs is significantly associated with decreased DFS; (C), High AgNORs count in MSGTs is significantly associated with poor OS; (D), Low DI (aneuploidy) in MSGTs is significantly associated with poor OS.

Table 5. Multivariate Analysis for Predictors of DFS and OS

| Parameters | DFS | | OS | |
|---|---|---|---|---|
| | HR (95% CI) | P | HR (95% CI) | P |
| Site | 3.338 (0.908 - 12.265) | 0.069 | 2.435 (1.172 - 5.058) | 0.017* |
| LVI | 0.046 (0.000- 2.097) | 0.962 | 0.775 (0.000 - 4.634) | 0.958 |
| LNM | ----------- | ----- | 1.423 (0.328 - 6.179) | 0.638 |
| AgNOR count | 1.006 (0.259 - 3.898) | 0.993 | 1.781 (0.440 - 7.201) | 0.418 |
| DNA index | 0.284 (0 .068 - 1.179) | 0.083 | 0.417 (0.148 - 1.179) | 0.099 |

P, P value; HR, Hazard Ratio; CI, confidence interval; DFS, disease free survival; OS, overall survival; LNM, lymph node metastasis

Unfortunately, superimposition of AgNORs may appear, and continuous deposition of silver for a long time may lead to the fusion of small AgNORs. One of the limitations of AgNORs staining may be the underestimation of AgNORs number (Mohanty et al., 2020).

Concerning DNA ploidy, 40.4% of the MSGTs examined were aneuploidy in the present study, which aligns with Vargas et al., (2007), who observed that 44% of high-grade salivary carcinomas analyzed were aneuploidy. These results also agree with the study of Monteiro et al., (2009) as they found that 45% MSGTs had aneuploidy. Another study done by Zahran et al., (2018) showed that 66% of MSGTs were aneuploidy. These minor differences may depend on different histological types, the percentage of each type MSGTs, and the number of sections from FFPE blocks obtained within the sample included in the study.

The association between DNA ploidy and clinicopathological variables in MSGTs is frequently debated. The present study demonstrated that DNA ploidy was statistically significant with MSGT type, site, and tumor size. The findings of Monteiro et al. (2009) are consistent with the current study's findings as they showed that DNA ploidy was statistically significant with both MSGT type and tumor size (Monteiro et al., 2009), while Zahran et al., (2018) found no significant association between DNA ploidy and tumor site in MSGTs. This discrepancy may be due to the type of tissue sections included in the study. In the Zahran et al., (2018), fresh tissues were used, yet FFPE tissue sections were used in the current study. In addition, different MSGT types and percentages were included in their study.

In the current work, the TPSF was significantly associated with LVI. SPF represented a continuous variable associated with actively proliferating cells. Therefore, it is considered a surrogate parameter of

malignancy and metastatic potential (El-Deftar et al., 2012). Odell et al., (2021) proposed that aneuploidy was a predictor of malignant transformation in oral potentially malignant disorders (OPMDs) (Odell, 2021). Many authors found that DNA aneuploidy has been a valuable predictor in OSSC. Furthermore, Monteiro et al., (2009) and Zahran et al., (2018) revealed that DNA aneuploidy is a common finding in MSGTs.

The DNA content and the rapidity of cell turnover are reflected in the ploidy status and SPF of the tumor (Khanna et al., 2010). Using FCM to measure DNA content provides important information on cell distribution throughout the cell cycle phases (G0/G1, S-phase, and G2/M), disclosing the DNA ploidy of the studied cell population and calculating the frequency of apoptotic cells. Furthermore, it can measure a substantial number of separate cells in a short time (seconds to minutes) (Tabll and Ismail, 2012). El-Deftar et al., (2012) revealed that DNA ploidy was heterogeneous within the single tumor, and when several tissue samples from the same tumor were analyzed, the incidence of aneuploidy increased (El-Deftar et al., 2012). Furthermore, chromosomal aberrations may be below the detection limit of FCM. This finding is attributed to a decrease in the sensitivity to clumps of DNA caused by the FFPE specimens, representing one of the significant disadvantages of FCM. Modified techniques were applied to eliminate paraffin wax from nuclei retrieved from FFPE histopathological material before FCM steps (Leers et al., 1999).

A moderate positive correlation was present between AgNORs count and TSPF in the present study, which is compatible with the study done by Lorand-Metze et al., 2004; Mahmoud et al., 2017; Lorand-Metze et al., 2004; Mohamed Mahmoud et al., 2017). However, Eminovic-Behrem et al., (2000) revealed no significant correlation between AgNORs count and TSPF in colorectal carcinoma (Eminović-Behrem et al., 2000). A moderate negative correlation was detected between DNA ploidy and TSPF. El-Deftar et al., (2012) found that there is a borderline significant correlation between SPF and DNA ploidy in primary OSCC. The negative correlation can be attributed to the aneuploidy type in our study being hypoploidy. The current study revealed no significant correlation between AgNORs count and ploidy analysis. Many studies found that AgNOR counting reflects the proliferative activity rather than ploidy (Mikou et al., 1993; Celikel et al., 1995).

Regarding survival analysis, the present study revealed that the presence of LNM was associated with shorter OS and DFS, which agrees with those of Monteiro et al., (2009). Increased AgNORs count associated with short DFS and poor OS. This finding was in keeping with several studies done by Jagtap et al., 2020; Mohanty et al., 2020; Jagtap et al., 2020; Mohanty et al., 2020). However, Gundog et al. (2016) found an inverse correlation between AgNORs count, OS, and DFS in rectal cancer (Gundog et al., 2015).

In the current study, a statistically significant association has been revealed between MSGTs site and DFS, as recurrence was detected in 32.6% of the examined cases. This finding is in agreement with the studies done by Monteiro et al., (2009; El-Deftar et al., 2012; Monteiro et al., 2009; El-Deftar et al., 2012).

The current work showed that MSGTs with DNA aneuploidy showed poor OS and shorter DFS. These findings are inconsistent with those of Yang et al., (2020), that found no significant difference in OS between diploid and non-diploid patients of colon cancer.

Regarding multivariate analysis for OS and DFS, the tumor site is an independent predictor for OS and showed borderline significance as a predictor for tumor recurrence. Additionally, DI displayed borderline significance as a predictor for OS, aligning with Lorand-Metze et al., (2004) investigation in Non-Hodgkin Lymphoma.

The present work inferred that AgNORs are simple, reliable, easily applicable, and frugal quantitative markers for assessing cellular proliferation through NORs counting. The high AgNORs count, DNA aneuploidy, and TSPF had a poor influence on MSGTs prognosis. Consequently, AgNORs count, DNA ploidy status, and TSPF could be beneficial markers for predicting MSGTs prognosis. Further investigations with a more significant number of MSGTs cases with various histological types are recommended to elucidate the relation between AgNORs and DNA aneuploidy.

## Author Contribution Statement

Conceptualization: MTH, MAM, SFE, EAA; Data curation: MTH, MAM, SFE, EAA; Formal analysis: MTH; Methodology: MTH, MAM, SGM, DFT; Resources: MTH, MAM, EAA; Writing – original draft: MTH.

## Acknowledgments

*Conflict of interest*
he authors declare that they have no potential conflicts of interest to disclose.

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