# Evaluation of AgNOR Count asDiagnostic Marker of Prognosis and Proliferative Activity in Thyroid Neoplasm

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**Abstract**— Thyroid cancer is the most common endocrine malignancy. The aim of this study was to assess the diagnostic and prognostic significance of argyrophilic nuclear organizer region (AgNOR) counts in the cells of malignant and benign thyroid cancer. We analyzed paraffin-embedded tissue sections, from One hundred and two tissue samples were: 20 cases of normal thyroid tissue as control, 34 cases of thyroid adenoma, and 48 cases of thyroid carcinoma.Counting AgNORs helps with the differential diagnosis of thyroid cancers. AgNOR counts in control group ranged from 1.2 to 1.6 with a mean of 1.4± 0.01. In all the 34 cases of thyroid adenoma the AgNOR counts ranged from 3.2 to 4.1 with a mean of 3.65±0.58, which was significantly higher than that in the control group (P<0.05) .In 48 patients with thyroid carcinoma, the AgNOR value ranged from 5.2 to 8.45 (mean 6.82 ± 0. 36), which were significantly (P <0.05) higher than the values obtained from the control group and from the patients with thyroid adenoma. AgNOR counts were also compared in the different grades of thyroid cell carcinoma. A progressive significant increase in the counts was observed with increasing grades of carcinoma and noted a significant difference (P <0.05) in AgNORs between the three grades. These findings strongly support the view that the use of The AgNOR number, as cell proliferative activity marker, can be recorded as an inexpensive and reliable diagnostic method and prognostic tool in assessment of thyroid neoplasm. And by use of nucleolar AgNOR staining we could distinguish different grades of thyroid carcinoma.

**Index Terms**—: AgNOR s, Thyroid cancer, Prognosis, Cell roliferative activity, Cancer diagnostics, Tumors.



## 1 INTRODUCTION

The thyroid is the largest endocrine gland and by far the most common site of all primary endocrine cancers, therefore thyroid cancer is the most common malignancy of the endocrine system, its incidence had been increasing over the past 20 years and it was the sixth most common cancer among women [1], [2].

Assessment of the proliferative activity markers have been applied to evaluation of malignancy of many neoplastic lesions, such as PCNA, Ki-67 (they are nuclear protein involved in DNA synthesis), and AgNORs (loops of DNA)[3], [4].

Of the various techniques used for assessing proliferating cells of tumor tissue based on nuclear studies, staining of the nucleolar organising regions by silver compound (AgNOR) has become popular for its simplicity, low cost, and its good correlation with other proliferative markers [5].

Nucleolar organiser regions (NORs) are DNA sequences on the short arms of acrocentric chromosomes (13, 14, 15, 21 & 22), which involved in ribosomal synthesis [6].Associated with NORs there are some nucleolar proteins, which have a strong affinity with silver (argyrophilic proteins). Since NORs are closely related to the ribosomal protein synthesis, they are claimed to reflect cellular proliferative activity [7].

Nucleolar organizer regions may be visualized by number of ways. The simplest and most widely used method is silver colloid impregnation. This technique has been shown to be specific for carboxyl and sulphydryl-rich proteins associated with NOR (Nuclear organizer region).These groups have been proposed as reaction sites and reduced the silver solution forming deposits (black dots) of silver visible at low microscopic magnification [8].

In normal cells, the AgNORs are tightly packed in the nucleoli and are indiscernible. In rapidly proliferating cells such as neoplastic cells, nucleolar disaggregation may take place resulting in dispersion of individual AgNOR [9]. Pich *et al*. [10], in other study, provided that, the expression of AgNOR proteins is associated with several

biological properties of neoplastic cells: metabolic activity, DNA content, histological grade of differentiation and, especially, the rapidity of cellular proliferation.

Many studies [11], [12], [13], [14],[15] showed that AgNORs were significantly higher in malignant cells as compared to benign cells.AgNOR number and distribution in the nucleus (configuration) were useful in the detection of the proliferative activity and prognosis of some neoplasias, such as urinary bladder [16], breast [17], colorectal carcinoma [18], bone tumors [19], and lung cancers[20].

In thyroid cancer, many studies evaluated AgNOR in patients with benign and malignant tumors. Kawasaki *et al.*[21] detected AgNORs, as a well-known indicator for proliferative potential of the cancer cells, in 89 cases of DTC and found that AgNOR score might be clinically applicable as a useful indicator for disease recurrence in DTC. Mehrotra *et al*. [22] proved that AgNOR counting is more sensitive, simple and cost effective as compared to Ki-67 for differentiating between benign and malignant thyroid follicular neoplasms.

Słowińska-Klencka *et al*. [23] results recorded the usefulness of silver staining of nucleolar organizer regions (AgNORs) in the preoperative diagnosis of follicular lesions in the thyroid. Camargo *et al*. [24] correlated the subjective AgNOR counting method with histologic diagnoses of thyroid cancer and invasion. Finally, Eroz *et al.*[25] recorded AgNOR staining as an easy and reliable method for evaluating proliferation activity of cells in malignant and benign thyroid lesions.

## 2 METHODOLOGY

This study was carried out in thelaboratory of Histopathology in Alsader Teaching Hospital in Al-Najaf province.

### 2.1 Sampling of Cases

a) Study group: Paraffin blocks of eighty two (70 females and 12males) cases with the thyroid tumors; 34 cases with thyroid adenoma, and 48 cases with thyroid carcinoma were included in this study. These samples were collected from laboratory of histopathology in Alsader Teaching Hospital and from some private laboratories in this governorate.

b) Control group: Twenty samples with normal thyroid tissues were considered as control group for this study.

### 2.2Histopathological Examination

Five µm–thick sections were obtained from formalin fixed –paraffin embedded tissue blocks. Sections were stained with the routine haematoxylin and eosin (H&E) staining method [26]. Then examinations of these histological sections were doneunder the supervision of pathologist, who determines the grades of cancer according to the American Joint Commission on Cancer Staging Manualfor grading thyroid tumors [27].

### 2.3Standardized AgNOR Staining Technique

According to the recommendations of Treré[28], the "International Committee on AgNOR Quantitation" guidelines for AgNOR protein evaluation must be considered as standard method. This "standardized" silver-staining protocol, proposed by the Committee, consisted of staining the 5 micron paraffin sections in the dark at a constant temperature of 37$^{o}$C using pre-warmed solutions. A very important point in these guidelines was the introduction of a "retrieval" method for formalin-fixed samples based on the exposure of sections to high temperatures using a wet autoclave, microwave oven or pressure cooker. The result of staining is observed under the light microscope as black dots scattered all over the nucleus and the nucleolus, dots of silver (AgNOR dots or AgNORs).

### 2.4Quantitative Analysis of AgNOR Proteins (The Counting Method):

AgNORs were counted as brown-black dots in the nuclei of cells (within the yellowish background of the nucleus) using an oil-immersion lens.100 cells (chosen randomly) were studied in each case; the mean AgNOR per cell was calculated.AgNORs were counted in the normal tissue, benign and malignant tumors.

### 2.5Statistical Analysis

Statistical analyses of all results were carried out by the help of SPSS version 17 software statistical package using chi square (P value at level of significance less than 0.05.Data were recorded as mean± SD (standard deviation), range or percentage.

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## 3EXPERIMENTALRESULTS

### 3.1Histopathological variants of thyroid tumors

During this study, out of 82 cases with thyroid tumors, benign tumors have been found in 41.4% of cases, whereas thyroid carcinomas in 58.5% of cases.

The tumors with highest frequency of occurrence were papillary carcinomas (51.22 %) followed by follicular adenoma (40.24%) (results are shown in Fig. 1.).

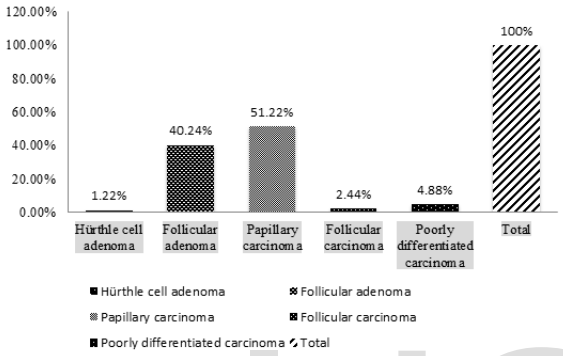


Fig.1 .Histopathologicalvariants of thyroid tumors.

Grading of the presented malignant cases was assessed according to the American Joint Commission on Cancer grading system of carcinoma which shown in Fig 2;revealing that grade I was reported in 34 (70.83%) cases, grade II 10 (20.83%), while those of grade III were 4 (8.34%) cases.

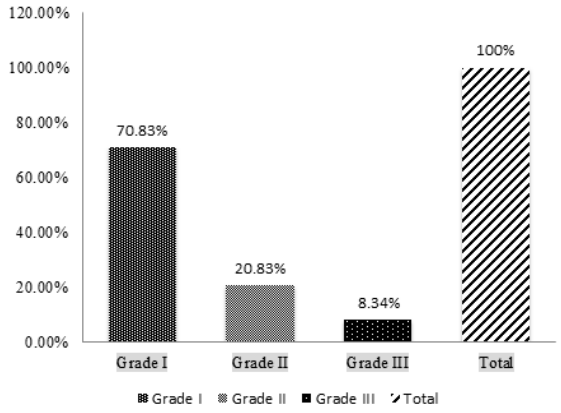


Fig.2.The percentage of grades of thyroid carcinoma patients.

### 3.2AgNOR Counts as Diagnostic Marker

The values of the mean AgNOR (mAgNOR) counts in nuclei of normal thyroid cells, adenoma cells, and thyroid carcinoma cells are presented in table 1.

AgNOR counts in control group ranged from 1.2 to 1.6 with a mean of 1.4± 0.01. In all the 34 cases of thyroid adenoma the AgNOR counts ranged from 3.2 to 4.1 with a mean of 3.65±0.58, which was significantly higher than that in the control group (P<0.05) (Table1).

In 48 patients with thyroid carcinoma, the AgNOR value ranged from 5.2 to 8.45 (mean 6.82 ± 0. 36) (Table1), which were significantly (P <0.05) higher than the values obtained from the control group and from the patients with thyroid adenoma.

Table 1
Distribution of AgNORs in Normal and Neoplastic Thyroid Tissue (Benign and Malignant)

| Thyroid Cells | No. of Cases | AgNOR Count/Cell | |
|---|---|---|---|
| | | Range | Mean |
| Normal cells | 20 | 1.2-1.6 | 1.4 |
| Adenoma cells | 34 | 3.2-4.1 | 3.65* |
| Carcinoma cells | 48 | 5.2-8.45 | 6.82* |

*P< 0.05 significant

### 3.2AgNOR Counts as Prognostic Marker

Mean AgNOR values were calculated for the three grades of thyroid cell carcinomas, well differentiated (GI), moderately differentiated (GII), and poorly differentiated (GIII).

AgNOR counts were also compared in the different grades of thyroid cell carcinoma(Table 2). A progressive significant increase in the counts was observed with increasing grades of carcinoma. Counts of 5.65± 0.62 were recorded in well-differentiated carcinoma while moderately and poorly differentiated carcinomas yielded counts of 6.87 ± 0.18 and 7.67± 0.02 respectively. The difference was statistically significant (P<0.05) between the three groups.

Table 2

Distribution of AgNORs Accordingto Grade of Thyroid CarcinomaPatients.

| Grade | No. of Cases | AgNOR Count/Cell | |
|---|---|---|---|
| | | Range | Mean |
| Grade I | 34 | 5.2-6.1 | 5.65 |
| Grade II | 10 | 5.9-7.85 | 6.87* |
| Grade III | 4 | 6.9-8.45 | 7.67* |

*P< 0.05 significant

## 5 Conclusions

It has been found that the means of AgNOR counts were the highest in malignant tumor cells and the lowest in non-neoplastic thyroid cells.

Our results were agreed with many studies, that AgNOR count are useful in discriminating between benign and malignant conditions being significantly higher in malignant cells than in normal cells.

The same results were found by Bukaeva*et al*. [29], their data indicated that AgNORs counts can be used as differential diagnosis between thyroid carcinoma and adenoma. Also Mehrotra *et al.*[22] had used AgNOR to differentiate hyperplastic nodules (HPN), follicular adenoma (FA) and follicular carcinoma (FCA). Their result proved that AgNOR counting is more sensitive, simple and cost effective for differentiating between benign and malignant thyroid follicular neoplasms.

Lewy-Trenda *et al*. [30] found that only AgNOR numbers (in comparing with other proliferations markers) were helped in differentiation between thyroid nodules and follicular carcinoma.

Eroz *et al*. [25] recorded that Patients with PTC had significantly ($p<0.001$) higherAgNORcount ($4.6 \pm 1.2\%$) than in the patients with benign lesions ($2.0 \pm 0.5\%$), also they mentioned that AgNOR staining was an easy and reliable method for evaluating proliferation activity of cells in malignant and benign thyroid lesions.

The results of this study were in agreement with previous data that had been recorded by Slowińska-Klencka *et al*. [31]; they demonstrated that the mean values with at least five AgNORs allowed for differentiation between malignant and benign follicular lesions.

Cornianu*et al*. [32] study had indicated that the AgNOR methods were commonly used as the markers of proliferative activity, and mentioned that there were a good correlation between the mean number of NORs nucleus and the rate of cell proliferation appreciated by mitotic index.

Since their number corresponds to the proliferation rate, they can be used for tumor grading, i.e. the number of nucleolar AgNOR dots increased as the histological grading increased [33].

Kawasaki *et al*. [21] study had shown that in tumors with hematological metastasis, AgNORs score was significantly higher than in tumors without metastasis, as well as, higher risk for disease recurrence was demonstrated in those cases with high AgNOR score than cases with lower score.

In this study, the Mean AgNOR values had calculated for the three grades of thyroid cell carcinomas and noted a significant difference ($P <0.05$) in AgNORs between these three grades (Table 4.28).

In conclusion we believed that by use of nucleolar AgNOR staining we could distinguish different grades of thyroid carcinoma.

These findings strongly support the view that proliferative activity and malignant potential of neoplastic lesions of the thyroid increase progressively as the grade of the lesion becomes higher.

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## REFERENCES

[1] AmericanCancer Society. Cancer Facts & Figures 2008. Atlanta: American Cancer Society; 2008.
[2] Jemal A, Siegel R, Xu J, Ward E. Cancer statistics. CA Cancer J Clin. 2010;60:277–300.
[3] Krohn K, Stricker I, Emmrich P, Paschke R. "Coldthyroidnodules show a markedincreaseinproliferationmarkers". Thyroid. 2003; 13(6): 569-75.
[4] Augustynowicz A, Dzięcioł J, Barwijuk-Machała M, Dadan J, Puchalski Z and Sulkowski S. "Assessment of proliferative activity of thyroid Hürthle cell tumors using PCNA, Ki-67 and AgNOR methods". Folia HistochemicaetCytobiologica. 2004; 42(3): 165-168.
[5] Pich A, Chiusa L, Margaria E. "Prognostic relevance of AgNORs in tumor pathology". Micron. 2000; 31: 133-141.
[6] Kalmárová M, Smirnov E, Masata M, Koberna K, Ligasová A, Popov A, Raska I. "Positioning of NORs and NOR-bearing chromosomes in relation to nucleoli". J Struct Biol. 2007; 160(1):49-56.
[7] Raĭkhlin NT, Bukaeva IA, Probatova NA, Smirnov EA. "Argyrophilic proteins in theregionsofnucleolarorganizersare markers of cell proliferation rate". ArkhPatol. 2006; 68(3):47-51.

[8] Sirri V, Roussel P, Hernandez-Verdun D. "The AgNOR proteins: qualitative and quantitative changes during the cell cycle". Micron. 2000; 31(2): 121-6.
[9] Bukhari MH, Niazi S, Khan SA, Hashmi I, Perveen S, Qureshi SS, Chaudhry NA, Qureshi GR, Hasan M. "Modified method of AgNOR staining fortissueandinterpretation in histopathology". Int J ExpPathol. 2007; 88(1):47-53.
[10] Pich A, Margaria E, Chiusa L. "Significance of theAgNORin tumor pathology". Pathologica. 2002; 94(1):2-9.
[11] Raĭkhlin NT, Bukaeva IA, Baronin AA, Probatova NA, Smirnova EA, Bronshteĭn MI. "Expression of argyrophilicproteins from the nucleolar organizer regions as an index of maturity degree of benign and malignant adrenal tumors". ArkhPatol. 2002; 64(3):26-30.
[12] Vacharadze K, Burkadze G, Turashvili G, Kiria N. "Argyrophilic nucleolar organizer regions in benign and malignant mesothelial lesions". Georgian Med News. 2005; 128:91-3.
[13] Eslami B, Rahimi H, Rahimi F, Khiavi MM, Ebadifar A. "Diagnostic value of silver nitrate staining for nucleolar organizer regions in selected head and neck tumors".J CancerRes Ther. 2006; 2(3):129-31.
[14] Stemberger-Papić S, Stanković T, Vrdoljak-Mozetic D, Versa-Ostojić D, Krasević M, Stifter S, Audy-Jurković S. "Morphometry and digital AgNOR analysis in cytological imprints of benign, borderline andmalignantserous ovarian tumours". Cytopathology. 2006; 17(6): 382-9.
[15] Hussein HG, Ali HH. "Value of the silver-stained nucleolar organizer regions technique in the differentiation between benign and malignant lesions in urine cytology".SaudiMed J. 2009; 30(5):719-21.
[16] Cucer N, Imamoglu N, Tozak H, Demirtas H, Sarac F, Tatlisen A, Oztürk F. "Two-dimensional AgNOR evaluation as a prognostic variable in urinary bladder carcinoma: a different approach via total AgNOR area/nucleus area per cell". Micron. 2007; 38(6):674-9.
[17] Kidogawa H, Nanashima A, Yano H, Matsumoto M, Yasutake T, Nagayasu T. "Clinical significance of double staining of MIB-1 and AgNORs in primary breast carcinoma". Anticancer Res. 2005; 25(6B): 3957-62.
[18] Santacroce L, Bufo P, Gagliardi S, Mastropasqua MG, Losacco T. "Argyrophilic nucleolar organizerregions(AgNORs)asmalignancy biomarkers in colorectal neoplasms".Clin Ter. 2001; 152(2):91-3.
[19] Arora B, Jindal K, Kumar S, Rekhi B, Arora H, Arora DR. "Quantitative evaluation of AgNORs inbonetumours".Pathology.2003; 35(2):106-8.
[20] Gulati A, Sharma J, Sharma BB, Kaushik R, Kashyap S. "Evaluation of AgNORs in pulmonary lesions: a cyto-histopathological correlation". Indian J PatholMicrobiol. 2002; 45(3):289-92.
[21] Kawasaki F, Onoda N, Ishikawa T, Ogawa Y, Ikeda K, Sugano S, Kato Y, Chung KH. "Evaluation of argyrophilic nucleolar organizer regions (AgNORs) in differentiated thyroid carcinoma as an indicator for disease recurrence". Oncol Rep. 2000; 7(4):853-7.
[22] Mehrotra A, Goel MM, Singh K. "Ki-67 andAgNORproliferativemarkers as diagnostic adjuncts to fine needle aspiration cytology of thyroid follicular lesions". Anal Quant CytolHistol. 2002; 24(4): 205-11.
[23] Słowińska-Klencka D, Klencki M, Popowicz B,LewińskiA. "AgNOR quantification in the diagnosis of follicular pattern thyroid lesions". Anal Quant CytolHistol. 2003; 25(6):347-52.
[24] Camargo RS, Maeda MY, di Loreto C, Shirata NK, Anselmo Garcia E, Filho AL. "Is AgNOR and DNA ploidy analysis useful for evaluating thyroid neoplasms?" Anal Quant CytolHistol. 2005; 27(3): 157-61.
[25] Eroz R, Cucer N, Karaca Z, Unluhizarci K, Ozturk F. "The evaluation of argyrophilic nucleolar organizing region proteins in fine-needle aspiration samples of thyroid". EndocrPathol. 2011; 22(2):74-8.
[26] Woods AE, Ellis RC. Laboratory Histopathology. 1st edition. Churchill-Livingston. London. 1994; pp 800.
[27] Greene FL, Page DL, Fleming ID, Fritz AG, Balch CM, Haller DG, Morrow M .American Joint Committee on Cancer. AJCC Cancer Staging Handbook: TNM Classification of Malignant Tumors. 6th ed. New York: Springer-Verlag, Inc. 2002.
[28] Treré D. AgNOR staining and quantification. Micron J. 2000; 31: 127–131.
[29] Bukaeva IA, Smirnova EA, Pavlovskaia AI, Makanin MA, Ol'khovskaia IG, Raĭkhlin NT. "Significance of argyrophilic proteins of the nucleolar organizer region in differentiation of benign and malignant growth of thyroid epithelial tumors". ArkhPatol. 2001; 63(3):15-8.
[30] Lewy-Trenda I, Janczukowicz J, Wierzchniewska-Lawska A. "Practical application of proliferation markers' (MIB-1, PCNA, AgNOR) expression analysis for differential diagnostics of nodular thyroid lesions". WiadLek. 2006; 59(1-2):32-7.
[31] Slowińska-Klencka D, Klencki M, Popowicz B, Sporny S, Lewiński A. "Multiparameter analysis of AgNOR in thyroid lesions: comparison with PCNA expression". HistolHistopathol. 2004; 19(3):785-92.
[32] Cornianu M, Milos IN, Golu I, Taban S, Milos A. "Proliferative activity of thyroid Hurthle cell tumors". Acta Endo 2006, 2 (3): 269-281.
[33] Omidia S N. "Evaluation of AgNOR staining in human bladder transitional cell carcinoma". Iran J Basic Med Sci. 2003; 6 (17):4-8.