*International Journal of Pathology; 2005; 3(2): 86-90*

# AgNOR Proliferative Index in Malignant Pleural and Peritoneal Effusions

**Shazia N. Ibnerasa, Naseer Ahmed Chaudhry* and Saeed Akthar Khan****

Department of Pathology, Postgraduate Medical Institute, Lahore
* Department of Pathology, University of Health Sciences, Lahore
** Department of Pathology, Lahore Medical & Dental College, Lahore.

**Introduction:** The inability to decide on the presence or absence of malignant cells in cytologic specimens casts a difficult problem in clinical management. Over the last decade, silver staining of the nucleolar organizer regions-associated proteins (AgNORs) in interphase cells has become a widely used alternative method for assessing proliferation in tumour pathology. A great deal of work has been done to evaluate the usefulness of AgNOR counts in the study of various neoplasms. It has been established that new predictor of biologic aggressiveness in a neoplasm, AgNOR proliferative index (pAgNOR) correlates with the proliferative activity of a cell. In this study, AgNOR silver staining was done on 40 cases of cytologically positive malignant effusions and both mean AgNOR (mAgNOR) and pAgNOR counting was done.

**Objectives:** To measure AgNOR counts and their distribution in malignant effusions in relation to a new parameter, AgNOR proliferative index (pAgNOR) which reflects the proliferative activity of a cell.

**Place and Duration of Study:** The study was carried out in the Department of Pathology, Postgraduate Medical Institute Lahore while the cases were collected from Surgical, Medical, Gynaecological and Oncology units of Services hospital, Mayo Hospital, Gulab Devi hospital and Lahore General hospital from March 1997 to November 1999.

**Patients and Methods:** Forty patients were selected with different types of malignancies and having cytologically positive effusions. Pleural and peritoneal fluid was collected aseptically and smears were made from the cell button after centrifugation. AgNOR silver staining technique was performed on each smear.

**Results:** Using the criteria of Ahsan et al 91-92, in our study highly significant ($p < 0.001$) difference was noted when AgNOR size of malignant effusions between 2+ and 3+ was compared with 0 – 1+. Similar significant relationship was seen ($p < 0.001$) when AgNOR distribution of 2+ and 3+ was compared with 0 – 1+ distribution. Most of the cases with malignant effusions had AgNOR proliferative index above 90%. However, when AgNOR proliferative index between pleural and peritoneal effusions were compared, the difference was not significant.

**Conclusion:** The two simple AgNOR counting methods can be used reliably to evaluate tumour cell kinetics especially in situations in which the tissue is insufficient for flow cytometry, for example, in small biopsies and limited needle aspirates.

**Key words:** Effusions, malignant, AgNOR, AgNOR Proliferative index.

## Introduction

The identification of mesothelial cells in cytologic samples is often a diagnostic challenge. This is particularly true in potentially malignant effusions in which reactive mesothelial cells may simulate adenocarcinoma cells[1]. The inability to decide on the presence or absence of malignant cells in cytologic specimens casts a difficult problem in clinical management[2]. Recently, interest has been focused on the assessment of cell kinetics or oncogenes when evaluating the biologic aggressiveness of malignant tumours and a variety of methods can be used to estimate the proliferative activity of the cells[3]. Over the last decade, silver staining of the nucleolar organizer regions-associated proteins (AgNORs) in interphase cells has become a widely used alternative method for assessing proliferation in tumour pathology. AgNOR quantity is the only parameter that allows, estimation of the rapidity of the cell cycle in routinely processed histological sections[4]. The nucleolar organizer regions (NORs) are loops of ribosomal DNA, which are acted upon by RNA polymerase-I to produce rRNA. NORs are located on the short arm of five pairs of acrocentric chromosomes

13,14,15,21,22[5]. The argyrophilic proteins include RNA polymerase I, topoisomerase IIa, transcription factor UBF and nucleolin. Heitz first recognized the correlation between certain chromosome regions and nuclei in 1931, and it was McClintock who in 1934, first called these regions as "nucleolus organizing". This work was ignored until Good Pasture and Bloom (1975), used a silver staining technique for the visualization of NORs (AgNORs). The recent outbreak of literature came when, Ploton et al in 1986, first described the current one-step method and applied it for supplemental diagnosis of malignancy[3]. A great deal of work has been done to evaluate the usefulness of AgNOR counts in the study of various neoplasms. A variety of benign and malignant tumors using either biopsy or cytology specimens have been analyzed by AgNOR technique[5-12]. The study by Khalid et al (1996) showed that AgNOR count in the nuclei of reactive mesothelial cells was significantly lower ($p<0.001$) than that observed in malignant cells. The results of study by Gul Naz Akhtar et al 2003[9] show that the AgNOR number permits a clear distinction to be made between malignant and benign cells. Reactive mesothelial cells have a lesser number of AgNORs as compared to malignant cells.

NORs are believed to be responsible for transcriptional activity and the increased number of AgNOR has been correlated with high proliferative activity. Since ploidy (DNA content) and proliferative activity are independent prognostic indicators, it was necessary to separately measure them to study their individual effect on the biologic behaviour of tumours[7].

Mourad et al in 1992 came up with another parameter in AgNOR counting, the AgNOR proliferative Index. It was expressed as the percentage of cells with more than five AgNOR per nucleus. His study concluded that mean AgNOR count correlate with the DNA mass or ploidy whereas, AgNOR proliferative index reflects proliferative activity of a cell. In a comparative study of proliferating cell nuclear antigen (PCNA) and AgNORs in transitional cell carcinoma of the urinary bladder, Khan 2001[11] reported that AgNOR proliferative index (pAgNOR) in grade III tumours was significantly higher ($p<0.05$) as compared with grade II tumours.

In the present study, this technique has been applied to compare AgNOR scores and AgNOR proliferative Index (pAgNOR) in malignant pleural and peritoneal effusions.

## Materials and Methods

A total of forty cases having malignant pleural and peritoneal effusions previously diagnosed and positive for malignant cells were included in this study. The cases were collected from medical, surgical, gynecological and oncology units of Mayo Hospital, Services Hospital, Gulab Devi Hospital and Lahore General Hospital, Lahore. The relevant clinical information was gathered from the hospital notes and the respective registrar of the ward. About 40 – 50 ml of the fluid was collected in a clean dry container and the collected fluid was immediately transported to the pathology laboratory of Post Graduate Medical Institute, Lahore.

Rest of the fluid was poured into clean, dry 15 ml centrifuge tubes. Centrifugation was carried out at 2000 rpm for 5 minutes. A cell button was formed in most instances at the bottom. The supernatant was poured off. A tiny portion of the cellular deposit was transferred to clean dry glass slides. Smears were made by either pull-apart method or by spreading sediments with the help of a wire loop on to the slides. The smears were air-dried and were fixed in 95% ethanol for 15 minutes. After fixation, one of the fixed smears was stained with Haematoxylin and Eosin and used for microscopy in order to confirm the nature of malignant process. The other fixed smear was used for AgNOR staining, by adopting the methodology of Khalid et al 1996[8].

Gelatin was dissolved in 1% formic acid to make a 2% solution. 50% aqueous silver nitrate was then added in a proportion of 1:2 to obtain the working solution. The smears were postfixed in 3:1 ethanol:acetic acid mixture. They were brought to water through graded alcohols, covered with filter paper and soaked drop-wise by the working solution. The smears were kept in the dark for 30 minutes in a humid chamber, washed with deionised water, dehydrated, taken to xylene and mounted.

AgNOR stained smears were examined under the light microscope. The areas having groups of malignant cells in the smears were selected. AgNOR counting was performed under 100*x* objective using oil immersion.

The nuclei stained light yellow and the AgNORs were visualized as brown-black discrete dots of variable sizes within the nuclei. AgNORs in 100 cells were counted by two pathologists independently and then compared. The size variation and distribution of AgNORs were performed by the following criteria used by Ahsan et al (1991-92)[13].

**Size variation grading:**

0 = More or less uniform in size.
1+ = Two different sizes.
2+ = More than two different sizes (but not those of 3+).
3+ = All grades and sizes including too minute to be counted.

**AgNOR distribution in the nuclei:**

0 = Limited to nucleoli.
1+ = Occasional dispersion outside nucleoli.
2+ = Moderate dispersion outside nucleoli.
3+ = Widely dispersed throughout the nucleus.

**AgNOR proliferative Index (pAgNOR):**

This was counted as described by Mourad et al 1992[7], as, the percentage of tumour cells with more than 5 AgNORs per nucleus.

The statistical methods Khan 2002[21] were employed to evaluate the observations and the results.

**Table No: 1: Comparison of AgNOR Counts in Malignant Effusions**

| Group | mAgNOR/Cell | |
|---|---|---|
| | Range | Mean + S.D |
| Pleural Effusions n=17 | 7.75 - 19.72 | 12.65 ± 3.60 |
| Peritoneal Effusions n=23 | 12.65 ± 3.60 | 12.08 ± 3.61 |
| **p value** | **NS** | |

## Results

In the present study mean AgNOR count in malignant pleural effusion was 12.65 ± 3.60 while in malignant peritoneal effusions, the mean AgNOR count was 12.08 ± 3.61. When AgNOR size was compared using the criteria cited by Ahsan et al (1991-92)[13], most of the cases of malignant effusions were between 2+ and 3+ as compared with 0 – 1+. The difference was highly significant ($p < 0.001$). However, the difference between the AgNOR size in pleural and peritoneal effusions was not significant (table 2).

Similar relationship was seen when AgNOR distribution was compared using the criteria, cited by, Ahsan et al (1991-92). Most of the cases with malignant effusions had AgNOR distribution of 2+ and 3+ as compared with 0 – 1+, and the difference was highly significant ($p < 0.001$). The difference in the AgNOR distribution between pleural and peritoneal effusions was not significant (table 3).

**Table No: 2: Comparison of AgNOR Size in Cases with Malignant Pleural and Peritoneal Effusions**

| Group | 0 – 1+ | 2+ - 3+ | Total |
|---|---|---|---|
| Pleural Effusions | 2 | 15 | **17*** |
| Peritoneal Effusions | 6 | 17 | **23*** |
| **Total** | **8**** | **32**** | **40** |

***p = NS***
****p = <0.001***

***AgNORs size variation***

*0 = More or less uniform*
*1+ = Two different sizes*
*2+ = More than two different sizes (but not those of three)*
*3+ = All grades and sizes including too minute to be counted*

**Table No: 3: Comparison of AgNOR Distribution in Cases with Malignant Pleural and Peritoneal Effusions**

| Group | AgNOR Distribution | | |
|---|---|---|---|
| | 0 - 1+ | 2+ - 3+ | Total |
| Pleural Effusions | 2 | 15 | **17** |
| Peritoneal Effusions | 0 | 23 | **23** |
| **Total** | **2** | **38** | **40** |

***p = NS***

***AgNOR Distribution:***

*0 = Limited to the nucleoli*
*1+ = Occasional dispersion outside the nucleoli*

*2+ = Moderate dispersion outside the nucleoli*
*3+ = Widely dispersed throughout the nucleus*

Most of the cases with malignant effusions had AgNOR proliferative index above 90% (figure 1). However, when AgNOR proliferative index between pleural and peritoneal effusions were compared, the difference was not significant (table 4).

**Table No: 4: Comparison of AgNOR Proliferative Index in Cases with Malignant Effusions**

| Group | AgNOR Proliferative Index (pAgNOR) %age | |
|---|---|---|
| | Range | Mean ± S.D. |
| Pleural Effusions (n = 17) | 68 – 100 | 85.47 ± 9.47 |
| Peritoneal Effusions (n = 23) | 55 – 100 | 83.83 ± 12.55 |
| **p Value** | **NS** | |

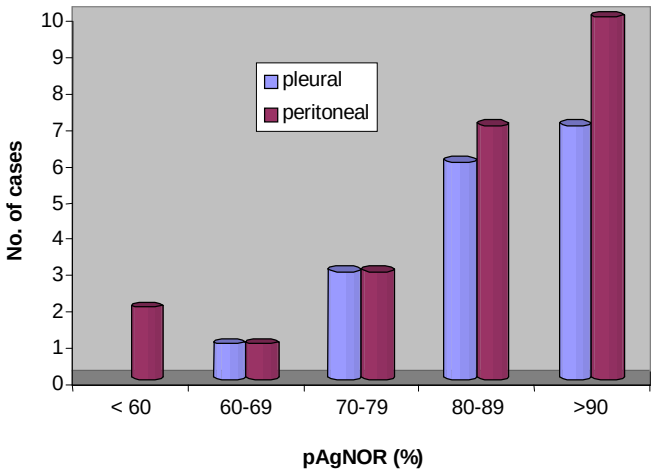


**Fig. 1: AgNOR Proliferative Index (pAgNOR) in Cases with Malignant Pleural and Peritoneal Effusions**

# Discussion

Over the past 12 years, "AgNOR method" has been applied in tumour pathology for both diagnostic and prognostic purposes and it has become a widely used alternate method for assessing proliferation in tumour pathology [4,14,15,16]. AgNOR analyses have been found to be a useful adjunct to other methods in the routine diagnosis of malignant serous effusions.[17, 18, 19]

In the present study mean AgNOR count in malignant pleural effusion was 12.65 ± 3.60 while in malignant peritoneal effusions, the mean AgNOR count was 12.08 ± 3.61. This is in accordance with Rocher et al (2001)[18]. Moreover, it was demonstrated that AgNORs in neoplastic cells were more numerous and of variable sizes whereas these were fewer and of uniform sizes in benign cells [20,17].

In the present study AgNOR size and distribution was compared using the criteria cited by Ahsan et al 1991-92[13]. Most of the cases of malignant effusions were between 2+ and 3+ as compared with 0 and 1+. This difference was highly significant ($p < 0.0001$) (tables 2 & 3). The results were in accordance with Khalid et al (1996)[8] who reported a highly significant difference ($p < 0.001$) in both the size and distribution of AgNORs between benign and malignant effusions.

Similarly, in a study conducted by Akhter (2000)[9], AgNORs in malignant cells were greater in number, irregularly distributed and heterozygous in size as compared to mesothelial cells, which were categorized by a small number and uniform size.

In addition, the present study also evaluated AgNOR proliferative Index (pAgNOR), in malignant effusions as described by Mourad et al (1992)[7]. Most of the cases of malignant effusions had pAgNOR above 90% (figure 1). This was in accordance with the findings of Khan (2001)[11].

Thus, the two simple AgNOR counting methods can be used reliably to evaluate tumour cell kinetics especially in situations in which the tissue is insufficient for flow cytometry, for example, in small biopsies and limited needle aspirates.

# References

1. Fetsch PA, Simsir A, Abati A. Comparison of antibodies to HBME – 1 and Calretinin for the detection of mesothelial cells in effusion cytology. Daig Cytopathol 2001; 25: 158-161.
2. Ko EC, Jhala NC, Shultz JJ, Chhieng DC. Use of a panel of markers in the differential diagnosis of Adenocarcinoma and reactive mesothelial cells in fluid cytology. Am J Clin Pathol 2001; 116: 709-715.
3. Suto T, Sugai T, Nakamura S-C, Funato O, Nitta H, Sasaki R et al. Assessment of the expression of p53, MIB-1 (Ki-67 Antigen), and

Argyrophillic nucleolar regions in carcinoma of the extrahepatic bile duct. Cancer 1998; 82: 86-95.
4. Bankfalvi A, Ofner D, Schmind KW, Schmitz KJ, Breuklemann D, Krech R et al. Standardized in-situ AgNOR analysis in breast Pathology: Diagnostic and cell kinetic implications. Pathol. Res. Pract. 1999; 195: 219 – 229.
5. Chern J-H, Lee Y-C, Yang M-H, Chang S-C, Perng R-P. Usefulness of argyrophillic nucleolar organizer regions score to differentiate suspicious malignancy in pulmonary cytology. Chest. 1997; 111: 1591-96.
6. Lim SM, Duggan MA, Ruff M, Rahim S, McGregor SE, Green FHY. Morphometric analysis of nuclear organizer regions in benign and malignant peritoneal effusions using backscattered electron microscopy. J Pathol 1992; 166: 53-60.
7. Mourad WA, Erkman-Balis B, Livingstone S, Shoukri M, Cox CE, Nicosia SV et al. Argyrophillic nucleolar organizer regions in breast carcinoma. Cancer 1992; 69: 1739-1744.
8. Khalid AW, Khan SA, Chaudrhy NA, Tayyab M, Tehseen S. Silver staining nucleolar organizer region (AgNOR) counts in begin and malignant effusions. Pak. Postgraduate Med Journal 1996; 7: 3-4
9. Akhter GN. p53 Immunostaining in benign and malignant effusions. [Thesis]. Lahore: University of the Punjab; 2000.
10. Parveen S. p53 and argyrophillic nucleolar organizer regions in normal, cirrhotic and carcinomatous liver [Thesis]. Lahore: University of the Punjab; 2000.
11. Khan AH. Comparative study of Proliferative nuclear cell antigen (PCNA) and Argyrophillic nucleolar organizer regions in transitional cell carcinoma [Thesis]. Lahore: University of the Punjab; 2001
12. Pich A, Chiusa L, Formiconi A, Galliano D, Bortolin P, Comono A et al. Proliferative activity is the most significant predictor of recurrence in noninvasive papillary urothelial neoplasms of low malignant potential and grade 1 papillary carcinomas of the bladder. Cancer 2002; 95: 784-90.
13. Ahsan S, Tayyab M, Chaudhry NA, Khan SA. Silver staining nucleolar organizer region (AgNOR) typing in nodular hyperplasia of the prostate. Pak Postgraduate Med J 1991-92; 2-3: 67-72.
14. Trere D. AgNOR staining and quantification. Micron 2000;31:127-31.
15. Santacroce L, Bufo P, Gagliardi S, Mastropasqua MG, Losacco T.[Argyrophillic nucleolar organizer regions (AgNORs) as malignancy markers in colorectal neoplasms]. Clin Ter 2001; 152: 91-3.
16. Chen M, Lee JC, Lo S, Shern J. Argyrophillic nucleolar organizer regions in nasopharyngeal carcinoma and paraneoplastic epithelia. Head Neck 2003; 25(5): 395-9
17. Sujathan K, Kannan S, Pillai KR, Chandralekha B, Amma NS, Nair MK. Significance of AgNOR count in differentiating malignant cells from reactive mesothelial cells in serous effusions. Acta Cytol 1996; 40: 724-8.
18. Rocher AE, Blanco AM, Palaoro LA. Usefulness of AgNOR technique in the interpretation of serous effusions. Rev Med Chil 2000; 28: 963-8.
19. Pomjanski N, Motherby H, Buckstegge B, Knops K, Rohn BL, Bocking A. Early diagnosis of mesotheliomas in serous effusions using AgNOR analysis. Anal Quant Cytol Histo 2001;23:151-60.
20. Derenzini M, Betts CM, Eusebi V. Distribution of interphase nucleolar organizer regions and diagnosis of malignancy [Letter]. The Lancet 1987; ii: 286.
21. Khan SA. Thesis writing. 2nd Edition. Lahore: Department of Pathology, Postgraduate Medical Institute; 2002: 37-66.