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Diagnostic Utility of AgNORs Staining of Serous Effusion among Sudanese Patients

Dr Muaz Osman Mohamed Fagere
Assistant Professor of Histopathology & Cytopathology
Albaha University, Faculty of Applied Medical Sciences, Laboratory Medicine Department K.S.A
Omdurman Islamic University, Faculty of Medical Laboratory Sciences, Department of Histopathology & Cytopathology

1. INTRODUCTION

Serous effusion is a condition of excess accumulation of fluids in serous cavities i.e. (Pleural, Peritoneum, Pericardium) due to different underlying pathological conditions, which vary from inflammatory conditions to primary or secondary malignancies. Some reports suggest that as many as 50% of the patients with lung cancers or breast cancers will develop pleural effusion [1]. The commonest primary malignant tumors causing metastases to the serous cavities are adenocarcinoma of the breast, lung, ovary, stomach, large intestine, pancreas, thyroid, kidney, sarcoma and malignant thymomas [2]. In Sudan the most common aetiology of accumulation of benign serous effusions are inflammatory conditions while metastatic malignancies are the major cause of malignant effusions, while primary malignant mesothelioma is very rare condition, few cases are reported in areas with endemic exposure to asbestos. After aspiration of effusion a set of laboratory investigations should be conducted to identify the nature of the effusion constituents, and it is chemical composition. Effusion sample must be submitted to cytopathology lab to identify its cellular component, which contributes to the identification of malignant cells involving the serous cavities, and is usually made by conventional Papnicolaou (Pap) staining technique. Ancillary techniques such as image analysis and flow cytometry have proved useful in the distribution of benign and malignant fluids but, they are not readily available in most laboratories in the Sudan. Immunocytochemistry (ICC) is probably the most frequently used ancillary technique applied to effusion diagnosis; it can provide reliable insights into various diagnostic dilemmas in effusion cytology. So far many antibodies have been used in serous effusions to enhance the diagnosis with varying degree of efficacy [3].The serous effusions represent a common and challenging diagnostic problem with diverse and non-similar aetiology. Current methods which applied in this task are either insufficient or invasive. Immunocytochemistry plays an important role in diagnosis and patients management through its ability in determining the nature of the cells that encountered in the effusions and the discrimination between reactive and neoplastic cells that underlying the disease. In certain conditions benign serous effusions, produce reactive mesothelial cells, mimicking the morphology of the neoplastic cells, which increase the difficulties of diagnosing of metastatic carcinomas which is often associated with the reactive mesothelial hyperplasia, and the morphological variations of the mesothelial cells offer great potential for negative diagnosis, and false positive diagnosis which can have a real disastrous consequences for patients [4]. A comparatively simpler technique used for this purpose is the silver staining of nucleolar organizer regions (NORs). Which attempts to add useful diagnostic criteria especially in the differentiation between benign and malignant effusions [5]. A comparatively simpler technique Interphase AgNORs are the structural and functional units of the nucleolus which contain all the essential components for the synthesis of ribosomal RNA. In the human karyotype, NORs are located in each of the short arms of the acrocentric chromosomes 13, 14, 15, 21 and 22.[6] The two argyrophilic proteins which are associated with rRNA transcription and processing are nucleolin and nucleophosmin.[7] These proteins are argyrophilic and are easily stained by silver stains. After silver-staining, the NORs can be identified as black dots present throughout the nucleolar area. The number and size of NORs reflect cell activity, proliferation and transformation and help to distinguish benign from malignant cells.[8] Evaluation of the quantitative distribution of AgNORs has been applied in tumour pathology both for diagnostic and prognostic purposes. A number of studies carried out in different tumour types demonstrated that malignant cells frequently present a greater AgNOR count than corresponding non-malignant cells.[9,10] In the present study, this technique has been applied to differentiate malignant cells from reactive mesothelial cells in pleural and peritoneal effusions.

2. MATERIALS AND METHODS

2.1. Study Design:

This is a prospective study aimed to assess the Diagnostic utility of AgNORs staining of serous effusion among Sudanese patients, the study was conducted in Khartoum state hospitals, in the period from September 2013, to May 2015.

2.2. Study Sample:

Eighty three cytological materials (effusion) were collected from patients previously diagnosed as having serous effusion. The cytological smear were processed and stained according to (Pap) and AgNORs staining methods.

2.3. Specimen Collection:

The effusions specimens were collected by needle aspiration from eighty three patients with serous effusion manifestation, and then it had been delivered to the Histo/Cyto pathology laboratory.

2.4. Sample Processing:

2.4.1. Fixation:

The effusion samples were centrifuged at 1500 rpm for 10 minutes, from the deposited cells smears were prepared and fixed according to the method of staining. Smears for conventional Pap method were fixed while it was wet by 95% ethanol. AgNORs smears were post-fixed in 3:1 ethanol : acetic acid mixture.

2.4.2. Staining Procedures:

2.4.2.1. Conventional Papnicolaou (Pap) Staining Procedure:

The alcohol fixed smears were hydrated through descending grades of alcohol concentrations 95% through 85% through 75% to distilled water for 2 minutes in each stage. For staining the nuclei, the smears were treated with Harri´s Hematoxlyin for 5 minutes; differentiated in 0.5% aqueous hydrochloric acid for 10 seconds, rinsed in distilled water. Then the smear were blued in alkaline water for 4 seconds, and then dehydrated in ascending grades of alcohol concentrations 75% through 85% though 95% for 2 minutes in each stage. For the cytoplasmic counter stain smears were treated with Orange G6 (O.G6) for 2 minutes, rinsed in 95% alcohol then treated with Easoin Azour (EA50) for 3 minutes, Dehydrated, Cleared, and Mounted in DPX.

2.4.2.2. AgNORs staining Procedure:

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 post-fixed in 3:1 ethanol : acetic acid mixture. They were brought to deionized distilled 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. Only nuclei of mesothelial , epithelial or malignant cells were evaluated. Inflammatory cells (PMNs, lymphocytes and macrophages) were excluded. 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 individuals independently and then compared. The size variation and distribution of AgNORs were performed by the following criteria used by Ahsan et al [11].

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.

AgNORs distribution in the nuclei:

0 = Limited to nucleoli.

1+ = Occasional dispersion outside nucleoli.

2+ = Moderate dispersion outside nucleoli.

3+ = widely dispersed throughout the nucleus.

2.5. Statistical analysis

Analysis was performed using statistical software SPSS(Statistical package for the Social Sciences) version 18. Preliminary analyses were done such as; descriptive statistics, frequencies, cross tabulation, and compare Means.

2.6. Ethical Considerations

The aims and benefits of this study were explained to the participants. Informed consents were obtained from all members who involved in this study. Health education was provided each participant.

2.7. Method of data collection

Data concerning patients involved in this study such as age, sex, and the results of effusion diagnosis were collected by check list method.

3. RESULTS AND OBSERVATIONS

In this study, the diagnostic utility of AgNORs staining of serous effusion among Sudanese patients were assessed. These assessment were evaluated among eighty three patients with serous effusion . Their ages ranging from 20 to 72 with a mean age of 52 years old. Table 1. Fig 1, show that females 45(54.2%) were the major population in the study while males constituted 38(45.8%).

Table 2. Fig 2, represent that the dominant age of the study population were among the age group of 51+ which constituted 26(31.3%), followed by the age group of 31-40 which constituted 22(26.5%), followed by the age group of 41-50 then 20-30 which constituted 20(24.1%) and 15(18.1%) respectively. As shown in Table 3. Fig 3, the majority of the study population were females with age group 50+ which constituted 15(18.1%), followed by males with age group 41-50 which constituted 13(14.4%). Table 4. Fig 4, represent the description of the study population by effusion origin , its apparently that the majority of effusion origin were from pleural cavity which constituted 46(55.4%), followed by peritoneum and pericardium cavities which constituted 28(33.7%) and 9(10.9%) respectively. As shown in Table 5. Fig 5, concerning the description of the study population by effusion type the majority were benign effusion constituting 57(68.7%) and malignant effusion were 26(31.3%). Concerning Table 6. Fig 6, which showing the description of the study population by etiology Tuberculosis was the major cause of benign effusions constituting 28(33.7%), followed by Pneumonia, Rheumatoid Arthritis, and Chronic Heart Failure which constituted 11(13.2%), 10(12%), and 8(9.6%) respectively. While Breast Cancer was the major cause of malignant effusions constituting 10(12%), followed by Ovarian Cancer, Lung Cancer, and Non-

Hodgkin's Lymphoma which constituted 8(9.6%), 5(6%), and 2(3.6%) respectively. As shown in Table 7 Fig 7, the majority of the malignant effusions were peritoneal effusion constituting 13(15.6%), followed by pleural, pericardium effusions which constituting 10(10%) and 3(3.6%) respectively. Table 8 Fig 8, shows the Mean ± SD of AgNORs count/100 cells, the count were significantly higher in malignant effusions (Mean ± SD = 13.52±4.21) compared to benign ones (Mean ± SD = 4.16±.86), with *P Value* of (P< 0.001) which prove the presence of a statistical significant difference between them. Table 9 Fig 9, shows the grade of AgNORs dispersion pattern, malignant effusions showed higher grade (21 sample with grade 3+), compared with benign effusion which showed only 1 sample with the same grade, this results also indicates the statistical significance difference between malignant and benign effusions concerning grades of dispersion pattern. Table 10 Fig 10, shows the grades of AgNORs size, malignant effusions showed higher grade (17samples with grade 3+), compared with benign effusion which showed only 2 samples with the same grade, this results also indicates the statistical significance difference between malignant and benign effusions concerning grades of AgNORs size.

Table 1. Description of the study population by gender

Gender	Frequency	Percentage
Female	45	54.2%
Male	38	45.8%
Total	83	100%

Table 2. Description of the study population by age

Grouped age	Frequency	Percentage
20-30	15	18.1%
31-40	22	26.5%
41-50	20	24.1%
51+	26	31.3%
Total	83	100%

Table 3. Description of the study population by gender and age

	20-30		31-40		41-50		50+		Total	
	No	%	No	%	No	%	No	%	No	%
Female	12	14.4%	11	13.2%	7	8.4%	15	18.1%	45	54.2%
Male	3	3.6%	11	13.2%	13	15.7%	11	13.2%	38	45.8%
Total	15	18.1%	22	26.5%	20	24.1%	26	31.3%	83	100%

Table 4. Description of the study population by effusion origin

Effusion origin	Frequency	Percentage
Pleural	46	55.4%
Peritoneum	28	33.7%
Pericardium	9	10.9%
Total	83	100%

Table 5. Description of the study population by effusion type

Effusion type	Frequency	Percentage
Benign effusion	57	68.7%
Malignant effusion	26	31.3%
Total	83	100%

Table 6. Description of the study population by aetiology

Aetiology	Frequency	Percentage
Tuberculosis	28	33.7%
Pneumonia	11	13.2%
Rheumatoid arthritis	10	12%
breast ca.	10	12%
Chronic heart failure	8	9.6%
ovary ca.	8	9.6%
Lung ca.	5	6%
Non-Hodgkin's	2	3.6%
Total	83	100%

Table 7. Description of the study population by effusion origin and effusion type

	Benign		Malignant		Total	
	No	%	No	%	No	%
Pleural	36	43.4%	10	12%	46	55.4%
Peritoneum	15	18.1%	13	15.6%	28	33.7%
Pericardium	6	7.2%	3	3.6%	9	10.9%
Total	57	68.7%	26	31.3%	83	100%

Table 8. Mean±SD of AgNORs count / 100 cells

Effusion type	Mean ±SD AgNORs count / 100 cells
Malignant effusion	13.52±4.21*
Benign effusion	4.16±.86

*$P < 0.001$

Table 9. Description of AgNORs dispersion in malignant and benign effusions

Effusion type	0	1+	2+	3+
Malignant effusion	1	1	3	21*
Benign effusion	48	6	2	1

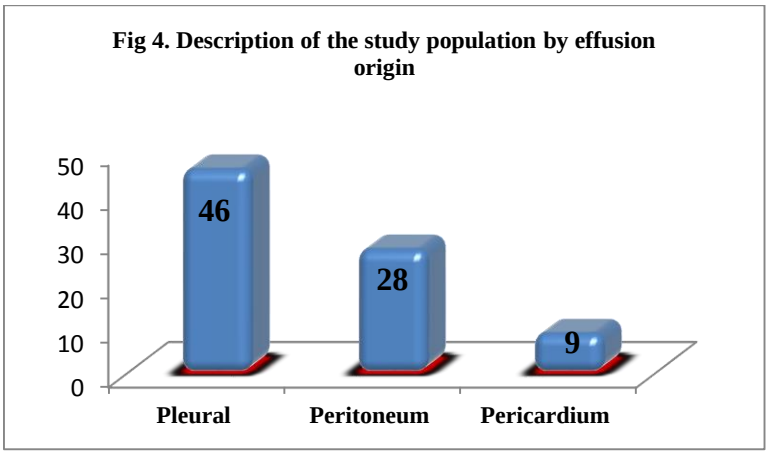
*$P < 0.001$

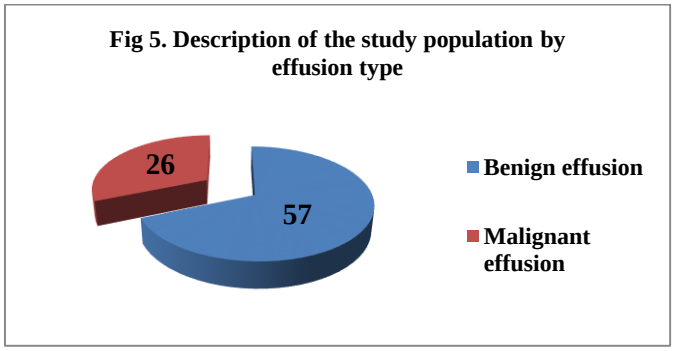
Table 10. Description of AgNORs size in malignant and benign effusions

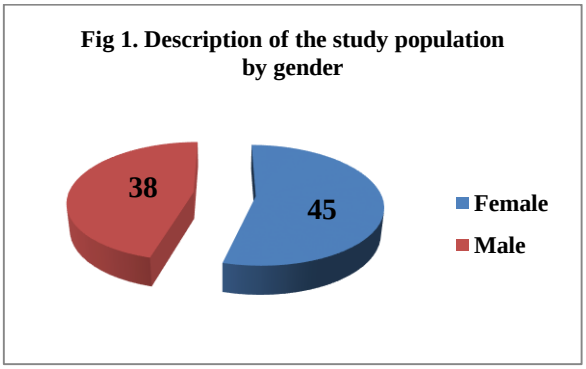
Effusion type	0	1+	2+	3+
Malignant effusion	1	3	5	17*

Benign effusion	45	6	4	2

*$P < 0.001$







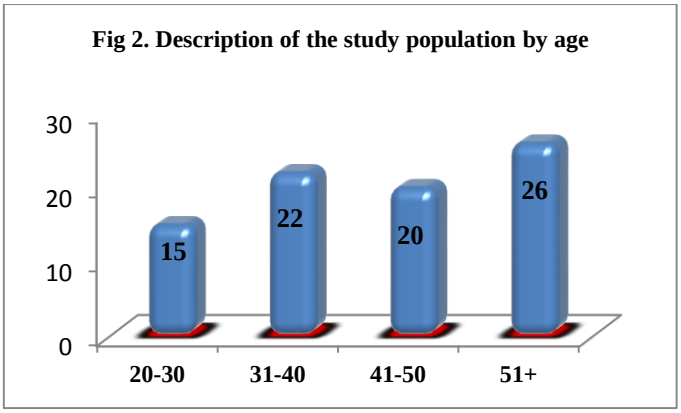


Fig 6. Distribution of the study population by aetiology

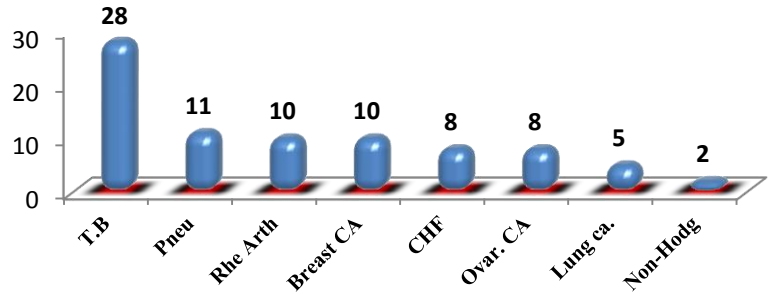


Fig 7. Description of the study population by effusion origin and effusion type

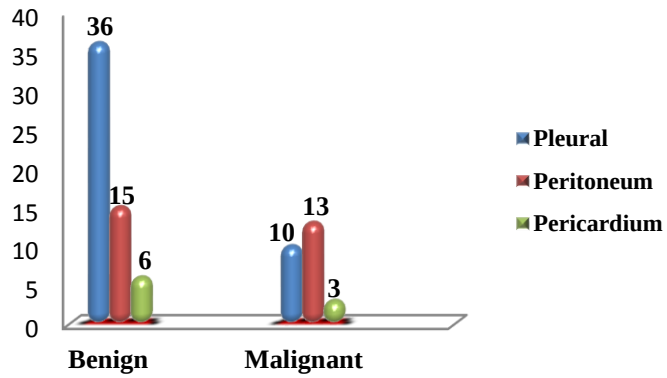
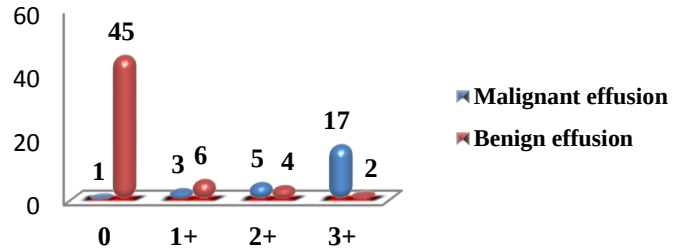
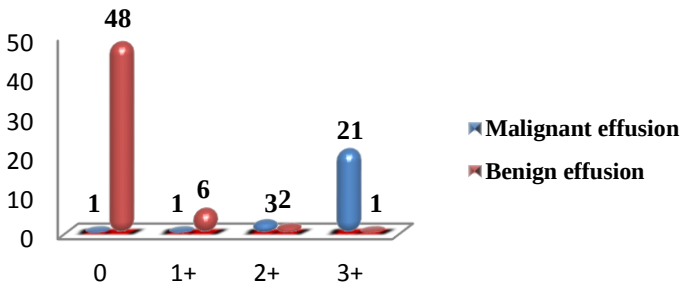
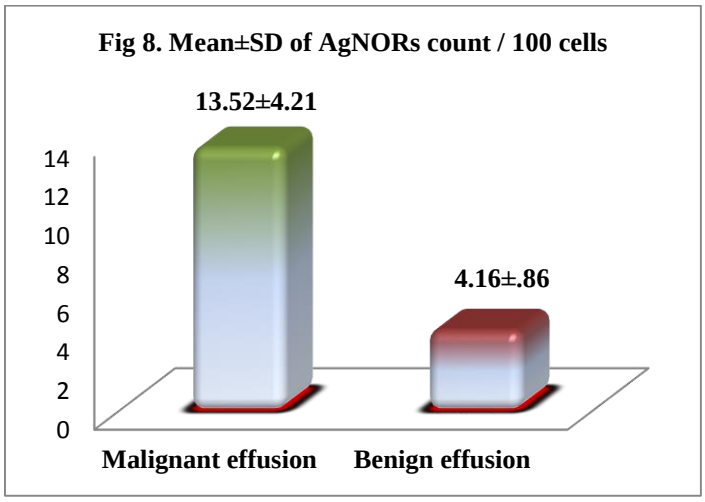


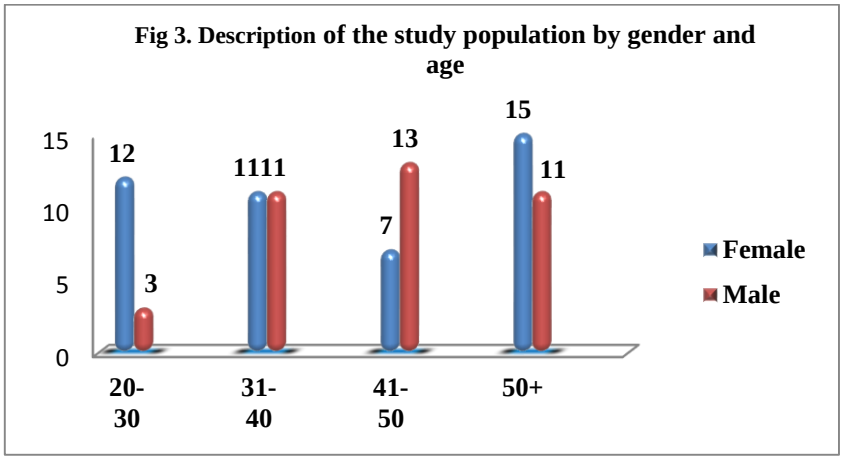
Fig 10. Description of AgNORs size in malignant and benign effusions



Fif 9. Description of AgNORs dispersion in malignant and benign effusions







4. DISCUSSION

The present study validates the diagnostic utility of AgNORs staining of serous effusion among Sudanese patients . Out of 83 (100%) patients with accumulated serous effusion, The majority of these patients were females there ages were among the group age of 50+. Malignant effusions were detected in 26 (31.3%) patients mainly found to be caused by ovarian cancer. In this study Tuberculosis was frequently observed as a major aetiological factor of the benign effusions this finding is similar to the result obtained by many studies Liam, et al. [12], Kalaajie WK [13], Who suggested that the most common cause of benign exudative effusion was tuberculosis.

During this study, the Mean ± SD of (AgNORs count/100 cells) in malignant effusion samples were found to be higher than benign effusion ones, this result indicates that there is a statistical significant differences between the two Means. This increased count of AgNORs in malignant cell is mainly attributed to the high proliferative activity and other requirement of ribosomes biogenesis, since NORs are believed to be responsible for transcriptional activity [14]. This finding supports the study by[15]. Moreover, many studies demonstrated that AgNORs in neoplastic cells were more numerous and of variable sizes whereas these were fewer and of uniform sizes in benign cells [17],[18].

In the present study the AgNORs size and dispersion patterns were significantly higher graded in the malignant effusion samples than benign ones, this results is supported by the study of Gul Naz Akhtar et al. [19] who concluded that AgNOR size and distribution patterns were of a significantly higher grade ($p<0.001$) in the malignant pleural and peritoneal effusions as compared with non-malignant effusions.

5. CONCLUSION AND RECOMMENDATIONS

On the basis of this study and review of other studies, it could be concluded that:

1. Analysis of AgNORs count, size, and dispersion pattern of in effusion samples found to be rapid, cost-effective, and useful diagnostic tool in the differentiation of malignant serous effusions from the other pathological conditions underlying the accumulation of effusions.

2. Malignant serous effusion was frequently observed almost in all body cavities and generally caused by different metastatic malignant cell from distance origins.

3. Females with ovarian cancer represented the majority among the patients with malignant effusion.

4. Peritoneum cavity was the most common site for accumulated effusion.

5. Following the criteria devised by Ahsan et al, we found that the AgNORs in malignant cells were greater in number, irregularly distributed throughout the nucleus and heterogenous in size.

6. On the other hand, benign cells were characterized by a lesser number of small, homogenously sized, regularly clustered AgNORs.

It's recommended:

1. That AgNORs count, size, and its dispersion pattern routinely used in the laboratory to differentiate between malignant and benign serous effusions.

2. That additional ancillary techniques such as flow cytometry, automated image morphometry should be incorporated in the diagnosis of doubtful effusions so as to help solving the everlasting dilemma of the malignant effusions.

3. That AgNOR counting methods used to evaluate tumour cells especially when the tissue is insufficient for diagnosis, i.e. in small biopsies and limited needle aspirates.

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