EVALUATION OF SEROUS EFFUSIONS USING AgNOR COUNT- A STUDY IN A TERTIARY CARE CENTRE OF SAHARANPUR

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ABSTRACT

BACKGROUND

Abnormalities of nucleolar morphology are constant features of cancer cells. The importance of the nucleolus for the diagnosis of malignancy has been reconsidered after the observation that structural and morphological reorganisation is a consequence of the redistribution of the fibrillar components within the nucleolus. The nucleolar fibrillar components (fibrillar centre and associated dense fibrillar component) are the sites where ribosomal genes are located during interphase. They therefore represent the interphase counterpart of metaphase Nucleolar Organizer Regions (NOR's). The use of this parameter relative to interphase, NOR distribution has become of great importance after the finding that interphasic NOR's can be clearly visualised at the light microscopic level using a silver reaction, which stains the acidic proteins of the NOR's (AgNOR Proteins) on routinely prepared histo- and cytological samples. By means of this technique, silver-stained interphasic NOR's has been used for the diagnosis of malignancy of numerous neoplastic lesions. Cancer cells in fact have a greater number of silver-stained NOR's than the corresponding benign cells. The diagnosis of malignancy in cytologic preparations of pleural and peritoneal effusions is not always easily performed. Interest therefore has focussed on identifying reliable methods of supplementing conventional cytological methods to differentiate malignant cells from benign cells. Recently, AgNOR's (Silver Staining of Nucleolar Organizer Regions) have come to limelight. NOR's are being increasingly used in diagnosing a variety of tumours.

Aims and Objectives:

The objectives of the present study were

1. To identify reliable methods of supplementing conventional cytological methods to differentiate malignant cells from benign cells in serous effusions.
2. To study various serous effusions by conventional cytological methods.
3. To differentiate benign and malignant serous effusions on the basis of conventional cytological methods.
4. To study AgNOR count in differentiating malignant cells from benign cells in serous effusions and
5. To find out the utility of AgNOR counts in malignant vs benign cell differentiation in fluids.

MATERIALS AND METHODS

This is a descriptive study. We studied 65 cases of serous effusions over a period of one year from December 2016 - December 2017. Samples for this study were collected from various outpatients and inpatients admitted in Medicine and Surgical wards.

Staining and counting procedure.

1. Fluids collected were subjected to-
 - Pap staining.
 - 'AgNOR' staining.
2. Cytopathological evaluation of smears was done using Papanicolaou staining and further differentiation using AgNOR count.
3. The number of AgNOR's present in each nucleus were counted. Two hundred nuclei were taken into account. The mean AgNOR count of the specimen was then calculated. The AgNOR's were visualised using oil immersion objective and to avoid biased results, counts were done by two different observers.

RESULTS

A total of 43 cases (66.15%) of benign fluids were examined. Low mean AgNOR counts (less than 4) were seen in 41 cases (95.35%), whereas 2 (4.65%) benign cases had high (more than 4) mean AgNOR counts. Of the 22 (33.85%) malignant fluids encountered, low mean AgNOR counts (less than 4) were observed only in one case with a percentage of 4.54%, whereas high mean AgNOR counts were observed in 21 cases with a percentage of 95.46%. Cytologically, all the malignant cases 22 (100%) were diagnosed as adenocarcinoma with mean AgNOR counts as in previous paragraph.

CONCLUSION

The mean AgNOR counts have a definite role in differentiating benign from malignant effusions. They can be used as supportive test to increase the value of routinely used Papanicolaou staining methods.

KEY WORDS

AgNOR, Serous Effusion, Conventional Cytology.

HOW TO CITE THIS ARTICLE: Sharma R, Sharma V, Goyal N. Evaluation of serous effusions using AgNOR count- a study in a tertiary care centre of Saharanpur. J. Evolution Med. Dent. Sci. 2018;7(47):5057-5062, DOI: 10.14260/jemds/2018/1125

'Financial or Other Competing Interest': None.
Submission 04-10-2018, Peer Review 02-11-2018,
Acceptance 09-11-2018, Published 19-11-2018.

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DOI: 10.14260/jemds/2018/1125

BACKGROUND

Accumulation of fluid in a serous cavity in excess of the normal small amount is referred to as an effusion. The likely benign causes of effusions included in the pleura are congestive heart failure, pulmonary infarcts, inflammatory processes including viral pneumonia and tuberculosis and cirrhosis of liver causing peritoneal effusion.

Aspiration of serous cavities is a simple and relatively non-invasive technique to achieve a diagnosis. In malignant conditions, accumulation of serous fluid is more commonly due to secondary involvement of the serous membranes than the primary malignant tumour of the mesothelium-malignant mesothelioma. The diagnosis of malignancy in cytologic preparations of pleural and peritoneal effusions is not always easily performed. Malignant cells in effusions take on some characteristics of in-vitro cultural cells: in particular they frequently lose their relationship with surrounding structures.

Abnormalities of nucleolar morphology are constant features of cancer cells. The importance of the nucleolus for the diagnosis of malignancy has been reconsidered after the observation that structural and morphological reorganisation is a consequence of the redistribution of the fibrillar components within the nucleolus. The nucleolar fibrillar components (fibrillar centre and associated dense fibrillar component) are the sites where ribosomal genes are located during interphase. They therefore represent the interphase counterpart of metaphase nucleolar organizer regions (NOR's)

The use of this parameter relative to interphase NOR distribution has become of great importance after the finding that interphasic NOR's can be clearly visualised at the light microscopic level using a silver reaction, which stains the acidic proteins of the NOR's (Ag-NOR Proteins) on routinely prepared histo- and cytological samples. By means of this technique, the quantity of silver-stained interphasic NOR's has been used for the diagnosis of malignancy of numerous neoplastic lesions. Cancer cell share in fact have a greater number of silver-stained NOR's than the corresponding benign cells.

MATERIALS AND METHODS

Study Design

The present descriptive study was undertaken at Department of Pathology of a tertiary care centre, conducted over a period of 1 year from December 2016 to December 2017.

Study Population

A total of 65 cases of body effusions were included in the present study. Samples for this study were collected from various outpatients and inpatients admitted in Medicine and Surgical wards of our institution.

Collection of Data

Clinical data such as site of effusion, radiological findings and provisional clinical diagnosis were obtained from the patient's medical records.

Sample Evaluation

Collection of Sample: - Samples from patients for this study were collected under strict aseptic precautions, were processed as soon as possible. 10% formalin in equal amount was used as preservative for fluid. Fluid so collected were subjected to-

- Pap staining.
- 'AgNOR' staining.

Improved Silver Technique for AGNOR Staining-Silver Colloid Solution:

- 2 g/dL gelatin dissolved in 1 g/dL aqueous formic acid.
- The above-mentioned solution was mixed just before staining in a ratio of 1: 2 volumes with a 20 gm/dL aqueous silver nitrate solution.

Technique

1. Fix the smear in 3: 1 ethanol-acetic acid solution for thirty minutes.
2. Rinse briefly in absolute alcohol for 5 seconds.
3. Rinse briefly in 70 percent alcohol for 5 seconds.
4. Rinse in running water for ten minutes and then washed three times in deionized distilled water.
5. Slides are then placed in freshly prepared staining solution for 30 minutes at room temperature in the dark.
6. Sides are then washed 6 times with distilled water.
7. Dehydrated by passing three or four baths of absolute alcohol.
8. Cleared in xylene.
9. Mounted with DPX mountant.

RESULTS

AgNOR's appeared within nuclei as dark brownish dots of varying sizes against yellow background.

Counting Procedure

The number of AgNOR's present in each nuclei are counted. Two hundred nuclei are taken into account. The mean AgNOR count of the specimen is then calculated. The AgNOR's were visualised using oil immersion objective and to avoid biased results the counts were done by two different observers.

Cytopathological evaluation of smears was done using Papanicolaou staining and further differentiation using AgNOR count.

Statistical Analysis

The data was entered in MS-Excel and analysed using Epi-Info 7 software. Frequencies and means of finding were calculated and appropriate test of significance (Fisher Exact Test) were applied.

The present study comprised of Sixty five (65) cases of different body effusions over a period of one year from Dec. 2016 to Dec. 2017, received in the Department of Pathology. SMMH Medical College, Saharanpur, collected from various outpatients and inpatients admitted in Medicine and Surgical wards of this medical college, hospital. Following observations were made.

Sl. No.	Age in Yrs.	No. of Cases	(%)
1.	0-15	02	3.07%
2.	16-30	13	20.00%
3.	31-45	09	13.85%
4.	46-60	28	43.07%
5.	61-75	12	18.46%
6.	76-90	01	1.55%

Table 1. Age-Wise distribution of Cases Analysed

Table 1. Shows age-wise distribution of total cases analysed.

Sl. No.	Sex	No. of Cases	(%)
1.	Females	34	52.31%
2.	Males	31	47.69%

Table 2. Sex-Wise distribution of Cases Analysed

Table 2. As is evident from the table, most of the patients were females 34 (52.31%) and rest 31 (47.69%) were males.

Sl. No.	Type of Fluid	No. of Cases	(%)
1.	Peritoneal Fluid	38	58.46%
2.	Pleural Fluid	23	35.38%
3.	Pericardial Fluid	04	06.16%
4.	Synovial Fluid	-	-
5.	Others	-	-

Table 3. Different types of Body Fluids/ Effusions Analysed

Table 3. Shows different types of fluids analysed in the present study. In all Ascitic fluids (peritoneal fluids) were 38 (58.46%), pleural fluid were 23 (35.38%) and only 4 cases (6.16%) of pericardial fluid were encountered and analysed.

Sl. No.	Cytological Diagnosis	No. of Cases	Mean AgNOR Count		
			Low (< 4)	High (> 4)	
1.	Benign	43 (66.15%)	41 (95.35%)	02 (04.65%)	P value < 0.0001 Highly significant
2.	Malignant	22 (33.85%)	01 (4.54%)	21 (95.46%)	
	- Adenocarcinoma	22 (100%)	01 (4.54%)	21 (95.46%)	
	- Squamous cell Ca	-	-	-	
	- Lymphoma	-	-	-	
	- Melanoma	-	-	-	
	- Others	-	-	-	

Table 7. Mean AgNOR Counts in Body Fluids Analysed

Table 7. Shows analysis of body fluids and their evaluation on the basis of AgNOR counts. A total of 43 cases (66.15%) of benign fluids were encountered with low mean AgNOR counts (less than 4) in 41 cases (95.35%), whereas 2 (4.65%) benign cases had high (more than 4) mean AgNOR counts.

In all, 22 (33.85%) malignant fluids were encountered. Low mean AgNOR counts (less than 4) were observed only in one case with a percentage of 4.54%. Whereas high mean AgNOR counts were observed in 21 cases with a percentage of 95.46%.

Sl. No.	Type of Fluid	No. of Cases	(%)
1.	Transudate	36	55.38%
2.	Exudate	29	44.62%

Table 4. Distribution of Various Fluids Encountered in the Study

Table 4. Shows distribution of total cases on the basis of their protein contents. Mostly 36 (55.38%) fluids were transudates and rest 29 (44.62%) were exudates.

Sl. No.	Nature of Effusions	No. of Cases	%
1.	Benign	43	66.15%
2.	Malignant	22	33.85%

Table 5. Benign/ Malignant Nature Wise distribution of Cases Analysed

Table 5. As the table shows, in the entitled study there were 43 cases (66.15%) of benign effusions and malignant effusions were 22 (33.85%).

Sl. No.	Cytological Diagnosis	No. of Cases	%
1.	Benign	43	66.15%
2.	Malignant		
	(a) Adenocarcinoma	22	33.85%
	(b) Squamous cell carcinoma	22	100%
	(c) Lymphoma	-	-
	(d) Melanoma	-	-
	(e) Others	-	-

Table 6. Cytological Diagnosis of Different Body Fluids

Table 6. Shows cytodiagnosis in total cases analysed. Out of 65 cases, 43 cases (66.15%) were benign and 22 (33.85%) cases were diagnosed as malignant as regard to malignant effusions. Cytologically, all cases were adenocarcinomas with an incidence of 100%. No case of squamous cell carcinoma, lymphoma and melanomas were seen.

DISCUSSION

The present work on Silver staining of "Nucleolar Organiser Region's" (AgNOR's) in body effusions has been carried out in Pathology Department of SMMH Medical College, Saharanpur, to evaluate their role in differentiating benign and malignant effusions. A total of sixty-five body fluids as effusions including benign and malignant were analysed.

Howell et al (1975)^[1] pointed out an important and surprising feature of NOR's. They pointed out that some of the NOR's are completely negative for Ag-As staining. The property of NOR's to stain with Ag-As staining depends also upon the physiological activity of NOR's apart from presence of rDNA. The silver staining of NOR's in chromosomes has drawn attention on few important points-

1. Only active NOR's can be stained (Miller et al, 1978).^[2]
2. Evidence to date supports the view that each individual has a characteristic or model number of AgNOR's (Bloom and Good Pasture, 1976).^[3] This raises the prospect of determining the familial transmission of individual acrocentric chromosomes.
3. The silver positive material is located around the proposed NOR site, but never directly on the chromatin segment (Schwarzacher et al, 1983).^[4]
4. Tendency of acrocentric regions to associate through satellite regions randomly. The AgNOR technique demonstrate the presence of physical connections indicating that the respective chromosomes participated together in nuclear organisations.

The distribution of NOR's in cytologic preparations of human serous effusions with the aim of differentiating malignant and non-malignant reactive cells was studied by Derenzini et al (1989).^[5] He suggested that malignant cells were characterised by an irregularly scattered distribution of AgNOR's were determined by NOR's and by a heterogeneity in the size of NOR's in addition to an increased number of NOR's.

Carrillo et al (1994)^[6] in thirty eight pleural and peritoneal effusions from patients with malignant solid tumours. Cytologically, 13 effusions were benign, 23 malignant and 2 indeterminate. The mean AgNOR counts for benign, malignant and indeterminate specimens were 2.99, 3.94 and 3.50 respectively.

Ayres et al (1988)^[7] observed a mean AgNOR value of 1.04 for normal and 1.75 for reactive mesothelium in biopsy samples.

In a similar study, Trevisan et al (1993) found a mean count of 1.64 for normal reactive mesothelium. Sujathan et al (1996)^[8] studied one hundred aspirated samples of benign and malignant effusions using the one-step silver staining method. AgNOR study by Sujathan et al (1996) concluded that in malignant cells the mean AgNOR count was 4.72 and the AgNOR's were irregular in shape, while in benign mesothelial cells AgNOR's were comparatively larger, single dots with a mean count of 1.92, cytologically atypical samples, five were in the malignant range, the others were within benign limits. In malignant lesions, the highest mean AgNOR count was seen in Lymphoma (5.44) and lowest observed in adenocarcinoma (4.57).

The results of study of Sujathan et al (1996)^[8] suggest that the AgNOR method can be valuable as an additional diagnostic tool for use on ascitic or pleural fluid.

Peritoneal fluids constituted as the largest number of effusions. Pleural fluid and pericardial fluid cases were small in number. Similar types of fluids have been included and analysed by the studies of Derenzini et al (1989)^[5] and Carrillo et al (1994).^[6] Surprisingly, they have not included pericardial effusions in their study.

Most of the body fluids were transudates and exudates were small in number. Present study showed mostly benign effusions as compared to malignant effusions. The ratio was 2:1. In the study of Carrillo et al (1994),^[6] the ratio of benign-to-malignant was reversed, i.e. 13 were benign and 23 were malignant, whereas 2 cases were indeterminate. Sujathan et al (1989)^[8] in their study again showed a more or less increased preponderance of malignant cases over benign with a ratio of (1.2: 1). The present reversal of ratio in our study can be explained on account of, since it was a small study and the time period again was small.

Cytodiagnosis of malignant effusions showed adenocarcinoma. Squamous cell carcinoma, lymphoma and melanomas were not observed. Similar studies have been conducted by Derenzini et al (1989),^[5] who analysed 100 cases of malignant effusions that included 45 metastatic pleural effusions and 25 peritoneal effusions as metastatic adenocarcinoma. This study also included mesothelioma cases and reactive pleural effusions. In another study carried out by Carrillo et al (1994)^[6] has also reported adenocarcinomas along with squamous cell carcinoma and a fair number of cases of melanomas. In the study of Sujathan et al (1989),^[8] in the analysis of 55 cases included 45 adenocarcinoma along with cases of lymphoma and melanomas and also anaplastic carcinoma.

So our findings are in accordance with the study by Derenzini et al (1989),^[5] Carrillo et al (1994)^[6] and Sujathan et al (1989)^[8] as regards to maximum number, 01 case of adenocarcinoma.

The mean AgNOR counts measured in different body fluids showed a mean AgNOR count of less than 4 in benign effusions and mean AgNOR counts of more than 4 in malignant effusions. Similar findings about mean AgNOR counts have been observed by Carrillo et al (1994),^[6] Ayres et al (1988)^[7] and Sujathan et al (1989).^[8]

Carrillo et al (1994)^[6] has showed a lower mean AgNOR count value of less than four per nucleus in benign effusions. Surprisingly, some of the malignant cases had also been reported having less than four counts, whereas majority of malignant effusions had mean AgNOR count more than four. No satisfactory explanation has been offered by the author for this discrepancy.

Ayres et al (1988)^[7] in his study has reported mean AgNOR value of 1.04 for normal and mean AgNOR value of 1.75 for reactive mesothelium, similarly Trevisan et al (1993) has reported mean count of 1.64 for normal reactive mesothelium. When compared our findings are in accordance with the findings of Sujathan et al (1989).^[8]

While differentiating benign effusions from malignant effusions some workers have taken into account AgNOR dot sizes, larger dots or smaller dots. In malignant forms, dot size has been smaller as compared to dot size in benign effusions. In one single case of moderately differentiated adenocarcinoma effusion, the AgNOR dots were very large and were lesser in number. It might be that the size was definitely increased or it was due to groups of nucleolar

organizer regions which could not be visualised separately at provided optical level. Similar relationship of AgNOR dot sizes and degree of malignancy has been stressed upon by Derenzini et al (1987)^[9] and Crocker et al (1988).^[10] Somewhat opposite observations as regard to AgNOR dots have been reported by Crocker and Egan (1988),^[11] who have reported that AgNOR dot size increases with increasing degree of malignancy. Here, our findings are different from the above observations.

It was noticed that NOR dots could be easily visualised within the nucleoli in most of the cases. Similar observational views have been put forward by Crocker et al (1989)^[12] who was of the opinion that AgNOR dots are well seen lying within the nucleoli in the tumour cells in contrast to resting cells where they are not easily seen.

The mean AgNOR counts increases significantly as we move from well-differentiated carcinomas to poorly differentiated carcinomas. These findings can be explained on the basis of the speculations given by Crocker et al (1988),^[10] who observed a linear correlation between the mean numbers of AgNOR per nucleolus and percentage of S phase cell and suggested that the numbers on AgNOR's in a lymphoma may be related to the dividing fraction of the cell rather than to the ploidy alone.

Jan-Mohamed (1989)^[13] stated that AgNOR count may be increased due to increased number of chromosomes or increased cell turnover. Underwood and Giri (1988)^[14] states that increase in AgNOR count is not an absolute increase in the number, but rather an index of dispersion.

Different cytogenetics explained differently the causes of increased NOR counts in malignancy. It may be due to increase in the chromosome number, increasing the chromosome arms containing NOR's (Schwarzacher et al, 1988),^[4] gene amplification (Roberts et al, 1987),^[15] but it is most significantly related to proliferation (Cairns et al 1989).^[16]

The AgNOR dots have been utilised for differentiating various histological types of tumours in biopsy specimens as in diagnosing malignancies of breast (Smith et al, 1988). Endometrium (Hansen et al, 1990),^[17] cervix (Egan et al, 1990,^[18]), ovaries (Griffiths et al, 1989),^[19] pleural mesothelium (Ayres et al, 1988)^[7] and skin (Egan MJ et al, 1988).^[20]

The value of AgNOR dots in relation to differentiate various types of malignancies in effusions has been evaluated by Ayres et al (1988), Derenzini et al (1989)^[5] and Egan et al (1990).^[18]

Thus, it could be concluded that though NOR's have a definite role in differentiating benign from malignant effusions, they do not supersede the value of routinely used Papanicolaou staining methods as a considerable overlap is seen between the various grades of malignancy. Kodousek R and Dusek J (1991)^[21] also points out that AgNOR technique does not exceed the value of routinely stained smears of good quality. However, in spite of these limitations, AgNOR staining methods seem to represent a valuable contribution as early when a cytologic diagnosis is difficult.

The present study shows significant association between malignancy and high AgNOR counts. The results of the study cannot be generalised due to a small sample size.

The AgNOR count if combined with other cell proliferation indices such as DNA flow cytometry, Ki-67

immunohistochemistry can lead to better understanding of neoplasms and then AgNOR counts may also assist in the grading of neoplasm and their prognosis.

CONCLUSION

Although, the mean AgNOR counts have a definite role in differentiating benign from malignant effusions, they do not however supersede the value of routinely used Papanicolaou staining methods, but definitely have a supportive role.

The clinical implication of AgNOR's determination in the evaluation of serosal effusions remains supportive. However, above study indicates that AgNOR counts have supportive practical value which can be utilised in differentiating malignant effusions from benign ones, when AgNOR findings are correlated with conventional cytological findings, clinical findings and other laboratory parameters. However, an effort should be directed towards better standardisation of techniques and counting of AgNOR's.

Also, such studies should be conducted on larger number of patients, so that the results can be generalised and baseline data for further research can be established.

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