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Original Research Article

Silver stained Nucleolar organizer regions in nonneoplastic and neoplastic lesions of endometrium

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Abstract: Nucleolar organizer regions (NORs) are loops of DNA that transcribe to ribosomal RNA. They are associated with nonhistone nucleoproteins which can be stained with silver (AgNORs). The present retrospective study was done to study the efficacy of AgNOR technique in differentiating between non neoplastic and neoplastic lesions of endometrium and to assess the relationship between AgNOR counts and severity of hyperplasia and carcinoma. This study was carried out in the Department of Pathology, in a tertiary care teaching hospital from July 2009 to August 2011. Endometrial specimens received were processed and stained with H & E and AgNOR. Mean AgNOR counts were correlated with histopathological diagnosis. In results out of 100 cases of endometrial specimens studied, 40 were normal endometria (proliferative and secretory phase), 41 were nonneoplastic lesions (simple hyperplasia without atypia, complex hyperplasia without atypia, complex hyperplasia with atypia) and rest 19 were neoplastic lesions (Grade 1 and Grade 2 endometrioid adenocarcinomas). Significantly higher mean AgNOR counts were found in endometrial adenocarcinomas as compared to all hyperplasias and higher in hyperplasias as compared to normal proliferative phase and least in normal secretory phase. In conclusion AgNOR counts are reliable markers of endometrial proliferation and allow a clear distinction between normal, non neoplastic and neoplastic endometrial changes. Thus, AgNOR technique proves to be a useful adjunct to routine histopathology to evaluate endometrial lesions.
Keywords: Nucleolar organiser regions, AgNORs, Endometrium

INTRODUCTION

Nucleolar organiser regions (NORs) are segments of DNA, closely associated with nucleoli containing, coding genes for ribosomal RNA and contribute to regulation of cellular protein synthesis [1]. They are located on short arm of chromosomes 13, 14, 15, 21 and 22. Associated with these regions are certain acidic, argyrophilic and nonhistonic proteins called NOR-associated proteins (NORAPs). NORs can be rapidly identified in light microscopic sections by a simple, one-step, colloidal silver technique by staining their associated proteins with colloidal silver and these silver stained reaction products represent the AgNORs and appear as black dots within nucleus [2]. Quantification of AgNORs is a valuable parameter in tumour pathology because they preserve tissue architecture. An increased number of AgNORs is associated with increased tumor aggressiveness as the mean number of AgNORs per nucleus is higher in malignant than in benign tissues, higher in high grade malignancies and in tumors with a poor prognosis compared to those with good prognosis [3]. AgNORs also seem to correlate with other markers of proliferation activity, such as mitotic counts, immuno staining with monoclonal antibody (Ki67) and proliferating cell nuclear antigen (PCNA) as well as S cell fraction as assessed by DNA flow cytometry.

Under physiologic circumstances, the endometrium undergoes cyclic changes characterized by proliferation and subsequent secretory transformation. Several investigators have attempted to show presence of AgNORs in endometria and to characterize different aspects of endometrial proliferation [4].

This study was done to study the efficacy of AgNOR technique in differentiating between non neoplastic and neoplastic lesions of endometrium and to assess the relationship between AgNOR counts and severity of hyperplasia and carcinoma

MATERIALS AND METHODS

100 endometrial specimens obtained from hysterectomy and dilatation & curettage (D&C) sent for histopathological examination from July 2009 to August 2011 in a tertiary care hospital were routinely processed. Two paraffin sections were cut from each

paraffin block. One section was stained with hematoxylin and eosin (H& E) and the other section was subjected to AgNOR staining. AgNOR staining was performed using the technique described by Crocker and Nar[5]. Four-microns-thick sections were made for each case, deparaffinized in xylene, and hydrated through decreasing grades of ethanol to three changes of deionized water. The sections were then reacted with freshly prepared silver colloidal solution (containing one part by volume of 2% gelatin in 1% formic acid and two parts by volume of 50% aqueous silver nitrate solution) poured over the tissue sections and kept for 45 minutes at room temperature in dark room. The silver colloidal solution was washed with three changes of deionized water. The sections were then treated with 5% sodium thiosulphate for 5 min and washed in deionized water, dehydrated through increasing grades of ethanol, cleared in xylene, and mounted.

AgNORs were seen as brownish black dots in the nucleus i.e. both within the nucleolus and scattered within the nucleoplasm against a light yellow background. Under oil immersion lens, number of AgNORs within the nuclei of 100 endometrial glandular cells was counted and mean number of AgNORs per nucleus for each case was evaluated.

The mean AgNOR counts were correlated with the histopathological diagnosis and collected data was analyzed by unpaired t test, analysis of variance and chi square test. The p values less than 0.05 were considered significant.

OBSERVATIONS AND RESULTS

Out of 100 cases of endometrial specimens, the histopathological diagnoses were as follows: 40 were normal endometria (proliferative (n=24) and secretory phase (n=16), 41 were non neoplastic lesions (simple hyperplasia without atypia (n=20), complex hyperplasia without atypia (n=16), complex hyperplasia with atypia (n=5)) and rest 19 were neoplastic lesions (Grade 1 (n=15) and Grade 2 (n=4) endometrioid adenocarcinomas).

On subjecting the endometrial samples to AgNOR staining (Table 1 & 2 and Figures 1-3), it was observed that mean number of AgNORs in endometrial adenocarcinoma (5.62 ± 0.37) was significantly higher than that of complex hyperplasia with atypia (4.68 ± 0.23, $p < 0.0001$), complex hyperplasia without atypia (3.63 ± 0.23, $p<0.0001$) and simple hyperplasia without atypia (3.5± 0.63, $p<0.0001$).

Also, mean AgNOR counts in complex hyperplasia with atypia (4.68 ± 0.23) was significantly greater than that of complex hyperplasia without atypia (3.63 ± 0.23, $p<0.0001$) and simple hyperplasia without atypia (3.5± 0.63, $p<0.0001$).

Significantly, higher mean AgNOR counts in glandular cells of proliferative phase (3.42 ± 0.3) were observed as compared to secretory phase (2.66 ± 0.19, $p < 0.0001$).

A slight rise but no significant difference was seen between the mean AgNOR counts in endometrial adenocarcinomas. Out of the 19 cases of endometrioid adenocarcinomas, fifteen (n=15) were of Grade 1 showing mean AgNOR count of 5.57. Rest four (n=4) were of Grade 2 showing mean AgNOR count of 5.82.

Thus, mean AgNOR counts in endometrioid adenocarcinomas Grade 2(5.82 ± 0.22) was found to be slightly greater than that of endometrioid adenocarcinomas Grade1 (5.57 ± 0.39), but with no significant statistical difference ($p>0.05$).

The mean AgNOR scores were found to increase steadily from normal (3.11 ± 0.46), non neoplastic lesions comprising of hyperplasias (3.69 ± 0.59) and neoplastic lesions (5.62 ± 0.37).

The observations revealed statistically significant differences in values between normal, non neoplastic and neoplastic endometria ($p<0.0001$).

Table 1: AgNOR distribution in 100 endometrial specimens

Histopathological diagnosis	No of cases	AgNOR range	Mean AgNOR count	Standard deviation
Proliferative phase	24	3.1-4.2	3.42	0.3
Secretory phase	16	2.4 -3.1	2.66	0.19
Simple hyperplasia without atypia	20	2.5-4.5	3.5	0.63
Complex hyperplasia without atypia	16	3.2- 4.1	3.63	0.23
Complex hyperplasia with atypia	5	4.4 -4.9	4.68	0.23
Endometrial adenocarcinoma	19	4.9-6.2	5.62	0.37
Total	100	2.4-6.2	3.83	1.04

$P < 0.0001$

Table 2: AgNOR distribution in normal, non-neoplastic and neoplastic endometria

Endometrial specimens	No of cases	AgNOR range	Mean AgNOR count	Standard deviation
Normal endometrium	40	2.4-4.2	3.11	0.46
Non neoplastic lesions	41	2.5- 4.9	3.69	0.59
Neoplastic lesions	19	4.9-6.2	5.62	0.37
Total	100	2.4-6.2	3.83	1.04

$P < 0.0001$

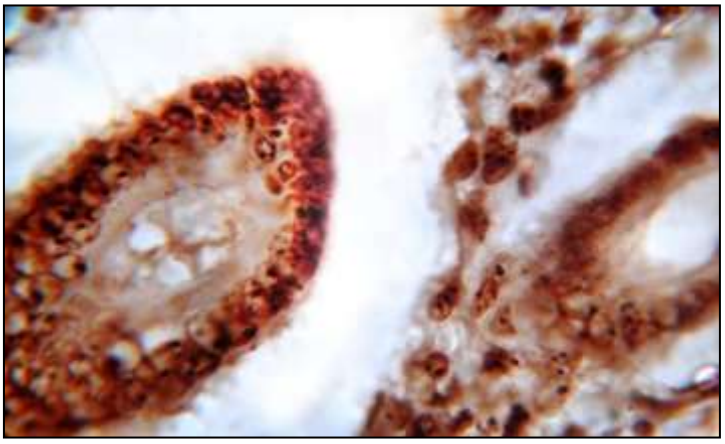

Fig-1: Photomicrograph showing proliferative phase with 3-4 AgNOR dots per nucleus. (AgNOR stain X 1000)

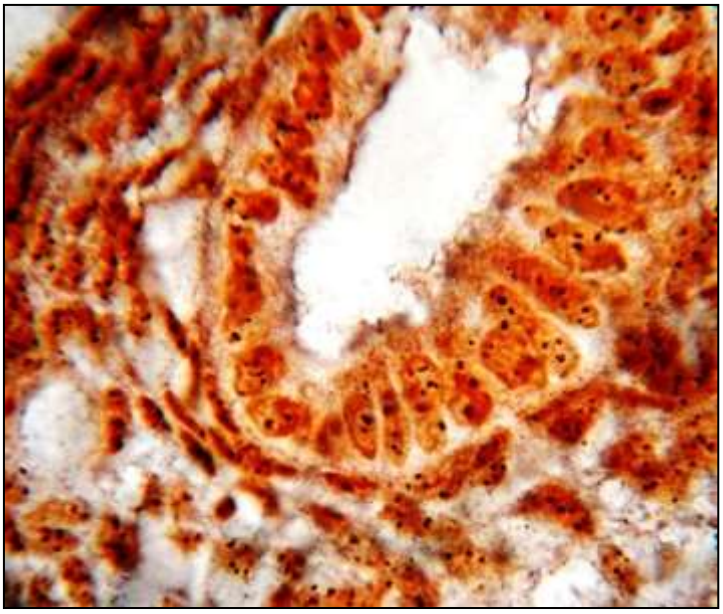

Fig-2: Photomicrograph showing complex hyperplasia with atypia with 4-5 AgNOR dots per nucleus. (AgNOR stain X 1000)

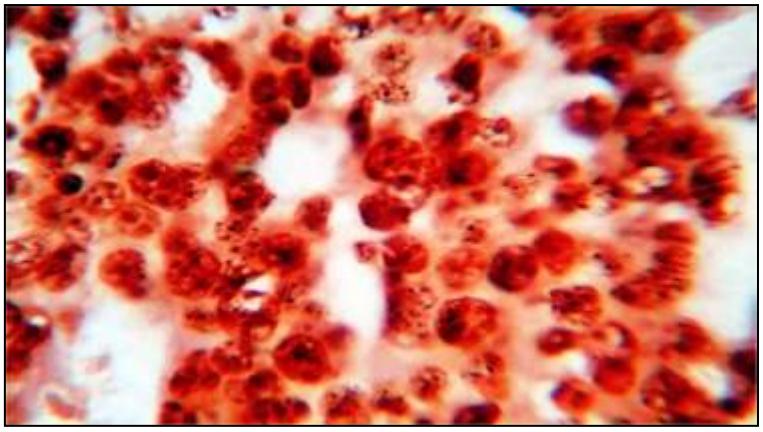

Fig-3: Photomicrograph showing endometrial adenocarcinoma with 5-6 AgNOR dots per nucleus (small sized AgNOR dots in scattered distribution). (AgNOR stain X 1000)

DISCUSSION

AgNOR staining has already been applied and shown to be of great value in differentiating benign from malignant lesions of breast, cervix, endometrium, oral cavity, thyroid, skin, soft tissue tumours, lymphomas and melanomas [5-13]. Several investigators have demonstrated that AgNOR counts are indeed correlated with the degree of endometrial proliferation [4, 14].

Endometrial adenocarcinomas show highest mean AgNOR counts as compared to all hyperplasias and higher in hyperplasias as compared to normal proliferative phase and least in normal secretory phase. Among non neoplastic endometria, mean AgNOR counts in complex hyperplasia with atypia are significantly greater than that of complex hyperplasia without atypia and simple hyperplasia without atypia. The number of AgNORs tends to increase with the advance of neoplastic changes. It is further suggested that in carcinomas, there is no clear cut relationship with histological grade based on AgNOR counts.

On comparing number of AgNORs, Brustmann H *et al.;* [4] got relatively increased number of AgNORs in comparison to our study. A study by Wilkinson N *et al.;* [17] concluded mean AgNOR count of more than 9 in curettage material from an atypical proliferative lesion of the endometrium as highly suggestive of invasive neoplasm. Studies conducted by Niwa K *et al.;* [15], Kaushik R *et al.;* [16] and Terlikowski S *et al.;* [3] found lesser number of AgNORs in comparison to our study. Reasons for varying NOR counts include different section thickness, different staining procedures and different counting methods. Prolonged fixation appears to cause the AgNORs to coalesce, thus resulting in a low count, especially in small biopsy specimens fixed in an excess of formalin overnight. Thus, it is not possible to directly compare counting results from different institutions [4].

Papadimitiou C S *et al.;* [18] observed the distribution of AgNORs. Malignant changes were characterized mainly by a high number of AgNORs which were small in size and showed a scattered distribution, hyperplastic and normal endometrium showed small numbers of large sized AgNORs in a clustered distribution. Similar findings were observed by Terlikowski S *et al.;* [3].

Although, our study shows a good correlation between NORs and stage of endometrial proliferation, it does not help to solve the most important diagnostic problem in this field, namely, differential diagnosis between atypical hyperplasia and beginning of invasive carcinoma. It would be farfetched and unjustified to make a diagnosis of carcinoma solely on the basis of one morphological characteristic. The same is true for AgNORs. The mean number of AgNORs in a specimen cannot provide a diagnosis of malignancy. However, seen in conjunction with morphological characteristics; AgNOR counts can provide additional and helpful information [4].

Despite its limitations, mainly related to section thickness, tediousness and time consuming counting, the silver staining NOR technique represents a potential tool for indirect evaluation of cell kinetics in already routinely fixed and paraffin embedded sections [3].

CONCLUSION:

AgNOR technique is simple, inexpensive and useful for evaluation of proliferative activity in human endometrium and can be used as an adjunct to routine histopathology to evaluate endometrial lesions.

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