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Original Article

Value of Argyrophilic Nucleolar Organizer regions in Benign, Premalignant, and Malignant Lesions of Cervix Uteri

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Abstract

Objectives: The objective of this study is to evaluate the correlation of argyrophilic nucleolar organizer region (AgNOR) count between benign, premalignant cervical intraepithelial neoplasia (CIN), and malignant lesions of cervix.

Method: A retrospective study of 150 cases of paraffin-embedded blocks were histologically grouped as (A): Normal cervix, (B): Benign lesions - chronic cervicitis without dysplastic change, (C): Cervix with CIN I and II, (D): CIN III (E): Squamous cell carcinoma - well-differentiated, (F): Squamous cell carcinoma - moderately differentiated, (G): Squamous cell carcinoma - poorly differentiated (small cell non-keratinizing), and (H): Adenocarcinoma of cervix. The paraffin blocks were further subjected to thin sections, and silver staining (AgNOR) was done in the dark at room temperature. The AgNOR counting was done under oil immersion (×100). The number of black dots per 100 cells was counted and averaged.

Result: In this study, the mean AgNOR count was found to be statistically significant (t = 3.5 –21.8) at a confidence limit <0.0, clearly proving proliferative activity of the benign, premalignant, and malignant nucleoli.

Conclusion: AgNOR counting progressively increases directly in proportion to increased proliferative activity of the cells: (A+B) normal cervix and chronic cervicitis without dysplasia mean AgNOR count 3.5, (C): Cervix with CIN I and II mean AgNOR count 6.9, (D+E): CIN III and squamous cell carcinoma - well-differentiated mean AgNOR count 10.3, (F+G): Squamous cell carcinoma - moderately and poorly differentiated mean AgNOR count 16.16, and (H): Adenocarcinoma of cervix mean AgNOR count 21.8

Key words: AgNORs staining technique, AgNORs counting procedure, Benign and Malignant lesions of cervix, Proliferative index

INTRODUCTION

For a long time, it has been known that abnormalities of nucleolus such as hypertrophy and irregular shape were constant features of cancer cells, but little attention was given to nucleolar morphology. However, recently, it has been proved at ultrastructural levels[1] that nucleolar morphology corresponds to the interphase counterpart of the nucleoli organizer regions (NORs) of metaphase chromosome, and hence, they are called interphasic NORs[2] It has been shown that these NORs can be visualized both at electron and light microscopic levels by a silver staining technique.[3] By means of this technique, the NOR proteins associated with ribosomal genes are potentially transcribable.[4] The NOR distribution pattern and their increased numbers have been successfully used to differentiate benign from malignant tumors in cytologic and histologic preparations.[5]

Carcinoma of the uterine cervix shares a large incidence of malignancies of the female genital tract.[6] Although the organ is readily accessible for biopsy, failure in early diagnosis, due to inadequate screening programs, and lack of awareness being the main cause for high mortality rate.

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Cervical intraepithelial neoplasia (CIN) is a stage that precedes full-fledged carcinoma and is considered as a very vital stage as the diagnosis at this stage affords a very favorable outcome. A number of methods have been tried in the past to diagnose early carcinoma cervix, and only recently, argyrophilic nucleolar organizer region (AgNOR) staining has been proved to be a spark in darkness and its value is consistently increasing in diagnosing early carcinoma cervix and also a variety of other malignancies already mentioned.

The current work is an inspiration from the work of Pratiba and Kuruvilla.[7] The study of AgNOR count on benign, premalignant, and malignant lesions of the cervix gained importance as an indicator of cell proliferation, thus differentiating benign from malignant lesion. It was found that AgNOR count increased progressively from normal to CIN Grade I, II, and III and Invasive carcinoma.

MATERIALS AND METHODS

The present study has been undertaken in the Department of Pathology, Netaji Subhash Chandra Bose Medical College and Hospital, Jabalpur. A total of 150 paraffin-embedded cervical biopsies were studied for 1 year. They were classified broadly into eight groups:

Group I	Histopathologically Normal cervix (control)
Group II	Chronic Cervicitis without dysplastic changes
Group III	CIN Grade I CIN Grade II
Group IV	CIN Grade III
Group V	Squamous cell carcinoma large cell keratinizing type (well differentiated)
Group VI	Squamous cell carcinoma large cell non-keratinizing type (moderately differentiated)
Group VII	Squamous cell carcinoma small cell non-keratinizing type (poorly differentiated)
Group VIII	Adenocarcinoma of cervix

All the paraffin blocks were cut at 4 mu thickness.

The sections were put on to slides and stained by the following methods:

1. Hematoxylin and Eosin (H & E stain) slides obtained from archives.
2. AgNOR staining method[8]:

AgNOR staining solution requires:

1. Silver colloid solution
   a. 2 g per dl powdered gelatin dissolved in 1 g per dl aqueous formic acid.
   b. 50 g per dl aqueous silver nitrate solution.

The above-mentioned solutions are freshly prepared just before staining in the ratio of 1:2 (1 volume of 2% gelatin in 1% formic acid and 2 volumes of 50% aqueous silver nitrate).

2. 5 g per dl sodium thiosulfate solution.
3. 0.5 g per dl methyl green solution (optional for counterstaining).

Steps of AgNORs staining:

1. 4 mu thick paraffin sections were deparaffinized in xylene.
2. Hydrated through descending grades of ethanol to deionized water.
3. Sections were then incubated under safelight condition (dark) at room temperature in a silver colloid solution for 40 min.
4. Sections were then thoroughly washed with 5% sodium thiosulfate to remove the excess of silver colloid in the sections.
5. The sections were throughly washed in deionized water.
6. Sections were counterstained with 0.5% methyl green for 5–10 s (optional).
7. Sections were dehydrated in ascending grades of ethanol and finally to absolute alcohol.
8. Sections were cleared in 2–3 baths of xylene.
9. Sections were mounted in DPX.

Morphology of AgNOR stained tissue under microscope:

AgNORs are visualized as dark-brownish dots arranged in clumps or in clusters within the nuclei against yellowish background. Methyl green counterstaining makes the nuclear outline prominent and thus helps in distinguishing AgNOR dots within the nucleus from extranuclear silver colloid impurities.

Counting Procedure

The number of AgNOR5 present in each nucleus is counted as proposed by Evans *et al.*1991.[9] 100 nuclei are taken into account, using ×10 eyepiece and ×100 oil immersion lens. At this magnification, the AgNORs are visible both within and outside the nuclei. The mean AgNOR count of the specimen is then calculated taking into account both intra- and extra-nucleolar AgNORs. The counting system is summarized as follows:

1. Nucleoli were counted as individual intra- and extra-nucleolar AgNORs.
2. Two "total AgNOR'S figures" were thus derived "total

A" excluding intranucleolar AgNORs and "total B" including intranucleolar AgNORs.

For each group of 100 nuclei counted, the mean standard deviation and range of numbers of AgNORs were determined. Using Student's *t*-test, the significance of differences in total AgNOR counts between the following groups was calculated:

Groups I and II: Normal cervix and chronic cervicitis without dysplastic changes.

Groups II and III: Chronic cervicitis and CIN Grade I and II.

Groups III and IV: CIN Grade I, II, and III.

Groups IV and V: CIN Grade III and squamous cell carcinoma large cell keratinizing type (well differentiated).

Groups V and VI: Squamous cell carcinoma well differentiated and moderately differentiated.

Groups VI and VII: Squamous cell carcinoma moderately differentiated and poorly differentiated (small cell non-keratinizing type).

Groups II and VIII: Chronic cervicitis and adenocarcinoma

Statistical Analysis

The results obtained in counting procedure were analyzed statistically by means of unpaired *t*-test.

Observation

A total of 150 cases of cervical biopsy were included to study the value of AgNOR count in benign, premalignant, and malignant lesions of cervix uteri. The cases were distributed in eight groups based on histopathological diagnosis [Table 1].[4]

Evaluation of AgNOR Count

AgNOR count was studied in a total of 150 cases distributed according to Table 1. Both intra- and extra-nucleolar AgNORs were counted at the magnification of ×1000 (oil immersion).[9,10] Well-defined argyrophilic dots were identified. The AgNOR count was done in 100 representative nuclei in each case, and mean AgNOR per nucleus was calculated. From the values thus obtained, standard deviation was calculated for each group in comparison [Table 2].

RESULT

On comparing the AgNOR count between Groups I and II, a statistically significant value is obtained ($t = 35$) at confidence limit <0.001. The AgNOR count in Groups II and III is also statistically significant ($t = 10.3$) at confidence limit <0.001. Similarly, on comparing the other groups, the AgNOR count was found to be statistically significant at confidence limit <0.001 [Table 3].

DISCUSSION

Although uterine cervix is readily accessible for biopsy, failure in early diagnosis is one of the main causes for high mortality rate. CIN is a stage that precedes full-fledged carcinoma cervix. This is considered a very vital stage as diagnosis at this stage affords a very favorable outcome. A number of methods have been tried such as DNA flow cytometry and markers of proliferative activity such as Ki-67 and BK.j9.9. The former is quite expensive and space consuming while the later cannot be applied to formalin-fixed tissue. Recently, AgNOR staining has been proved to be a spark in darkness as far as diagnosis of early cancer is concerned.[11] Its value is consistently increasing in diagnosing early carcinoma cervix and variety of other tumors.[12,13] It is considered to be a marker of proliferative activity of a cell, and its results are in parallel to those of other proved markers of proliferative activity such as Ki-67.[14]

In the present study, the relative incidence of different grades of cervical malignancy is shown in Table 4.

While chronic cervicitis was present in nearly 90% of biopsy, their relative incidence is restricted in this study just to avoid the overlap in AgNOR count and subsequent calculations. Mean age of cancer cervix in this study was

Table 1: Classification and number of cases studied

Group	Histopathological diagnosis	Number of cases
Group I	Normal cervix	10
Group II	Chronic Cervicitis without dysplastic changes	20
Group III	CIN Grade I CIN Grade II	30
Group IV	CIN Grade III	20
Group V	Squamous cell carcinoma large cell keratinizing type (well differentiated)	20
Group VI	Squamous cell carcinoma large cell non-keratinizing type (moderately differentiated)	20
Group VII	Squamous cell carcinoma small cell non-keratinizing type (poorly differentiated)	20
Group VIII	Adenocarcinoma of cervix	10

CIN: Cervical intraepithelial neoplasia

Table 2: Mean AgNOR count in various groups of lesions with S.D

Group	Histological diagnosis	Number of Cases	Range of AgNOR	Mean AgNOR Count	S.D
Group I	Normal cervix chronic	10	1.1–2.2	1.3	0.370
Group II	Cervicitis without dysplastic changes	20	1.4–3.2	1.9	0.476
Group III	CIN Grade I CIN Grade II	30	2.2–4.6	3.5	0.575
Group IV	CIN Grade III	20	3.7–5.8	4.71	0.640
Group V	Squamous cell carcinoma large cell keratinizing type (well differentiated)	20	4.4–7.6	6.40	0.852
Group VI	Squamous cell carcinoma moderately differentiated	20	8.6–14.8	9.4	0.917
Group VII	Squamous cell carcinoma small cell non-keratinizing (poorly differentiated)	20	14.2–20.6	11.6	1.319
Group VIII	Adenocarcinoma of cervix	10	6.4–13.2	10.6	1.74

CIN: Cervical intraepithelial neoplasia

Table 3: The *P* value for difference in count in various groups studied

Groups	Histological diagnosis	t value	*P* value
Groups I and II	Normal cervix and chronic cervicitis without dysplastic changes	3.5	<0.001
Groups II and III	Chronic cervicitis and CIN Grade I and II	10.3	<0.001
Groups III and IV	CIN Grade I, II, and III	6.9	<0.001
Groups IV and V	CIN Grade III and squamous cell carcinoma large cell keratinizing type (well differentiated)	13.3	<0.001
Groups V and VI	Squamous cell carcinoma well differentiated and moderately differentiated	10.75	<0.001
Groups VI and VII	Squamous cell carcinoma moderataly differentiated and poorly differentiated (small cell non-keratinizing)	6.16	<0.001
Groups II and VIII	Chronic cervicitis and adenocarcinoma	21.8	<0.001

CIN: Cervical intraepithelial neoplasia

Table 4: Different grades of cervical lesions

Histopathological Diagnosis	*n* (%)
Histopathologically normal cervix	10 (6.66)
Chronic cervicitis without dysplasia	20 (13.33)
CIN I and II	30 (20.00)
CIN III	20 (13.33)
Squamous cell carcinoma large cell keratinizing type (well differentiated)	20 (13.33)
Squamous cell carcinoma large cell non-keratinizing type (moderately differentiated)	20 (13.33)
Squamous cell carcinoma small cell non-keratinizing type (poorly differentiated)	20 (13.33)
Adenocarcinoma of the cervix	10 (6.66)

CIN: Cervical intraepithelial neoplasia

40 years. The most common clinical finding was DUB, leukorrhea, and post-coital bleeding.

Study of AgNOR

In normal cells, the AgNORs are tightly packed in the nucleoli and are indiscernible (mean AgNOR count in Group I lesion - 1.3). In rapidly proliferating cells such as neoplastic cells, nucleolar disaggregation may take place resulting in a dispersion of individual AgNORs (mean AgNOR count in Group III and IV lesions - 3.5 and 4.71, respectively).

Increase in cell ploidy and increased transcriptional activity may also result in higher AgNOR count (mean AgNOR count in Group II lesion - 1.9)

Therefore, benign and malignant lesion showed a significant difference in AgNOR counts. In this study, the mean AgNOR count was found to increase progressively from normal to CIN and invasive carcinoma. On comparing CIN with normal cervix and CIN with invasive carcinoma, there was a statistically significant difference in both the sets. These findings strongly support the view that proliferative activity and malignant potential of intraepithelial neoplastic lesion of the cervix increase progressively as the grade of the lesion becomes higher.

In small cell carcinoma of the cervix, AgNORs were dispersed as fine dots throughout the nucleus and could not be counted easily. This may indicate a very high proliferative activity of these tumours resulting in marked disaggregation of AgNOR dots thus indicating a poor prognosis (mean AgNOR count - 11.61).

CONCLUSION

AgNOR count progressively increases directly in proportion to increased proliferation of cells. In histopathologically normal cervical biopsy and chronic cervicitis without dysplasia, mean AgNOR count being 1.3 and 1.9, respectively, as compared to mean AgNOR count of 3.5-CIN I &II, 4.71-CIN III, 6.49-Squamous cell

carcinoma well differentiated, 9.4-Moderately differential and 11.6-Poorly differentiated and 10.6 Adenocarcinoma respectively.

The findings in this study indicate that NOR5 can be demonstrated by means of argyrophilia of their associated proteins using a simple silver staining method.[15] The technique can be used as an adjunct to routine histopathological examination of cervical lesions, especially for grading cervical intraepithelial neoplasia, thus rendering earlier diagnosis and better prognosis.

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