ISSN 2394–806X (Print), ISSN 2454-5139 (Electronic)
IJHRMLP, Vol: 02 No: 02, July, 2016
Printed in India

ORIGINAL PAPER

Value of AgNOR, in Malignant Lesions of Cervix

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***Received** on September 10, 2015; **editorial approval** on January 12, 2016*

ABSTRACT

***Objectives**: To evaluate the correlation of AgNOR count and malignant lesions of cervix.*

***Methods**: The material for the study was collected from patient with various lesions of cervix. After colposcopy, a cervical biopsy was done. Routine paraffin sectioning was done for these biopsy specimens. Histopathologic diagnosis was first established on these sections using the routine (H & E) stain. Then, further sections were cut from prepared blocks and were subjected to AgNOR staining technique. AgNOR count was taken as the mean number of black dots per 100 cells observed under a 100× oil immersion objective.*

***Results**: In this series biopsy specimens were obtained and subjected to routine method of haematxoylin and eosin staining which revealed 25% to be benign and 26.6% to be cervical intraepithelial neoplasia and 48.4% cases to be carcinoma.*

***Conclusion**: The results of the AgNOR when used can provide strength to the clinician and histopathologist in diagnosing early carcinoma in cases of suspicious cervix.*

***Keyword:** AgNOR staining technique, malignant lesion, Cervix*

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INTRODUCTION

Noncommunicable diseases are emerging as important health problems with changes in the lifestyles and demographic profiles of developing countries, which demand an appropriate control program before they assume epidemic proportions. One of these is the problem of cancer. In India, cervical cancer is one of the leading causes of cancer deaths in women and is the fourth most common cancer among women all over the world.[1]

In India, it is most common in Bangalore and Chennai and the second most common in Mumbai and Thiruvananthapuram, followed by Dibrugarh in the 3rd place.[2]

Invasive cancer of cervix has been considered a preventable cancer because it has a long pre-invasive state and treatment for pre-invasive lesion is effective. Thus, early diagnosis and essential for prevention of disease progression to invasive cancer.[3] An adequate aimed biopsy and preoperative staging is necessary in deciding on treatment of these pre-invasive lesion.

In the last few decades a new technique, AgNOR technique has achieved much attention because of its special characteristic of discriminating benign lesion from malignant lesions.

Nuclear organizer regions (NOR) are loops DNA that encodes ribosomal RNA. The 10-acrocentric chromosomes in man [pairs 13, 14, 15, 21 and 22] have nuclear organizer region or [NOR] on the short arm.[4]

The amount of AgNOR staining reflects the activity of rRNA genes. Ultra structurally the silver is usually localized to the febrile component of the nucleoli.[5]

However, a recently described one-step Silver staining technique[4] has aroused considerable interest amongst

tumor Pathologist, with a claim that there might be a possible association between high AgNOR count and malignant transformation. Since then many workers studied AgNOR in various cervical lesions with conclusion that there was a statistically significant difference of AgNOR count in benign and malignant lesions of cervix exist.

The normal (typical) transformation zone on the cervix represents the area of physiologic (normal) metaplastic epithelium that has replaced the columnar epithelium. This site is of the greatest interest as it has the potential for developing neoplasia of the cervix. The introduction of carcinogens at this point results in an atypical transformation zone.[6]

In the present study, an attempt has been made to study the correlation of AgNOR count in the case of suspicious cervix.

MATERIALS AND METHODS

The present study was carried out in the department of pathology, Gauhati Medical College and Hospital (GMCH), Guwahati. The material for the study was collected from patients with various lesions of cervix attending the Gynae OPD of GMCH.

Scheme of study

The cases were studied in detail according to pretested proforma.

Cervical punch biopsy: The patient is put in lithotomy position and the cervix is exposed with a vaginal speculum site of the biopsy is determined by Schiller's test. Schiller's test- Grams iodine was applied to the cervix which results in mahogany brown staining of the epithelium of the portio vaginalis except in areas of carcinoma, erosion, ulceration, atrophy, hyperkeratosis, parakeratosis, metaplasia, etc. A piece of cervical epithelium and underlying stroma is removed with cervical biopsy forceps.

Cervical conization: Cervical conization is removal of a cone shaped portion of the cervix including the entire transformation zone and a variable length of a cervical canal. The procedure is carried out under general anesthesia.

Tissue processing for Histopathological examination:

Step I: A trimmed piece of tissue was kept in 10% formalin saline for over 1-2 hours for fixation. A label was attached for identification.

Step II: The formal saline was when washed running tape water for 15

Step III: Tissue was then treated with ascending grades of alcohol 50% for 2 hours, 70% for 2 hours, 90% for 2 hours, 95% for 24 hours and 95% for overnight.

Step IV: The tissue was then transferred to absolute alcohol for 2 hours, till when complete hydration was achieved.

Step V: After that the tissue was then kept in xylol for overnight.

Step VI : Again the tissue was cleaned in xylol for 2, hours and kept in paraffin bath 57ºC for 4 hours with the label.

Step VII: Tissue was then taken out of the paraffin bath and block was made Luck hart's L piece by putting melted paraffin.

Step VIII: After that the block thus prepared was put in a freezer for hardening.

Step IX: After preparation of the block, it was trimmed and section was made in rotary microtome (3-5i) and ribbon obtained was placed in water bath at 56ºC and two sections were lifted in albumenized slides each block. Two slides were than prepared, one for H & E stain and other for AgNOR staining.

Step X: Slides are kept in a incubator for over night.

AgNOR staining was done with the following reagents:

(a) 50 silver nitrate solution
Silver nitrate -50gm.
Distilled water -100ml.

(b) 2% gelatin solution in 1% formic acid Gelattin -2 gm
Distilled water -100gm.
To this added
Formic acid -1 ml.
Distilled water -99ml.

(c) 5% Thiosulphate solution
Thiosulphate -5gm.
Distilled water -100ml.

(d) Working solution

(e) Reagent (a) + Reagent (b) in a ratio of 2.1.

AgNOR stained section was first examined under low objective and area without overlapping of cells was

selected AgNOR courting was done under oil immersion objective (X100).

AgNOR Examination: 100 cells from selected area, there is no overlapping of cells examined under oil immersion objective (100 X). All the sliver structures, which appears as brownish block dots counted (i.e. both intra and nuclei). The mean count was evaluated as below.

$$\text{Mean AgNOR count} = \frac{\text{Total No of AgNOR}}{\text{100 cells}}$$

In case, where AgNORs appear as a cluster and cannot be counted separately it is regarded as single granule.

RESULTS AND OBSERVATION

In the present sixty biopsies of uterine cervix were studied by the AGNOR method that identifies the nuclear organizer regions. In all the cases after obtaining the biopsy specimen histological diagnosis was confirmed by routine haematoxylin and eosin method. After that next AgNOR staining was done the result was correlated with histopathological diagnosis.

Distribution of cases: In this series of biopsy specimen obtained from all the cases, were finally subjected to routine method of haematxoylin and eosin staining which revealed 25% cases to be benign, 26.6% cervical intraepithelial neoplasia and 48.4% cases to be carcinoma. The most commonly encountered benign lesion chronic, exocervicitis (46.7%) followed by endocervicitis (33.3%) and squamous metaplasia (20.0%).

Cervical intraepithelial neoplasia (CIN) grade –II was found to be common about (43.8%), followed CIN –III (37.4%) and CIN –I (18.8%).

The malignant squamous lesions are narrated in **Table 1**.

Table 1 Distribution of morphologic sub type of squamous cells carcinoma

Type of malignant squamous lesion	No of cases	Percentage
L.C.N.K	6	20.69%
K.S.C.C.	16	55.17%
S.C.C	7	24.14%
Total	29	100%

Age wise distributions of cervical lesions are shown in **Table 2**.

Table 2 Age wise distributions of cervical lesions

Age group	Benign lesion	Percen-tage	CIN	Percen-tage	Malig-nant	Percen-tage
25-34	5	33.3%	2	12.5%	2	6.9%
35-44	8	53.3%	12	75.05	11	37.9%
45-54	1	6.7	2	12.5%	9	31.1%
55.64	1	6.7%	-	-	4	13.8%
65-74	-	-	-	-	30	10.3%
Total	15	100%	16	100%	29	100%

AgNOR counting

AgNOR were visualized as brownish black both within the nucleolus (intra-nucleolar) and elsewhere in the nucleoplasm (extra-nucleolar). 100 nuclei were selected randomly and numbers of AGNOR per nucleus were calculated under X 100 oil immersion objective.

Table 3 AgNOR mean count in benign CIN and malignant lesion

Category	No of cases	AgNOR Mean count		
		Mean	S. Deviation	Range
Benign lesion	15	1.33	0.019	1.10 -1.75
CIN	16	3.90	0.044	2.88 -5.01
Malignant	29	5.6	0.016	5.04 – 7.3

AgNOR Mean counts in different morphologic subtypes of squamous cells carcinoma are shown in **Table 4**.

Table 4 AgNOR Mean counts in different morphologic subtypes of squamous cells carcinoma

Category	No of cases	AgNOR Mean count		
		Mean	S. Deviation	Range
LKSCC	16	5.71	0.549	5.06-7.3
LCNK	6	5.64	0.37	5.18-6.19
SCC	7	5.41	0.211	5.31-5.75

Significance of the test: The significance of AgNOR mean counts observed in identifying various benign, intraepithelial malignant lesion of the cervix was calculated by student's t test.

Benign lesion versus malignant lesion

$$\text{Student 't' test} = \frac{X - Y}{SD \quad \frac{1}{} + \frac{1}{\sqrt{n_1 n_1}}}$$

$$T = 5.59 \frac{-1.40}{0.224} = \frac{4.19}{0.224} = 18.71 \; [P < 0.0001 \text{ with } 42 \text{ d.f}]$$

The calculated value of **t** for d. f. has been found to be a greater the table value of **t** at 0.00001 level of significance i.e. < 0.00001, which is very height significant

$$\text{Student 't' test} = \frac{X - Y}{SD \quad \frac{1}{} + \frac{1}{\sqrt{n_1 n_1}}}$$

$$T = 5.31 \frac{-2.4}{0.039} = \frac{2.91}{0.039} = 746$$

$$= 74.6 \; [P < .001 \text{ for } 31 \text{ d.f}]$$

Here the calculated value of t for 31 d. f. has been found to be greater the table value of **t** at 0.001 level of significance, i.e., P < 0.001 which is very highly significant.

CIN lesion versus malignant lesion

$$\text{Student 't' test} = \frac{X - Y}{SD \quad \frac{1}{} + \frac{1}{\sqrt{n_1 n_1}}}$$

$$T = 5.59 \; \frac{-5.31}{0.228} = \frac{0.28}{0.228} = 1.22 \; [P < \text{for d.f. } 45)$$

As P< 01 for 45 d. f. the test is highly significant.

The AgNOR mane count in benign lesion to be 1.5 (Range 1.1 -1.75 S.D. 0.57 and CIN 3.7 (range 2.8 -4.98, SD -6019] and malignant lesion 5.6 (range 5.06 6.16, SD 0.37]. In this of study cut off point for malignant lesion was set up 4

There was no significant difference in AgNOR count betweens squamous metaplasia and chronic cervicitis. The AgNOR mean count per nucleus significantly higher in CIN and malignant lesion as compared to benign lesion No significant different were noted between CIN II and III and different morphologic subtype of squamous carcinomas.

DISCUSSION

Cervical cancer is both preventable and curable if detected early in preinvasive or at an early invasive stage by screening procedures as it has a well-defined natural history and a long detectable preclinical phase. If detected and treated early in the preinvasive and early invasive stages, the disease has virtually a 100 % cure rate. However, in advanced cancers, the 5-year survival rate drops to less than 35 %. In countries where cervical programs have been established, the incidence of cervical cancer has markedly decreased.[7]

Among the benign lesions in my study chronic cervicitis, ectocervix (46.7%) was most commonly encountered. This was followed by endocervicitis 5 cases (33.3%) and squamous metaplasia 3 cases (20.0%). In some cases nebothian follicles formation was also seen unlike invasive carcinoma, epithelium in chronic cervicitis was found to be intact. In some cases necrosis and granulation tissue formation was also seen. In a total of 15 cases, 1 case initially was diagnosed as CIN-I and due to presence of nuclear atypia that later was excluded after examining serial section and diagnosed finally as chronic cervicitis (Ectocervix). The atypia was due to chronic inflammation (inflammatory atypia). Three cases diagnosed as squamous metaplasia; metaplastic epithelium observed was matured with an appearance indistinguishable from native squamous epithelium. Nabothian cyst formation was observed in all those three cases.

Cervical biopsy and AgNOR count add to the diagnostic accuracy. The number and size of NOR dots in the malignant cells are significantly different from those in normal and benign cells and reflect the current phase of transcription of the cells.

Out of 60 cases, 16 cases were **Cervical Intraepithelial Neoplasia (CIN)** category. 18.8% cases as CIN—1, 43.8% cases as CIN-II, 37.4% cases as CIN—III. The cases of CIN were observed mostly in the epithelial of the transformation zone.

AgNOR counts have been reported to consists in the distinction between high grade and low grade lymphoma, benign melanocytic lesion and malignant melanoma[8], various type of small round cell tumor of childhood[9], reactive mesothelial proliferative and mesothelioma, normal, cirrhotic and carcinoma of liver, distinction between oat cell and lymphocytes. The increased AgNOR count reflects increased cellular proliferative activity of cells.

In this study the AgNOR mean count in chronic cervicitis, ectocervix was found to be 1.33. The AgNOR within the nucleus are usually small and rounded. Present study co-related with Allen JP.[10] The AgNOR count in CIN lesion was found to the 3.7, the finding of which are tallied with Egan M[9] and others.[10]

In this study, out 60 cases 49.4% cases were found to be malignant. The mean AgNOR score in our study was 1.33 in chronic cervicitis, 1.55 in mild dysplasia, 3.7 in CIN lesion showing a progressive increase in the score. The differences in the AgNOR count between CIN-I and CIN-II and between CIN-II and CIN-III and between CIN-III and CIN-I were statistically significant. Egan et al.[9] observed that the mean AgNOR count increased steadily, whereas the mean size of the AgNOR dots decreased from CIN-I to CIN-III.

An Indian study done by Pratibha and Kuruvilla[11] on the role of AgNOR in diagnosing premalignant and malignant lesions of the cervix showed that the mean AgNOR count progressively increased from normal to CIN-I, CIN-II, CIN-III, and invasive carcinoma. The mean AgNOR per nucleus was 1.2, 1.8, 3.0 and 4.3 in the Normal cervix, CIN-I, II then CIN-III, and squamous cell carcinoma, respectively, in their study. The difference between counts in CIN-I and CIN-II and in the normal cervix and between counts in CIN-III and in invasive cancer was statistically significant.

Our figures matched with the AgNOR per cell quoted by Kaushik et al. [12] and Pratibha and Kuruvilla[11] for CIN-I, CIN-II, and III. But, for the AgNOR count in the normal cervix and in squamous cell carcinoma, our figures matched with Kaushik et al. The AgNOR count reported by Pratibha and Kuruvilla was higher (7.35) as compared to our figure, i.e., (3.7). The AgNOR count showed an increase from CIN to SCC in our study.

Correlation of AgNOR means count in benign and malignant lesion in present study series.

Student t test was performed and it was found that calculated value of t for 42 d.f. has been found to be greater than table value of t at 0.0001 level of significance i.e. $P < 0.0001$ which is very highly significant.

CONCLUSION

AgNOR method is a good method of screening cases of suspicious cervix and its diagnostic efficacy can be improved with the help of the AgNOR count. This simple silver staining technique can be used as an adjunct to routine histopathologic examination especially for grading dysplasia, thus rendering earlier diagnosis and treatment. AgNOR count can be used to assess the cellular proliferation rate, as there is an increase in the AgNOR count from chronic cervicitis to dysplasia and malignancy. To conclude, the results of AgNOR when used can provide strength to the clinician and histopathologist in diagnosing early carcinoma in cases of suspicious cervix.

Conflict of interest: None declared.

Ethical clearance: Taken.

Source of funding: Self.

Declaration of Authors: We declare that the author(s) named in this article did this work and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. The study was conceived and designed by Dr. Basanta Sonowal, who also collected and analyzed the data. Dr. Amitabh Handique contributed to analyze the data and designing the manuscript.

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