Original Research Article

Evaluation of AgNOR counts in pre-cancerous and cancerous cervix

Saraswathi D[1], Sathiyabama D[2*]

[1]Associate Professor, Karpagam Faculty of Medical Sciences and Research, Othakalmandapam, Coimbatore -641032, Tamil Nadu, INDIA.
[2]Associate Professor, Department of Pathology, IRT-Perundurai Medical College, Perundurai, Erode -638053, Tamil Nadu, INDIA.
Email: sarasgeethu@gmail.com

Abstract

Background: The AgNOR technique which was used extensively in cytogenetics earlier has now gained importance as an indicator of cell proliferation. cytology may fail to identify low grade and high grade squamous intraepithelial lesions which would progress to invasive cancers. In such cases, silver-stained nucleolar organizing regions (AgNOR) can be used. The study was conducted to evaluate AgNOR counts in pre-cancerous and cancerous cervix. **Material and Methods:** A total of 50 carcinoma cervix patients were included during the two years period from 2004 to2006. 25 cases served as control. The cervical and vaginal smears collected from controls and from patients were subjected to AgNOR staining. AgNOR counting was carried out as proposed by Chiu *et al*. **Results:** Most patients presented initially at an advanced stage (>IIB) moderately differentiated squamous cell carcinoma was the commonest type observed. AgNOR technique provided an index of cell proliferation. AgNOR count/cell increased gradually from normal to SIL changes to invasive carcinomas. The AgNOR count was high in poorly differentiated carcinomas and low in well differentiated carcinomas. The observations revealed significant increase of AgNOR count in cancerous lesions than in pre-cancerous lesions. **Conclusion:** AgNOR count can be used to assess the cellular proliferation rate. This simple silver staining technique can be used as an adjunct to routine cytological and histopathologic examination for early diagnosis.
Key Words: Carcinoma cervix, AgNOR count, precancerous lesion.

[*]**Address for Correspondence:**
Dr. Sathiyabama D, Associate Professor, Department of Pathology, IRT-Perundurai Medical College, Perundurai, Erode -638053, Tamil Nadu, INDIA.
Email: sathiyabamad@gmail.com
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INTRODUCTION

Cervical cancer, commonest malignancy in women in a developing country is a scourge of humanity. Early detection and management of squamous cell carcinoma is the best approach to achieve control over cancer cervix[1]. Cervical cancer can be prevented through both primary prevention and early detection using screening techniques due to its well-defined natural history and a long detectable preclinical phase. A variety of tests like PAP smear, cervicography, flow cytometry and immunohistochemical methods are now available for early detection of cervical cancer and its precursor lesions[2]. For a definitive diagnosis, a histopathologic examination is done, but they are more complicated and expensive than the silver stain used to show Nucleolar Organizing regions. The AgNOR technique which was used extensively in cytogenetics earlier has now gained importance as an indicator of cell proliferation. In normal cells, the AgNOR are tightly packed in the nucleoli and are indiscernible. In rapidly proliferating neoplastic cells nucleolar disaggregation takes place resulting in dispersion of individual AgNORs[3]. Pre-cancerous lesions precede a majority of cervical carcinoma and may exist in the non-invasive stage for longer periods and shed abnormal cells which can be detected by exfoliative cytology using papanicoloau stain. Papanicoloau smears remain a simple screening method for detecting the precancerous lesions of cervix in spite of limitations due

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to sampling and preparation errors. In invasive carcinoma of cervix, which are largely detected by biopsies, cancer cell proliferation greatly influences the clinical outcome of the patients. The available evidences indicate that evaluation of cell kinetic parameters may help surgical Pathologists and Oncologist to define the biological behaviors of cancer. Cytology may fail to identify high risk, low grade and high grade squamous intraepithelial lesions which would progress to invasive cancers. In such cases, molecular tumor markers can be used like silver-stained nucleolar organizing regions (AgNORs)[4]. There are also many studies using AgNOR conducted in preinvasive and invasive squamous epithelial lesions of cervix and AgNOR proved to be a simple, inexpensive and reliable proliferation marker in lesions of cervix. The study was conducted to evaluate AgNOR counts in pre-cancerous and cancerous cervix.

MATERIAL AND METHODS

A total of 50 carcinoma cervix patients were included during the two years period from 2004 to 2006. 25 cases served as control. The cervical and vaginal smears collected from controls and from patients were subjected to AgNOR staining. A detailed clinical history like age, parity, age at menarche, years of married life, years after last delivery, hematological investigation, review of previous cytology and histopathological examination, family status/ socio economic status was done in all cases. Cervical and vaginal smears were collected from controls and from each patient using Wooden Ayres Spatula. By putting the woman on the examination table in dorsal position speculum was introduced into the vagina exposing the cervix and Wooden Ayres Spatula was introduced into the posterior fornix. The Scraped material obtained was then spread on a slide. The pointed end of the Ayres Spatula was introduced into the cervical OS and rotated 360°, sampling the whole ectocervix. For each patient two slides were taken and they were fixed immediately with isopropyl alcohol. The cyto brush was also used in those patients to whom it is difficult to get endocervical samples and those who have stenotic cervical os especially in post-menopausal women. A cyto brush was introduced into the cervical os till few bristles were seen outside the cervical os. The brush was rotated 180° and then the brush was rolled over on a glass slide and specimen was fixed immediately. Single step AgNOR staining technique was employed for the demonstration of AgNOR's. The freshly prepared solution was poured onto the smears which were then left in dark at 37°c for 30 minutes. Slides were dehydrated in 3 changes of an acetone, cleared in Xylene and mounted in DPX. AgNOR counting was carried out as proposed by Chiu *et al*[5].

RESULTS

The prospective study included 50 exfoliative cytology specimens of clinically evaluated and diagnosed cases of squamous cell carcinoma cervix. This study also includes 25 control specimens for comparison and correlation of efficacy of AgNOR in cervical cancers. The patients initially diagnosed with Papanicoloau Stain as Cervical Cancer were divided into 6 groups according to age. There was increased incidence of cervical cancer observed in the age group of 41-50 years (38%) followed by 51-60 years (30%) and 31-40 years (20%) (Table 1).

Table 1: Age distribution of cervical cancer

Sr. No.	Age Group	No. of Cases	Percentage
1	20 – 30 years	2	4%
2	31 – 40 years	10	20%
3	41 – 50 years	19	38%
4	51 – 60 years	15	30%
5	61 – 70 years	3	6%
6	71 – 80 years	1	2%
	Total	**50**	**100%**

Most of the patients referred for Papanicoloau stain were belonging to surrounding villages of low socioeconomic status with poor hygiene and lived in overcrowded surrounding. Most of them presented with foul smelling discharge / post coital bleeding, bleeding PV for which initial papanicoloau cytological examination was undertaken. In all 50 cases of initially diagnosed cervical cancers with cytology further cervical biopsies were undertaken and histopathological examination was done. In this study most of the cases were moderately differentiated (37 cases, 74%), followed by well differentiated squamous cell carcinoma (9 cases, 18%). Poorly differentiated carcinoma constitute only 8% of total cases.

Table 2: Classification of cervical cancers (SCC) according to morphological subtypes

Sr. No.	Morphologic Subtype	No. of Cases	Percentage (%)
1	Non Keratinizing SCC	35	70
2	Keratinizing SCC	8	16
3	Small Cell SCC	5	10
4	Basaloid SCC	-	-
5	Verrucous SCC	-	-
6	Warty SCC	2	4
7	Papillary SCC	-	-
8	Lymphoepithelioma Like SCC	-	-

Most of the cases were Non Keratinizing Squamous Cell Carcinoma (35 cases, 70%) followed by Keratinizing Squamous Cell Carcinoma (33% cases, 9.11%). Small Cell, Non Keratinizing / Poorly differentiated / Neuroendocrine cancer constituted only 1.1% of cases during the prospective study period. Evaluation of

AgNOR counts in controls and in cancer cervix, AgNOR size variation and distribution was recorded according to the criteria provided by Ashan *et al*. Students 't' test was applied for the statistical analysis of results. Size variation was graded as 0= More or less uniform size; 1+=Two different sizes; 2+=More than 2 different sizes (but not those of 3+); 3+=All grades and sizes heterogenous. Distribution of AgNOR's in the nucleoli were graded as 0=Limited to nucleoli; 1+=Occasional dispersion outside the nucleoli; 2+=Moderate dispersion outside the nucleoli, 3+=Widely dispersed throughout the nucleoli.

Table 3: Comparison of AgNOR counts, size variation and distribution in control cases

Group	AgNOR count / cell		AgNOR size variation		AgNOR distribution	
	Range	Mean	0 - 1+	2+ - 3+	0 – 1+	2+ - 3+
Control (25 cases)	1 – 3	1.63	22	3	23	2

22 cases exhibited size variation of 0 to 1+ and 23 cases shows 2+ to 3+ size variation. The AgNOR distribution is also 0 to 1+ in 23 cases and 2 cases shows 2+ to 3+. The AgNOR count per cell in all 25 cases ranged 1-3 AgNOR dots/cell, with mean of 1.63. Similarly, the comparison was done in 50 cases of Carcinoma Cervix diagnosed by histopathological examination prior to radiation as given in Table 4.

Table 4: Comparison of Carcinoma Cervix diagnosed by histopathological examination

Grade	AgNOR count / cell		AgNOR size variation		AgNOR distribution	
	Range	Mean	0 - 1+	2+ - 3+	0 - 1+	2+ - 3+
Well differentiated SCC	3-8	3.76	2	7	1	8
Moderately differentiated SCC	3-5	3.84	3	34	2	35
Poorly differentiated SCC	3-9	4.12	-	4	-	4

In well differentiated squamous cell carcinoma (9 cases), the AgNOR count/cell ranged from 3-8 count/cell, with mean of 3.76, AgNOR size variation was 2+ to 3+ (7 cases), and 2 cases shows 0 to 1+, AgNOR distribution ranges 2+ to 3+ (8 cases), one case 0 to1+. In moderately differentiated squamous cell carcinoma (37 cases), the AgNOR count/cell ranged 3-5/cell with mean of 3.84, AgNOR size variation was 2+ to 3+ in 34 cases and 2 cases showed 0 to 1+, AgNOR distribution ranges 2+ to 3+ in 35 cases, two cases 0 to 1+. In poorly differentiated squamous cell carcinoma (4 cases), the AgNOR count/cell ranged from 3-8/cell with mean of 4.12, AgNOR size variation and distribution ranged 2+ to 3+ in all four cases.

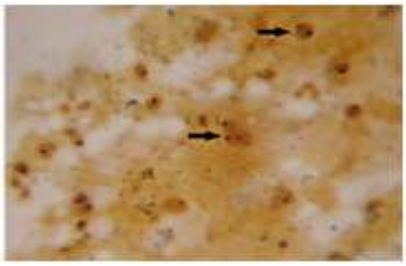
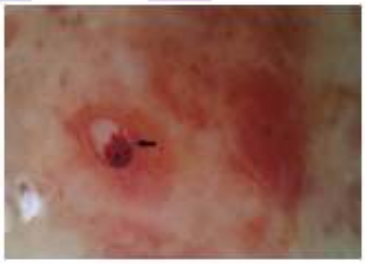


Figure 1:



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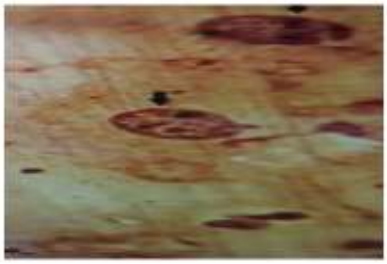

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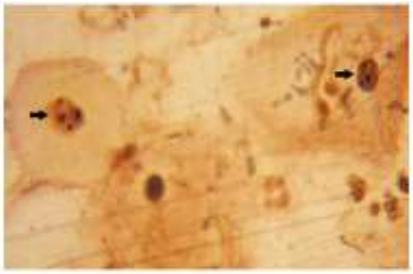

Figure 4:

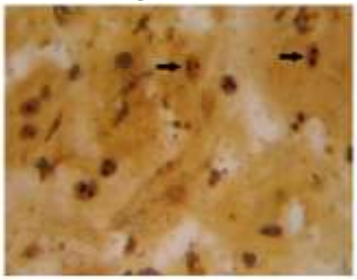

Figure 5:

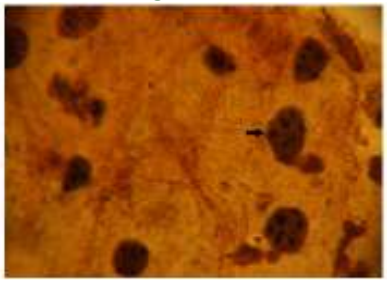

Figure 6:

Legend

Figure 1: AgNOR control smear, normal 1-2 dots/cell; **Figure 2:** AgNOR LSIL 2-3 dots/cell; **Figure 3:** AgNOR HSIL 3-4 dots/cell; **Figure 4:** AgNOR well differentiated SCC- 3-4 dots/cell; **Figure 5:** AgNOR Moderately differentaited SCC 3-5 dots/cell; **Figure 6:** AgNOR poorly differentiated SCC 3-5 dots/cell

DISCUSSION

Carcinoma cervix account for 80% of all gynecological cancers including breast cancer being commonest cancer in women in the developing countries. The most common cancer among women in India is cervical cancer. In India, carcinoma cervix continues to lead the list of cancers afflicting female genital tract because of poverty, poor socioeconomic status, wide spread ignorance, poor personal hygiene, early marriage and child bearing and high parity status continue to prevail in one country. Religious taboos and social traditions in still a false sense of modesty which inhibits women, particularly from rural backgrounds from seeking medical aid for what they consider a minor gynecological problem. The average age of women newly diagnosed with cervical cancer is between 50 and 55 years. This cancer rarely occurs in girls younger than 15 years. It begins to appear in women in their twenties. Cervical cancer is different from most cancers that tend to occur more often as people get older. Although cervical cancer does affect young women, many older women do not realize that their risk of developing cervical cancer does not go down as they age and that it is important for them to continue having Pap tests. In our study the youngest age group at which cervical cancer identified was 28 and 29 years and incidence is common between 41-50 years followed by 51-60 years. Our study contrast with other studies that the initial age of cervical cancer is 5-10 years earlier than the western population[1]. In our study above 74% of the cases were moderately differentiated squamous cell carcinoma followed by 18% well differentiated carcinoma. This distribution is well in correlation with various studies. In this study evaluation of cervical cancer with the AgNOR status was done with Pap smears after initial diagnosis with histopathological examination.

Table 5: Comparison of AgNOR count in SCC cervix with other study groups

Sr. No.	Study Group	Grade of SCC	AgNOR Count/Cell	
			Range	Mean
1	Akhtar K *et al*[6]	Well	3.7–3.77	3.74
		Mod	3.75-3.89	3.82
		Poor	3.86-4.2	4.03
2	Agarwal J andGupta JK[7]	Well		5.27
		Mod		5.41
		Poor		5.37
3	Prathiba D andKuruvilla S[8]	Well		4.2
		Poor		5.3
4	Kashyap S *et al*[9]	Well	2.17-7.52	3.66
		Mod	2.24-4.68	3.04
		Poor	3.01-3.89	3.45
5	Miller *et al*[10]	Well		2.9
		Poor		4.0
6	Present Study	Well		3.76
		Mod		3.84
		Poor		4.12

Our study correlates well with study conducted by Akhtar K *et al*[6], Prathiba D and Kuruvilla S[8] and Miller *et al*[10] that the count increases when the neoplasm becomes moderate to poorly differentiated. However, in contrast Agarwal J and Gupta JK[7] reported high AgNOR count in moderately differentiated squamous cell carcinoma followed by poorly differentiated squamous cell carcinoma. Likewise, Kashyap S *et al*[9] reported high AgNOR count in well differentiated followed by poorly differentiated and moderately differentiated squamous cell carcinoma. Rowland DC in a study of AgNOR in Cervical Intraepithelial Neoplasia (CIN) did not give any significant difference in AgNOR counts in normal squamous epithelium CIN I and CIN II, but there was a significant increase in CIN III group as well as invasive cancer[11].Crocker *et al*[12] who have done intensive work on NOR's in various tumors have observed three main types of AgNOR configurations in normal neoplastic cells. All three types of NOR patterns were observed in his study. Our study correlates with the other studies and literature that AgNOR count gradually increases from the normal to invasive cancer. In our study, the 25 control cases showed an AgNOR count/cell of 1-3 with a mean of 1.63 and 3 cases exhibited a size variation of 2+ to 3+ and distribution variation of 2+ to 3+ in 2 cases. This may be reflected as initial SIL changes / Koilocytic changes / as a response to intense inflammatory reaction. These cases are subjected for repeat Pap smears at 3 month intervals, revealed decreased in AgNOR counts to normal ratio at the end of one year. With the standardization of the silver staining technique diagnostic pathology has achieved a new milestone. The AgNOR's have been shown to reflect DNA transcriptional activity. Study of AgNOR's has been identified as a reliable indicator of cell proliferation and in turn of the malignant potential of a lesion. Malignant tumor cells are characterized by extremely large AgNOR's which show a random or scattered distribution. They are useful in discriminating between benign and malignant conditions being significantly higher in malignant cells than in normal cells. They also serve as a significant prognostic indicator in malignant lesions. AgNOR count can be used to assess the cellular proliferation rate. This simple silver staining technique can be used as an adjunct to routine cytological and histopathologic examination especially for grading dysplasias, thus rendering earlier diagnosis and treatment.

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