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ROLE OF AGNOR STAIN IN CERVICAL SMEAR: AS AN ADJUNCT TO PAP

Pathology

Dr. Kriti Bharadwaj Pathology Resident, TMMC&RC, Moradabad, Uttar Pradesh-244001

Dr. Seema Awasthi* HOD & Professor, Department of Pathology, TMMC&RC, Moradabad, Uttar Pradesh-244001.*Corresponding Author

Dr. Ankita Mittal Associate Professor, Department of Pathology, TMMC&RC, Moradabad, Uttar Pradesh-244001

ABSTRACT

Background: AgNOR special stain count is an easy, simple, potential and cost effective method that can be used as an adjunct for routine cytology and histopathology for diagnosis of cervical intraepithelial neoplasia mainly grey zone cases. **Aims:** To assess the AgNORs count as a cell proliferative activity marker in cervical smears and to correlate the count with the results of PAP stained smears. **Material & Methods:** It was a **c**ross-sectional descriptive study conducted in Department of Pathology. The study was conducted on 30 high-risk cases of cervical cancer. AgNOR staining was performed on cervical smear in the dark at room temperature. The AgNOR counting was performed under oil immersion (×100). The numbers of black dots per 100 cells were counted and average was derived. **Results:** Mean AgNOR count was found to increase progressively from normal to SIL and invasive carcinoma. Sensitivity, specificity, PPV and NPV of AgNOR score was found to be 91.6%, 100%, 100% and 97.7% respectively. **Conclusion:** AgNOR count was found to increase progressively with the severity of the disease activity with a good diagnostic accuracy to predict malignancy.

KEYWORDS

AgNORs count, Pap smear, SIL

Introduction

Cervical cancer, one of the most common form of genital cancer in females, originates from the transformation zone of cervix. It is known for developing from premalignant lesions like LSIL, HSIL which progress into invasive carcinoma. Thus it becomes critical to identify accurately the precancerous lesion by appropriate methods so that definite treatment could be institutedat the appropriate time. [1]

Papanicolaou cervical smear screening method &histologic interpretation of cervical punch biopsy specimen has markedly reduced morbidity and mortality burden of cervical cancers. Cytology have few pitfalls as it fails to detect the grey zone cases i.e high risk low grade SIL (LSIL) and high grade SIL (HSIL) which further progress into invasive cancers. According to American Society guidelines of Colposcopy and Cervical Pathology (ASCCP), cervical punch biopsy confirmed cases as CIN 2 or CIN 3 have to go for excisional treatment so potential progression into invasive cancer can be halted. Accurate diagnostic interpretation of cervical punch biopsy to differentiate between the low-grade intra epithelial lesion & high grade intra epithelial lesion is very important as it can avoid overtreatment of false-positive cases and under treatment of false-negative cases. Hence there is need to have some additional tool which could be used as an adjunct. In view of same, simple cell proliferative markers have been employed such as AgNOR staining, Ki67 etc.[2]

AgNOR special stain count is an easy, simple, potential and cost effective method that can be used as an adjunct for routine cytology and histopathology for accurate diagnosis of cervical intraepithelial neoplasia mainly in grey zone cases.[3] Molecular tumour marker is AgNOR named as silver stained (Ag) nucleolar organizer regions (NORs). NORs are loops present in DNA lie inside the nucleus of cell on acrocentric chromosomes 13, 14, 15, 21 and 22. These region NORs are highly linked with argyrophilic protein having affinity for silver like polymerase C_{23} and B_{23}. Simple silver staining technique can recognize these argyrophil associated proteins as black dots which are seen after silver staining mainly in nucleolar and extra nucleolarregions[4]. Normal cell show 20 black dots of AgNORs stain (2 per arm of chromosome i.e. 2x 10 = 20) and show coarsely tightly packed arrangement [5]. As lesion is progressing from normal cells to dysplastic cells & further into malignant cells, DNA amount increases, thus total AgNOR dots also increases.[3]

Documented study reports that mild dysplasia having low AgNOR counts are more likely regress and fall back in normal conditions compared to the lesion having high AgNOR counts which can progress into malignant lesion like invasive cancer of cervix, hence it could also be a significant prognostic tool.[3,6-8]

Thus tocategorise cervical lesions intodefinite category to avoid overtreatment of false positive cases and under treatment of false negative cases; by meredepending on cytological findings could be misleading. Additional cell proliferative markers are used to observe better diagnostic accuracy. Hence this study was planned to assess the AgNORs count as a cell proliferative activity marker in cervical smears and to establish a relationship with the results of PAP stained smears.

Subjects and Methods

Study design, settings and participants:

It was a hospital based cross-sectional study conducted over a period of one year in Department of Pathology of a tertiary care teaching hospital in Uttar-Pradesh, India. All suspected women who were at high risk of cervical cancer presenting to Gynaecology department constituted the study population. Known cases of carcinoma Female genital tract other than cervix were excluded from the study. A total of 30 high risk cases were enrolled for study.

Data collection

All selected patients were approached, personally briefed about the study. After taking an informed written consent, a detailed past history was taken from the selected patients.

The cervical smears of all the cases for the study were collected from the squamocolumnar junction of the cervix by a gynaecologist and one of the smear was stained according to Papanicolaou's technique (Pap smear) & the other with AgNOR stain while cervical biopsieswere done and these were considered as gold standard. These cervical lesions were categorized according to Bethesda classification on conventional Pap smear basis.

AgNOR STAINING METHOD[9]:

AgNOR staining solution requires:

1. Preparation of staining solution:

- Solution A: 2% gelatin in 1% formic acid
- Solution B: 50% aqueous silver nitrate solution

One part of solution A mixed with two parts of solution B to prepare working solution.

2. Staining methods:

- The collected material was smeared onto the slides and is fixed immediately in 95% ethanol.

- The slides were then stained subsequently with AgNOR stain.
- The working solution mixture A & B are layered over the slides and kept in a dark room for a period of 50 – 60 minutes.
- The silver colloid was then washed off with deionised water.
- The smears were then dehydrated through alcohol and then cleared in Xylene.
- Mounted using DPX mounting medium.

Morphology of AgNOR stained tissue under microscope:

- AgNORs are visualised as blackish or brown dots in a pale yellow background, both in the nucleolus and within the nucleoplasm. The following AgNOR parameters were calculated.
- **Mean AgNOR count:** The number of AgNORs within the nuclei of 100 neoplastic cells were calculated using a 100X objective. The mean numbers of NORs per nucleus were then calculated and results were expressed as a mean count +/- Standard Deviation.
- **Distribution and size variation of AgNORs:** The size variation and the distribution of the AgNORs within the nucleus were evaluated and given a score of 0, 1+, 2+, 3+ according to the criteria given in Table 1.

Table 1: AgNORs size variation & Distribution in the nuclei grading [10]

AgNOR Size Variation	Score
More or less uniform	0
Two different sizes	1+
More than two different sizes (but not those of 3+)	2+
All grades and sized including too minute to be counted	3+
AgNOR distribution-nuclei	Score
Limited to nucleoli	0
Occasional dispersion outside nucleoli	1+
Moderate dispersion outside nucleoli	2+
Widely dispersed throughout the nucleus	3+

Statistical analysis

Data were analysed and statistically evaluated using SPSS software, version 17.[11] Quantitative data was expressed in mean, standard deviation while qualitative data were expressed in percentage. Statistical differences between the mean of two group were compared by Mann Whitney U test. Sensitivity, specificity, Positive predictive value (PPV) and Negative predictive value (NPV) of AgNOR count was calculated taking histopathology as gold standard. 'p' value less than 0.05 was considered statistically significant.

Ethical issues

All participants were explained about the purpose of the study. Confidentiality was assured to them along with informed written consent. The study was approved by the Institutional Ethical Committee. There was no interference or influence of research process on the treatment of the patient.

RESULTS

Mean age of study subjects was 40.6±10.9 years. Pap smear examination found 9 (30.0%) having NIELM, 11 (36.7%) participants having LSIL, 6 (20.0%) having HSIL and 6 (13.3%) had Squamous Cell Carcinoma (SCC) while based on histopathology results,19 cases were found premalignant and 11 (36.7%) were malignant in which 8 cases were (72.7%) well differentiated while 1 (9.7%) each was reported as moderately differentiated, non-keratinizing SCC & adenocarcinoma. Mean AgNOR score in different diagnosis on Pap smear is shown in Table 2. Mean AgNOR count was found to increase progressively from normal to SIL and invasive carcinoma. Sensitivity, specificity, PPV and NPV of AgNOR score was found to be 91.6%, 100%, 100% and 97.7% respectively.

DISCUSSION:

Although cervical cancer is the most common form of genital cancer of females, its anatomy of origin make it an ideal site for the easy screening of the premalignant conditions. The progression of cervical cancer can be halted in early stages before its progression towards the severity or invasion i.e. from the pre-neoplastic lesion to malignant lesion of cervix, with the help of proper screening test and accurate diagnosis.

Because of easy availability, simplicity & being cost effective (pocket friendly), screening by pap smear method is accepted as the most preferred screening test for cervical cancer globally. Despite of its being very accurate there are some lacunas in the reporting & subjective variations & variability has been observed mainly in grey zone cases where making accurate diagnosis is challenging and to assign the case as high risk or low risk is of utmost importance. So these under diagnosed & over-diagnosed errors could be minimized by the introduction of some proliferative marker along with Pap smear such as AgNOR stain. Silver stained nucleolarorganiser region that easily identify argyrophil associated proteins like – polymerase C23 and B23. The AgNOR counts show a linear increase with the progression of the severity of disease. AgNOR count increases as

Normal Cell ⟶ Dysplastic cell ⟶ Malignant Cell

Present study showed an increase in AgNOR count as the severity of the cervical lesions increases (Inflammatory ⟶ LSIL ⟶ HSIL Carcinoma). AgNOR count found to increase progressively with the severity of the disease activity. As the total amount of DNA increases from premalignant cases to malignant lesions the number of total AgNOR dots also increases. It also showed mild increase in AgNOR counts between inflammatory lesions & LSIL but there was obvious and significant difference was observed in counts between the categories of LSIL to HSIL or HSIL to frank malignant lesions.

Out of total 30 high risk cases,17 cases in the present study were diagnosed as premalignant on conventional pap screening i.e LSIL or HSIL but same case on AgNOR staining showed higher AgNOR count indicating the higher degree of lesion. When these grey zone cases were biopsied and compared with histopathology (gold standard) they were found to be falling in severe lesion category as compared to the results of PAP. Thus this emphasizes the importance of using AgNOR count as adjunct with conventional Pap smear for more accuracy.

Our study results well correlated with the studies conducted by Akhter K et al[6], Prathiba D et al[7] and Miller B et al[8] where an increase in AgNOR count observed as the severity of the lesions increased. They also observed increase in count between well differentiated and poorly differentiated squamous carcinoma. However Agarwal J et al[12] found highest AgNOR count in moderately differentiated category followed by poorly differentiated squamous cell carcinoma. Another study by Kashyap S et al[13]observed highest count in well differentiated SCC followed by poorly differentiated and moderately differentiated SCC. Our study showed increase in AgNOR count from inflammatory to invasive cancer similar to Pelusi G et al[14] but there was no statistically significant increase between the well & poorly differentiated SCC. There was statistically significant increase was observed in AgNOR count between LSIL and HSIL category (p value<0.001).

The AgNOR stained cervical smears sensitivity and specificity was 91.6% and 100% as compared to histopathology (gold standard) of these cases cervical biopsy.

Conclusion and recommendations

AgNOR count was found to increase progressively with the increase in severity of the disease activity and diagnostic accuracy was increased with dual evaluation for accurate staging. Thus we emphasize the use of AgNOR stain as an adjunct to conventional pap smear to increase the diagnostic accuracy and efficacy and can be used as an additional screening tool which will help in early diagnosis and better prognosis.

Limitations

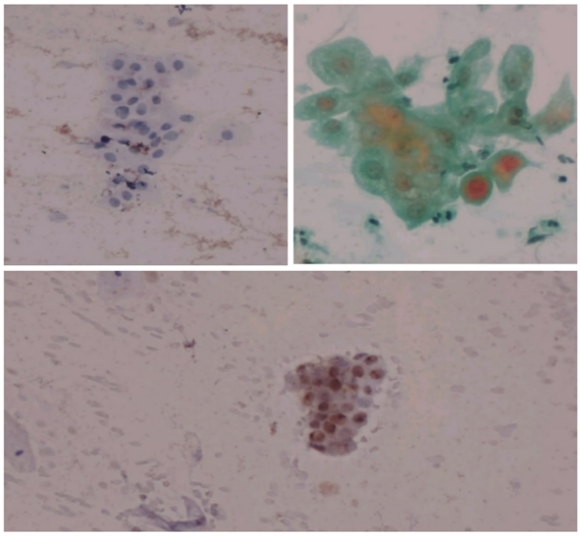
Our study should be viewed with the following limitations in mind :

- There was no comparison group of healthy mothers to see AgNOR count.
- Due to its cross-sectional nature, changes over time in different premalignant and malignant lesion could not be discerned
- Sample size was small

Table 2: Reported mean AgNOR counts in different diagnosis by Pap test (N=30)

	NIELM	LSIL	HSIL	SCC
AgNOR score	2.73±0.46	4.0±1.38	5.85±0.93	6.08±0.39
P value with NIELM		0.98	<0.001	<0.001
P value with LSIL	0.98		0.99	0.99
P value with HSIL	<0.001	0.99		0.53

Figure 1: Under 400 magnification left side upper show AgNor stained LSIL cervical smear, right side upper show PAP stained HSIL cervical smear and lower half show AgNor stained HSIL cervical smear



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