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Original Research Article

Study of the proliferative activity of various premalignant & malignant cervical lesions with a correlation to HPV status

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Abstract: Preinvasive & invasive cervical lesions are a major public health concern, though it is a preventable problem to a great extent. At present few reports have been published concerning the variability of proliferative activity in cervical biopsy in women with cervical neoplastic damage. Again though the association between HPV & neoplastic cervical lesions is well known, only a few studies have been published comparing the status of HPV with proliferative activity in south Bengal, India. The objectives of present study is to find the association of HPV status &proliferative activity (AgNOR count) in premalignant & malignant cervical lesions & to help the clinicians &histopathologists in cases of diagnostic dilemma. A study was conducted in 50 women residing in south Bengal. The patients were divided into two groups: Control group (patients with chronic cervicitis=8) & study group (patients with premalignant & malignant cervical lesions=42).HPV-16 status was determined by DNA PCR. AgNOR staining was performed according to standard guidelines. Counting the number & shape of AgNOR was done under 100 x objectives in 100 cells in each slide & mean AgNOR count obtained. Statistical significance was established at $P< 0.05$. The mean percentage of HPV positivity increased significantly with severity of lesions (P=0.004). Proliferative activity of various cervical lesions was also significantly associated with HPV status (P=0.0048). HPV positivity was seen to be significantly associated with age at menarche (p=0.0265) & age at first pregnancy (p=0.019). In present study, a direct association of proliferative activity with HPV status was found.
Keywords: Proliferative activity; AgNOR; cervical neoplasm; HPV; DNA PCR.

INTRODUCTION

Invasive cervical cancer is one of the most common cancers among women from developing countries [1].Worldwide it is the 2nd most common cancer among women [2]. Every year in India, 122,844 women are diagnosed with cervical cancer and 67,477 die from the disease. India also has the highest age standardized incidence of cervical cancer in South Asia at 22, compared to 19.2 in Bangladesh, 13 in Sri Lanka, and 2.8 in Iran[3]. The epidemiology of cervical cancer shows wide geographical variation in its occurrence. These differences are related to social and economic conditions, as well as to religion, and the influence of these factors on sexual practices [4]. The occurrences of cancer in cervical epithelium, in most of the cases, are followed by HPV infection, koilocytic changes & preinvasive squamous epithelial lesions. A number of systems are there to describe preinvasive lesions; the oldest is the dysplasia-carcinoma in situ (CIS) system, with mild dysplasia on one end and severe dysplasia/CIS on the other. Another is the CIN classification, with mild dysplasia termed CIN 1(cervical intraepithelial neoplasia 1) and CIS lesions termed CIN 3. Still another system reduced these entities to two: low-grade squamous intraepithelial lesion (LSILs) and high-grade SILs (HSILs) [5].

Among various etiologic factors, Human Papilloma Virus (HPV) infection is considered to be the most important agent in cervical oncogenesis. Young women are mostly vulnerable to HPV infection, but most are transient & resolve spontaneously in 6 to 24 months [6]. Only a small percentage will lead to precursor cervical lesions & only those that persist long term, pose a risk for the development of cancer [6]. Nearly all cervical cancers are caused by persistent infections with one of around 15 carcinogenic types of HPV [7]. HPV 16 & 18 demonstrates the strongest carcinogenic potency, accounting for 70% of cancers [8].

Cytopathic effect of HPV increases the life span as well as proliferative activity of cervical epithelial cells. Many authors have demonstrated that the study of proliferative activity can be of prognostic significance in early detection of premalignant & malignant cervical lesions. Proliferative activity can be assessed by study of nucleolar organizer regions (NORs) [9] which are loops of DNA encoding ribosomal RNA. Argyrophilic proteins associated with NORs are selectively identified by a silver colloid staining technique & visualised as dark intranuclear dots under optical microscope [10]. From various studies it has been described that argyrophilic nucleolar organizer regions (AgNOR) in cervical biopsies can be applied as a useful proliferative marker in different premalignant & malignant lesions, but few of them demonstrate the relationship between proliferative activities & HPV status.

Therefore we evaluated the proliferative activity (by AgNOR study) of different cervical lesions along with the role of HPV in cervical carcinogenesis.

MATERIALS & METHODS:

This prospective study was carried out for duration of one & half year. Total 50 women, who presented at the outpatient department (OPD) of a tertiary care hospital with relevant history like vaginal bleeding, contact bleeding, whitish discharge & cervical mass lesions were included in this study. Informed written consent was taken from all patients before inclusion. Detailed history& relevant clinical information were obtained from each patient. We have designated cases & controls for this study as following:-

Cases

Patients with histologically proven preinvasive & invasive malignant cervical lesion.

Controls

Patients with chronic cervicitis.

After initial screening cervical biopsy (punch or wedge biopsy) were taken from the patients suspected of having a cervical pathology. One part of the biopsy material was taken in 10% formalin & sent to Department of Pathology for histopathological examination. Remaining part was collected in a sterile tube, placed in dry ice & sent for HPV-16 detection.

Formalin fixed paraffin embedded blocks were prepared from the biopsy material. In each case 3-4 micron sections were taken. Two sets of slides were made. One set were stained with Haematoxylin & Eosin for histological examination & other set with silver stain for AgNOR protein. AgNOR sites are visible under microscope as intranuclear black dots whereas background as pale yellow. Proliferative activity was assessed by counting the number of AgNOR dots under oil immersion objective. The count was done in 100 cells per slide. As all the argyrophilic dots visible in the nucleus are nucleolar organizer regions (NOR), all the silver-stained extra and intranucleolar structures were counted. All the pleomorphic and single small dots were counted individually, and the mean number of AgNORs per nucleus was calculated for each slide.

HPV detection

As HPV-16 is more common in India compared to other serotypes; the current study was undertaken for its detection. It was detected from formalin free tissue. DNA was isolated according to standardised protocol from tissue embedded in tissue freezing medium &cryo-sectioning in -28^0C. After checking the stock DNA it was diluted to 50 ng/ulconc^n for PCR analysis. HPV was screened by PCR using MY09/11 primer obtained from consensus L_1 region of the HPV genome. Further genotyping was done for HPV-16 designed from E_6 region. [Fig 1]

Statistical analysis:

Percentage of HPV-16 positivity was determined in various group of cervical pathology ranging from chronic cervicitis to invasive carcinoma. Relation of other patient-related factors to HPV positivity was also evaluated. Microsoft Office Excel (XP) was used for tabulating & comparing data. SPSS version 16 software was used for analysis of data. Chi-square test was done for association between two categorical variables. A confidence level of 95% i.e. p value < 0.05 was considered statistically significant.

Ethical approval was sought and obtained from institutional ethical committee.

RESULTS & ANALYSIS:

Out of total 50 cases, 8 cases were of chronic cervicitis, 11 cases had preinvasive cervical lesion (CIN) & 31 had invasive malignancy. Out of 11 preinvasive lesions 3 cases were of CINI, 2 of CINII & 6 of CINIII. Among invasive malignancy, squamous cell carcinoma (SCC) were commonest (58%) followed

by adeno carcinoma (22.6%) & adenosquamous carcinoma (19.4%).

2 out of 8 cases of chronic cervicitis show HPV-16 infection, whereas 8 out of 11 cases of CIN & 26 out of 31 cases of invasive malignancy demonstrate HPV-16. So HPV-16 positivity is significantly associated with severity of the lesions (p=0.004) (Table no.1)

Out of 31 cases of invasive malignancy, adenosquamous carcinoma showed maximum HPV-16 positivity followed by squamous cell (94.4%) & adeno carcinoma (43%).

All cases of chronic cervicitis (8 out of 8) show mean AgNOR count between 1-3. [Fig 2(a)] Out of 11(eleven) cases of CINIII, 5(five) cases show mean AgNOR count between >3-6 & 6(six) cases between 1-3. None of them show mean AgNOR count of >6. [Fig 2(b)] Out of 31 cases of carcinoma cervix, 19 cases have AgNOR count between >3-6 & 12 cases >6 which shows a highly significant relationship. (p=0.0001)(Table no.2) [Fig 2(c)].

Lesions having mean AgNOR count between 1-3 show 42.8% HPV-16 positivity whereas lesions of >6 AgNOR count demonstrate 100% HPV-16 positivity. Association of proliferative activity with HPV-16 positivity is also highly significant (p=0.0048) (Table no.3)

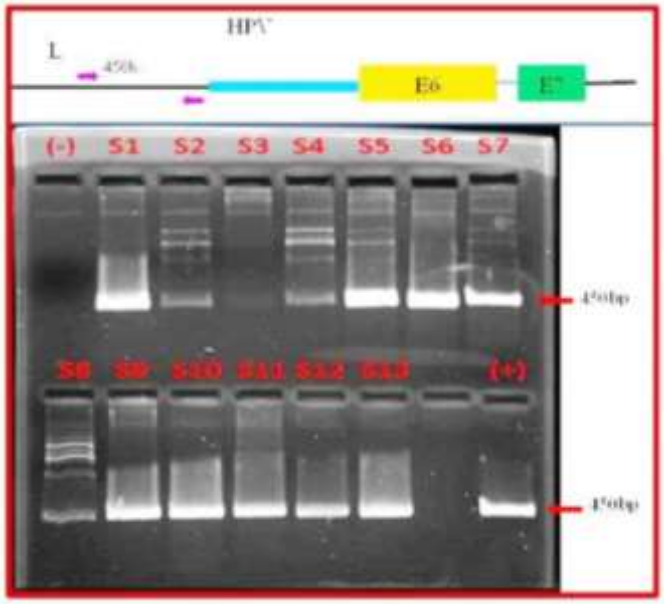
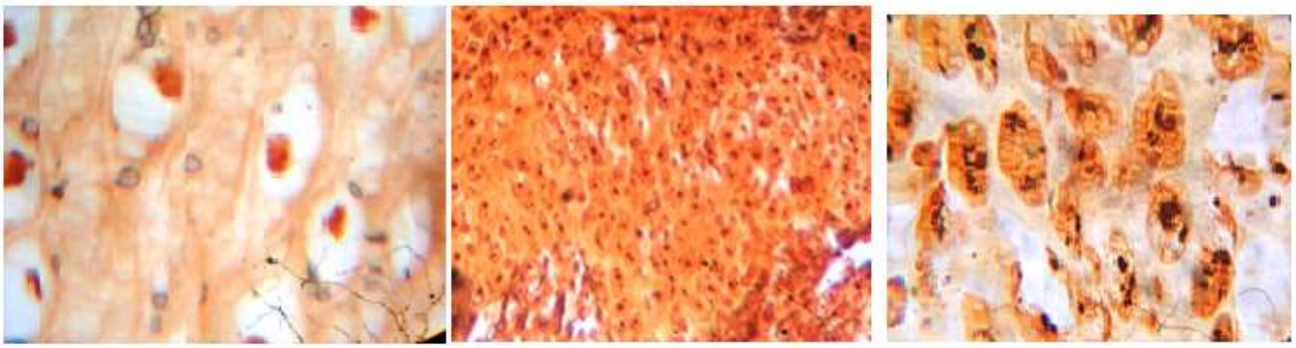


Fig-1: Representative agarose gel along with positive (HPV16 plasmid) and negative (water) controls. Detection was performed using primers MY09/11 which generates a PCR product of 450bp.



2(a) 2(b) 2(c)

Fig-2(a): Microphotograph showing AgNOR stain in chronic nonspecific cervicitis(400X).
Fig-2(b): Microphotograph showing AgNOR stain in CINIII (400X).
Fig-2(c): Microphotograph showing AgNOR stain in SCC of cervix(400X).

Table -1: Demonstration of human papilloma virus (HPV) type-16 positivity among cases of chronic cervicitis, preinvasive & invasive lesions of cervix

	Total no of cases	HPV-16 positive	% of HPV-16 Positivity
Chronic cervicitis	8	2	25
Preinvasive lesion	11	8	73
CA Cervix	31	26	83.8

p = 0.004

Table-2: Proliferative activities of various cervical lesions

	Proliferative Activity (Mean AgNOR count)		
Cases	1-3	>3-6	>6
Chronic cervicitis	8	0	0
CIN	6	5	0
Carcinoma cervix	0	19	12

p=0.0001

Table- 3: Demonstration of human papilloma virus (HPV) type-16 positivity in relation to proliferative activity of various cervical lesions

Proliferative activity(Mean AgNOR count)	Total no cases	HPV-16 Positive	% of HPV-16 Positivity
1-3	14	6	42.8
>3-6	24	18	75.0
>6	12	12	100

p=0.0048

DISCUSSION

Cases of preinvasive lesions & carcinoma cervix showed 73% & 83.8% HPV-16 positivity respectively whereas 25% HPV-16 positivity were reported in healthy subjects.(P = 0.004). % of HPV positivity in control group was higher in our study compared to study by ICO information centre on Human Papilloma Virus (HPV) in India. They found about 7.9% of women in the general population to harbour cervical HPV infection at a given time, and 84.1% of invasive cervical cancers are attributed to HPV 16 or 18 [11]. A population based study conducted by Dutta S et al. [12]in Eastern India found population prevalence of HPV was 9.9%. They also found that gradual increase in HPV copy numbers was associated with progressive cytologic severity.

The present study revealed increasing AgNOR count with severity of histopathological lesions (P=0.0001) & SCC cases show highest mean AgNOR count. The study assumes importance with the report of Alarcon-Romero et al. [13] that found a statiatically significant difference of AgNOR dots associated with infection of high risk HPV types. Finding of the present study is also corroborated with the result of the study conducted by Kaushik R et al. [14] & Singh M et al. [15] who concluded that mean AgNOR counts in cervical epithelium showed progressive & statistically significant increase from chronic cervicitis to CIN I, II & III& invasive ca.

Proliferative activity or mean AgNOR count of various lesions is also corroborated with HPV status. Lesions having mean AgNOR count between 1-3 show 42.8% HPV-16 positivity whereas lesions of >6 AgNOR count demonstrate 100% HPV-16 positivity (P=0.0048). So it is seen that infection with high risk HPV types like HPV-16 induces greater proliferative activity of cervical epithelium. Martial Guillaud et al. evaluated impact of HPV infection on cell proliferation in epithelial layers in cervical neoplasia. They observed increased cell proliferation and decreased epithelial thickness with increased disease grade (when analyzing the epithelium at full thickness). Analysis within individual cell layers showed a ≥50% increase in cell proliferation for CIN2 vs. CIN1 lesions in higher epithelial layers. Higher rates of proliferation for HPV-positive vs. -negative cases were seen in epithelial layers beyond the basal/parabasal layers in normal and CIN1 tissues [16]. Isacson C et al. evaluated the kinetic indices of cell proliferation and apoptosis in a histopathological spectrum of cervical neoplasia and compared low-versus high-risk HPV-associated lesions. They found no significant difference in the proliferative and apoptotic indices in similar grade lesions when stratified into low-versus high-risk HPV types. These findings suggest that apoptosis in HPV-infected lesions

correlates with proliferative activity rather than HPV types [17].

HPV infection of the cervical epithelium depends on some other factors like age at menarche, age at marriage or first sexual intercourse, parity, socioeconomic status, use of contraceptive etc. Our study revealed a significant relationship of HPV infection with age at menarche& age at first pregnancy but no such relationship was established with age of the patient & parity.

CONCLUSION

So to conclude, in this single institution-based study, we have studied risk factors, proliferative activity of premalignant & malignant cervical epithelial lesions & prevalence of HPV-16 infection in those lesions. Statistically significant association were found between percentage of HPV-16 infection with severity & proliferative activity of various cervical squamous epithelial lesions.

Cervical cancer is unique among cancers in that it can largely be prevented through screening and removal of precursor lesions. It is evident that Papanicolaou smear screening has increased the detection of this potentially curable cancer as well as detection & eradication of preinvasive lesions. However, cellular abnormalities may be missed or may not be sufficiently distinct. A patient with mild dyskaryosis may have high grade lesion on subsequent colposcopy & biopsy. Early detection of high risk HPV types from cervical tissue may improve triage, treatment & follow up in infected patients.

In this context it will be logical to mention the role of HPV vaccine. The importance of HPV-vaccination programmes targeting young adolescents before first sexual intercourse can have a great effect in decreasing the incidence of cervical cancer [18].Additional efforts are necessary in sexual education & family planning to decrease the incidence of HPV infection & associated premalignant & malignant cervical lesions.

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