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A Study to Evaluate the Evidence of Human Papilloma Virus Infection in Preinvasive and Invasive Cancerous Lesions of Cervix in Different Epidemiological Background in Eastern India

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Abstract:
Objective: *The objective was to study the evidence of Human Papilloma Virus 16 (HPV 16) in pre-invasive and invasive cervical neoplasia. Different epidemiological factors in relation to HPV 16 infection were also observed.*
Materials and Methods: *Immunohistochemical detection of HPV 16 was done using anti-HPV 16 mouse monoclonal antibody against L1 fusion protein in cervical scraping or biopsy specimens obtained from subjects (50 cases, 15 controls) after confirmation of diagnosis by Papanicolaou's stain or Hematoxylin & Eosin stain.*
Results: *HPV 16 positivity in patients (56%) was extremely significant (p=0.0008) compared to healthy controls (6.67%). HPV positivity was inversely related to grade of lesion and age of patient, the grades being found increasing with age. No relation was found of HPV infection with parity and age at menarche. Higher HPV positivity with lower ages of first sexual intercourse and first child birth was found but not statistically significant (p=0.89 & p=0.94 respectively). HPV positivity was highest among OCP users (77.78%) and lowest among condom users (33.33%), though the difference was insignificant (p=0.75).*
Conclusion: *The study suggests the association of HPV 16 in cervical neoplastic lesions with possible role in progression, though no epidemiological factors were related. So, an effective HPV 16 vaccine may reduce the cervical cancer burden.*
Keywords*: Human Papilloma Virus 16, cervical neoplasia, Immunohistochemistry*

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I. Introduction

Cancer cervix is the fourth most common cancer in women worldwide, and nearly 528,000 new cases and 266,000 deaths are reported each year (in 2012).[1] Almost 87% of cervical cancer deaths occur in developing countries, and more than one-fifth of all new cases are diagnosed in India.[1] The incidence of cervical cancer shows marked geographical variation. The incidence of CA cervix is 15% of all new cancers in women in developing countries, whereas it is only 3.6% in the developed world.[2] In India, 122,844 women are diagnosed with cervical cancer and 67,477 die from the disease every year.[3] It is the second most common cancer in women aged 15–44 years in India.[3] Cervical cancer is the third largest cause of cancer mortality in India accounting for nearly 10% of all cancer-related deaths in the country.[4]

The infection with human papillomavirus (HPV) plays a major role in the development of cervical cancer.[5] More than 200 different types of HPV have been identified on the basis of genomic differences [6]. Specific subtypes of HPV that carry a considerable risk of causing malignant progression in the uterine cervix are termed high-risk HPV.[7, 8] HPV 16 is the most commonly occurring subtype in cervical neoplasia. [8]

Other than HPV infection, so many risk factors, from epidemiological to etiological theories, have been identified for cervical cancer.[9] Among them sexual factors (multiple sexual partners and lower age of first intercourse), smoking, OCP use, diet and immunosuppression are the important ones.[9]

A wide variation in the prevalence of HPV positivity ranging from 6% to 38% has been reported in the general population from different geographical regions.[10] This difference in positivity may be due to the difference in various diagnostic methods and difference in various epidemiological factors among population in a particular geographic area.

Testing for high-risk HPV has a better sensitivity and reproducibility than cytological screening and would theoretically serve as a better primary screening test. [11]

High-risk HPV types were found in 87.8% of the squamous cell carcinomas in one study from Andhrapradesh in India. [12] Among the HPV positive cancers, the major high-risk HPV type was detected as HPV 16 (66.7%).[12] Hence, our study was aimed at detecting only HPV16, as it is the most relevant one in our limited resources.

As there is scarcity of information regarding prevalence of HPV 16 infection in the eastern part of India, there is a scope to study the evidence of HPV 16 in cervical cancer patients by a relatively simple and specific method (Immunohistochemistry)

II. Materials and method

This study was carried out in the department of Pathology, Institute of Post Graduate Medical Education and Research (IPGMER), Kolkata, a tertiary care medical institute. The work was done in collaboration with the department of Gynaecology & Obstetrics, IPGMER.

Study Population:

The subjects of our study were recruited from the patients attending the OPD or being admitted in the indoor of the department of Gynaecology & Obstetrics, IPGMER, Kolkata. This study population was represented from the city of Kolkata and adjacent districts of West Bengal, India.

Sample Size of the study:

Total 65(sixty five) subjects were included in this study, out of which 50 (fifty) subjects were cases and the rest 15 (fifteen) subjects were controls. Among the total 50 cases, 5(five) cases were HIV positive and among the total 15 controls 5(five) were HIV positive.

Study Design:

It was a prospective single blind unicentric study.

Study Duration:

Total period of the study was one year and eleven months.

Study Settings:

The screening, recruitment and relevant data collection followed by cervical scrapings or biopsy was obtained in the Dept. of Obstetrics & Gynecology, IPGMER, Kolkata. ELISA for HIV was done in the ICTC of IPGMER. The histopathological & cytological examination, immunohistochemistry, results interpretation and data analysis was carried out in the Dept. of Pathology, IPGMER, Kolkata.

Selection of Cases:

Inclusion Criteria:
1) Cytologically suggestive preinvasive and invasive cancerous lesions of cervix .
2) Histologically proven preinvasive and invasive cancerous lesions of cervix.
3) For HIV positive comparable group: Patients who are HIV positive (by ELISA), fulfilling the above mentioned inclusion criteria

Exclusion criteria:
1) Cytologically benign lesions of cervix.
2) Cytologically equivocal lesions of cervix.
3) Histologically benign lesions of cervix.

Stepwise Method of the Study:

1. Patients attending OPD or admitted in indoor with chief complains of menstrual abnormalities (contact bleeding , bleeding on straining, intermenstrual or post-menopausal bleeding), excessive/offensive vaginal discharge, pelvic pain, bladder/rectal symptoms etc. were examined and screened in the Dept. of Obstetrics & Gynecology.
2. Clinically suggestive patients (for AIDS or HIV infection) and/or with history of exposure was subjected to ELISA for HIV. Similarly HIV positive patients (by ELISA) with history and chief complain suggestive of cervical malignancy were also included in screening.
3. After initial screening, cervical scraping (for cytological examination) or punch/surgical biopsy (for histopathological examination) was obtained accordingly.
4. Samples for cytological examination were stained with Papanicolaou's (Pap) stain and Leishman-Giemsa stain and remainder of the sample was preserved.
5. From the cervical biopsy specimens two sets of slides were made. Some were stained with Hematoxylin & Eosin stain for histological examination and remainder was preserved.

6. After examination under microscope, the smears of cytologically suggestive cancerous lesions as well as the sections of histologicaly proven preinvasive and invasive malignant lesions was recruited in the study for immuno-histochemical (IHC) staining.
7. Fifteen subjects (among which five are HIV positive) with normal cervical biopsies without any evidence of malignant or premalignant lesions was used as control.
8. Detailed history and other relevant clinical data were collected according to a preset proforma.
9. Informed written consent was taken from all patients before inclusion in the study.
10. After completion of all formalities, the second group of samples from cytology and histology were used for detection of HPV antigen by IHC staining. IHC staining was also done amongst the controls.
11. Percentage of HPV positivity was determined both in cytological and histological specimens and compared with HIV positive group. Relation of other patient-related factors to HPV positivity was also evaluated.

Statistical analysis:
Chi-square (χ^2) test, the most important non-parametric test of significance, was used to detect the significance of HPV 16 positivity in different groups of sample.
Significant at 5% level ($p<0.05$) with degree of freedom 1 was chosen in this study (95% CI). To measure the strength of association of HPV infection with different epidemiological risk factors of cervical carcinoma, 'Odds ratio' was calculated. Graph Pad InStat software version-3 was used for statistical analysis.

III. Result

Table No. 1: Detection of human papillomavirus (HPV) type-16 by immunohistochemistry among healthy (control) subjects and cases of cervical carcinoma:

	No. of HPV Positive cases	No. of HPV Negative cases	total cases	% of HPV Positivity	OR	95% CI
Control	1	14	15	6.67	1	--
Cases with Cervical Lesions	28	22	50	56	17.82	2.17-146.19

Table No. 2: Demonstration of human papillomavirus (HPV) type-16 positivity among different types of pre-invasive & invasive lesions of cervix:

Cyto/Histological Diagnosis			Total no. of cases	Total no. of HPV positive cases	% of HPV positivity	
Pre-invasive lesions	ASCUS/ ASC-H		04	00	00	62.50
	LSIL		14	10	71.43	
	HSIL		06	05	83.33	
Invasive SCC lesions	Well Differentiated SCC		02	02	100	61.90
	Moderately Differentiated SCC		14	10	71.43	
	Poorly Differentiated SCC		05	01	20	
Others	Adeno Ca	3	05	00	00	
	AdenoSq Ca	1				
	MMMT	1				
Total			**50**	**28**	**56**	

SCC- Squamous cell CA, ASCUS- Atypical Squamous Cells of Undetermined Significance, ASC-H- Atypical Squamous Cells, cannot exclude high-grade squamous intraepithelial lesion, LSIL- Low Grade Squamous Intraepithelial Lesion, HSIL- High Grade Squamous Intraepithelial Lesion, MMMT- Malignant Mixed Mullarian Tumour

Table No. 3: Detection of human papillomavirus (HPV) type-16 in cervical lesions according to age of the patient:

Age groups (years)	Total no.of cases	No. of HPV Pos. cases	% HPV Positivity	OR	95% CI
< 25	01	00	00	1	--
25-34	09	06	66.67	5.57	0.18–176.42
35-44	14	08	57.14	3.92	0.14–112.99
45-54	19	11	57.89	3.32	0.12–91.68
≥ 55	07	03	42.86	2.33	0.07–76.73
χ^2 for trend				0.28	p = 0.59

Table No. 4: Detection of human papillomavirus (HPV) type-16 according to parity:

Parity	No of cases	No. of HPV Positive cases	% HPV Positivity	OR	95% CI
1-2	10	05	50	1	--
3-4	27	16	59.26	1.46	0.34–6.25
≥ 5	13	07	53.85	1.17	0.22–6.08
χ^2 for trend				0.02	p = 0.89

Table No. 5: Detection of human papillomavirus (HPV) type-16 according to age at menarche:

Age at menarche (years)	No.of cases	No. of HPV Pos. cases	% HPV Positivity	OR	95% CI
≤ 12	17	09	52.94	1	--
13–14	28	16	57.14	1.19	0.35–3.98
≥15	05	03	60	1.33	0.18 –10.13
χ^2 for trend				0.11	p = 0.74

Table No. 6: Detection of human papillomavirus (HPV) type-16 ccording to age at marriage/1st sexual intercourse:

Age at marriage/1st sexual intercourse (years)	No.of cases	No. of HPV Positive cases	% HPV Positivity	OR	95% CI
≤ 15	05	03	60	1	--
16–17	22	13	59.09	0.96	0.13–6.98
18–19	12	07	58.33	0.93	0.11–7.82
≥ 20	11	05	45.45	0.55	0.06–4.76
χ^2 for trend				0.46	p = 0.89

Table No. 7: Detection of human papillomavirus (HPV) type-16 according to age at first child birth:

Age at first child birth (in years)	No.of cases	No. of HPV Positive cases	% HPV Positivity	OR	95% CI
≤ 15	01	00	00	1	--
16–17	09	06	66.67	5.57	0.18–176.42
18–19	24	13	54.17	3.52	0.13–95.16
≥ 20	16	09	56.25	3.8	0.13–107.4
χ^2 for trend				0.0057	p = 0.94

Table No. 8: Detection of human papillomavirus (HPV) type-16 according to use of different types of contraceptive methods:

Contraceptive method	No.of cases	No. of HPV Pos. cases	% HPV Positivity	OR	95% CI
None	11	06	54.55	1	--
Tubal ligation	19	11	57.89	1.14	0.26–5.12
Condom	03	01	33.33	0.42	0.03–6.07
OCP	09	07	77.78	2.92	0.40–20.91
Cu-T	07	03	42.86	0.63	0.09–4.22
Others	01	00	00	0.28	0.009–8.43
χ^2 for trend				0.099	p = 0.75

Table No. 9: Distribution of human papillomavirus (HPV) type-16 according to different socioeconomic strata:

Socio-Economic strata	No. of cases	No. of HPV Positive cases	% HPV Positivity	OR	95% CI
Very low income group	26	14	53.85	1	--
Low income group	11	07	63.63	1.5	0.35–6.40
Lower middle income group	08	05	62.50	1.43	0.28–7.26
Middle income group	04	02	50	0.86	0.10–7.05
Upper middle income group	01	00	00	0.29	0.01–7.71
High income group	00	00	00	--	--
χ^2 for trend				0.08	p = 0.78

Very low income group: Yearly income: Urban < Rs.10000, Rural < Rs.5000
Low income group: Yearly income < Rs. 50,000
Lower middle income group: Yearly income Rs. 50,001-1,00,000
Middle income group: Yearly income Rs. 1,00,001-2,50,000
Upper middle income group: Yearly income Rs. 2,50,001-5,00,000
High income group: Yearly income >Rs. 5,00,000

Table No.10: Distribution of human papillomavirus (HPV) type-16 among HIV-positive and HIV-negative patients of cervical lesions:

HPV Positivity	HIV negative cases of cervical lesions		HIV positive cases of cervical lesions	
	No. of cases	% of total cases	No. of cases	% of total cases
Positive	27	60	1	20
Negative	18	40	4	80
Total	45	--	5	--

IV. Discussion

Invasive Cervical Carcinoma cases were associated with HPV16 in 51.0% cases and HPV18 in 16.2% cases infection[13] in one meta-analysis. The HPV16 family of viruses were more commonly found in SCC than ADC, whereas the HPV18 family were more common in ADC[13]. As in other developing countries, in India, squamous cell carcinoma predominates, whereas adenocarcinoma of the cervix accounts for a small percent of all cervical cancer[14].

Hence, in our study we have identified the evidence of HPV 16 by immunohistochemistry (L1 Capsid protein) method in both preinvasive and invasive carcinoma of cervix. In the present study we have also tried to find the relationship of different epidemiological aspects of cervical carcinoma with HPV 16 infection. In addition we have also tried to detect the HPV 16 positivity among HIV positive women with cervical cancerous lesions.

In our study, we found that 56% (28 out of total 50 cases) of patients with preinvasive or invasive cervical cancerous lesions were positive for HPV 16 infection, detected by immunohistochemical method. On the other hand, within the healthy control group HPV 16 positivity was only 6.67% (1 out of 15 controls). This

was extremely significant among the cervical lesion patients (OR 17.82 , 95% CI 2.17-146.19 & p = 0.0008) in comparison to healthy control subjects (Table-1). Sowjanya A P et al found the prevalence high-risk HPV types were 87.8% of which two-third case (58.53%) were positive for HPV 16 in cervical cancer patients in Andhra Pradesh, India[12]. The percentage positivity of HPV 16 found in our study is more or less same with the percentage found in this and other studies also.

The percentage of HPV 16 positivity was 62.50% among pre-invasive lesions and 61.90% among invasive lesions respectively (Table-2). All the HPV positive invasive lesions were squamous cell carcinoma, no HPV 16 was found among adeno & adeno-squamous carcinoma patients. HPV 16 positivity among different types was as followings, ASCUS/ ASC-H - 0%, LSIL - 71.43%, HSIL - 83.33%, well differentiated SCC - 100%, moderately differentiated SCC -71.43%, poorly differentiated SCC - 20% and others - 0%. The findings of our study corroborated with the other studies[15, 16, 17] in the view of higher percentage HPV 16 positivity in relatively low grade lesions and a lower percentage in high grade carcinomas, except in cases of ASCUS/ ASC-H (0%) and well differentiated SCC (100%) where unexpected values may be due to very low sample size. The percentage positivity in LSILs & HSILs confirms HPV16 infection as an early event and further indicates a role in progression of lesions. These results show a strong association between HPV infection and degree of differentiation and are consistent with the hypothesis that production of the HPV structural antigen requires a high degree of squamous cell maturation.

In our study the HPV 16 positivity was found maximum in lower age group (66.67% in 25-34 years, OR – 5.57, 95% CI 0.18–176.42) and decreases with increasing age (42.86% in ≥ 55 years,), though not statistically significant (p = 0.59 in χ^2 for trend), which can be explained by clearance of viral load with time (Table-3). Very small sample size is the probable explanation of finding no HPV positive cases in < 25 years age group. The findings of our study was consistent with the findings of a study by Evans MF et al.[17]

Most of the cervical lesions (27/50) and maximum HPV positivity (59.26%, OR 1.46) was seen among women who gave child birth of 3 to 4, less among parity less than 2 and no cases of cervical cancer found among nullipara (Table-4), but the difference in HPV positivity among different parity groups was not statistically significant (p = 0.89 in χ^2 for trend). This finding, which is consistent with other studies,[14, 18] suggest a positive relation between parity and cancer cervix & HPV positivity. The possible explanation of HPV positivity with increased number of child birth may be suggested as hormonal influences on biology of HPV and labour-related trauma.

HPV positivity was consistently increasing with the increase in age at menarche i.e. 52.94% in ≤ 12 years age group, 57.14% in 13–14 years age group and 60 % in ≥15 years age group (Table 5), though not statistically significant (p = 0.74 in X^2 for trend). It is very difficult to say any relation of HPV infection with increase age of menarche at this point of time.

It was seen in our study that, percentage of HPV positivity was inversely related to the age of marriage or 1st sexual intercourse (Table-6) i.e. higher positivity with lower age (60% in ≤ 15 years) and lower positivity in higher age (45.45% in ≥ 20 years, OR 0.55), yet this finding was not statistically significant (p = 0.89 in χ^2 for trend). So any relation of marriage / 1st sexual intercourse with HPV positivity could not be concluded.

The present study also observed the relation of age at first child birth with HPV positivity which was as following- 0% in ≤ 15 years, 66.67% in 16–17 years, 54.17% in 18–19 years and 56.25% in ≥ 20 years (Table 7). This finding of the apparent influence of early age at first pregnancy is consistent with the other studies[14, 18], but the difference in HPV positivity among different age groups at first child birth was not statistically significant (p = 0.94 in χ^2 for trend).

Among different contraceptive methods, tubal ligation was the most frequently (19/50) used method. An increased risk of HPV positivity was found among women with tubal ligation (57.89%) and women who used oral pills (77.78%), (OR= 1.14 & 2.92 respectively), whereas condom (33.33%) & Cu-T users (42.86%) had lower HPV positivity (Table-8), however this difference in HPV positivity among different methods of contraceptive use in comparison to those who have not used any contraceptive was not statistically significant (p = 0.75 in χ^2 for trend), but consistent with other studies.[14, 18] Hormonal effect causing increased viral transcription is the possible explanation for this positive association with OCP use. On the other hand, barrier method like condom use may have a protective effect against HPV transmission causing relatively lower percentage of HPV positivity.

When comparing the HPV positivity rate among different socioeconomic strata (Table-9), we can see that highest HPV positivity was seen in low income group (63.63%, OR 1.5) whereas middle and upper middle income group had shown lowest positivity (50%, OR- 0.86 & 0%, OR- 0.29 respectively). As the study population was taken from the patients who attended the outpatient department of a Govt. hospital, this sample population does not represent the whole population. Therefore, it is very difficult to come to an inference about the relation of HPV positivity with socioeconomic condition of the patient.

Regarding the relation of HIV infection with HPV positivity among patients with cervical cancerous lesions, only 5 patients were HIV positive among total 50 patients with cervical lesions, within which only one

patient (i.e. 20%) was HPV positive, whereas among the rest 45 HIV-negative patients 27 (60%) were HPV positive (Table 10). Strickler H D et al**.** found the prevalence ratio for HPV16 was low compared to other HPV types in both the HIV-seropositive cohorts of Women's Interagency HIV Study (WIHS, n=2058) and HIV Epidemiology Research Study (HERS, n=871)[19]. Our study finding was consistent with this low HPV 16 positivity, though most of the studies[20, 21] showed HIV infection and immunosuppression play an important role in modulating the natural history of HPV infection and an aggressive course of HPV infection in HIV-positive women. All the 5 patients in our study were of low-grade lesions, which may explain the low HPV 16 positivity among HIV positive cervical cancer patients.

V. Conclusion

From this study we may conclude that there is a strong relation between HPV 16 infection and cancerous lesions of cervix (both pre-invasive and invasive cervical cancer) in this eastern part of India, which is similar to that reported in other parts of India and worldwide. Most of the epidemiological factors of cancer cervix seem to be related to HPV infection, though statistical significance could not be established. We may also suggest that an effective vaccine targeting HPV 16 will reduce the cervical cancer burden and HPV detection may have a role in population based screening program.

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