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Original Research Article

A demographic and clinico pathological study of HPV associated cofactors in the pathogenesis of cervical cancer

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ABSTRACT

Aims: Cervical carcinogenesis is a multi-step process associated with refractory infection by high-risk human papillomavirus (HPV) types. Only a minority of HPV infected women develop cervical intraepithelial neoplasia (CIN) or cervical cancer, indicating that HPV infection is not the sole risk factor to induce cervical cancer. The present study aimed to identify the association of demographic and clinic pathological factors in persistent high risk HPV infections of cervix and progression to cervical cancer in East Indian women.

Materials and Methods: Study subjects comprised 71 women with histologically proven cervical cancer, and 100 women with benign cervical lesions. The case group included HPV 16 +ve subjects with malignancy. Among subjects with benign lesions, 43 were HPV 16 +ve (intermediate group) and 57 were HPV – ve (control group). Demographic factors like age at diagnosis, age at first childbirth, parity, postmenopausal status, literacy, smoking and clinic pathological factors like type of cervical cancer and grading were correlated with HPV infections and cervical cancer. HPV status and HPV Type was confirmed by PCR based method, using specific primers.

Results: There was significant association between HPV +ve individuals (both cases and intermediates) and increased parity, post menopause, increased age at diagnosis, decreased age at first childbirth, illiteracy and smoking. All individuals of the case group were HPV +ve, type 16 and most had poorly differentiated and stage III squamous cell carcinoma of the cervix.

Conclusion: Our study suggests that demographic factors should be taken into account when screening for HPV infectivity and cervical cancer.

1. Introduction

Cervical cancer is the fourth most frequent cancer in women worldwide with 570,000 new cases in 2018 representing 6.6% of all female cancers. Approximately 90% of deaths from cervical cancer occurred in low- and middle-income countries. In India, about 60,078 cervical cancer deaths occur annually (estimates for 2018), responsible for 6.6% of all female cancer. Mortality from cervical cancer in India is 4.2 million, and cervical cancer ranks as the second leading cause of female cancer deaths in India in the 15 to 44 years age group.[1]

Cervical carcinogenesis is a multi-step process and persistent infection by certain oncogenic Human Papilloma Virus (HPV) types has been found to be the necessary cause of most premalignant and malignant cervical epithelial lesions. There are more than 100 known HPV genotypes, at least 15 of which are oncogenic. HPV 16 and 18, the two most common oncogenic types, cause approximately 70% of all cervical cancers worldwide.[2]

The immune response of the host plays a crucial role in the progression or regression of hrHPV infection of the uterine cervix. An effective immune response promotes spontaneous clearance of the virus, while a compromised immune response often starts up the pathological process, developing it into the higher grade cervical dysplasia. The

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HPV oncoproteins E6 and E7 play a key role in cervical carcinogenesis via the disturbance of apoptosis, the cell cycle, and adaptive immune surveillance.

It takes 15 to 20 years for cervical cancer to develop in women with normal immune systems. It can take only 5 to 10 years in women with weakened immune systems, such as those with untreated HIV infection. Apart from innate immunity, certain demographic and socio-economic factors are associated with persistence of HPV infection and cervical carcinogenesis.

A wareness of risk factors for cervical cancer, particularly the role of HPV, has been low in the general population for a long time.[3–5] Today, population cervical screening programmes are increasingly adopting HPV-based screening.[6] Identification of HPV cofactors, which in addition to HPV infection increase the risk for precursor lesions of cervical cancer, will allow a better understanding of the natural history of HPV and cervical carcinogenesis.[7]

The common sexually transmitted high-risk human papillomavirus (Hr HPV) is found to be a necessary factor for cervical cancer.[8] Most newly acquired HPV infections are transient, but a small fraction of persistent infections are involved in the development of cervical cancer and its immediate precursor lesions.[9,10]

2. Aims and Objectives

2.1. Aim

To understand the role of demographic factors on the development of HPV related cervical cancers.

2.2. Objectives

1. To estimate the prevalence of HPV16/18 infections in various categories of cervical samples (grouped according to histopathology, presence/absence of HPV infection).
2. To check the association of HPV infections with clinicopathological and demographical factors and assess their potential as early markers of development of cervical cancer.

3. Materials and Methods

3.1. Recruitment of subjects

This is a case - control study, conducted in the period fr om October 2015 to April 2017 on East Indian women of the state of West Bengal, India, who attended Saroj Gupta Cancer Centre Research Institute, West Bengal, Jwaharlal Nehru Medical College, West Bengal and Gynecology OPD of Kolkata Medical College, West Bengal Study sample included 171 women; case group comprised 71 women, control group comprised 100 women, of which 43 were found to have HPV infection and 57 were HPV–ve. Women with history of chronic or recurrent pruritus vulvae and leucorrhoea, persistent abnormal vaginal bleeding (like postcoital, post menopausal bleeding or menorrhagia) and persistent cervical lesions (cervical hypertrophy, erosion, ulceration, cervical growth) were included in the study. Women with history of recent childbirth, miscarriage/abortions (within previous 4 months), menstruation at the time of visit, history of prior treatment for cervical malignancy, pregnant & unmarried women were excluded from the study. A questionnaire was used to collect information from patients on clinical history, demographic data, life style and reproductive factors. Intervention was per speculum cervical examination, with cervical smear or cervical punch biopsy. Histopathological report of cervical biopsy samples, were recorded in the questionnaire form.

3.2. Ethical clearance

All samples were collected from the study participants with informed consent approved by the Institutional Ethical Committee.

3.3. Sample collection

Ecto-cervical and endo-cervical tissue samples were collected from subjects for cytopathological examination.

3.4. Detection of HPV positivity

DNA was isolated from all cervical tissue samples using the QIAamp DNA mini kit according to the manufacturer's protocol. All samples were screened for the presence of HPV infection with PCR, using L1 consensus primers: MYO11 and MYO9. L1 negative samples were reamplified with nested GP 5/6 primers for further HPV screening. The amplified PCR products were subject to electrophoresis on a 2% agarose gel and amplified bands (150bp) were visualized under UV light after staining with ethidium bromide. (Figures 1 and 2) The primer sequences are shown in Table 1. The samples that were negative for both the primers were considered to be HPV-ve. The samples that were positive for either primer were considered to be HPV +ve.

3.5. Detection of HPV type 16 and 18

HPV +ve samples were typed by specific primers homologous to the E6 region of HPV-16 and 18. (Figures 3 and 4)

HPV 18 +ve were few, so the study was concentrated on HPV 16 +ve and HPV 16 –ve samples. Samples which were histopathologically confirmed squamous cell carcinoma were classified as the case group, non-malignant HPV-ve samples were classified as the control group and non-malignant HPV 16 +ve samples were classified as the intermediate group.

Table 1: Primer sequences

Target Name	Primer sequence	Product length
L1	Forward primer : 5' GCM CAG GGW CAT AAT AAY CC-3' Reverse primer: 5'- CGT CCM ARR GGA WAC TGA TC-3'	454bps
GP5/6	Forward primer: 5'TTG GTT ACT GTG GTA GAT ACT AC-3' Reverse primer 5' GGA AAA TAA ACT GAT AAT CAT ATT C3'	150bps
HPV16	Forward primer 5'TCA AAA GCC ACT GTG TCC TG 3' Reverse primer 5' CGT GTT CTT GAT GAT CTG CA 3'	116bps
Hpv18	Forward primer 5' ACC TTA ATG AAA AAC CAC GA 3' Reverse primer 5' CGT CGT TGG AGT CGT TCC TG 3'	100bps

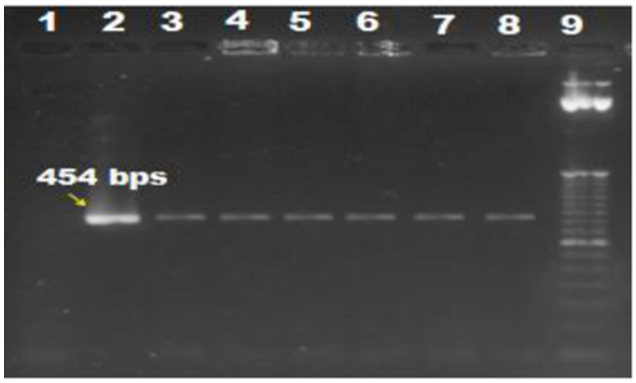


Fig. 1: Gel doc images of samples with L1 primers Lane 1 – negative control, Lane 2 –positive control, lane 9 – 50bp marker, Lanes 3 – 8 test samples. All showpositive bands at 450bp, thus all are HPV +ve.

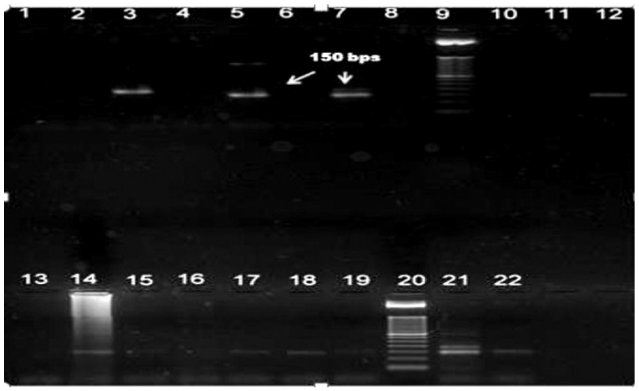


Fig. 2: Gel doc images of samples with GP5/6 primers Lane 1-negative control; lanes 2 to 8, test samples; lane 9 - 50bp ladder; lanes- 10 to 19 test samples and lane 21,22- positive controls (cell line DNA Caski, SiHa).

3.6. Statistical analysis

The number and percentage of patients were compared across the groups using Pearson's Chi Square test for Independence of Attributes (software: SPSS version 20). The changes in expression levels were tested for statistical significance using non-parametric test (Mann-Whitney U Test). (SPSS version 20). $P < 0.05$ was considered as statistically significant.

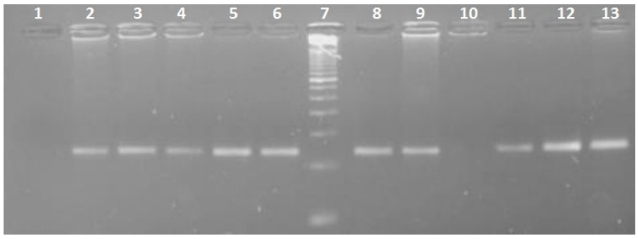


Fig. 3: Gel doc images of samples with HPV 16 primers Lane 1-negative control; lanes 2 to 6 test samples; lane 7- 50bp ladder; lane 8 to 11- test samples; lane 12,13 positive controls (HPV16 +ve cell line DNAs- SiHa, Caski).

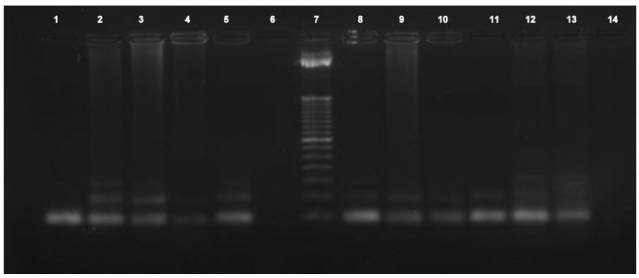


Fig. 4: Gel doc images of samples with HPV 18 primers Lane 1-negative control; lanes 2 to 6test samples; lane 7- 50bp ladder; lane 8 to 12- test samples; lane 13 positivecontrol (HPV 18 +ve cell line DNA-HeLa).

4. Results

Demographic characteristics have been depicted in Tables 2 and 3. The age at diagnosis, menopausal status, age at menopause, age at first childbirth, parity, education, and smoking habit have been considered.

The mean age of subjects with malignancy appeared to be significantly greater (p value .009) compared to controls, but there was no significant difference between case and intermediate (HPV+ve control) or between intermediate and control (HPV–ve).

The age at first childbirth is significantly lower among cases than among controls (p value < 0.001) and intermediates (p value 0.001)

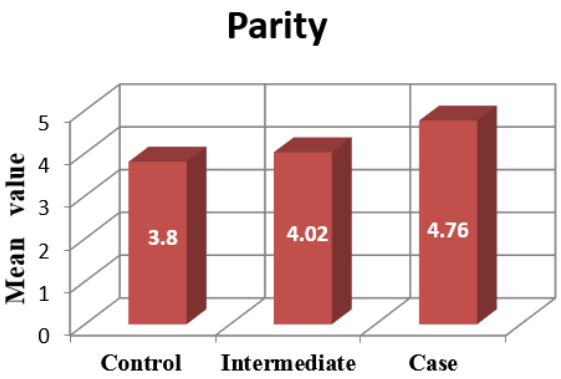
Age at menopause does not appear to be significantly different among the cases, intermediates or controls.

Parity among cases is higher than controls (p value 0.002) and also higher than in intermediates (p value 0.028).

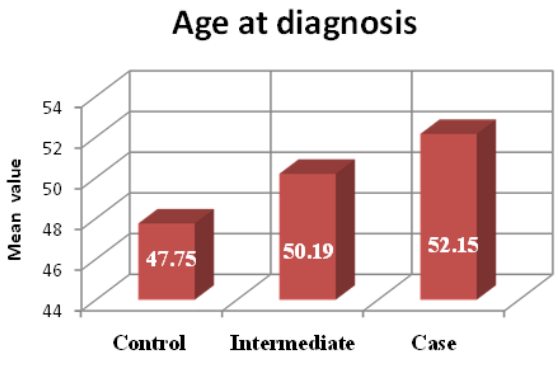
Illiteracy is significantly higher among case than controls (p value <0.001) and also between case and intermediates (p value 0.001) but there was no significant difference between intermediates and controls.

Regarding menopause at the time of diagnosis, post-menopausal women were significantly more in the case group than in the control group or the intermediate group (p value <.001 and p value .038 respectively.

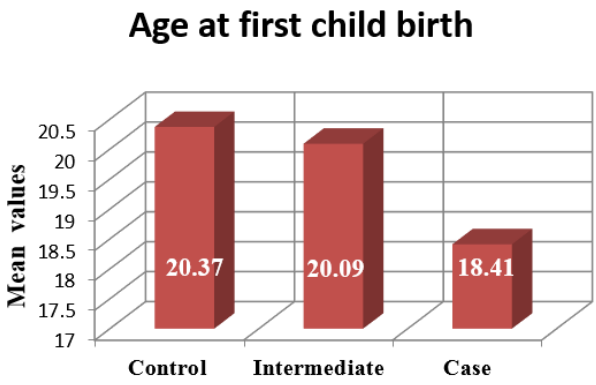
Smoking history shows significant difference among case and control (p value <0.001) and among intermediate and control (p value < 0.001) but no significant difference among case and intermediate (p value-0.309).



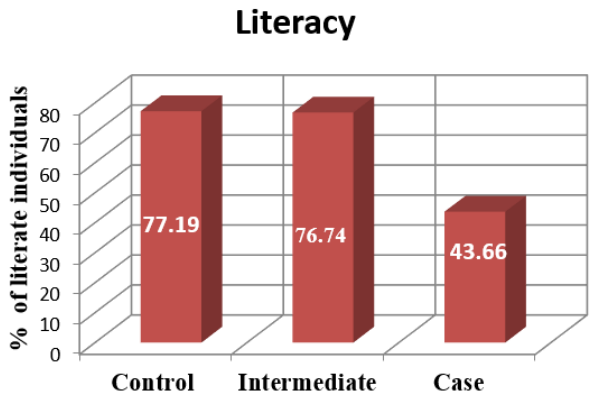
Graph 1: Showing correlation between parity and case, intermediates and controls



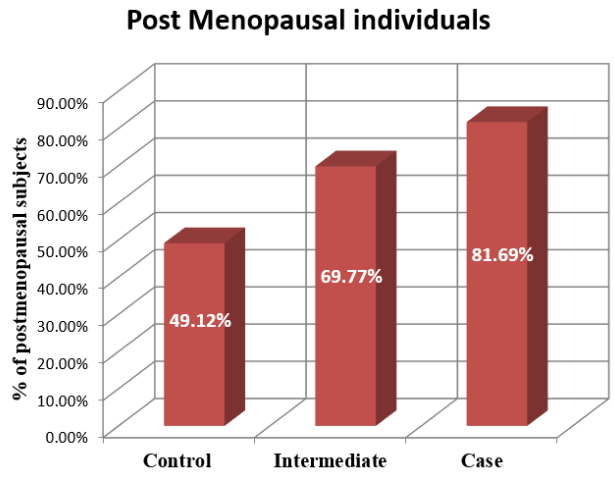
Graph 2: Showing correlation between age at diagnosis and case, intermediates and controls



Graph 3: Showing correlation between age at first child birth and case, intermediates and controls



Graph 4: Showing correlation between literacy and case, intermediates and control



Graph 5: Showing correlation between post menopausal status and case, intermediates and controls

Table 2: Demographic table

Attibutes	Cases n=71		HPV+ve nonmalignants n=43		Controls n=57		
	Range	Mean +/-SD	Range	Mean +/-SD	Range	Mean +/-SD	Significant P value
Age at diagnosis (years)	32-73	52.15 ± 10.02	35-75	50.19 ± 9.29	32-70	47.75 ± 10.12	Case vs control 0.009
Age at First Childbirth	13-21	18.41 ± 2.31	15-24	20.09 ±2.09	15-24	20.37 ± 2.06	Case vs control < 0.001 Case vs HPV +ve nonmalignant 0.001
Parity	2-11	4.76+/-1.89	0-9	4.02 +/- 1.87	1-9	3.80+/- 1.93	Case v control 0.002 Case v HPV +ve nonmalignants 0.028
Age at menopause	34-57	46.14 ± 5.11	33-50	44.66 ± 4.12	38-55	46.45 ± 3.76	Not significant

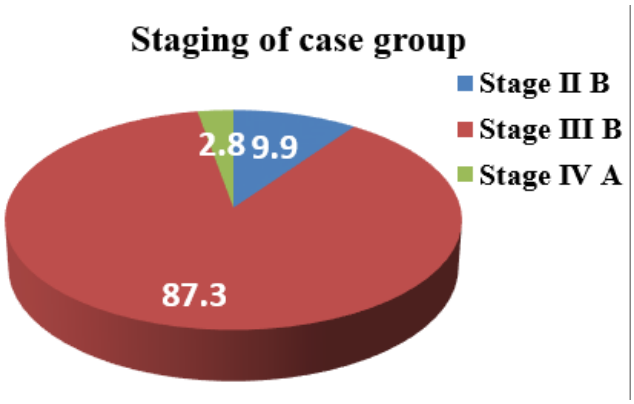
Table 3: Demographic table (Contd)

Attributes		Case n (%)	Intermediate n (%)	Control n (%)	Sig. P value
Literacy	Illiterate	40(56.34)	10(23.26)	13(22.81)	Case vs Control <0.001 Case vs Intermediate 0.001
	Literate	31(43.66)	33(76.74)	44(77.19)	
Menopause	premenopause	13(18.31)	13(30.23)	29(50.88)	Case vs Control <0.001 Intermediate vs Control 0.038
	postmenopause	58(81.69)	30(69.77)	28(49.12)	
Smoking	nonsmokers	44(61.97)	24(55.81)	10(17.54)	Case vs Control <0.001 Intermediate vs Control <0.001
	smokers	14(19.72)	6(13.95)	18(31.58)	
	Unknown	13(18.31)	13(30.23)	29(50.88)	

4.1. Analysis of the data by their clinico pathological and HPV status

Cases were grouped according to the stage and grade of cervical carcinoma as per pathologist's report and all belonged to the squamous cell carcinoma type. Of the 71 cases, none were in stage I, 7 (9.9%) were in stage II, 62 (87.3%) were in stage III and only 2 (2.8%) were in stage 4. After grading, it was found that 13 (17.57%) were well differentiated cancers, 8 (10.81%) were moderately differentiated and 50 (71.62%) were poorly differentiated.

HPV positivity was tested in all 171 samples – all the case samples were found to be HPV positive, while 43 of the nonmalignant samples were found to be HPV positive, (the intermediate group) and the remaining 57 were HPV –ve and taken as controls. The HPV positive samples were further classified according to types and all the intermediates were HPV 16 positive; 65 cases (91.55%) were HPV16 +ve and 6 cases (8.45%) were HPV18 +ve.

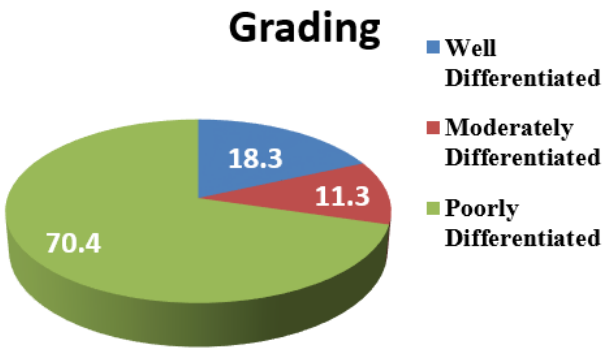


Graph 6: Pie chart for stage of the cases

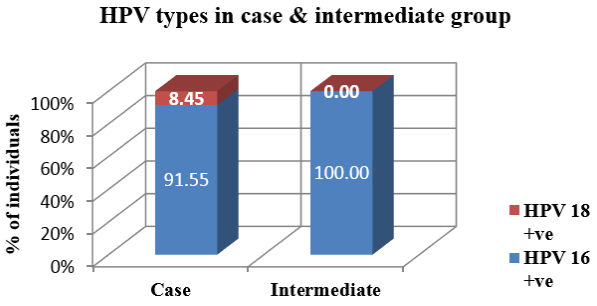
5. Discussion

The results of this study has been analyzed on demographic factors of the 3 different categories (Malignant group, HPV +ve nonmalignant group and HPV –ve nonmalignant control group), and the clinico-pathological factors in the malignant group.

Previous studies found sexual activity at a younger age to be a risk factor for persistent and recurrent HPV infection, which lead to cervical cancer.[11] In our country, age at marriage can be taken as age of first exposure to sexual activity in the general population. It is possible that age at first intercourse is related to ICC risk through HPV acquisition, perhaps due to early exposure to HPV infection and prolonged duration of infection.[12] A second possibility is that early first intercourse is a marker of high-risk sexual behavior for HPV exposure. This would be consistent with the high incidence of HPV infection in young women shortly after the first sexual intercourse.[13] A third possibility is that younger women are more susceptible to HPV infection.[14,15] Studies have also shown that greatest



Graph 7: Pie chart for grade of the cases



Graph 8: Graph for HPV 16 & 18 among cases and intermediates

metaplastic activity occur at puberty and pregnancy at the transformation zone of the cervix, leading to increased HPV infection, but progression from infection to malignancy is a slow process.[16] A steady increase in Invasive Cervical Cancer (ICC) risk with decreasing age at first intercourse has been observed. However, early age at menarche has not been implicated with persistent HPV infection and progression to ICC. This conclusion is also supported by a prospective study of HPV infection risk in the first years after menarche.[17]

In our study, the age at first childbirth is significantly lower among the malignant group than the HPV +ve nonmalignant group and the controls; Jenson et al, in his study of high-risk HPV+ women, found childbirth but not pregnancy to be predictive of CIN3+ during 13 years of follow-up.[18]

The effect of parity was particularly evident when the study population was restricted to women with persistent high-risk HPV infection. Several mechanisms such as increased hormone levels and impaired immune response have been suggested to explain the increased risk for precursor lesions or cervical cancer in relation to pregnancy and childbirth.[19] Furthermore, since the transformation zone remains on the ectocervix for a longer period in multiparous women, direct exposure to HPV is facilitated.[20]

Our study has found that parity was significantly higher among the malignant group, when compared to the control group and the non malignant HPV +ve individuals.

The peak age of occurrence of cervical cancer in India is between 55 and 59 years, and the highest age-adjusted rates are in Aizawl in the north-eastern part of India at 24.3 per 100,000 women.[21] In our study, the age at diagnosis is significantly higher among the malignant individuals compared to control group. However, the age at menopause in our study population is almost similar among the malignant, the nonmalignant HPV + ve and the control groups. Our study also reveals a significantly larger number of postmenopausal women among the malignant group than among either controls or the non malignant HPV + ve individuals. A similar observation has also been presented by Sabrina Zidi in a study in Tunisia, in 2014.[22] Postmenopausal women are infected with persistent oncogenic HPV at a high rate, highlighting the need for continued screening in postmenopausal women to detect preneoplastic genital lesions.[23]

Our study reveals that illiteracy is significantly more in the malignant individuals than in either controls or the non malignant HPV + ve individuals. Illiteracy is closely linked with multiparity and younger age at first child birth, thus explaining this demographic factor in our study results.

Jensen et al had previously identified heavy smoking to increase the subsequent risk for high-grade cervical lesions given persistent HPV infection.[24] Our study could not take smoking as co factor into consideration since most wome in all 3 categories (case, intermediate, control) denied

HPV-16 is the most commonly identified genotype in CIN-2+ lesions diagnosed in Chinese women with LSIL cytology, whereas HPV-18 is the most commonly identified genotype in patients with CIN-1 lesions.[25] In a study based in Northern Ireland, majority of the study population (64.5%) having high-risk (HR) HPV infection; 37.4% were positive for HPV-16 and 5.1% for HPV-18. HPV type-specific prevalence was 48.1%, 65.9%, 81.3%, 92.2%, and 64.3% among cervical intraepithelial neoplasias (CIN) Grades I-III, squamous cell carcinomas (SCC) and adenocarcinoma (AC) cases, respectively.[26] The clinico-pathological results of our study reveals that whole of the malignant group is HPV 16 or HPV 18 positive, which shows the close link between malignancy of the cervix and infection with high risk HPV (HPV 16 and 18). G.Y.F. Ho et al (1995) also found persistent HPV 16/18 infection to be the most important risk factor for progression to HSIL and cervical cancer.[27] All our malignant individuals belonged to squamous cell carcinoma group. Most were in stage III and poorly differentiated grade, indicating that cervical cancer is maximally detected in advanced stage and grade.

6. Summary

Of the 171 individuals included in the present study, 71 belonged to the malignant group, and the remaining 100 constituted the non- malignant group, which was further subdivided into a HPV +ve nonmalignant (n= 43) and a HPV –ve control group (n= 57). All the malignant cases were histologically proven squamous cell carcinoma and 91.55% belonged to stage III, 71.62% were of poorly differentiated grade. All were found to be HPV16 +ve,. Most malignant individuals were postmenopausal (81.69%) compared to controls (49.12%) and HPV +ve nonmalignants (69.77%). Mean age at diagnosis was higher (52.15yrs) in malignant group, compared to control group (47.75yrs) and HPV +ve nonmalignants (50.19yrs). Mean age at first child birth was lower in malignants (18.41yrs) than in controls (20.37yrs) and HPV +ve nonmalignants (20.09yrs). Malignant individuals had higher parity (mean value= 4.76) than controls (mean= 3.8) and the HPV 16+ve group (mean =4.0). Illiteracy among malignant group is 56.34% compared to 22.81% in controls and 23.26% in HPV +ve nonmalignants.

7. Conclusion

Our study shows that age of first childbirth, multiparity, illiteracy are important risk factors for progression of HPV infection to cancer, and screening for HPV infection should start from the time of marriage and continue even after menopause.

8. Acknowledgements

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9. Source of funding

None.

10. Conflict of interest

None.

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