Seminars /Conferences / Workshops attended
Seminar/Conference/Workshop attended:

1. Awarded for Best Poster Presentation in the 9th Annual Conference of Indian Society of Colposcopy and Cervical Pathology (ISCCP), from 22nd - 23rd February, 2014, held at Kolkata.

2. Presented poster at the 32nd Annual Convention of Indian Association for Cancer Research & International Symposium on "Infection & Cancer", from 13-16th February 2013, held at ACBR, New Delhi.


4. Presented poster at the 31st Annual Convention of Indian Association for Cancer Research & International Symposium on ‘Cancer Genomics and its impact in the clinics’ from 26th-29th January 2012, held at ACTREC, Navi Mumbai.


6. Attended the “Fifth Workshop on Genetic Epidemiological Methods for Dissecting of Complex Human Traits” organized by TCG-ISI centre for population genomics (CpG) and University of Pittsburgh, USA at Kolkata from 17th February-24th February 2010.

7. Presented poster at the “Symposium on Cervical Cancer and its Control in India”, organized by the Cancer Foundation of India, held on 4 December, 2009.


Prevalence of Human Papillomavirus in Women Without Cervical Cancer: A Population-based Study in Eastern India

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Summary: Despite the high incidence of cervical cancer, population-based data on prevalence of human papillomavirus (HPV) are limited in India. This study aimed to evaluate the prevalence of any HPV type and type-specific prevalence of HPV 16/18 in women without cervical cancer. HPV viral load was measured and correlated with cytologic abnormalities of the cervix. A total of 2501 women between 25 and 65 years of age and without cervical cancer were screened by pap smear cytology. HPV DNA was detected from cervical scrapes by nested polymerase chain reaction. Detection of HPV 16/18 was carried out by polymerase chain reaction using type-specific primers and was confirmed by Southern hybridization. Viral load was determined by absolute real-time polymerase chain reaction. Population prevalence of any HPV was found to be 9.9%. The risk of HPV infection was higher in women aged 25 to 34 years (odds ratio, 1.11), in married women below 20 years of age (odds ratio, 1.80), and in women with parity Z 4 (odds ratio, 1.04). Prevalence of HPV 18 (1.4%) was greater than that of HPV 16 (0.6%) in the overall screened population. High-grade squamous intraepithelial lesion cytology was more frequent in women infected with HPV 16 than in those infected with HPV 18 and other types. A gradual increase in HPV copy numbers was associated with progressive cytologic severity. In this study, HPV prevalence is comparable to HPV prevalence reported by other studies among Indian and Asian women. Although the prevalence of HPV 18 was more than that of HPV 16, type 16 infection was associated with higher oncogenicity. Key Words: Cervical cancer—HPV—Population prevalence—Viral load.
hospitals and have small sample size (4–6). The single community-based study with the highest sample size (N = 1799) was conducted in South India by Franceschi et al. (7). There is paucity of similar data from the eastern part of India.

The present study was undertaken to evaluate the overall HPV prevalence in women from eastern India who did not have cervical cancer. The study also aimed to determine the prevalence of HPV types 16 and 18, which are the most oncogenic of all HPVs and the primary targets of HPV vaccines. The relationship between HPV viral load and the severity of cervical cytologic abnormalities was also studied.

MATERIALS AND METHODS

Selection of Study Subjects

Women between 25 and 65 years of age and ever married, who underwent cervical cancer screening from September 2007 to March 2010 in a community-based demonstration screening program, were invited to participate. The screening program was conducted in the rural districts surrounding the city of Kolkata in eastern India. Women who had visible growth or ulcer in the cervix, had undergone hysterectomy, were pregnant, had cytologic suspicion of invasive cancer, or colposcopic/histologic diagnosis of invasive cancer at the time of screening were excluded. Written informed consent was obtained from the eligible women before recruitment into the study. The study was approved by the Research Ethics Committee of Chittaranjan National Cancer Institute. An enrollment questionnaire containing demographic characteristics was filled up for each participant by a health worker before screening.

Collection of Cervical Samples

Cervical smears for cytology were collected on glass slides using wooden Ayre spatulas and fixed immediately in 90% ethanol. The same spatulas were used to obtain repeat cervical scrapings that were placed in 2 mL of cold phosphate-buffered saline for HPV DNA testing. The fixed slides were sent to the Department of Pathology for pap stain/cytology, and the samples in phosphate-buffered saline were stored at 4°C for further processing.

Evaluation of Cervical Cytology

Cervical smears were stained with papanicolaou stain according to the standardized procedure. The pathologist interpreted and reported the slides according to Bethesda, 2001 classification. Cytologic diagnosis of atypical squamous cell of undetermined significance, atypical glandular cell of undetermined significance, or worse was considered to be abnormal and the women were referred for colposcopy.

Detection of HPV DNA

DNA was isolated from the cervical scrapes according to the procedure described by Singh et al. (8). Concentration of DNA was measured using an UV Spectrophotometer (Cary 100, Middleburg, The Netherlands). The mean DNA concentration of the samples was 1.45 ± 0.5 μg/μL. Integrity of DNA was ensured by polymerase chain reaction (PCR) for β Globin gene (9). The isolated DNA samples were tested for the presence of HPV by PCR in GeneAmp PCR System 9700 (Applied BioSystems, Foster City, CA) using primers from the L1 consensus region of the HPV genome and AmpliTaq Gold Taq DNA polymerase (10). MY 09/11 primers of the L1 region were used to detect 450 bp amplicons of HPV (11). The PCR products were electrophoresed in 2% agarose gel and stained with ethidium bromide for visualization under ultraviolet light and photographed. For confirmation of HPV presence in the samples, nested PCR was carried out by taking 2 μL of the PCR product and GP5+/6+ primers having complementary sequence within the amplicons of MY 09/11. The samples that showed PCR products of 155 bp size in the agarose gel electrophoresis as mentioned above were confirmed as HPV positive. In all the cases, HPV 16 and HPV 18 plasmids were used as positive controls.

Determination of HPV Types

The HPV-positive samples were then tested for the presence of HPV type 16/18 by PCR using HPV 16 type-specific primers from the E6 region and HPV 18-specific primer from the long control region (12). The PCR products were electrophoresed in 2% agarose gel and stained with ethidium bromide for visualization under ultraviolet light and photographed.

To confirm the HPV types, the PCR products in the agarose gels were transferred to a gene screen nylon membrane for Southern hybridization using 32P-labeled HPV type-specific probes (13).

Estimation of HPV Copy Number

HPV copy numbers in the HPV-positive samples were determined by the Power SYBR Green absolute real-time PCR method (Applied Biosystems) using
primers GP5+/6+ in ABI Prism 7500 (Applied Biosystems). The reaction was carried out in a 25 μL reaction volume having 12.5 μL of 2 × SYBR Green, 1 μL each of 10 pmols GP5+/6+ primers, and 100 ng of template DNA. The cycling condition was as follows: initial activation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 30 seconds, 48°C for 30 seconds, and 72°C for 1 minute; plate read. SDS 7500 raw data were analyzed by Microsoft Excel. For standard calibration, serial dilution of HPV-16 plasmid was used in 10-fold dilution series of 500 pg/μL, 50 pg/μL, 5 pg/μL, 50 fg/μL, 5 fg/μL, and 0.5 fg/μL; for negative calibration, β Globin primers were used.

Statistical Analysis
Univariate analysis was performed to estimate the risk factors associated with HPV prevalence, such as age, age at marriage, parity, and religion; odds ratios (OR) with 95% confidence intervals (CI) were calculated. All variables from the univariate analysis were entered in a multiple logistic regression model to adjust for all variables, and corresponding OR values with 95% CI were calculated for multivariate analysis. The χ² for linear trend was calculated for risk of HPV infection in cytologically normal and abnormal women. Epi Info, Centers for Disease Control and Prevention, was used for univariate analysis of data. For multivariate logistic regression analysis, the SPSS (SPSS Inc., Chicago, IL) statistical program was used.

RESULTS
The demographic characteristics of the study population are described in Table 1. The mean age of the women was 36.8 ± 7.7 years. The population prevalence of any HPV was estimated to be 9.9%, irrespective of cervical cytologic status. HPV prevalence by age, age at the time of marriage, and parity is shown in Table 2. Younger women (aged between 25 and 34 yr) showed relatively higher risk of HPV infection (OR, 1.11; CI, 0.85–1.47), although there was no statistically significant variation in HPV prevalence between the age groups. Women who were of younger age at the time of marriage (< 20 yr) and those having high parity (≥4) also had higher risk of HPV infection in the cervix. Lower HPV prevalence was observed among Muslim women compared with Hindu women (7.6% vs. 10.6%; P < 0.05), possibly because of the protection offered by circumcision of Muslim men.

Although 9.2% of women with normal cervical cytology were positive for any HPV, the prevalence of any HPV was 20.6% in women with abnormal cytology. Among women with different grades of cytologic abnormalities, the risk for HPV infection was highest in those having high-grade squamous intraepithelial lesion pap smear (OR, 10.76; CI, 3.52–33.24), with 53.3% of HPV prevalence (Table 3). A significant linear trend (χ² for trend 28.17, P < 0.000) of increasing risk of infection with increasing grades of cytologic abnormalities was observed.

<table>
<thead>
<tr>
<th>TABLE 1. Demographic characteristics of the screened population</th>
</tr>
</thead>
<tbody>
<tr>
<td>All women (N = 2501)</td>
</tr>
<tr>
<td>Age group</td>
</tr>
<tr>
<td>25–34 (1084) 113 (10.4) 1.11 (0.85–1.47)</td>
</tr>
<tr>
<td>35–44 (973) 92 (9.5) 0.94 (0.71–1.25)</td>
</tr>
<tr>
<td>45–54 (330) 31 (9.4) 0.94 (0.62–1.42)</td>
</tr>
<tr>
<td>55–65 (114) 11 (9.6) 0.87 (0.42–1.76)</td>
</tr>
<tr>
<td>Age at marriage (N)</td>
</tr>
<tr>
<td>&lt; 20 yr (1655) 190 (11.5) 1.80 (1.30–2.48)</td>
</tr>
<tr>
<td>20–25 yr (613) 49 (8.0) 0.74 (0.53–1.04)</td>
</tr>
<tr>
<td>&gt; 25 yr (233) 8 (3.4) 0.30 (0.14–0.64)</td>
</tr>
<tr>
<td>Parity (N)</td>
</tr>
<tr>
<td>0–1 (358) 41 (11.5) 0.82 (0.57–1.19)</td>
</tr>
<tr>
<td>2–3 (1492) 140 (9.4) 0.87 (0.66–1.55)</td>
</tr>
<tr>
<td>≥4(651) 66 (10.1) 1.04 (0.76–1.42)</td>
</tr>
</tbody>
</table>

CI indicates confidence interval; OD, odds ratio; HPV, human papillomavirus.
Interactions between participants’ age, age at the time of marriage, parity, and religion were considered as confounding variables for HPV infection among all women on multivariate analysis. After adjusting for the confounding variable, the risk for HPV infection was 1.2 times (OR, 1.22; CI, 0.84–1.76) in the age group of 25 to 34 years compared with other age groups. In addition, the risk for HPV infection was 6.8 times (OR, 6.81; CI, 3.80–12.19) among women who were aged <20 years at the time of marriage and 4.5 times (OR, 4.48; CI, 2.59–7.76) among women with parity ≥4.

HPV types 16 and 18 accounted for 21.0% of all HPV infections in the study population. The prevalence of HPV 18 (1.4%) in the screened women was greater than that of HPV 16 (0.6%) (Table 4). The highest prevalence of HPV 16 was observed in the 55-to-65-year age group, whereas HPV 18 was most frequently seen in a relatively younger age group of 45 to 54 years. However, no statistically significant trend with respect to age groups was found for either type 16 ($\chi^2$ for trend 0.01, $P = 0.913$) or type 18 ($\chi^2$ for trend 0.24, $P = 0.626$).

The prevalence of HPV 16 (0.5%) was lower than that of HPV 18 (1.3%) in women with normal cytology as well. Among women older than 44 years with a normal cytology (N = 462), none had HPV-16 infection and only 3 had HPV 18 infection.

Figure 1 shows the relative proportion of cytologic abnormalities in women infected with HPV 16, HPV 18, and HPV types other than 16/18. Although the prevalence of HPV 18 (4%) is higher than that of HPV 16 (2.7%) in women with an abnormal cytology, the prevalence of high-grade squamous intraepithelial lesion cytology was significantly higher in women infected with type 16, substantiating the higher oncogenic nature of type 16.

A gradual increase in HPV copy numbers was also observed with progressive cytologic severity (Fig. 2).

**DISCUSSION**

In an earlier study in the population residing in the same areas where the present study was conducted, we demonstrated that the population prevalence of oncogenic HPV by Hybrid Capture 2 method was 6.1% (14). In the present study, we demonstrated that the population prevalence of all types of HPV among women without cervical cancer was 9.9%.

The prevalence of HPV in women with a cytologically normal cervix (9.2%) in our study was lower than that of 11.7% in the same study (13). The higher prevalence of HPV 16 in the screened women in the 55-to-65-year age group, whereas HPV 18 was most frequently seen in a relatively younger age group of 45 to 54 years. However, no statistically significant trend with respect to age groups was found for either type 16 ($\chi^2$ for trend 0.01, $P = 0.913$) or type 18 ($\chi^2$ for trend 0.24, $P = 0.626$).

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**FIG. 1.** Relative proportion of cytologic abnormalities in women infected with HPV 16, HPV 18, and HPV types other than 16/18.

**TABLE 3.** Prevalence of human papillomavirus by cytology diagnosis

<table>
<thead>
<tr>
<th>Cytology</th>
<th>n (%</th>
<th>HPV positive (%)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>2313 (93.9)</td>
<td>212 (9.2)</td>
<td>1</td>
</tr>
<tr>
<td>ASCUS/AGUS</td>
<td>105 (4.3)</td>
<td>20 (19.0)</td>
<td>2.25 (1.31–3.83)</td>
</tr>
<tr>
<td>LSIL</td>
<td>30 (1.2)</td>
<td>3 (10.0)</td>
<td>1.02 (0.24–3.53)</td>
</tr>
<tr>
<td>HSIL</td>
<td>15 (0.6)</td>
<td>8 (53.3)</td>
<td>10.76 (3.52–33.24)</td>
</tr>
<tr>
<td>Total (N)</td>
<td>2463*</td>
<td>243</td>
<td></td>
</tr>
<tr>
<td>$\chi^2$ for trend: 28.173, $P &lt; 0.000$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*In 38 (1.5%) samples, cytology was unsatisfactory, and these were excluded from analysis. Four of them were HPV positive, of which 1 was HPV 18 positive.

AGUS indicates atypical glandular cell of undetermined significance; ASCUS, atypical squamous cell of undetermined significance; CI, confidence interval; HSIL, high-grade squamous intraepithelial lesion; LSIL, low-grade squamous intraepithelial lesion; OR, odds ratio.

**TABLE 4.** Human papillomavirus type 16 and 18 prevalence among all screened women and cytologically normal women, stratified by age groups

<table>
<thead>
<tr>
<th>Age group</th>
<th>n</th>
<th>HPV 16 (%)</th>
<th>HPV 18 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All screened women</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25–34</td>
<td>1084</td>
<td>6 (0.6)</td>
<td>18 (1.7)</td>
</tr>
<tr>
<td>35–44</td>
<td>973</td>
<td>8 (0.8)</td>
<td>11 (1.1)</td>
</tr>
<tr>
<td>45–54</td>
<td>530</td>
<td>1 (0.3)</td>
<td>6 (1.8)</td>
</tr>
<tr>
<td>55–65</td>
<td>114</td>
<td>1 (0.9)</td>
<td>1 (0.9)</td>
</tr>
<tr>
<td>Total</td>
<td>2501</td>
<td>16 (0.6)</td>
<td>36 (1.4)</td>
</tr>
<tr>
<td>Women with normal cytology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25–34</td>
<td>995</td>
<td>6 (0.6)</td>
<td>15 (1.5)</td>
</tr>
<tr>
<td>35–44</td>
<td>856</td>
<td>6 (0.7)</td>
<td>11 (1.3)</td>
</tr>
<tr>
<td>45–54</td>
<td>346</td>
<td>0</td>
<td>3 (0.9)</td>
</tr>
<tr>
<td>55–65</td>
<td>116</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>2313</td>
<td>12 (0.5)</td>
<td>29 (1.3)</td>
</tr>
</tbody>
</table>

HPV indicates human papillomavirus.
observed that the HPV-16 copy number increases with increasing epithelial abnormalities from normal cytology to cervical intraepithelial neoplasia I, II, III, or rapid neoplastic progression of cervical intraepithelial neoplasia I cases (20,21). In conformity with such findings, we also observed a linear correlation between all HPV viral load and grades of cervical abnormality.

The major limitation of our study is that we looked only for types 16 and 18 as they are the most oncogenic types. We propose to analyze stored positive samples to identify genotypes other than types 16 and 18.

In conclusion, the HPV prevalence in the studied population from eastern India is similar to that of other parts of the country, although HPV 18 was more frequent than HPV 16. The significance of such a finding has to be investigated further.

REFERENCES


**FIG. 2.** Box plot showing distribution of human papillomavirus (HPV) copy number in normal cervix (N = 207), atypical squamous glandular cell of undetermined significance (AGUS)/atypical glandular cell of undetermined significance (AGUS) (N = 20), low-grade squamous intraepithelial lesion (LSIL) (N = 3), and high-grade squamous intraepithelial lesion (HSIL) (N = 8). Copy numbers were plotted in the graph as log (HPV copies/100 ng of cellular gDNA). The median value of each sample group, connected through a polynomial trend line, showed gradual increase in HPV copy number with progressive cytologic severity.

The population prevalence of HPV 16 in our study (0.6%) was less compared with that reported in Asian (1.7%) and Indian populations (3.8%) (7,15). Interestingly, our study observed higher prevalence of type 18 compared with type 16 in women with normal cervical cytology. However, in women with high-grade cytologic abnormalities, the proportion of HPV type 16 was double that of type 18. A study conducted in the north-eastern part of India also observed higher prevalence of type 18 compared with type 16 in the population (18).

The viral load of HPV plays a cofactorial role in cervical carcinogenesis (19). Earlier studies have comparable to overall HPV prevalence among Asian women with no cervical disease (8.7%) reported by the pooled meta-analysis from the International Agency for Research on Cancer (15). The study by Franceschi et al. (7) observed a higher prevalence (14.2%) among Indian women possibly because their samples included women of younger age compared with ours. Similar to other studies in India, we also did not observe any variation in HPV prevalence with age (3,14). In the population we studied, male circumcision is a common practice among those belonging to the Muslim religion. The protective effect of male circumcision on the risk of HPV transmission to female partners that was reported by some authors has been reflected in the significantly lower HPV prevalence among the Muslim women in our study (16,17). Lower age at the time of marriage and high parity were observed as significant risk factors for HPV infection.

The population prevalence of HPV 16 in our study (0.6%) was less compared with that reported in Asian (1.7%) and Indian populations (3.8%) (7,15). Interestingly, our study observed higher prevalence of type 18 compared with type 16 in women with normal cervical cytology. However, in women with high-grade cytologic abnormalities, the proportion of HPV type 16 was double that of type 18. A study conducted in the north-eastern part of India also observed higher prevalence of type 18 compared with type 16 in the population (18).

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*S. DUTTA ET AL.*

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GENETIC AND EPIGENETIC PROFILING OF HIGH RISK HPVS IN NORMAL WOMEN AND CERVICAL CARCINOMA PATIENTS OF INDIA

Thesis Abstract:

Aim of this study was to understand how the genetic and epigenetic profile of High Risk Human Papillomavirus (HR HPV) varies during development of uterine cervical carcinoma (CaCx) from asymptomatic infection in the cervical epithelium. For this, at first prevalence of HR HPV infection was detected in women population and CaCx patients of India, followed by identification of susceptible immunogenetic alleles for persistent viral infection in cervix. Then, changes in genetic (physical status, viral copy number, sequence variation of the HR HPV16 genome) and epigenetic (methylation of the viral enhancer and the early-late promoters) profile of HR HPV16 genome were analyzed from asymptomatic infection to pre-neoplastic to CaCx samples. In addition, to understand the prognostic importance of these profiles of HR HPV16, comparative analysis was made in post-therapeutic cervical swabs and Circulating Tumor Cells (CTC) in plasma in pre and post therapeutic conditions.

In population based screening (N=4500), the prevalence of HPV infection in cervix was 7.6% and prevalence of HR HPV16 and HR HPV18 were 2.7% and 1%, respectively. The HPV viral load gradually increased with cervical abnormalities. The persistence of HR HPV16/18 might be due to the presence of IL-1β -511T-allele and HLA-DQB1*03 A-allele in cases because of its association with the development of pre-neoplastic cervical lesions and/or CaCx. More importantly, the women containing both the polymorphic A and T-alleles were more susceptible to develop CaCx.

During development of CaCx, the episomal form of HPV16 was prevalent in asymptomatic (N=93) and pre-neoplastic (N=28) samples followed by significant increase (p=0.01) of integrated form in CaCx samples (N=98). The methylation profile of integrated HPV16 in asymptomatic infection, pre-neoplastic and CaCx samples showed similar pattern, with comparatively lower early-promoter methylation and higher late-promoter methylation. In contrary, the asymptomatic and pre-neoplastic samples with the episomal form showed higher early-promoter methylation and lower late-promoter methylation.

Prevalence of HPV16 in pre/post-therapy plasma was significantly associated (p=0.03) with high viral load in the corresponding primary tumor site of cervix. In the plasma of pre or post-therapy CaCx patients, integrated form of HPV16 was predominant with significantly increased hypomethylation of early promoter (p<0.01) and hypermethylation of late promoter (p<0.01), than the corresponding cervical samples. After therapy, the integrated form was also more persistent in cervical swabs, with hypomethylation of the early enhancer (p<0.05) and late-promoter (p<0.01). Importantly, the patients with differential methylation of HPV16 promoters in plasma and higher viral-load in primary tumor site showed poor prognosis with metastasis in distant organs.

Thus, our data indicated that the persistent HR HPV16 infection in the asymptomatic women followed by its changes in genetic and epigenetic profile occurred during development of CaCx. Moreover, the presence of HR HPV16 in plasma and its profile in pre and post therapeutic CaCx patients has prognostic importance.