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Prevalence of Human Papillomavirus, Cytomegalovirus and *Chlamydia trachomatis* among Women with Normal Cervical Cytology and their Impact on TLRs Expression

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ABSTRACT

To study the prevalence of HPV infection and its associated co-infection among sexually active women with normal cytology and to correlate the infection status with TLR expression. In a population based study, first voided urine samples were collected from 370 sexually active women with normal cervical cytology. The presence of HPV, CMV and CT infections were by PCR based approach. The expression pattern of TLR2 and TLR4 was analyzed in formalin fixed cervical tissue samples by immunohistochemistry. An overall HPV, CMV and CT prevalence of 22.6, 6.8 and 2.3%, respectively was reported among the study population. Further it was observed that women with HPV infections were more prone to CMV (OR: 1.79, 95% CI 0.69-4.63) and CT (OR: 22.41, 95% CI 2.65-189.48) infections. The immunostaining of the formalin fixed blocks with anti-TLR2 and TLR4 showed surface expression of TLRs in cervical squamous with no infection and TLR2 and TLR4 immunoreactivity in infected cervical samples were markedly negative. The infection of the cervix with HPV, CMV and CT decreases TLR2 and TLR4 expression in cervical squamous and thus abolishes the innate immune responses. The abolished TLR response may be a crucial step in the carcinogenic events mediated by infectious agents.

Key words: HPV, normal cytology, prevalence, TLR2, TLR4

INTRODUCTION

Cancer of the uterine cervix is the second most common cancer among women worldwide. It is the leading cancer among Indian women and considered as a serious public health problem of global importance (Gheit *et al*., 2009, 2014; Bhatla and Maheswari, 2009). Epidemiological and experimental data have shown cervical cancer to be linked to infection with certain genotypes of Human Papilloma Virus (HPV) (Van Tine *et al*., 2004). Acquisition of HPV is especially common among young sexually active women with 3-year cumulative incidence estimated to be more than 40% (Ho *et al*., 1998; Woodman *et al*., 2007). A cross-sectional evaluation of the HPV prevalence in 13 countries estimated that 6.6% of women in the age range of 15-74 years with normal cytology are carriers of HPV DNA, with marked variation within and between world regions range (1.4-25.6%). The crude and adjusted HPV prevalence estimates in 157879 women

with normal cytology, stratified by 15 world regions showed crude HPV prevalence among women with normal cytology to be 10%. The corresponding adjusted prevalence estimate was 10.4, 95% CI 10.2-10.7 (Vinodhini *et al*., 2012a).

However, most HPV infections spontaneously regress and only in a small percentage of cases the infection persists and causes low-grade intraepithelial lesions (LSIL) that progress to high-grade intraepithelial lesions (HSIL) and ultimately, develop into invasive cervical carcinoma (Hariri *et al*., 2012). It is now accepted that persistent infection by high-risk HPVs is a necessary but nonsufficient condition for the development of cervical cancer (Burd, 2003). Several epidemiological studies have shown concurrent bacterial or viral infections with increased HPV persistence to be closely associated to the progression of cervical carcinoma.

Cytomegalovirus (CMV), a herpesvirus frequently infects the cervix and produces persistent infections of the genitourinary tract. *Chlamydia trachomatis* (CT) infection was examined as a cause of Invasive Cervical Cancer (ICC) among women with HPV infection. The infection with CT has been observed to increase the risk of squamous cervical cancer among HPV-positive women. Generally, CMV and CT are found to be the predominant co-infecting partners of HPV. Thus the presence of CT and CMV co-infections might promote HPV persistence or enhance its oncogenicity. With this view point sexually active women with normal cervical cytology were screened for the presence of HPV infection and for their co-infections with CMV and CT. Further studied whether persistent infection of the cervix epithelium induces TLR-mediated immune response which might be a critical cofactor for the development of cervical lesions.

MATERIALS AND METHODS

Study sites: A population based study was conducted as a part of our screening program to estimate the prevalence of HPV and its associated co-infections including CT and CMV among sexually active women with normal cytology from three taluks (Musiri, Mannachanallur and Tiruchirapalli) of Tiruchirappalli district, Tamilnadu, India between January 2013 and April 2013.

Study subjects: Approximately 120 first voided urine samples were collected from each stratum (taluks) making a total of 370 samples from sexually active women aged between 20 and 65 years. Apart from this, three cervical biopsy samples from women undergoing hysterectomy for reasons other than anogenital tract cancers and confirmed to have normal pathology were collected from Annal Mahatma Gandhi General Hospital, Tiruchirapalli and was used as control. The inclusion criteria for the study subjects were: (1) Negative diagnosis for any kind of cancer, (2) No history of hysterectomy or cervical colonization, (3) No physical or mental problems, (4) not pregnant, (5) not received antibiotics for the past six months. The study has been approved by the Institutional Ethics Committee of Bharathidasan University (DM/07/101/373).

Sample collection: To reach women from each stratum, collaboration with the local health care centers and Governmental Hospitals was set up. The public health nurses invited participation by visiting women at home and informed them about the risk factors, prevention, early detection and pretreatment of cervical carcinoma. The purpose and procedures of the study were explained to study subjects and informed written consent was obtained from all the participants. The women who gave consent to participate were included and visual inspection of the cervix was done for all participating women and a smear was diagnosed by cytoscreeners to confirm normal cytology. First voided urine samples were collected from participants in a 50 mL falcon tubes and the samples

were immediately transported to lab on ice. The samples were refrigerated and stored at 4°C before processing. The women included in the study were interviewed for characteristics such as age, age at first sexual intercourse, number of sexual partners, number of pregnancies and paan (Betal leaf combined with areca nut cured tobacco) chewing habits.

Sample processing: The collected urine samples were centrifuged at 3000 g for 20 min. After centrifugation the supernatant was discarded and the resultant cell pellets were washed twice in ice-cold 1X PBS and stored at -20°C until use. The collected biopsy samples were placed immediately in ice cold PBS and transported to the laboratory on ice and stored at -20°C until use. DNA was extracted from cellular pellets by guanidium thiocyanate method (Thilagavathi *et al*., 2012) and from biopsy samples by phenol-chloroform method (Vinodhini *et al*., 2012b). To test the integrity of DNA obtained, a PCR for human genomic β-globulin gene using specific primers was performed.

Detection of HPV, CMV and CT infection: A PCR based detection for HPV, CMV and CT infection was adopted (Shanmughapriya *et al.,* 2012). The DNA samples were tested by the MY09/11 PCR protocol. The samples negative for MY09/11 were further assessed for the presence of HPV DNA with general $GP5^+/6^+$ PCR system. CT infection was detected by a nested PCR based assay using KL5/KL6 and KL1/KL2 primers complementary to sequence of the cryptic plasmid. CMV infection was detected by a nested PCR based assay using external and internal primers specific for CMV glycoprotein B gene. The expected product size of 150, 350 and 150 bp for HPV, CT and CMV respectively was visualized on a 2% agarose gel stained with ethidium bromide.

Immunohistochemistry: Immunohistochemistry analysis was performed to correlate the status of TLR responses in cervical tissues during the studied bacterial and viral infections. Immunohistochemistry using formalin fixed paraffin embedded tissue blocks corresponding to the biopsy samples used for PCR based detection was carried out. The sections were deparaffinised followed by antigen retrieval (autoclave retrieval at 121°C for 10 min in 10 mM sodium citrate buffer, pH 6.0). Polyclonal antibodies to TLR-2 and TLR-4 (Biovision, USA) was diluted in 1:50 in blocking solution and reacted overnight at 4°C. An anti-mouse IgG HRP detection system with diaminobenzidine tetrachloride (Sigma Aldrich, USA) was used. The sections were counter stained with hematoxylin and embedded. The results of immunohistochemistry were interpreted as strongly positive (+3) only when >10% of the tumour cells demonstrated a strong complete membrane staining as weakly positive (+2) when weak to moderate complete membrane staining was observed in >10% of the tumour cells and as negative (0/+1) when a barely perceptible or no membranous staining was observed.

Data analysis: All data were reviewed for completeness at the end of each interview. The data were coded and analyzed using the SPSS package version 11.0. Univariate analysis was under taken to screen potentially significant risk variables for HPV, CMV and CT infections. The odds ratio and the corresponding 95% confidence intervals were calculated by unconditional logistic regression and maximum likelihood estimation. Tests of statistical significance were based on difference in the log likelihoods and all p values are 2-sided.

RESULTS

A total of 370 first voided urine samples were obtained from women who met the inclusion criteria. Of the 370 samples, 47 missed the cellular pellets and hence discarded. DNA was extracted

from the remaining 323 samples and the quality of the DNA was analyzed on a 0.8% agarose gel stained with ethidium bromide. The integrity of the extracted DNA samples was analyzed by PCR for the β-globulin gene. Of the 323, 13 samples were excluded because they were found negative for β-globulin. The final study group comprised of 310 women with no abnormal cervical cytology.

The GP5+/6+PCR based analysis to study the prevalence of HPV infection among 310 women showed 70 (22.6%) samples to be positive for HPV infection, with Manachanallur showing the highest prevalence of 27.2%, followed by Musiri (25.2%) and Tiruchirapalli (11.5%). The nested PCR based analysis to study the prevalence of CMV infection showed 21 (6.8%) to be positive, with Tiruchirapalli showing the highest prevalence of 10.3%, followed by Musiri (7.2%) and Manachanallur (3.7%). Among 310 samples, 7 (2.3%) were CT positive, with Manachanallur showing the highest prevalence of 3.7%, followed by Musiri (2.4%) and none from Tiruchirapalli (Table 1).

Table 2 summarizes the major factors associated with HPV infection. Early age at first coitus (OR 2.67, 95% CI 0.95-7.38), being never pregnant (OR 3.08, 95% CI 0.89-10.57), paan chewing (OR 3.59, 95% CI 0.70-18.42) and nulliparity (OR 2.05, 95% CI 0.57-7.32) showed significant association with HPV infection.

The study was extended to evaluate CMV and CT co-infections among HPV positive women. To examine mutual relationships between HPV and CMV or HPV and CT infections, we calculated Odds Ratios (ORs) with their 95% confidence intervals (95% CI). Accordingly we found that women with HPV infections (10%, 7/70) were more likely to be co-infected with CMV than those without HPV infections (5.6%, 14/240), with an odds ratio of 1.79 (95% CI 0.69-4.63). Similarly CT co-infection was more predominant among women with HPV infections (8.6%, 6/70) than those without HPV infections (0.42%, 1/240), with an odds ratio of 22.41 (95% CI 2.65-189.48) (Table 3).

Next we hypothesized whether persistent bacterial and viral infection of the cervical epithelium induces TLR2 and TLR4-mediated inflammation, a critical cofactor for cervical lesion. To analyze the correlation between different bacterial and viral infection with TLR2 and TLR4 response the formalin fixed samples from patients undergoing hysterectomy for reasons other than anogenital cancer were used. The corresponding fresh biopsy samples of the formalin fixed blocks were analyzed for HPV, CMV and CT infection by PCR based approach as described earlier. Of the three samples analyzed one each were found to be positive for none, HPV and HPV/CMV infection. The immunostaining of the formalin fixed blocks with anti-TLR2 and anti-TLR4 showed surface expression of TLR2 and TLR4 in cervical squamous with no infection (Fig. 1a). Surprisingly no staining with TLR2 and TLR4 was detected in cervical columnar epithelial cells infected with HPV alone as well as in HPV and CMV co-infection (Fig. 1b and c). TLR2 and TLR4 immunoreactivity in infected cervical samples was markedly negative. Although sample size was limited, the differences of TLR2 and TLR4 expression levels between normal cervical epithelia with no infection and with various bacterial and viral infections was found to be statistically significant ($p<0.001$).

Table 1: Prevalence of HPV, CMV and CT infections among women with normal cervical cytology

	Musiri (n = 125)				Manachanallur (n = 107)				Tiruchirapalli (n = 78)				Over all (n = 310)			
	Positive		Negative		Positive		Negative		Positive		Negative		Positive		Negative	
Types of infection	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
HPV	31	25.0	94	75.0	30	27.8	77	72.2	9	11.5	69	88.5	70	22.60	240	77.4
CMV	9	7.1	116	92.9	4	4.2	103	95.8	8	9.6	70	90.4	21	6.70	289	93.3
CT	3	2.4	122	97.6	4	4.2	103	95.8	0	0.0	78	100.0	7	2.40	303	97.6

Table 2: Risk factors associated with HPV infection among sexually active women with normal cervical cytology

	Overall (N = 310)					
	HPV positive (n = 70)		HPV negative (n = 240)			
Parameters	No.	%	No.	%	OR	95% CI
Occupation						
House Wife	36	51.4	100	41.6	1.46	0.76-2.81
Manual Laborer	22	31.4	96	40.0	0.69	0.39-1.21
Others	12	17.2	44	18.4	0.90	0.46-1.86
Age at first coitus						
≤15	10	14.3	15	6.3	2.64	0.95-7.38
16-20	27	38.6	156	65.0	0.33	0.17-0.65
21-25	33	47.1	69	28.8	1.52	0.74-3.1
No of pregnancies						
0	8	11.4	9	3.7	3.08	0.89-10.57*
1-3	46	65.7	152	63.4	1.00	Ref
>3	16	22.9	79	32.9	0.62	0.29-1.32
Sexual intercourse						
Often	21	30.0	67	28.0	1.09	0.54-2.23
Rare	49	70.0	173	72.0	1.00	Ref
No of sexual partner						
0-1	66	94.3	225	93.8	1.00	Ref
≥2	4	5.7	15	6.2	1.03	0.27-3.91
Abortion						
Yes	21	30.0	76	31.7	0.83	0.41-1.71
No	49	70.0	164	68.3	1.00	Ref
Cervical screening						
Yes	4	5.7	12	5.0	1.00	Ref
No	66	94.3	228	95.0	0.77	0.2-3.01
Pan chewing						
Yes	4	5.7	5	1.9	3.59	0.70-18.42*
No	66	94.3	235	98.1	0.28	0.05-1.48
Menopause						
Yes	15	21.4	36	14.9	1.54	0.68-3.51
No	55	78.6	204	85.1	1.00	Ref
Types of delivery						
Normal	55	78.6	203	84.5	0.65	0.29-1.49
Cesarean	15	21.4	37	15.5	1.53	0.67-3.49
No of Children						
0	6	8.6	10	4.3	2.05	0.57-7.32*
1-2	48	68.6	148	61.5	1.00	Ref
≥3	16	22.9	82	34.2	0.59	0.28-1.25

Table 3: Correlation between HPV and CMV infection and HPV and CT infection among women with normal cervical cytology

	HPV			
Other infections	Positive (n = 70)	Negative (n = 240)	OR	95% CI
CMV				
Positive (n = 21)	7	14	1.79	0.64-4.63
Negative (n = 289)	63	226		
CT				
Positive (n = 7)	6	1	22.86	2.70-193.33
Negative (n = 303)	64	239		

DISCUSSION

The development of HPV vaccines holds tremendous promise in developing countries like India where cervical cancer is the most common malignancy among middle-aged women, particularly in rural areas. By the effort and under the auspices of the Indian Council of Medical Research (ICMR) HPV vaccination trial is being carried out in India. To maximize the cost effectiveness of the HPV vaccination programmes in India, it is important to understand the prevalence of HPV infection

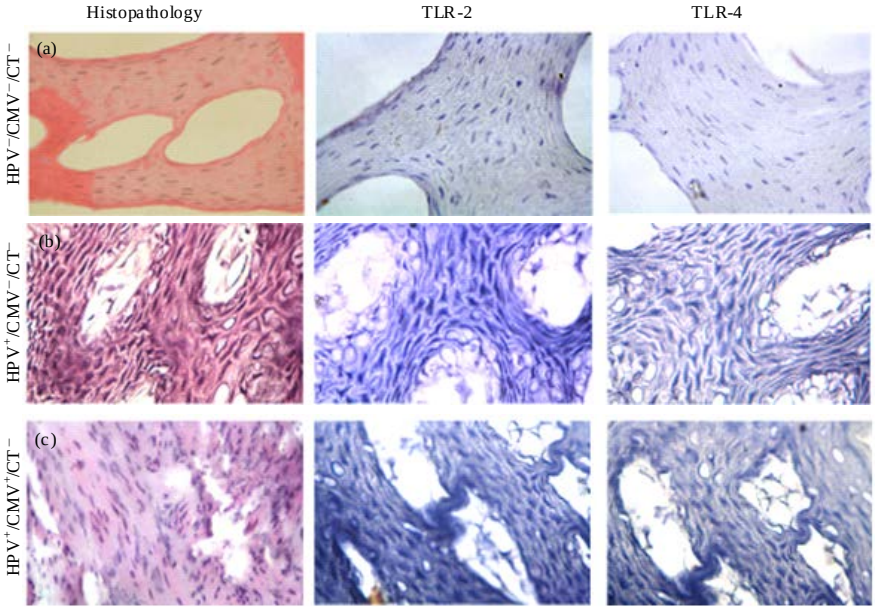

Fig. 1(a-c): TLR2 and TLR4 expression status in women with normal cervical cytology (a) Cervical tissue with normal cytology and $HPV^{-}/CMV^{-}/CT^{-}$, (b) Cervical tissue with normal cytology infected with $HPV^{+}/CMV^{-}/CT^{-}$, (c) Cervical tissue with normal cytology and $HPV^{+}/CMV^{+}/CT^{-}$

in various geographical regions. In this regard, as a part of our surveillance in Tiruchirapalli district we sought to evaluate the prevalence of HPV infection among sexually active women with normal cytology from three major taluks of Tiruchirapalli district.

The present population based screening showed an overall HPV prevalence of 22.6%, which is comparably greater than two large populations based screening from Osmanabad District of West India (10.3%) and Dindigul district of South India (9.6%) (Sankaranarayanan *et al*., 2005, 2009; Franceschi and Mahe, 2005). Thus envisaging an increase in the prevalence of HPV infection among women and hence Tiruchirapalli can be classified as region with high HPV prevalence according to Clifford *et al*. (2005).

We then asked whether the sexually active women have other bacterial and viral co-infections with HPV. As expected women with HPV infections were more prone to CMV and CT infections with an odds ratio of 1.79 (95% CI 0.69-4.63) and 22.41 (95% CI 2.65-189.48) respectively. Thus it can be postulated that HPV infection is necessary but nonsufficient to develop invasive cervical carcinoma. Our results have been well supported by several epidemiological studies that show concurrent bacterial or viral infections with increased HPV persistence to be closely associated to the progression of cervical carcinoma. The CMV has been isolated from cell cultures derived from biopsies of cervical carcinomas (Melnick *et al*., 1978) and has been shown to induce transformation of cells *in vitro* (Albrecht and Rapp, 1973; Geder *et al*., 1976). In some seroepidemiological studies, a higher frequency of circulating antibodies against CMV has been found in women with cervical carcinoma than in women with other cancers or without malignant disease (Vestergaard *et al*., 1972). Similarly CT infection was examined as a cause of Invasive Cervical Cancer (ICC) among women with HPV infection. CT increased the risk of squamous cervical cancer among HPV-positive

women. Case-control studies for HPV infection status have demonstrated an association between detection of antibodies to CT and the development of cervical cancer (Anttila *et al*., 2001; Koutsky *et al*., 1992; Wallin *et al*., 2002), providing considerate evidence for this hypothesis. A retrospective study demonstrated an increased risk of cervical cancer in women who had a history of CT infection (Wallin *et al*., 2002).

After considering the cervix of sexually active women to be infected with various bacterial and viral infections we next explored whether their persistent infection could trigger TLR immune response. Thus it is important to determine TLRs' effects on preneoplasia CINs and cervical cancer. In the present study, demonstrated TLR2 and TLR4 expression to be down regulated during infection of the cervical epithelium with CMV, CT or HPV.

The mammalian TLRs are key components in the innate immune response to infection and play a crucial role in the defense against microbial invasion. Several studies has shown TLR4 to be induced during viral and bacterial infections (Ramphal *et al*., 2008; Wieland *et al*., 1998; Song *et al*., 2007a, b; Tulic *et al*., 2007; Delgado-Lopez and Horwitz, 2006). Consistent with the previous study we initially expected TLR2 and TLR4 to be induced in response to bacterial and viral infection of the cervical epithelium and TLR2 and TLR4-mediated inflammation might be a critical cofactor for cervical lesion. But to our surprise, we observed a down-regulation of TLR2 and TLR4 expression. Our observations are supported by several findings, where TLR9 expression has been suppressed in HPV16-positive cancer-derived cell lines and primary cervical cancers. The suppressive effect was mainly attributed to HPV16 E6 and E7 proteins that inhibited TLR9 transcription (Hasan *et al*., 2007). Furthermore, our study has also been substantiated by a report where Hepatitis B Virus (HBV) is able to suppress the TLR-induced antiviral activity of liver cells (Wu *et al*., 2009). However, current evidence indicates TLR4 gene expression is decreased in leukemic leukocyte populations (Webb *et al*., 2009).

CONCLUSION

The observation of the present study suggest that in addition to the screening for prevalence of HPV infections, CMV and CT infection should be investigated as an essential predisposing factor for cervical cancer. In addition, cervical cancer development is linked to the persistent infection of high-risk HPVs. Thus E6 and E7 the major oncoproteins of HPV play a key role in the tumorigenesis of cervical epithelium. An unknown mechanism can be used by HPV to suppress the host immune response probably by deregulating TLRs transcript. This indicates the innate immune responses to be abolished and thus may be a crucial step in the carcinogenic events mediated by HPVs. But the regulation of TLR expression in tumors has not been fully elucidated and the current evidence is controversial. Hence further studies have to be carried out to study the expression of TLRs in human cells with various bacterial and viral infections.

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