Journal of Medical Virology 84:1920–1927 (2012)

Relationship Between Cervical Disease and Infection With Human Papillomavirus Types 16 and 18, and Herpes Simplex Virus 1 and 2

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Persistent infection with high-risk HPV, particularly Type HPV 16 and 18, is necessary in the development of cervical cancer, but apart from HPV infection, other causative factors of most cervical cancers remain unknown. The aim of this study was to determine the prevalence of HPV 16 and HPV 18 and HSV 1 and HSV 2 in cervical samples, and to assess the role of HSVs in cervical carcinogenesis. Two hundred thirty-three healthy controls and 567 cases (333 of cervicitis, 210 of cervical intraepithelial neoplasia, and 24 of squamous cell carcinoma) in cervical exfoliative cells were tested for HPV 16, HPV 18, HSV 1, and HSV 2 DNA using the triplex real-time polymerase chain reaction method. In contrast to healthy women, positive rate of HPV is related significantly to cervical lesions (odds ratios (ORs) = 4.1, $P < 0.01$ for cervical intraepithelial neoplasia; ORs = 24.9, $P < 0.01$ for squamous cell carcinoma), but not cervicitis (ORs = 2.3, $P > 0.05$). HSV 2 prevalence in cervical intraepithelial neoplasia and squamous cell carcinoma was higher than in healthy women (ORs = 4.9, $P < 0.05$ for cervical intraepithelial neoplasia; ORs = 4.7, $P < 0.05$ for squamous cell carcinoma). HSV 2 coinfection with HPV in cervical intraepithelial neoplasia and squamous cell carcinoma was strongly higher than in healthy women (ORs = 34.2, $P < 0.01$ for cervical intraepithelial neoplasia; ORs = 61.1, $P < 0.01$ for squamous cell carcinoma). The obtained results indicated that the presence of HPV is associated closely with cervical cancer, and that HSV 2 infection or co-infection with HPV might be involved in cervical cancer development, while HSV 1 might not be involved. ***J. Med. Virol. 84: 1920–1927, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: human papillomavirus; herpes simplex virus type 1; herpes simplex virus type 2; cervical cancer; triplex real-time PCR assay

INTRODUCTION

Cervical cancer remains an important public health problem worldwide. Cervical cancer is the second most common cancer among women around the world [Ferlay et al., 2004]. It is well known that persistent infection with human papillomavirus (HPV) is the main cause of cervical intraepithelial neoplasia and uterine cervix cancer [Schiffman et al., 2007; Sankaranarayanan et al., 2009]. Over 100 HPV types have now been catalogued. Approximately 15 high-risk types are carcinogenic and associated with the development of cervical cancer [Munoz et al., 2004; Wentzensen et al., 2009]. Epidemiological surveys have shown that oncogenic HPV Type 16 and 18 account for 70% of cervical cancers [Bosch et al., 2002]. Natural history studies of HPV infection have shown that, in contrast to those that progress to cancer, most infections are transient and not associated with detectable cytological abhealthyities [Ho et al., 1998]. It has been assumed generally that the presence of HPV infection alone is unlikely to be sufficient for the development of cervical cancer. Other sexually transmitted infections, such as herpes simplex virus (HSV)

Grant sponsor: Major Program of Ministry of Science and Technology of China; Grant number: 2009ZX10004-107.

The authors declare no conflict of interest.

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Accepted 15 May 2012

DOI 10.1002/jmv.23353
Published online in Wiley Online Library (wileyonlinelibrary.com).

or *Chlamydia trachomatis*, may also be involved [Bosch et al., 2002; Golijow et al., 2005].

HSVs include two different genotypes, HSV type 1 and 2 (HSV 1 and HSV 2). Both HSV 1 and HSV 2 infections are acquired from contact with infectious secretions on oral or genital mucosal surfaces and cause a variety of clinical presentations. Previous studies have shown that HSV 1 interferes in cellular DNA repair mechanism and results in some genetic changes during the process of acute lymphoblastic leukemia [Wilkinson and Weller, 2006; Mahjour et al., 2010]. It has been shown to be associated significantly with papillary thyroid cancer and the presence of lymph node metastases [Jensen et al., 2010]. Epidemiologic studies have shown that genital HSV 1 infection increases with changes in human sexual behavior [Manavi et al., 2004], which represents primarily past exposure to nongenital infections. It warrants exploring, therefore, whether or not HSV 1 causes cervical cancer. HSV 2 infection was considered first a possible causal agent for cervical cancer in 1960s [Rawls et al., 1968], and has been investigated as a HPV co-factor for cervical carcinogenesis, however, its impact on the progression of HPV-infected cervical cells to cancer remains unclear [Smith et al., 2002]. Even the role of HSV 2 infection in cervical cancer is the subject of active controversy. Epidemiologic studies have reported an interaction between HSV 2 and HPV in increasing cervical cancer risk with serologic evidence for HSV 2 diagnosis [Smith et al., 2002; Munoz et al., 1995], however, no HSV 2 DNA sequences of HSV 2 have been found in cervical tissues [Zereu et al., 2007]. To date, only a few studies have explored the relationship between HSV DNA and cervical cancer using PCR-based methods, with variable results [Danh et al., 2003; Pierpaolo et al., 2008].

If HSV is indeed a co-factor for HPV in the progression of cervical lesions, HSV DNA should be detectable in cervical intraepithelial neoplasia and cervical cancer. To examine the prevalence of HPV 16, HPV 18, HSV 1, and HSV 2 infection and to investigate the association between HSVs and development of cervical cancer, a sensitive and specific triplex real-time polymerase chain reaction (RT-PCR) was used to detect simultaneously and quantitatively the presence of HPV 16, HPV 18, HSV 1, and HSV 2 DNA in cervical exfoliated cells obtained from women with healthy cervix, cervicitis, cervical intraepithelial neoplasia, and squamous cell carcinoma.

MATERIALS AND METHODS

Clinical Samples

A total of 2,773 women who sought routine cervical cancer screening from the Hubei Provincial Hospital of Traditional Chinese Medicine from November 2008 to June 2011 were collected. Cervical exfoliated cells were collected with a cytobrush from the uterus endocervix of each woman. A thin-prep microscopic Cytology Test (TCT) and a high-risk Human Papillomavirus (HR-HPV, 16, 18, 31, 33, 45, 52, 56, and 58) quantitative DNA assay (PCR Fluorescence, Guangzhou, China) were carried out on all women. Women were referred to colposcopy if cytology indicated undetermined significance, or higher abhealthy cytology, or positive of HR-HPV. The final clinical diagnosis of women with abhealthy cytology was made by histological evaluation of biopsy samples obtained at colposcopy. Eight hundred cervical samples were included in the study, corresponding to women aged 19–71 years, and comprising 233 healthy women, and 333 cases of cervicitis, 210 cases of cervical intraepithelial neoplasia, and 24 cases of squamous cell carcinoma. Samples were eluted in 5 ml of phosphate-buffered saline (PBS), and kept frozen at −60°C. Five hundred ninty-seven serum samples selected at random from the 800 study patients were obtained from 183 healthy women and 414 cases (213 cervicitis, 177 cervical intraepithelial neoplasia, and 24 squamous cell carcinoma).

All participants agreed to join in the present study and signed informed consent forms. The study project was performed according to the principles of the Declaration of Helsinki, and was approved by the Human Research Ethical Committee of Hubei Provincial Hospital of Traditional Chinese Medicine.

DNA Preparation

DNA was isolated from the cervical exfoliated cells using the QIAamp DNA Mini Kit according to the manufacturer's instructions (QIAGEN, Valencia, CA).

Simultaneous Detection of HPV16, HPV18, HSV 1, and HSV 2 DNA

HPV16, HPV18, HSV 1, and HSV 2 DNA were detected simultaneously in 800 cervical exfoliated cells by triplex real-time PCR assay using the sequences of HPV type-specific L1 primers and probes [Seaman et al., 2010] and the sequences of the HSV type-specific gB primers and probes [Sugita et al., 2008]. The sequences are: HPV 16-F (forward primer)-5′-TTGTTGGGGTAACCAACTATTTGTTACTGTT-3′, HPV 16-R (reverse primer)-5′-CCTCCCCATGTCTGAGGTACTCCTTAAAG-3′, and HPV 16-P (probe)-5′-6FAM–GTCATTATGTGCTGCCATATCTACTTC-BHQ-3′; HPV 18-F-5′-GCATAATCAATTATTTGTTACTGTGGTAGATACCACT-3′, HPV 18-R-5′-GCTATACTGC TTAAATTTGGTAGCATCATATTGC-3′, HPV 18-F-5′-6FAM-AACAATATGTGCTTCTACACAGTCTCCTGT-BHQ -3′ (HPV 16 and/or 18 were detected and quantified together); HSV-F-5′-CGCATCAAGACCACCTCCTC-3′, HSV-R-5′-GCTCGCACCAC GCGA-3′, HSV 1-P-5′-JOE-TGGCAACGCGGCCCAAC-BHQ-3′, HSV 2-P-5′-Cy-5-CGGCGATGCGCCCCAG-BHQ-3′. The triplex real-time PCR assay was optimized for simultaneous quantitative detection purposes, in terms of the reagent concentrations required to allow similar detection sensitivity and dynamic range for HPV16, HPV18, HSV 1, and HSV 2 (data not shown).

Triplex real-time PCR mixtures contained PCR (1×) buffer without $MgCl_2$, 3.5 mM $MgCl_2$, 0.5 mM deoxynucleotide mixture (dATP, dTTP, dCTP, and dGTP), 0.5 U Hotstart Taq DNA polymerase, 0.4 μM HPV 16 and HPV 18, 0.35 μM HSV 1 and HSV 2 primers, 0.25 μM HPV 16 and HPV 18, HSV 1, and HSV 2 probes, and 5 μl of DNA template. Cycling conditions were as follows: 94°C for 5 min, followed by 40 cycles of 95°C for 20 sec and 58°C for 40 sec. The ABI PRISM 7500 real-time analysis software (Applied Biosystems, Redwood, CA) read each sample every few seconds and computed a mean baseline for early PCR cycles. A sample was considered negative when its viral concentration was less 10 copies/reaction.

A quantitative detection system for a single-copy human CCR5 gene (located on chromosome 3 and encoding for CC chemokine receptor 5; GenBank accession no. NC000003) was also used to determine the number of cells present in the sample to healthyize the HPV and HSV viral load [Broccolo et al., 2005]. Since cervical samples differ widely in the amount of DNA present, DNA from cervical samples was considered suitable for HPV and HSV viral load determination if the number of human CCR5 copies per reaction was higher than 2×10^3 (corresponding to 10^3 cells per reaction).

HSV 1-IgG and HSV 2-IgG Detection

HSV 1-IgG and HSV 2-IgG were detected in 597 serum samples using an ELISA Kit, according to the manufacturer's instructions (Yanhui, Shanghai, China).

HPV 16-IgG and HPV 18-IgG Detection

HPV 16-IgG and HPV 18-IgG were detected in 597 serum samples using an ELISA Kit, according to the manufacturer's instructions (Kenqiang, Shanghai, China).

Statistical Analysis

Healthyization of HPV and HSV viral load was calculated as $VL = [Cn_{HPVorHSV}/(Cn_{CCR5}/2)] \times 10^3$ cells, where VL is the number of HPV or HSV genomes per 10^3 cells (corresponding to 2×10^3 CCR5 copies), $Cn_{HPVorHSV}$ is the number of HPV or HSV genomes and $Cn_{CCR5}/2$ is the number of cells (corresponding to CCR5 copies number × 2). The prevalences of HPV 16, HPV 18, HSV 1, and HSV 2 were compared in each grade of cervical disease by the Chi-square test. The relation of viral infections with cervical stage was performed using logistic regression analysis, controlling for the confounding effect of age. Statistical analysis was performed using the SPSS 17.0 statistical package (SPSS, Inc., Chicago, IL) and values with $P < 0.05$ were considered statistically significant.

RESULTS

Overall, 800 cervical exfoliated cells studied had human CCR5 copy numbers higher than 2×10^3 (corresponding to 10^3 cells per reaction). The cells were further analyzed for the presence of HPV16, HPV18, HSV 1, and HSV 2.

Table I shows the prevalence of HPV 16, HPV 18, HSV 1, and HSV 2 DNA for 24 cases of squamous cell carcinoma, 210 cases of cervical intraepithelial neoplasia, 333 case of cervicitis, and 233 healthy controls. Overall, HSV 2 prevalence was higher ($P < 0.01$) in cases of squamous cell carcinoma (37.5%, $\chi^2 = 16.5$) or cervical intraepithelial neoplasia (36.2%, $\chi^2 = 45.9$) or cervicitis (19.5%, $\chi^2 = 10.7$) than in healthy women (9.4%). HSV 1 prevalence was similar ($P > 0.05$) in cases of squamous cell carcinoma (4.2%, $\chi^2 = 0.6$), cervical intraepithelial neoplasia (10.5%, $\chi^2 = 0.5$), cervicitis (9.3%, $\chi^2 = 0.1$), and in healthy control (8.6%). A total of 20 (83.3%) cases of squamous cell carcinoma, 98 (46.7%) cases of cervical intraepithelial neoplasia, 105 (31.5%) cases of cervicitis, and 25 (10.7%) healthy cervixes were HPV 16 and/or HPV 18 DNA positive. Compared with healthy cervix, HPV 16 and/or HPV 18 prevalence increased with increasing severity of cervical lesions ($\chi^2 = 33.6$, $P < 0.01$ for cervicitis; $\chi^2 = 71.1$, $P < 0.01$ for cervical intraepithelial neoplasia; and $\chi^2 = 79.4$, $P < 0.01$ for squamous cell carcinoma). This indicated that HPV 16 and/or HPV 18 and HSV 2 infection was associated with cervical precancerous lesions and cervical cancer, but HSV 1 infection may not be.

TABLE I. Prevalences of HPV 16, HPV 18, HSV 1, and HSV 2 Infection Based on Histologic Diagnosis by Triplex Real-Time PCR

	No. (%) of DNA positive results				
	HCX, n = 233	Cases, n = 567	Cervicitis, n = 333	CIN, n = 210	SCC, n = 24
HPV 16 or/and HPV 18	25 (10.7)	223 (39.3)**	105 (31.5)**	98 (46.7)**	20 (83.3)**
HSV 1	20 (8.6)	54 (9.5)	31 (9.3)	22 (10.5)	1 (4.2)
HSV 2	22 (9.4)	150 (26.5)**	65 (19.5)**	76 (36.2)**	9 (37.5)**

HPV (HPV 16 and/or HPV 18) positivities in the cases were strongly statistically different ($P < 0.01$, $\chi^2 = 63.2$), compared with healthy cervix; HSV 2 positivities in the cases were also strongly statistically different ($P < 0.01$, $\chi^2 = 23.9$.); HSV 1 positivities were not statistically different between cervical lesions and healthy cervix.
HCX, healthy cervix; CIN, cervical intraepithelial neoplasia; SCC, squamous cervical carcinomas.
**$P < 0.01$.

TABLE II. Prevalences of HSV 1 and/or HSV 2 Co-Infection With HPV Among HPV 16 and/or HPV18 DNA-Positive Women by Stage of Cervical Disease

	No. (%) of positive results				
	HCX, n = 25	Cases, n = 223	Cervicitis, n = 105	CIN, n = 98	SCC, n = 20
HPV and HSV 1[a]	2 (8.0)	24 (10.8)	14 (13.3)	9 (9.2)	1 (5.0)
HPV And HSV 2[b]	1 (4.0)	57 (25.6)*	25 (23.8)*	27 (27.6)*	5 (25.0)*
HPV And HSVs[c]	1 (4.0)	6 (2.7)	4 (3.8)	2 (2.0)	—

HSV 2 co-infections with HPV in the cases were statistically different ($P < 0.05$, $\chi^2 = 5.6$), compared with HCX; HSV 1 co-infections with HPV were not statistically difference in cervical lesions ($P > 0.05$, $\chi^2 = 0.1$), compared with HCX. HSV 1, HSV 2, and HPV co-infections were not statistically different in cervicitis and CIN, compared with HCX.
HCX, healthy cervix; CIN, cervical intraepithelial neoplasia; SCC, squamous cervical carcinomas.
[a]Both HPV (HPV 16 and/or HPV 18) and HSV 1 DNA.
[b]Both HPV (HPV 16 and/or HPV 18) and HSV 2 DNA.
[c]HPV (HPV 16 and/or HPV 18) and HSV1 and HSV2 DNA.
*$P < 0.05$.

Table II shows the prevalence of HSV 1 and/or HSV 2 co-infections with HPV among the following HPV 16 and/or HPV 18 DNA-positive cases: 20 cases of squamous cell carcinoma; 98 cases of cervical intraepithelial neoplasia; 105 cases of cervicitis; and 25 healthy cervix. HSV 2 co-infection with HPV was significantly higher ($P < 0.05$) in cases of squamous cell carcinoma (25.0%, $\chi^2 = 4.2$), cervical intraepithelial neoplasia (27.6%, $\chi^2 = 6.3$) and cervicitis (23.8%, $\chi^2 = 5.0$) than in healthy cervix (4.0%). HSV 1 co-infection with HPV was similar ($P > 0.05$) in cases of squamous cell carcinoma (5.0%, $\chi^2 = 0.2$), cervical intraepithelial neoplasia (9.2%, $\chi^2 = 0.1$), cervicitis (13.3%, $\chi^2 = 0.5$), and healthy cervix (8.0%). Rates of HSV 1, HSV 2, and HPV co-infection were not significantly different in cervicitis and cervical intraepithelial neoplasia, compared with healthy cervix. This suggests that HSV 2 co-infection with HPV was associated with cervical intraepithelial neoplasia and squamous cervical carcinomas, but that this is not the case for HSV 1 coinfection with HPV.

Table III shows the risk estimation of HPV 16, HPV 18, HSV 1, and HSV 2 infections in cervical disease. Compared with healthy women, single HPV virus (HPV 16 and/or HPV 18) was associated with a diagnosis of cervical intraepithelial neoplasia and squamous cell carcinoma. The odds ratios (ORs) increased with advanced stages of cervical disease, with ORs of 4.1 (95% CI: 2.4–14.9, $P < 0.01$) and 24.9 (95% CI: 5.6–129.5, $P < 0.01$) for women with cervical intraepithelial neoplasia and squamous cell carcinoma, respectively. Similarly, single HSV 2 DNA was associated statistically with cervical cancer, with ORs of 4.9 (95% CI: 1.3–15.4, $P < 0.05$) and 4.7 (95% CI: 1.1–6.8, $P < 0.05$) for women with cervical intraepithelial neoplasia and squamous cell carcinoma, respectively. HSV 2 co-infection with HPV was associated strongly with cervical cancer, with ORs of 34.2 (95% CI: 4.6–254.3, $P < 0.01$) and 61.1 (95% CI: 6.8–549.6, $P < 0.01$) for women with cervical intraepithelial neoplasia and squamous cell carcinoma, respectively. As with cervical intraepithelial neoplasia, the ORs increased from 4.9 (99% CI: 1.1–20.7, $P < 0.01$) for single HSV2 to 34.2 (99% CI: 2.5–477.5, $P < 0.01$) for HSV2 co-infection with HPV, and squamous cell carcinoma from 4.7 (99% CI: 0.8–9.5, $P < 0.05$) for single HSV2 to 61.1 (99% CI: 3.4–1,096.2, $P < 0.01$) for HSV2 co-infection with HPV. In contrast, HSV 1 DNA or coinfection with HPV was not associated statistically with cervical cancer for women with cervical intraepithelial neoplasia ($P > 0.05$) and squamous cell carcinoma ($P > 0.05$), respectively. This indicated that HPV 16 and/or HPV 18 and HSV 2 infection or coinfection increases

TABLE III. Risk Estimation of HPV (HPV 16 and/or HPV 18), HSV 1, and HSV 2 Infections for Cervical Disease

	ORs* (95% CI) *P*-value				
	HPV[a]	HSV 1[b]	HSV 2[c]	HPV and HSV 1[d]	HPV and HSV 2[e]
HCX	1	1	1	1	1
Cervicitis	2.3 (0.8–9.1) > 0.05	0.5 (0.2–1.1) > 0.05	1.3(0.7–2.3) > 0.05	2.2 (0.2–12.2) > 0.05	2.8 (0.5–8.2) > 0.05
CIN	4.1 (2.4–14.9) < 0.01	0.7 (0.3–1.5) > 0.05	4.9 (1.3–15.4) < 0.05	2.8 (0.3–25.3) > 0.05	34.2 (4.6–254.3) < 0.01
SCC	24.9 (5.6–129.5) < 0.01	—	4.7 (1.1–6.8) < 0.05	5.0 (0.4–57.5) > 0.05	61.1 (6.8–549.6) < 0.01

HCX, healthy cervix; CIN, cervical intraepithelial neoplasia; SCC, squamous cervical carcinomas; ORs, Odd ratios; CI, confidence interval.
[a]Only HPV infection (HPV 16 and/or HPV 18 DNA).
[b]Only HSV 1 infection.
[c]Only HSV 2 infection.
[d]Mixed infection of HSV 1 with HPV (HPV 16 and/or HPV 18 DNA).
[e]Mixed infection of HSV 2 with HPV (HPV 16 and/or HPV 18 DNA).
*OR adjusted for age.

TABLE IV. Viral Load of HPV 16, HPV 18, HSV 1, and HSV 2 Infections for Stage of Cervical Disease

	N, DNA mean ± SE[a]						
	Single virus DNA			Mixed virus DNAs[b]		Mixed virus DNAs[c]	
	HPV[d]	HSV 1	HSV 2	HPV[d]	HSV 1	HPV[d]	HSV 2
HCX	21, 3.50 ± 1.27	17, 3.62 ± 1.39	20, 2.94 ± 0.72	3, 3.27 ± 1.20	3.40 ± 0.86	2, 3.30 ± 0.57	3.00 ± 0.14
Cervicitis	62, 4.04 ± 1.28	13, 4.29 ± 1.42	36, 3.38 ± 1.14	18,4.02 ± 1.21	3.98 ± 1.26	29, 3.93 ± 1.37	3.54 ± 1.25
CIN	60, 4.32 ± 1.51*	11, 3.76 ± 1.34	47, 4.25 ± 2.19*	11, 4.86 ± 1.25*	4.00 ± 1.61	29, 4.98 ± 1.14*	4.24 ± 0.82*
SCC	14, 4.98 ± 1.55**	0, 0	4, 4.50 ± 2.27*	1, 3.98	2.25	5, 4.94 ± 0.78*	4.48 ± 0.72*

Compared with HCX, the difference of single HPV viral load was significant in cervicitis and CIN ($P < 0.05$), and remarkable significant in SCC ($P < 0.01$); both single and mixed HSV 2 viral load were higher in CIN and SCC than in HCX ($P < 0.05$); both single and mixed HSV 1 viral load was not different in cervical lesions and in HCX.
HCX, healthy cervix; CIN, cervical intraepithelial neoplasia; SCC, squamous cervical carcinomas.
[a]$\log_{10}$ copies/reaction.
[b]HSV 1 coinfection with HPV.
[c]HSV 2 coinfection with HPV.
[d]HPV 16 and/or HPV 18 DNA.
*$P < 0.05$.
**$P < 0.01$.

the risk of cervical intraepithelial neoplasia and squamous cervical carcinomas, but that HSV 1 infection does not.

Table IV shows the viral load of HPV 16, HPV 18, HSV 1, and HSV 2 infections for the stage of cervical disease. Among the 494 specimens positive for HPV 16, HPV 18, HSV 1, or HSV 2 DNA, $\log_{10}$ copies/reaction were analyzed by calculating the average (mean) and standard error (SE). Single virus DNA (HPV 16 and/or HPV 18) load in the case samples was higher than in healthy cervix (mean ranging from 4.32 for cervical intraepithelial neoplasia to 4.98 for squamous cell carcinoma; $P < 0.05$, 0.01). Similarly, the viral load of women with both single and mixed HSV 2 DNA with HPV was slightly higher in cervical intraepithelial neoplasia and squamous cell carcinoma ($P < 0.05$) than in healthy cervix (mean ranging from 4.25 (single) and 4.24 (mixed) for cervical intraepithelial neoplasia to 4.50 (single) and 4.48 (mixed) for squamous cell carcinoma). In addition, compared with the healthy cervix, both single and mixed HSV 1 DNA load with HPV was not associated with the stage of cervical disease ($P > 0.05$). This suggests that the viral load of both single and mixed HPV 16 and/or HPV 18 and HSV 2 was associated with the stage of cervical precancerous lesions and cervical cancer, but that HSV 1 DNA concentration may not be associated with cervical disease.

Table V shows the prevalence of HPV 16-IgG, HPV 18-IgG, HSV 1-IgG, and HSV 2-IgG for 24 cases of squamous cell carcinoma, 177 cases of cervical intraepithelial neoplasia, 213 case of cervicitis, and 183 healthy controls. Analysis of 597 serological samples indicated that the prevalence of HPV 16-IgG or/and HPV 18-IgG was higher ($P < 0.01$) in cases of squamous cell carcinoma (87.5%, $\chi^2 = 75.8$), cervical intraepithelial neoplasia (47.5%, $\chi^2 = 56.4$), or cervicitis (28.2%, $\chi^2 = 16.9$) than in control cases (11.5%). The HSV 2 serological prevalence was higher ($P < 0.01$) in cases of squamous cell carcinoma (33.3%, $\chi^2 = 13.6$), cervical intraepithelial neoplasia (30.5%, $\chi^2 = 28.9$) or cervicitis (18.3%, $\chi^2 = 8.5$) than in control cases (8.2%). HSV 1 serological prevalence was similar ($P > 0.05$) in cases of squamous cell carcinoma (83.3%, $\chi^2 = 0.5$), cervical intraepithelial neoplasia (88.1%, $\chi^2 = 0.1$) and cervicitis (90.1%, $\chi^2 = 0.3$) than in healthy women (88.5%). At the same time, co-positive results of HSV 1-IgG and/or HSV 2-IgG with HPV 16-IgG and/or HPV 18-IgG were found in the current investigated clinical samples. These results also indicated that the serology of HPV 16 and/or HPV 18 and HSV 2 infection was

TABLE V. Prevalences of HPV 16, HPV 18, HSV 1, and HSV 2 Infection Based on Histologic Diagnosis by ELISA

	No. (%) of IgG positive results				
	HCX, n = 183	Cases, n = 414	Cervicitis, n = 213	CIN, n = 177	SCC, n = 24
HPV 16 or/and HPV 18	21 (11.5)	165 (39.9)**	60 (28.8)**	84 (47.5)**	21 (87.5)**
HSV 1	162 (88.5)	368 (88.9)	192 (90.1)	156 (88.1)	20 (83.3)
HSV 2	15 (8.2)	101 (24.4)**	39 (18.3)**	54 (30.5)**	8 (33.3)**

HPV (HPV 16 and/or HPV 18) positivities in the cases were strongly statistically different ($P < 0.01$, $\chi^2 = 47.7$), compared with healthy cervix; HSV 2 positivities in the cases were also strongly statistically different ($P < 0.01$, $\chi^2 = 21.3$); HSV 1 positivities were not statistically different between cervical lesions and healthy cervix.
HCX, healthy cervix; CIN, cervical intraepithelial neoplasia; SCC, squamous cervical carcinomas.
**$P < 0.01$.

associated with cervical precancerous lesions and cervical cancer, but that this may not be the case for HSV 1.

DISCUSSION

Determination of the presence of HPV 16 and HPV 18 in cervical exfoliated cells is important for evaluating the risk of developing cervical cancer. For example, HPV 16 and HPV 18 detection was carried out previously during a cervical cancer screening project by the National Comprehensive Cancer Network (Ncervical cancerN Guidelines™, 2011). In the current study, among 2,773 specimens, 567 cases representing various degrees of cervical lesions were obtained for quantitative testing of HPV 16 and HPV 18 DNA by triplex real-time PCR. The prevalence of HPV 16 and HPV 18 was associated with increased severity of cervical lesions, from 10.7% in healthy cervix to 31.5% in cervicitis, 46.7% in cervical intraepithelial neoplasia, and 83.3% in squamous cell carcinoma. The ORs increased with advanced stage of cervical disease, from 4.1 for cervical intraepithelial neoplasia to 24.9 for squamous cell carcinoma, and viral load showed an increasing trend from cervicitis to squamous cell carcinoma. Serological results showed that the prevalence of HPV 16-IgG or/and HPV 18-IgG were higher ($P < 0.01$) in cases of squamous cell carcinoma, cervical intraepithelial neoplasia or cervicitis than in healthy women. The data indicated that infection with high risk HPV remained associated significantly with the development of cervical neoplasia and cervical cancer. However, among women infected with high-risk types of HPV, only a small subset will develop cervical cancer, suggesting that other factors must be present for the development of malignancy [Ho et al., 1998].

This study contributes to understanding the role of HSV 1 and HSV 2 infection in the etiology of cervical intraepithelial neoplasia and cervical cancer. The experimental results showed that HSV 2 DNA and serological positivity showed an increasing trend from cases of cervicitis to cervical intraepithelial neoplasia to squamous cell carcinoma. In addition, the results also correlated with the risk of precancerous and cervical cancer increased, and that the HSV 2 viral load of cervical intraepithelial neoplasia and squamous cell carcinoma was higher than in healthy cervix and cervicitis. At the same time, the risk estimation of HSV 2 co-infection with HPV showed a greater correlation with cervical intraepithelial neoplasia and squamous cell carcinoma than for single HPV or HSV 2. The results suggest that genital HSV 2 infection may act in conjunction with HPV infection to increase modestly the risk of cervical intraepithelial neoplasia and cervical cancer. However, HSV 1 DNA and viral load and serological positivity were not associated with cervical intraepithelial neoplasia and cervical cancer, and HSV 1 infection may not be involved in the development of cervical cancer. Similarly, HSV 1 coinfection with HPV was not correlated with increased risk of developing cervical disease.

Initial epidemiological studies indicated that HSV 2 infection was a potential etiologic factor for invasive cervical cancer, based on data that compared HSV 2 seropositivity between patients and control subjects [Brinten, 1992]. Inactivated HSV 2 has been shown to transform cells in vitro, and it has been demonstrated that HPV immortalized epithelial cells transfected with HSV 2 DNA become tumorigenic in nude mice, with several possible mechanisms of interaction [Galloway and McDougall, 1983; Guibinga et al., 1995; Munoz et al., 1995]. The role of HSV 2 in the development of cervical cancer has been the subject of debate however. Many studies [Munoz et al., 1995; Smith et al., 2002] have examined the role of HSV 2 infection in the etiology of invasive cervical cancer, and found a statistically significant association between invasive cervical cancer and HSV 2 seropositivity, after controlling for the presence of HPV DNA. However, other studies [Peng et al., 1991; Ferrers et al., 1997; Zereu et al., 2007] controlling for the presence of specific HPV DNA types, found that HSV 2 was not a statistically significant risk factor for invasive cervical cancer. These discrepancies may arise for a variety of reasons. Firstly, HSV 2 seropositivity in different geographical populations ranges from 15.6% (14/90) in Spain, to 37.8% (59/156) in Morocco, to 61.5% (48/78) in Colombia [Smith et al., 2002]. The serologic testing for the presence of antibodies to HSV 2 was performed at the same laboratory. Secondly, different cases of cervical disease, including squamous cell carcinoma, adenocarcinoma and adenosquamous cell carcinoma of the uterine cervix, have different results. The largest study showed that HSV 2 seropositivity was 30.8% (99/321) in squamous cell carcinoma, compared to 15.2% (5/33) in adenocarcinoma in Philippines. In addition, HSV2 seropositivity was 55.2% (80/145) in squamous cell carcinoma, compared to 43.8% (7/16) of adenocarcinoma in Brazil [Smith et al., 2002]. Thirdly, it is possible that methodology used has influenced results, since serologic methods do not discriminate between current and past infections, nor between genital and extragenital infections. Moreover, potential cross-reactivity between HSV 1 and HSV 2 may lead to HSV 2 mis-classification. A PCR-based assay is more sensitive than in situ hybridization assay [Luis et al., 2006], but its accuracy, sensitivity and specificity are inferior to that of real-time PCR [Tang et al., 2011]. Fourthly, the type of specimens tested, such as serum, cervical exfoliative cells, or pathologic tissue, including paraffin-embedded tissue, may influence the results. Although paraffin-embedded tissue constitutes an important source of materials for retrospective studies, it is well known that the time of fixation and the type of fixative used can affect considerably the quality of extracted DNA [Yang et al., 2004; Melo et al., 2005]. Zereu et al. [2007] raised the possibility that conditions as well as time of storage may affect HSV 2

DNA stability for PCR detection, when specimens were formalin-treated and paraffin-embedded for an extended period. Finally, the "hit-and-run" mechanism suggests that the expression of HSV genes would be necessary for the initiation of the transformation process, but not for its progression [Galloway and McDougall, 1983].

This study has several strengths. To our knowledge, this is the first report of simultaneous quantitative detection of HPV 16, HPV 18, HSV 1, and HSV 2 DNA in cervical exfoliative cells among women with healthy and cervical disease, using sensitive and specific triplex real-time PCR. First is the choice of the most specific, sensitive and accurate method available currently for DNA testing, namely real time PCR. This technique avoids false positive results due to possible cross-contamination, and can quantify significantly the viral load of HPV and HSVs, which is valuable for investigating the relationship between cervical cancer progression and viral load. Most of the clinical specimens were tested for HPV and HSV using the triplex RT-PCR and ELISA in this study. The results of the two methods majorly accorded with each other and both showed that HPV and HSV2 were associated with cervical cancer, but ELISA cannot examine the viral load and current infection. For example, the prevalence of HSV1-IgG was high than 85%, but the prevalence of HSV1 DNA was about 10%. It is obvious that HSV1-IgG in serum could not distinguish the time of infection and infection sites. Second is the selection of cases including cervicitis, cervical intraepithelial neoplasia and squamous cell carcinoma, which have gone through the process of development of cervical cancer, and the exploration of viral carcinogenesis. Thirdly, cervical exfoliative cells were selected as testing specimens, overcoming problems arising from time of fixation, type of fixative and viral distribution within the cervical pathologic and paraffin-embedded tissue, since invasion of cervical cancer occurs from the outside to the inside of the cervix. Fourth, the viral genotypes are meaningful. They include HPV types 16 and 18, the two main etiologic factors for cervical cancer, HSV 2, whose role in cervical cancer development remains conflicting and can further be investigated, and HSV 1, the infection of which is associated significantly with other cancers [Jensen et al., 2010] and increasing in the genital system [Manavi et al., 2004].

This study also has a potential limitation, namely that in the case of HSV coinfections with HPV, coinfection specimens and squamous cell carcinoma may have led to biased results.

In conclusion, the results suggest that HPV 16 and HPV 18 are important factors for cervical carcinogenesis, and that viral load may be associated with the degree of cervical disease. Moreover they show that HSV 2 infection is associated with increased risk of cervical carcinogenesis, and that coinfection with HPV may be associated with increasing severity of cervical lesions. Finally, HSV 1 infection was shown to have no effect on cervical intraepithelial neoplasia and cervical cancer. Future studies will evaluate the impact of sexually transmitted diseases other than HPV in cervical carcinogenesis.

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