

Correlation of viral factors with cervical cancer in Taiwan

Yu-Yen Yang^{1,2}, Lim-Woh Koh³, Ju-Hsin Tsai⁴, Chung-Hung Tsai⁵, Eric Fook-Chuen Wong¹, Shyh-Jye Lin⁶,
Chi-Chiang Yang⁶

Departments of ¹Medical Research, ²Clinical Laboratory and ³Obstetrics and Gynecology, Show Chwan Memorial Hospital, Changhua; Departments of ⁴Surgery and ⁵Pathology, Chung Shan Medical University Hospital, Taichung; and ⁶School of Medical Technology, Chung Shan Medical University, Taichung, Taiwan, ROC

Received: January 19, 2004 Revised: April 7, 2004 Accepted: May 7, 2004

The correlation of viral factors with cervical cancer was investigated. 27 cervical cancer biopsies and 29 normal cervical scrapings were determined by polymerase chain reaction method for 6 viruses, including human papillomavirus (HPV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus (HSV)-1, HSV-2, and human herpes virus (HHV)-8. Among 27 biopsies of cervical cancer, HPV was identified in 18. Of these HPV-positive specimens, 9 cases of HPV type 16 were identified, 2 cases of HPV type 18 and 1 case of mixed infection with HPV types 16 and 18 were identified. Among the HPV types detected, type-16 is the most closely associated with cervical cancer and type-18 ranks second. Of the remaining 6 cases, 1 case of HPV-45, 1 case of mixed infection with HPV type 35, CMV and HSV-2, and 4 cases of unidentified HPV type were also found. EBV, HSV-1 and HHV-8 were not found in the cervical cancer samples and might have no or little relationship with cervical cancer. Among the 29 specimens in the normal female control group, no viral infection was detected. The correlation of HPV with cervical cancer was significantly different between frozen tissues and paraffin-embedded tissues. Other viruses such as HSV-2 and CMV are not predictive of cervical cancer. They might not be involved in the oncogenic processes directly but might enhance the possibility of oncogenesis or infect cancer tissues opportunistically.

Key words: Cervical neoplasms, human papillomavirus, polymerase chain reaction, risk factors, tumor virus infection

Cervical cancer is common among women in Taiwan. It is estimated that approximately 3000 cervical cancer patients are diagnosed and 1000 die of the condition in Taiwan annually [1]. Cervical neoplasia is known to be induced in different ways, such as via sexual activity, multiple sex partners, multiple pregnancies, radiation, smoking and viral infection [2].

Human papillomavirus (HPV) is now classified in the *Papillomaviridae* family. It has been known for many years that HPV infection plays an important role in the carcinogenesis of cervical cancer [2]. HPV has also been investigated in patients with oral cancer [3]. HPV can infect the squamous epithelium of both the uterine cervix and the oral mucosa, leading to the formation of benign or malignant tumors [4]. Herpes simplex virus (HSV), cytomegalovirus (CMV), Epstein-Barr virus (EBV) and human herpes virus (HHV)-8 are members of the *Herpesviridae* family. HSV-1 infection occurs mainly in the mouth and lips, while HSV-2 infection usually

occurs in the genital tract [5]. However, there is a great deal of overlap between the epidemiology and clinical manifestations of infections. HHV-8 is considered to be causally associated with Kaposi's sarcoma [6]. CMV is a common human pathogen and spread mainly by the sexual route and transfusion route. It was suggested to associate with cervical cancer [7]. EBV is the causative agent of infectious mononucleosis and is closely associated with Burkitt's lymphoma, nasopharyngeal carcinoma, and opportunistic B-cell lymphoma in immunocompromised hosts [8-10].

The purpose of this study was to investigate the correlation of virus factors with cervical cancers in middle Taiwan. We studied HPV, CMV, EBV, HSV-1, HSV-2 and HHV-8 to investigate the different factors involved in oncogenesis of cervical cancer.

Materials and Methods

Samples

Twenty seven cervical cancer biopsies and 29 normal cervical scrapings were collected. Nine fresh biopsies of cervical tissue were stored at -80°C . The patients

Corresponding author: Dr. Chi-Chiang Yang, School of Medical Technology, Chung Shan Medical University, 110, Section 1, Chien-Kuo North Road, Taichung, Taiwan 402, ROC.
E-mail: cyang@csmu.edu.tw

from whom the biopsies were taken were between 30 and 66 years of age, with an average age of 45 years. Cytopathologically, 2 specimens were classified as CIS (carcinoma in situ), 5 were classified as CIN (cervical intraepithelial neoplasia) grade I, 1 as grade II and 1 as grade IV.

In the remaining 18 cases, 3 sections of 0.5- μ m thickness from paraffin-embedded cervical specimens were used for polymerase chain reaction (PCR) analysis. The patients from whom these specimens were taken were between 40 and 76 years of age, with an average age of 57 years. Clinical cancer stage classifications of 14 were classified as stage I, 3 as stage II, and 1 as stage III. The ages of the 29 patients providing normal cervical scraping specimens for the control group ranged from 21 to 56 years, with an average of 39 years. The results of cytopathological examinations of this normal group were class I. All the samples were collected at Chung Shan Medical University Hospital, Taichung city and Show Chwan Memorial Hospital, Changhua city from 1997 to 2001. Both hospitals are located in central Taiwan.

DNA extraction and PCR assay

DNA extraction and PCR assay were performed using the protocols reported previously [11]. Briefly, DNA was extracted according to the traditional phenol/chloroform method [12]. For the 9 frozen cervical cancer specimens, 50 mg of each was homogenized in 1.5 mL Eppendorf tubes. After treatment with proteinase K at 56°C for 60 min, DNA was extracted using the phenol/chloroform procedure and purified by ethanol precipitation. For the PCR assay, the dried DNA was resuspended with 20 μ L of deionized water. Paraffin-embedded specimens were dewaxed with xylene and ethanol, followed by DNA extraction. Target DNA was amplified in a 20 μ L reaction mixture containing 2 μ L of DNA sample, 10 \times Taq polymerase buffer (20 mM KCl, 10 mM Tris-HCl at pH 8.3, 1.5 mM MgCl₂, 0.1% Triton-100), 2.5 U of Taq polymerase (Promega, Madison, WI, USA), 200 pmol of each primer, 2.5 mM of deoxyribonucleoside triphosphate and 50 μ L of mineral oil. Thermal cycles were performed in a programmable PCR thermal cycler. Primers and thermal cycler programs for identification of each of the viruses are listed in Table 1. Nine pairs of primers were

Table 1. Polymerase chain reaction primers, product length and programs

Virus/gene	Region	Primers	Product length	PCR program
β -Globin		PC04: 5'-CAACTTCATCCACGTTACC-3' GH20: 5'-GAAGAGCCAAGGACAGGTAC-3'	268 bp	95°C 1', 55°C 1', 72°C 1'; \times 40
CMV	IE2	IE2-1: 5'-TCCTCCTGCAGTTCGGCTTC-3' IE2-2: 5'-TTTCATGATATTGCGCACCT-3'	240 bp	95°C 1', 50°C 1', 72°C 1'; \times 40
EBV	<i>Bam</i> HI-W fragment	a: 5'-TCGCGTTGCTAGGCCACCTT-3' b: 5'-CTTGGATGGCGGAGTCAGCG-3'	296 bp	95°C 1', 58°C 1', 72°C 1'; \times 40
HSV-1	DNA polymerase	a: 5'-ATGGTGAACATCGACATGTACCG-3' b: 5'-CCTCCCCTTCGTCCTCGTCTCC-3'	469 bp	96°C 1', 67°C 2', 72°C 3'; \times 40
HSV-2	DNA polymerase	a: 5'-ATGGTGAACATCGACATGTACCG-3' c: 5'-CCTCCTTGTCGAGGCCCCGAAAC-3'	391 bp	96°C 1', 67°C 2', 72°C 3'; \times 40
HHV-8	Putative minor capsid	KS1: 5'-AGCCGAAAGGATCCACCAT-3' KS2: 5'-TCCGTGTTGTCTACGTCCAG-3'	233 bp	95°C 1', 55°C 1', 72°C 1'; \times 40
HPV	Late 1	MY9: 5'-CGTCCMARRGGAWACTGATC-3' MY11: 5'-GCMCAGGGWCATAAYAATGG-3'	450 bp	95°C 1', 55°C 1', 72°C 1'; \times 40
HPV typing		Sense: MY9 5'-CGTCCMARRGGAWACTGATC-3' Antisense: HPV-6: 5'-ATCCGTAACATCATCTTCCACATACACCAA-3' HPV-11: 5'-ATCTGTGTCTAAATCTGCTACATACACTAA-3' HPV-16: 5'-GTCATTATGTGCTGCCATATCTACTTCAGA-3' HPV-18: 5'-TGCTTCTACACAGTCTCCTGTACCTGGGCA-3' HPV-31: 5'-TGTTTGTGCTGCAATTGCAAACAGTGATAC-3' HPV-33: 5'-TTTATGCACACAAGTAAGTACTAGTACAGTAC-3' HPV-35: 5'-GTCTGTGTGTTCTGCTGTGCTTCTAGTGA-3' HPV-45: 5'-ACACAAAATCCTGTGCCAAGTACATATGAC-3' HPV-58: 5'-ATTATGCACTGAAGTAAGGAAGGTAC-3'	358 bp 356 bp 432 bp 108 bp 254 bp 111 bp 360 bp 402 bp 364 bp	95°C 1', 55°C 1', 72°C 1'; \times 35

Abbreviations: CMV = cytomegalovirus; EBV = Epstein-Barr virus; HSV = herpes simplex virus; HHV = human herpes virus; HPV = human papillomavirus; PCR = polymerase chain reaction

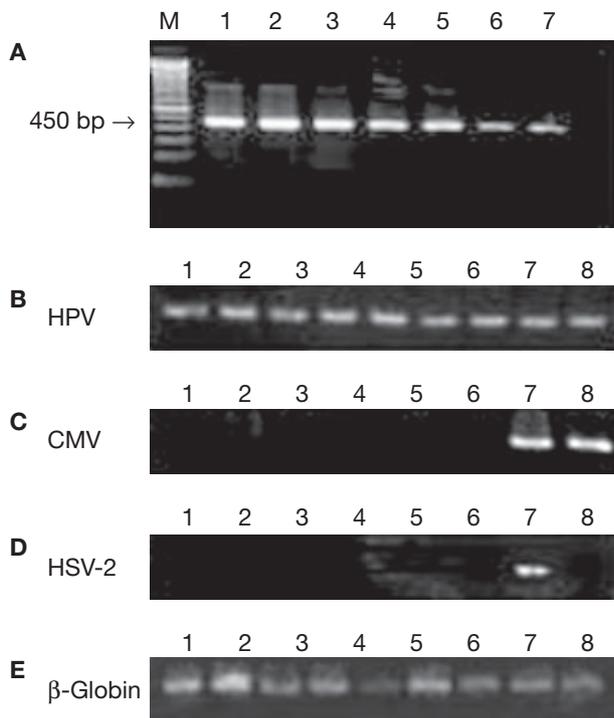


Fig.1. The results of 2% agarose gel electrophoresis after polymerase chain reaction. A) Lanes 1-7: human papillomavirus (HPV)-18-plasmid 0.1 ng, 20 pg, 4 pg, 0.8 pg, 160 fg, 32 fg, and 6.4 fg, respectively. B) HPV; C) cytomegalovirus (CMV); D) herpes simplex virus (HSV)-2; E) β -globin as internal control. B)-E): Lanes 1-8 are different cervical cancer samples.

used for HPV typing. β -Globin, HPV-18-plasmid, clinical CMV, EBV, HSV-1, HSV-2 and HHV-8 positive samples were also used as controls. PCR products were examined by direct agarose gel electrophoresis stained with ethidium bromide.

Statistical analyses

The presence of viral DNA in cervical cancer tissues was analyzed by Fisher’s exact test. The Fisher’s exact test was also used to test the relation of cytopathological cancer stage classifications with PCR-positive percentage between frozen and paraffin-embedded sample groups. *p* value <0.05 or <0.01 was taken to be significant in all analyses.

Results

Nine frozen tissues and 18 paraffin embedded specimens, for a total of 27 specimens, were chosen for cervical viral DNA study (HPV, CMV, EBV, HSV-1, HSV-2 and HHV-8). Different amounts of the HPV-plasmid DNA were serially diluted and analyzed by PCR. The sensitivity test suggested that 6.4 fg to 0.1 ng of viral DNA was detectable by 40 cycles of PCR (Fig. 1A). The amount of DNA template was adjusted within the sensitivity range. Fig. 1 demonstrates the results of agarose gel electrophoresis after PCR. HPV DNA was identified in 18 (66.7%) of 27 specimens: 9 frozen tissues (9/9, 100%) and 9 paraffin embedded tissues (9/18, 50%). For the 9 frozen tissues, CMV DNA was identified in 3 specimens (33.3%) and HSV-2 DNA was identified in 1 (11.1%). EBV, HSV-1 and HHV-8 DNA were not found in either frozen or paraffin-embedded tissues. Among the 9 HPV-positive frozen tissues, 3 (33.3%) were identified as HPV-16, 2 (22.2%) were identified as HPV-18 and 1 specimen (11.1%) contained both HPV-16 and HPV-18, 1 (11.1%) was identified as HPV-35, 1 (11.1%) as HPV-45 and 1 (11.1%) was unidentified HPV (data not shown). Among the 9 HPV-positive, paraffin embedded tissues,

Table 2. Detection of viruses in cervical cancer samples by polymerase chain reaction

Virus	Frozen tissue n = 9	Paraffin embedded tissue n = 18	Cervical cancer specimen n = 27	Control n = 29
HPV (+)	9 (100%)	9 (50%)	18 (66.7%) [<i>p</i> <0.001]	0
HPV-16	3 (33.3%)	6 (33.3%)	9 (33.3%)	
HPV-18	2 (22.2%)	0	2 (7.4%)	
HPV-16,-18	1 (11.1%)	0	1 (3.7%)	
HPV-35	1 (11.1%)	0	1 (3.7%)	
HPV-45	1 (11.1%)	0	1 (3.7%)	
Indeterminate	1 (11.1%)	3 (16.7%)	4 (14.8%)	
CMV (+)	3 (33.3%)	0	3 (11.1%) [<i>p</i> <0.05]	0
EBV (+)	0	0	0	0
HSV-1 (+)	0	0	0 (0%)	0
HSV-2 (+)	1 (11.1%)	0	1 (3.7%) [<i>p</i> >0.05]	0
HHV-8 (+)	0	0	0 (0%)	0

Abbreviations: HPV = human papillomavirus; CMV = cytomegalovirus; EBV = Epstein-Barr virus; HSV = herpes simplex virus; HHV = human herpes virus

Table 3. Presence of different viruses in samples of different stages of cervical cancer

Stage	No. of cases		HPV					Indeterminate	CMV	HSV-2
	+	-	16	18	16,18	35	45			
	CIS	2		1						
I	12	7	5	2		1		4	2	1
II	3	1	3							
III		1								
IV	1				1					
Total	18	9	9	2	1	1	1	4	3	1

Abbreviations: HPV = human papillomavirus; CMV = cytomegalovirus; HSV = herpes simplex virus; + = positive; - = negative; CIS = carcinoma in situ

6 (33.3%) were identified as HPV-16 and 3 (16.7%) were indeterminate types (data not shown). Table 2 summarizes the results. Among the 29 normal cervical scraping specimens from the normal controls, no viral DNA was detected.

Table 3 lists the presence of different viruses in the different stages of cervical cancer. Of the 18 HPV-positive samples, 2 out of 2 were in CIS, 12 out of 19 (67.0%) in stage I, 3 out of 4 (75.0%) in stage II, and 1 out of 1 in stage IV.

Discussion

Epidemiological and molecular studies show that cervical infection by certain types of human HPV is a precursor event in the genesis of cervical neoplasia [13]. Walboomers et al reported that the association between HPV and cervical cancer is high, up to 99.7% [14]. Several methods for detection of viral DNA have been used, including PCR, in situ hybridization, immunohistochemical staining, dot blotting and Southern blotting. As our sensitivity test result revealed that 6.4 fg to 0.1 ng of viral DNA was detectable by 40 cycles of PCR, it suggests that PCR is the most sensitive method of virus identification among these methods. In this study, the presence of viral DNA was thus identified using the PCR method. Recently, biotinyl-tyramide-based in situ hybridization methodology was used to investigate the prevalence of HPV integration in cervical intraepithelial neoplasia [15]. This method was demonstrated to be able to detect integrated viral DNA by punctuate signals within the nucleus and episomal viral DNA by a diffuse signal throughout the nucleus. For HPV PCR, the primers we selected were within gene late 1 region, which is a conserved region. In order to rule out the possibility that the HPV late 1 region did not integrate into the cellular genomic DNA, and

therefore could not be detected by PCR, the DNA of the oral specimens was treated with topoisomerase I to loosen supercoiled DNA according to the method described by Laghi et al [16]. However, still no difference of positive/negative results was detected (data not shown).

Over 30 types of genital HPV are reported to be oncogenic. The highly oncogenic genital HPV types include 16, 18, 31, 33, 52, 58, 35, 39, 45, 51, 56, 59, 66, and 68 [17]. Our findings demonstrated that 18 HPV-positive among 27 cervical cancer specimens (66.7%) were associated with cervical cancer ($p < 0.001$). These findings are compatible with those reported by others in Taiwan, suggesting HPV is the major risk factor for cervical neoplasia [18,19]. HPV-16 was the most prevalent type among HPV-positive specimens (9/18), and was distributed differently in different cervical CIS/CIN classes (Table 3). The study by Liaw et al [19] also showed that HPV DNA was found in 92% of high-grade cases (CIN 2-3 and invasive cancer); 54% of low-grade cases (CIN 1); and 9% of controls.

In the present study, analysis by Fisher's exact test suggests that there was no association between the sensitivity of HPV detection and cancer severity. However, only types 16, 18, 35, and 45 were identified in this study except for 4 samples that were indeterminate. Among the HPV types detected, type-16 is the most closely associated with cervical cancer and type-18 is the second. The study by Wu et al [18] demonstrated that HPV-16 and/or -18 (HPV-16/18) were detected in 71% (34 of 48) of neoplastic lesions of patients from Taiwan. Our data showed that HPV-16/18 were detected at a rate of 66.7% (12 of 18). This result is also consistent with that reported by An et al [20].

As some of the specimens collected were formalin-treated and paraffin-embedded, we considered the possibility that storage might have affected the DNA

stability. As shown in Table 2, HPV was detected in all 9 frozen tissues, but was detected in only half of the 18 paraffin-embedded tissues with cervical cancer. This indicates that the correlation of HPV with cervical cancer was significantly different between frozen tissues and paraffin-embedded tissues ($p < 0.05$). For paraffin-embedded oral cancer tissues, only 1 case was detected to be positive with HSV-1 DNA. Taken together, it was suggested that formalin treatment and paraffin embedding might have affected the quality of DNA during storage for PCR detection. In frozen cervical tissue, different HPV types were identified, with the exception of 1 indeterminate case. Among these tissues, CMV was found in 11.1% (3/27) of specimens and HSV-2 was found in 3.7% (1/27). Both viruses were co-infected with HPV. Analysis by Fisher's exact test suggested that CMV may be a cofactor in the oncogenesis of cervical cancer and HSV-2 a bystander. However, this finding is controversial since it has been reported that CMV is a bystander rather than a cofactor ($p < 0.05$) [21,22] and HSV-2 increases the rate of development of cervical neoplasia [23]. Moreover, EBV, HSV-1 and HHV-8 were not detected using PCR method, which suggested that these 3 viruses might have little or no relationship with cervical cancer.

In summary, viral factors, especially HPV, are closely related to cervical cancer. Other viruses such as HSV-2 and CMV are not predictive in this context. They might not be involved in the oncogenic processes directly but might enhance the possibility of oncogenesis or infect cancer tissues opportunistically. Our findings agree with reports that HPV infection is correlated with cervical cancer.

Acknowledgments

We thank Professor Dr. Ravindra M. Shah, Faculty of Dentistry, University of British Columbia, for proofreading. This study was funded by a grant (CSMC88-OM-B-035) from the Chung Shan Medical University, Taichung, Taiwan, ROC.

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