DETECTION AND TYPING OF HUMAN PAPILLOMAVIRUS IN CERVICAL INTRAEPITHELIAL NEOPLASIA GRADE III IN THAI WOMEN

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Abstract. Infection of human papillomaviruses (HPVs) has been shown to play an important role in the development of cervical cancer from precancerous lesions known as cervical intraepithelial neoplasia (CIN-I, CIN-II and CIN-III). In Thailand, cervical cancer is the most common cancer in women. Fifty tissue samples diagnosed as CIN-III and 50 tissues of normal histopathologic appearance as controls were examined for the presence of HPV-DNA and HPV typing using PCR and dot hybridization (DH) methods. All specimens used in this study were formalin-fixed paraffin embedded tissues. HPV-DNA was detected in 74% (37/50) of CIN-III and 6% (3/50) of the control group giving a crude odd ratio of 44.58 (95% confidence interval of 15.2-130). Among the CIN-III group, the most prevalent type was HPV-16 ; 48.65% (18/37) followed by HPV-18 ; 16.2% (6/37) and HPV-33 ; 10.8% (4/37). Mixed infection was identified in 4 specimens, *ie* HPV-6/16, HPV-16/18, HPV-16/33, and HPV-16/18/33. Twelve samples were untyped. In the control group, only one sample (33.3%) was detected to contain HPV-6 DNA and the remaining ones were untyped. Our results revealed infection with HPV, especially HPV-16 and HPV-18, to be strongly associated with CIN-III in Thai women.

INTRODUCTION

Cervical cancer remains an important health problem for women worldwide, especially in developing countries including Thailand. Approximately 33% of all cancers occurring in Thai women is cervical cancer (National Cancer Institute Thailand, 1995). Cervical cancer develops from precancerous lesions known as cervical intraepithelial neoplasia (CIN). The CIN is divided into 3 grades, *ie* CIN-I, CIN-II, CIN-III, which describe the various stages of cellular atypia seen in abnormal cervical epithelium. Epidemiologic studies have documented that human papillomaviruses (HPVs) play an important role in cervical cancer and CIN. Approximately 90-100% of all cervical cancers are caused by infection with HPV, whereas the prevalence of HPV in each grade of CIN varies widely. It ranges from 25- 90% according to the severity of the lesion (Cornelissen *et al,* 1992 ; de Villiers *et al,* 1987; Kalantari *et al,* 1997; Liaw *et al,* 1995; Olesen *et al,* 1995; Wu *et al,* 1994).

Nowadays, more than 70 genotypes of HPV are recognized. Corresponding to an association with cervical cancer and CIN, three groups can be separated, *ie* low risk group (HPV-6, 11, 42, 43,and 44), intermediate risk group (HPV-31, 33, 39, 51, 52 and 66), and high risk group (HPV-16, 18, 45, and 46). Many studies have shown the progression of low grade CIN (CIN-I) to high grade CIN (CIN-II, III) or invasive cancer to depend on the respective HPV types. For example, the patients infected with the high risk HPV (HPV-16/18) have a rapid progression to invasive cancer when compared to those with low risk HPV (HPV-6/11) (Campion *et al,* 1986). A recent prospective cohort study of women who were cytologically negative at the study inception, but HPV 16/18 positive, revealed CIN II or III in 39% within a 2 year follow-up, but in only 3% negative for all HPV types (Koutsky *et al,* 1992). In this present study, an independent cross-sectional study was designed to investigate the prevalence of HPV infection and HPV types in CIN-III patients compared to the control group by using PCR and hybridization methods.

MATERIALS AND METHODS

All samples used in this study were formalin-fixed paraffin embedded tissues. Fifty samples with

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a histopathologic diagnosis of CIN-III and 50 samples obtained from cases of chronic cervicitis with a normal histopathologic appearance were used. Both were selected from outpatient women attending the Department of Obstetrics and Gynecology of Chulalongkorn Hospital, Bangkok, Thailand, during the year 1995-1996.

Detection of HPV-DNA was carried out by PCR amplification of HPV-L1 region, using consensus primers MY11 and MY09, in parallel with the human beta-globin primers GH20 and PC04 (Bauer *et al,* 1991). The DNA extracted from the tissue was prepared as described in Bhattarakosol *et al,* (1996). Amplification of DNA was carried out in a 50 µl total reaction mixture containing 50 mM KCl, 10 mM Tris, pH 8.5, 4 mM $MgCl_2$ 200 µM each dNTPs, 25 pmole of each HPV-L1 primers, 2.5 pmole of each beta-globin primer, 1.25 unit of Taq DNA polymerase (BRL,USA). After adding one µl of DNA sample, 30 µl of mineral oil were overlayed to prevent evaporation. The DNA amplification was performed using an automated thermal cycler (Perkin-Elmer Cetus, USA). The DNA was denatured at 95°C for one minute, annealed at 50°C for one minute, and extended at 72°C for 2 minutes. The step was repeated for 40 cycles followed by a final extension at 72°C for 10 minutes in the last cycle. The amplified HPV-L1 product is approximately 450 bp, whereas the beta-globin product is 268 bp. Successful amplification of beta-globin indicated the sample to be adequate for HPV analysis and no inhibitors to be present in the PCR reaction. HeLa-DNA was used as a positive control. HeLa cell is a continuous cell line, originally derived from a human cervical carcinoma and it contains HPV-18 DNA sequences, about 10-30 copies per cell (Bauer *et al,* 1991). Human DNA, extracted from human white blood cells, was used as an HPV-DNA negative and beta-globin as an internal control.

To type the HPV-DNA, dot hybridization (DH) was performed. Five purified plasmids containing HPV-6, 11, 16, 18 and 33 prepared by mean of miniprep technic (Davis *et al,* 1994) were used as type-specific HPV-DNA standards. The remaining aqueous reaction mixture from the PCR reaction was extracted with chloroform to remove the mineral oil. One microgram of the purified PCR product was dotted on a Hybond™ N^+ membrane (Amersham, England). Membrane preparation and hybridization were performed according to the manufacturer's specifications. To determine the presence and type of HPV, two generic oligonucleotide probes (GP01 and GP02) specific for the amplified HPV-L1 product and type specific oligonucleotides (MY12, MY13, MY14, WD74 and MY16) specific for HPV 6,11,16,18 and 33, respectively, were used as probes (Bauer *et al,* 1991). The probes were labeled using the enhanced chemiluminescence (ECL) non-isotope labeling kit purchased from Amersham, England. For hybridization, the amounts of probe used in the reaction were varied depending on each kind of probe. The GP01 and GP02 mixture, MY13 and MY16 were used at a 0.5 pmole concentration while MY 12, MY 14, MY16 and WD 74 were used at a 5 pmole concentration, each. The hybridization conditions and washing temperatures were 40°C and 40°C for MY12, MY13 and MY16; 42°C and 45°C for GP01 and GP02 mixture; 50°C and 55°C for MY14; and 55°C and 60°C for WD74, respectively. Hybridization was allowed for one and a half hours. Then, the membrane was washed twice with 5x SSC (0.75M NaCl, 0.075M Na citrate, pH 7.0) containing 0.1% SDS for 15 minutes. Hybridized DNA was detected using an ECL detection kit (Amersham, England). The ECL labeling and detection system utilizes the enhanced chemiluminescence associated with the horseradish peroxidase catalyzed oxidation of luminol to detect the oligonucleotide tailed at the 3' end with fluorescein-dUTP hybridized to the target sequence on the membrane.

The relationship between HPV infection and CIN-III were analysed by using the odds ratio (OR) and a 95% confidence interval (CI) (Mortan and Hebel, 1979)

RESULTS

Tissue samples collected from 50 CIN-III cases (mean age 39.14 years; SD 7.69 and median age 38.5 years) and 50 cases of the control group (mean age 43.86 years; SD 8.48 and median age 44.5 years) were examined for the presence of HPV-DNA by PCR amplification using an HPV-L1 consensus primer and the DH method employing generic probes (GP01 and GP02). The results revealed that 74% (37/50) of CIN-III and 6% (3/50) of the control group were HPV-DNA positive. This result yields an OR of 44.58 (95% CI= 15.2-130) supporting the association of CIN-III with HPV infection (Table 1).

After typing by DH using type specific probes (see materials and methods), it was found that HPV-16 is the most prevalent type among CIN-III cases (48.65%, 18/37) followed by HPV-18 (16.2%,6/37), HPV-33 (10.8%,4/37) and HPV-6 (5.4%, 2/37). There were 4 (10.8%) CIN-III cases with mixed infection between types and 12 (32.4%) cases could not be typed. In contrast, only one HPV-6 was typed among 3 HPV-DNA positive samples in the control group and the other 2 were untyped (Table 1).

Table 1

The distribution of HPV type (s) in CIN-III and control groups.

Samples	Total	HPV		HPV types (%)								Untype
		- (%)	+ (%)	6	11	16	18	33	6/16	16/18	16/33	16/18/33 (%)
CIN-III	50	13	37	1	-	18	6	4	1	1	1	1 12
		(26)	(74)	(2.7)		(48.65)	(16.2)	(10.8)	(2.7)	(2.7)	(2.7)	(2.7) (32.4)
Control	50	47	3	1	-	-	-	-	-	-	-	- 2
		(94)	(6)	(33.3)								(66.7)

DISCUSSION

Cervical dysplasia or neoplasia is a serious threat to women's health. Several factors are involved in the development of the disease, for example, sexual behavior (including sexually transmitted diseases), socioeconomic status, smoking etc, including HPV infection (Bornstein *et al,* 1995). Epidemiologic studies on the association between HPV infection and cervical dysplasia have been performed in many countries. The results show an HPV prevalence of approximately 25- 50% in CIN-I, 60-80% in CIN-II and 60-90% in CIN-III, respectively (Arends *et al,* 1991; Liaw *et al,* 1995; Wu *et al,* 1994).

In 1996, our group has reported the prevalence of HPV infection in cervical cancer in Thai women: HPV-16,18 and 33 were found in 61% of HPV positive cases (Bhattarakosol *et al,* 1996). In the present study, detection and typing of HPV in CIN-III patients have been performed in order to compare the results to those of our previous report. Moreover, histologically normal tissues of a control group also have been included in this study.

Our results show the prevalence of HPV-DNA in CIN-III patients to amount to 74% (37/50), while in the control group only 6% (3/50) were detected by PCR amplification and DH using generic probes. This result hints at a strong association between HPV-infection and CIN-III (OR=44.58; 95% CI= 15.2-130).

Typing of HPV-DNA by DH using type specific probes, we revealed that the type most prevalent in positive CIN-III patients was HPV-16 (48.65%,18/37) whereas HPV-18 was detected in 6 samples (16.2%), HPV-33 in 4 samples (10.8%) and HPV-6 in 2 samples (5.4%) (Table 1). Three samples showed double infection and one triple infection

(Table 1). These data strongly support those of our previous report on cervical cancer patients *ie* HPV-16; 42.7%, HPV-18; 20.7% and HPV-33; 3.6% (Bhattarakosol *et al,* 1996)

Although the two studies were performed in different groups of patients and in different years, the results indicate the same pattern of HPV-type infection. CIN-III represents the precancerous stage which can progress to the cancerous stage, that is cervical cancer. Therefore, we confirmed that HPV-16, 18 and 33 play an important role in CIN-III and cervical cancer in Thai women. Moreover, we also examined 50 patients with chronic cervicitis whose tissues appeared normal for the presence of HPV-DNA. This group acts as a control group. It was found that only 6 % (3/50) contained HPV-DNA and one of them was identified as HPV-6, a low risk HPV. Although the other 2 samples could not be typed they were not among HPV-16,18 and 33. When the result of the control group was compared to that of the CIN-III group, HPV infection in CIN-III was 12 times greater than in the control group. Detection of HPV-DNA in control patients has been reported in approximately 9 - 15 % (Liaw *et al,* 1995; Olesen *et al,* 1995).

In our study, HPV-L1 consensus primers were used for PCR amplification. The L1 region encodes the major capsid protein and is known to be conserved among HPV-types. At least 25 types of anogenital HPV can be amplified by these primers (Ting *et al,* 1990). Therefore, untyped samples may either be one of those 25 types or new types. Moreover, those negative for HPV cannot be excluded from possibly having HPV- infection. Since the majority of CIN-III harbor high risk HPV (HPV-16 and 18), detection and typing of HPV-DNA at various stages of CIN may be helpful in isolating high risk women with a chance to develop cervical cancer and it will possibly play an adjunctive role in population screening.

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