DETECTION AND TYPING OF HUMAN PAPILLOMA VIRUS DNAs IN NORMAL CERVIX, INTRAEPITHELIAL NEOPLASIA AND CERVICAL CANCER IN BANGKOK

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Abstract. We detected and typed HPV-DNA by polymerase chain reaction (PCR) in cervico-vaginal lavages of 102 women with normal cervical cytology, 57 patients with cervical intraepithelial neoplasia (CIN), and 23 cervical cancer patients. HPV-DNA detection and typing by *in situ* hybridization were also performed in cervical biopsies from CIN lesions and cancers. Five percent of women with normal cervical cytology, 46% of CIN, and 61% of cervical cancer were positive for HPV-DNA. Of CIN cases with positive HPV-DNA, 69, 15, 8, 4 and 4% were HPV-16, -33, -18, -11 and -16/33 respectively. Of cervical cancer cases with positive HPV-DNA, 86% were HPV-16, 7% were HPV-16/33, 7% were HPV-18/31. HPV typing was performed in biopsies from 37 CIN and 18 cervical cancers by *in situ* hybridization. By this method, 38% of CIN were HPV-DNA positive, of which 71% were HPV-16 and 7% were each of HPV-11, -18, -31 and -33. Thirty-nine percent of cevical cancers were positive, of which 71% and 29% were HPV-16 and HPV-16/18 respectively.

INTRODUCTION

Cervical cancer is the most common malignancy in Thai women (The Fact Finding Commission, National Epidemiology Board of Thailand, 1987; Vatanasapt *et al*, 1990). Infection by certain HPV types as shown by presence of HPV-DNA in cervix is a major risk factor for development of malignancy. HPV-16, -18, as well as HPV-31, -33, -35 are HPVs with carcinogenic potential, whereas HPV-6 and -11 are generally associated with premalignant lesions and low risk of progression (de Villiers, 1989; Jenson and Lancaster, 1990). Detection of high risk HPVs may be helpful in identifying individuals with higher risk of malignant progression (Lorincz, 1992; Meijer *et al*, 1992). HPV-DNA can be detected in cervico-vaginal specimens successfully by either hybridization technics such as dot blot hybridization, *in situ* hybridization, and Southern blot hybridization or by polymerase chain reaction (PCR). Amplification of the viral DNA by PCR provides higher sensitivity and improves significantly the detection rate (Ting and Manos, 1990). Prevalence and typing of HPV-DNA in cervico-vaginal specimens from Thai women has not been extensively studied. We analyzed the presence of HPV-DNAs of various types in vaginal washes from individuals with normal cervical cytology, CIN, and cervical carcinoma by PCR, and in tissue sections from CIN and cervical carcinoma patients by *in situ* hybridization.

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MATERIALS AND METHODS

Subjects and specimen collection

Cervico-vaginal lavages were collected from 102 women (age 16-59 years, mean ± SD: 32 ± 10 years), who attended the Gynecology Clinic at Siriraj Hospital for cervical cancer screening and had normal cytology in cervical smears, and 89 women (age 16-77 years, mean ± SD: 37 ± 11 years), who had abnormal cytology indicative of CIN or invasive cancer. The lavages were performed at the next visit after cervical smear with patients' consent. The lavages were done by gently washing the vaginal canal with 10 ml of normal saline. The lavages were then centrifuged at 3,000 rpm for 10 minutes, and the pellels were resuspended in 1 ml of phosphate buffered saline (PBS) and stored at -70°C.

Cervical biopsies were performed in patients with cervical lesions suggestive of neoplasia. A total of 55 biopsies were obtained, of these 46 cases had cervico-vaginal lavages. The biopsy specimens of 3-5 mm^3 in size were fixed in 10% buffered formalin and embedded in paraffin blocks. Five micron thick sections were placed on 2% aminopropyltriethoxysilane coated slides, dried at 37°C for 30 minutes, baked sequentially at 75°C for 1 hour and at 60°C overnight, and stored at room temperature before used for *in situ* hybridization.

HPV-DNA detection

PCR was performed as described (Ting and Manos, 1990). Briefly, the lavages were digested with 100 μg/ml of proteinase K in 1% Tween-20 at 55°C for 1 hour and the proteinase K was inactivated by heating at 95°C for 10 minutes. Ten microliters of the lysate was then subjected to PCR reaction by the primers MY11 and MY09, which are conserved primers that amplify L1 region of HPVs. The PCR was carried out with 95°C 1 minute, 55°C 1 minute, 72°C 1 minute for 35 cycles. To control for the adequacy of DNA in the samples, β-globin primers (GH20 and PC04) were included in the reaction. The PCR products were visualized in ethidium bromide stained agarose gel. The positive products were dot-blotted onto nylon membranes and hybridized with 3'-fluorescein labeled group-specific and type-specific oligonucleotide probes in order to confirm the positivity and determine the HPV type. The group-specific probes, which hybridize to all HPV type were MY1019 and MY18. The type-specific probes were MYB12, MYB13, MYB95, MYB130, MYB92, MYB64, and MYB117 for HPV-6, -11, -16, -18, -31, -33, -35, respectively. The hybridization signals were visualized by horseradish peroxodase labeled anti-fluorescein and enhanced chemiluminescence reaction (Amersham).

In situ hybridization was performed as described (Wolber and Clement, 1991). Briefly, the sections were deparaffinized in xylene, washed in ethanol and running water, and then digested with 0.5 μg/ml of proteinase K in 2mM $CaCl_2$, 20 mM Tris. Cl pH7.5 at 37°C for 20 minutes. After washing in 0.2% glycine/PBS, the slides were refixed in PBS containing 4% paraformaldehyde and 5 mM $MgCl_2$ for 7 minutes, washed again in 2% glycine/PBS, and dehydrated through graded ethanol/H_2O. For hybridization, the slides were overlaid with small drops of hybridization solution (50% formamide, 2xSSPE, 10% dextran sulphate, 10 mM Tris buffer pH 7.5, 1x Denhardt's solution, 500 μg/ml tRNA, 100 μg/ml salmon sperm DNA, 0.1% SDS) containing 5 μg/ml of type-specific biotinylated probes at 37°C overnight. To prepare probes, HPV-plasmids were purified by agarose gel electrophoresis after digesting with restriction enzymes to get rid of vector sequences. The HPV inserts were biotinylated by nick translation labeling. After hybridization, the sections were washed in 0.5x PBS/30% formamide at 37°C for 30 minutes, 0.5x PBS/10% formamide at 37°C for 30 minutes, 2x SSC for 15 minutes at room remperature. The signals were developed by incubating with streptavidine-alkaline phosphatase, washing, and subsequently incubating with the substrate NBT/BCIP to obtain purple color signal.

RESULTS

Sensitivity of PCR

To determine the sensitivity of the PCR, we performed PCR on serial dilution of HeLa cell line, which harbors 10-50 copies of HPV-18 DNA per cell. A 450 bp fragment was amplified in PCR reaction with a minimum of 5 HeLa cells per reaction. The lower limit of our PCR for detecting HPV-DNA was therefore 50-250 copies.

Detection and typing by PCR

Table 1 shows the detection rate of various HPV type by PCR. In women with normal cervical cytology, HPV-DNA could be detected in only 5 out of 102 cases. Of these 2 were HPV-6, one was HPV-11, one was HPV-16, and the other was HPV-18. In CIN and cervical cancer patients, HPV-DNA was detected in 46% and 61%, respectively. HPV-16 was the major type found in these groups.

Detection and typing by *in situ* hybridization

By *in situ* hybridization HPV-DNA was detected in 38% and 39% of CIN and cancer cases respectively. Majority of the HPV type was HPV-16 (Table 2). To compare the results between PCR

Table 1

Detection and typing of HPV-DNA in cervico-vaginal lavages by PCR and dot blot hybridization.

Histological diagnosis	No. cases	No. positive (%)	No. of cases with HPV-DNA type								
			6	11	16	18	31	33	35	16/33	18/31
CIN	57	26 (46)	0	1	18	2	0	4	0	1	0
CIN I	12	5 (42)	0	1	3	1	0	0	0	0	0
CIN II	10	0	0	0	0	0	0	0	0	0	0
CIN III	45	21 (47)	0	0	15	1	0	4	0	1	0
Cancer	23	14 (61)	0	0	12	0	0	0	0	1	1
Microinvasive	5	3 (60)	0	0	2	0	0	0	0	1	0
Invasive	16	10 (63)	0	0	10	0	0	0	0	0	0
Adeno	1	1	0	0	0	0	0	0	0	0	1
Adeno-squamous	1	0	0	0	0	0	0	0	0	0	0
Normal cervix	102	5(5)	2	1	1	1	0	0	0	0	0

Table 2

Detection and typing of HPV-DNA in cervical biopsies by *in situ* hybridization.

Histological diagnosis	No. cases	No. positive (%)	No. of cases with HPV-DNA type							
			6	11	16	18	31	33	35	16/18
CIN	37	14 (38)	0	1	10	1	1	1	0	0
CIN I	5	1	0	0	1	0	0	0	0	0
CIN II	0	0	0	0	0	0	0	0	0	0
CIN III	32	13 (41)	0	1	9	1	1	1	0	0
Cancer	18	7 (39)	0	0	5	0	0	0	0	2
Microinvasive	4	1	0	0	0	0	0	0	0	1
Invasive	12	6 (50)	0	0	5	0	0	0	0	1
Adeno	1	0	0	0	0	0	0	0	0	0
Adeno-squamous	1	0	0	0	0	0	0	0	0	0

and *in situ* hybridization, we analyzed the results from 46 cases that both cervico-vaginal lavages and cervical biopsies were available. PCR could detect HPV-DNA in 50% of cases (47% of CIN and 57% of cancer), whereas *in situ* hybridization could detect only 35% (31% of CIN and 43% of cancer). In cases that both test were positive, results of the HPV-DNA typing by both tests was the same.

DISCUSSION

As has been well documented, the presence of HPV-DNA associated with CIN and invasive cervical cancer in our study population. The prevalence in women with normal cervical cytology has been shown to vary substantially among studies and with respect to geographical area. Prevalence rates of 3-

50% were reported in various populations (de Sanjose *et al*, 1992). In our study population we found 5% of women with normal carvical cytology to be positive for HPV-DNA. Both low risk (6, 11) and high risk (16, 18) HPV types were found in this group. In contrast to our results, prevalence of HPV in women with normal cervical cytology in a northeast Thailand province (Khon Kaen) has been reported to be 18.8% (Ekalaksnanan *et al*, 1992). This higher prevalence might be due to socioeconomic and behavioral differences between this study population and ours or it could be due to a difference in examination of cervical smears. Diagnosis of cervical neoplasia by microscopic examination of cervical smear is rather subjective, and the results could vary considerably among examiners. Underdiagnosis of cytological abnormality would result in including individuals with cervical neoplasia in the normal group. This in turn results in an artifactually high prevalence of HPV. As expected, we found a high prevalence of HPV in patients with cervical neoplasia. The prevalence in the invasive cancer group was higher than in the CIN group. PCR showed a higher detection rate than *in situ* hybridization indicating higher sensitivity. The prevalence in our study was comparable to other studies (Nimmanahaeminda *et al*, 1994; Thirapakawong *et al*, 1989). The fact that nearly 40% of invasive cancers were negative for HPV-DNA by PCR suggests that either the test was not sensitive enough to detect all the infections or there might be different etiologic agents for HPV-negative cancer, for example, diverse strains of HPV that could not be detected by the primers used.

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