MATERIALS AND METHODS

1. Study Population

A total of 214 women who attending the outpatient gynecological clinic of Maharaj Nakorn Chiang Mai Hospital during January to August 1998 were enrolled in this study. They were cytologically and pathologically diagnosed as normal, low-grade squamous intraepithelial lesions (LGSIL), high-grade squamous intraepithelial lesions (HGSIL), squamous cell carcinoma, and adenocarcinoma.

2. Specimen Collection and Storage

Either cervical cell scrapes or tissue biopsies were collected and used for this study. Cervical cell scrapes were collected from women who were clinically diagnosed as normal or preinvasive cancer stage while tissue biopsies were obtained from women with invasive stages. Cervical cell scrapes were collected by using an Ayer spatular scraped at the exocervical and endocervical area. After scraping, cervical cells were smeared on a glass slide and sent for cytological diagnosis, and the rest were placed in 3 ml of cold steriled normal saline. When arriving at the laboratory, the samples were centrifuged and the cervical cell pellets were collected. Then, 0.5 ml of Trizol™ reagent (Gibco BRL, USA) was added to lyse those cells in the pellet and they were kept at –70°C until used.

For invasive carcinoma, tissue biopsies were collected. The tissues were placed in a sterile plastic box, which was kept in an icebox. When arriving at the laboratory, they were transferred to a freezer and kept at –70°C.

3. RNA and DNA Extraction

3.1 Cervical cell scraped samples

Both RNA and DNA were extracted simultaneously from a single tube by using the Trizol™ reagent (Gibco BRL, USA). Briefly, samples were thawed and incubated on ice for 5 minutes. A 100 µl of chloroform were added and shaken

vigorously. The samples were centrifuged at 7,826xg (10,000 rpm) for 10 minutes at 4°C. The aqueous phase was transferred to a new tube. The RNA was precipitated by adding 250 μl of isopropyl alcohol. The samples were incubated for at least 1 hour at –70°C and centrifuged again. The supernatant was removed and the RNA pellet was washed twice with 500 μl of 75% ethanol. The extracted RNA was kept in 75% ethanol at –70°C until used.

For the remaining interphase and lower organic phase, DNA were extracted further by precipitating in 150 μl of absolute ethanol and mixed by inversion. The mixture was stored at –70°C for 1 hour, and then the DNA were sedimented by centrifugation at 2,000xg (5,055 rpm) for 5 minutes at 4°C. The organic supernatant was removed and the DNA pellet was washed twice in 500 μl of 0.1M sodium citrate in 10% ethanol. At each wash, the DNA pellet was stored in washing solution for 30 minutes at room temperature and centrifuged at 2,000xg (5,055 rpm) for 5 minutes at 4°C. Then, the DNA pellet was washed twice in 1 ml of 75% ethanol and again stored in solution for 20 minutes at room temperature after each wash, and centrifuged afterwards at 2,000xg (5,055 rpm) for 5 minutes at 4°C. The DNA pellet was dried briefly at room temperature and dissolved in 8 mM NaOH. The pH was adjusted to around 8.0 by using 0.1M HEPES, and the extracted DNA were kept in solution at –70°C until used.

3.2 Tissue biopsy samples

The tissue sample was cut into small pieces of approximately 50–100 mg or 0.5 x 0.5 x 0.5 cm^3 while still frozen. The sample was put into a mortar, and ground in the presence of liquid nitrogen until it became powder. Then, 1.0 ml of Trizol™ reagent (Gibco BRL, USA) was added and mixed by pipetting through a pipette tip. The sample was incubated for 5 minutes on ice. After that, 200 μl of chloroform were added and shaken vigorously. The samples was centrifuged at 7,826xg (10,000 rpm) for 10 minutes at 4°C. The aqueous phase was transferred to a new tube. The RNA was precipitated by adding 500 μl of isopropyl alcohol and the sample was incubated for at least 1 hour at –70°C before being centrifuged again. The supernatant was removed and the RNA pellet

was washed twice with 1,000 µl of 75% ethanol. After washing, the extracted RNA was kept in 75% ethanol at -70°C.

The remaining DNA were extracted by the same procedure, as explained in 3.1.

4. Oligonucleotide Primers

4.1 Primers for HPV L1 gene detection

The oligonucleotide primers used for amplification of the L1 gene are L1C1 and L2C2, which are common to all HPV genotypes (Yoshikawa *et al*, 1991). The amplified product was approximately 250 bp. The following are nucleotide sequences of the L1C1 and L2C2 primers:

L1C1 = 5' CGT AAA CGT TTT CCC TAT TTT TTT 3'

L2C2 = 5' TAC CCT AAA TAC TCT GTA TTG 3'

4.2 Primers for HPV 16/18 E6/E7 mRNA detection

Two pairs of oligonucleotide primers, P16A, HPV16R (Hsu) (Hsu,1992), and P16A, P16B, were used to amplify E6/E7 mRNA of HPV-16 and –18, respectively. The group in this study originally designed the primers P16A and P16B. These were designed from the established sequence of E6/E7 ORFs of HPV-16 and -18. Unfortunately, the P16B could only annealed and amplify efficiently with the E6/E7 ORFs of HPV18 DNA. So, we decided to search for another primer to amplify the E6/E7 of HPV 16. It was found that the HPV16 reverted primer described by Hsu (Hsu and McNicol, 1992) together with P16A primer could amplify E6/E7 ORFs of HPV16 efficiently. Thus, the primer P16A and HPV16R(Hsu) for HPV16 E6/E7 amplification was used in this study. The amplified products generated from primer the P16A-HPV16R(Hsu) were 600, 400, and 279-bp. The 600-bp fragment was represented the full-length E6/E7 transcription, while the 400 and 279-bp fragments represented a splice transcripts generated from the same splice donor site at nt226 and different splice acceptor site at nt408 and nt526, respectively (Smotkin and Wettstain, 1986). The amplified products generated from primer the P16A-P16B were 440 represented the full-length E6/E7 transcripts and 256-bp

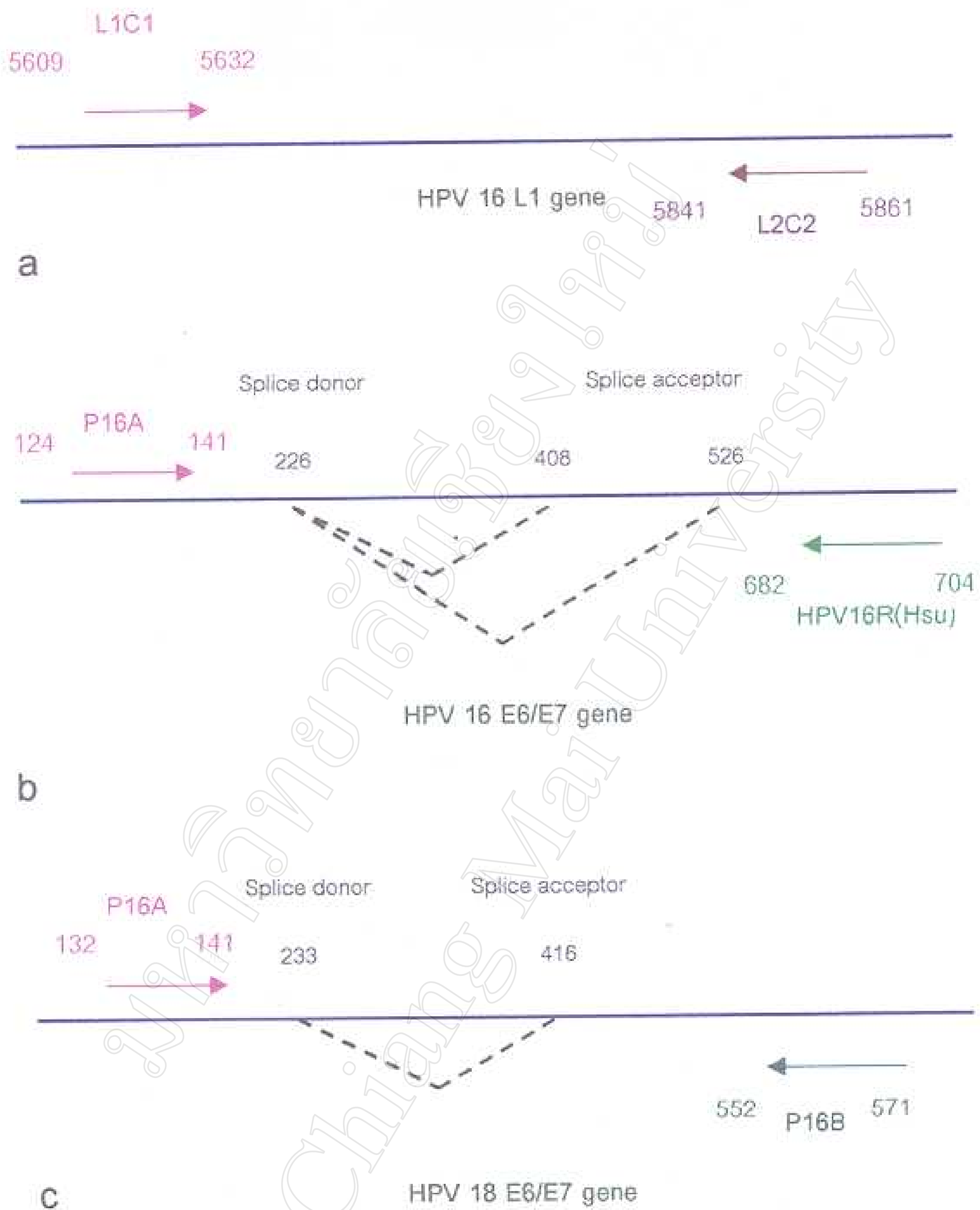

Figure 17 a) The position of L1C1 and L2C2 primers on the HPV 16 L1 ORF

b) The position of P16A and HPV16R(Hsu) primers and splice site on the HPV- 16 E6/E7 ORF

c) The position of P16A and HPV16B primers and splice site on the HPV-18 E6/E7 ORF

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splice transcript generated from splice donor site at nt233 and acceptor site at nt416 (Czegledy *et al*, 1994). Primer 16A was labeled with fluorescent dye for the aid of quantitative analysis. The following are the nucleotide sequences of P16A, P16B and HPV16R(Hsu);

P16A = 5' GCG ACC CXG CAA GXT ACC 3'

P16B = 5' GTT TCT GTG XAX CGA TGG XG 3' (X = inositol)

HPV16R(Hsu) = 5' TCC GGT TCT GCT TGT CCA GCT GG 3'

5. HPV DNA Detection by Using PCR

The PCR was carried out in a total volume of 50 μl reaction mixture, containing 2.5 mM of each dNTP (dATP, dCTP, dGTP, and dTTP), 10 mM Tris-HCl (pH 8.0) and 50 mM KCl, 2 mM $MgCl_2$, 1.25 unit Taq DNA polymerase. Twenty five pM of each L1C1 and L2C2 primers, and 0.5-1.0 μgm of sample DNA, HPV 33 plasmid DNA and distilled water were used as a positive and negative control, respectively.

The reaction was allowed to amplify for 40 cycles in a thermocycler (GeneAmp PCR system 2400, Perkin Elmer USA). Each amplification cycle comprised of denaturation at 94°C for 1 minute, primer annealing at 50°C for 1 minute and primer extention at 72°C for 1 minute. After the last cycle, all reaction mixtures were incubated further at 72°C for 7 minutes to ensure that the extension step was completed. They were then held at 4°C until picked-up for further analysis.

Ten microliters of amplification product was detected by 1% agarose gel and electrophoresis at 80 volts for 45 minutes (ethidium bromide was added to the gel at 40 μg%). The band of DNA was visualized under a UV transilluminator. The size of the DNA band was determined by comparison with a DNA marker 1 Kb ladder plus (Gibco BRL, USA).

6. HPV Genotyping by Restriction Fragment Length Polymorphism (RFLP)

The PCR amplified products from the positive samples were digested by a set of restriction endonuclease enzymes (*Rsa* I, *Dde* I, *Hae* III, and *Hinf* I). The reaction

was performed in a total volume of 20 μl. Each enzyme digesting reaction mixture contained 1-2 μgm of L1 gene amplified product, 2 μl of 10x enzyme reaction buffer, 1 μl (10 unit) of the enzyme, and distilled water to make a total volume of 20 μl. The mixtures were incubated at 37°C for 2 hours.

Ten microliters of digested product were analyzed on 6% polyacrylamide gel electrophoresis at 100 volts for 45 minutes. After electrophoresis, the gel was stained in 0.5 μgm/ml of ethidium bromide for 15 minutes and destained in distilled water for 10 minutes. It was then photographed under a UV transilluminator and the patterns of digested DNA fragments were analyzed by comparison with the genotye patterns that were constructed from the reference HPV genotypes as a standard table (Table 1). In the case of HPV-6 and –55, which gave a similar pattern, the extra restriction enzyme (*Eco* RII) would be added to the series with the same procedure in order to differentiate both types.

7. E6/E7 mRNA Detection by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The samples that were identified as HPV 16 or HPV 18 genotype were selected to determine the level of E6/E7 mRNA expression further by using the RT-PCR technique. The RNA precipitate, which was stored in 75% ethanol at -70°C, was centrifuged, and the supernatant was discarded. The RNA pellet was dried briefly, then dissolved in DEPC treated distilled water to determine the RNA concentration by using the UV spectrophotometric method.

7.1 cDNA synthesis

The RT-PCR was performed to amplify the cDNA by using the ThermoScript™ RT-PCR System (Gibco BRL, USA). Briefly, the reaction was performed in a total volume of 20 μl. Prepared the reaction mixture which comprised of 10 μM of appropriated primers, (HPV16R(Hsu) for HPV16 or P16B for HPV 18) and 1-5 μgm of total RNA, then adjusted volume to 10 μl with DEPC treated distilled water. The mixture was heated at 65°C for 5 minutes and then placed on ice immediately. Ten μl of master

Table 1. The L1 RFLP patterns of the reference HPV genotypes (ปราณี, 2539).

HPV type	Digested fragment product (bp)				
	Rsa I	*Dde* I	*Hae* III	*Hinf* I	*Eco* RII
6	250	250	207	250	250
55	250	250	208	250	195
51	250	250	250	250	
42	244	244	140	250	
18	250	216	210	141	
44	244	207	208	244	
16	250	169	200	250	
11	204	250	207	147	
31	216	250	122	250	
35	214	170	250	194	
52	190	180	190	250	
58	195	170	204	256	
54	182	166	208	140	
61	160	259	215	259	
33	141	250	112	250	
57	138	160	122	253	
59	115	132	253	253	
66	74	250	122	250	

reaction mixture that consisted of 4 μl of 5x cDNA Synthesis buffer, 0.1 μM DTT, 40 units of RnaseOUT, 20nM dNTP mix, 15 units of ThermoScript RT and 1 μl of DEPC treated distilled water, were pipetted into a reaction tube that was placed on ice. The sample was incubated at 50°C for 60 minutes and the reaction terminated by incubating at 85°C for 5 minutes. One μl of RNase H was added and incubated at 37°C for 20 minutes.

7.2 cDNA amplification by PCR

The PCR was carried out in a total volume of 50 μl. The reaction mixture, containing 2.5 mM of each dNTP, 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 1.25 units of Taq DNA polymerase, 25 pM of each appropriated primer P16A (fluorescent-dye labeled) and P16B or HPV16R(Hsu), and 2 μl of cDNA. The HPV 16 or HPV 18 plasmid DNA was used as a positive control, and distilled water for the negative control.

The reaction was allowed to amplify for 40 cycles in a thermocycler. Each cycle comprised of denaturation at 94°C for 1 minute, primer annealed at 50°C for 1 minute, and primer extended at 72°C for 1 minute. After the last cycle, all reaction mixtures were incubated further at 72°C for 7 minutes. They were then held at 4°C until picked-up and kept in a freezer at -20°C until analyzed.

7.3 Amplified product analysis

Ten microliters of amplified product were detected by 1% agarose gel containing 40 μgm% of ethidium bromide and electrophoresis at 80 volts for 45 minutes. The DNA band from the RT-PCR was visualized under the UV transilluminator and the DNA fragment size was determined by comparison with a DNA standard marker.

The relative amount of mRNA was analyzed by an automatic genetic analyzer, as the procedure recomended by the company. Briefly, 1 μl of fluorescent labeled amplified product was mixed with 12 μl of deionized formamide and 1 μl of GeneScan size standard (GeneScan 1000, Perkin Elmer USA). The mixture was heated at 95°C for 3 minutes, then placed on ice for at least 5 minutes before subjected to the capillary electrophoresis installed in the machine.

When the dye-labeled DNA fragments electrophoresed through the polymer (POP-6™, Perkin Elmer USA) in the capillary, the DNA fragments were separated according to size. As the labeled samples traveled past the window , the fluorescent dye was exited by the laser and emitted light at a specific wavelength and separated by a spectrograph. The signals were collected onto a charge-couple device (CCD) camera. The data collection software collected the light intensities using software selectable filters and stored them as electrical signals for eventual processing. At the end of a run, the computer automatically analyzed the collected data by using the software (GeneScan™ Analysis 2.1 Software, Perkin Elmer USA). The software automated the entire process of quantifying and sizing the DNA fragment by comparison with the GeneScan size standard (GeneScan 1000, Perkin Elmer USA).

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