CHAPTER III

MATERIALS AND METHODS

Samples

Samples in this study were the tissue of 100 patients with histologic examination as CIN-III and 100 samples chronic cervicitis (with a normal squamous epithelial cells by histologic technique) as a control group. The tissue obtained from patients who attended the Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn University, during September,1994 to November, 1996. All tissues are formalin-fixed paraffin embedded tissues. The histologic examination were evaluated by Gynecologist using 5 μm Hematoxylin-eosin tissue section staining.

DNA extraction

DNA extraction of paraffin embedded tissue was followed the method of Wu, et al. (1990) [97]. Twenty μm section of paraffin embedded tissues were cut by microtome section with disposable blade to prevent cross-contamination between specimens. Twenty pieces of that were placed in 1.5 ml microcentrifuge tube. Then 1.4 ml of xylene was added and mixed gently for 5 minutes to ensure deparafinization. The tube was spun in microcentrifuge at high speed (10,000 rpm) for 2 minutes, then the xylene supernatant was removed. This process was performed 3 times followed by 2 similar washes in absolute ethanol and final wash with 0.1 M EDTA, 0.05 M Tris-HCl, pH 8.0 buffer. After the buffer was discarded, the deparaffinized tissues were then lysed with 400 μl of lysis buffer (0.1 M EDTA, 0.05 M Tris-HCl, pH 8.0 containing 0.5% SDS and 0.5 mg/ml of proteinase K). Digestion was carried out at 55°C for 48 hours. On the following day, DNA was extracted twice with

phenol : chloroform : isoamyl alcohol (25 : 24 : 1). DNA was precipitated by adding 1/10 volume of 3 M NaAc and 2 volume of cold absolute ethanol, stored at -70°C for 1 hour. After that, the tube was spun in microcentrifuge at high speed for 10 minutes followed by washing in 70% cold ethanol. DNA was resuspended in 100 μl of TE buffer (10mM Tris-HCl, 0.1 mM EDTA, pH 8.0) and kept at -20°C until use.

Preparation of standard DNA

In this study, HeLa cell was used as a positive control. This is a continuous cell line, originally derived from human cervical carcinoma. It contains approximately 10-30 copies of HPV-18 DNA [98]. Human DNA extracted from human white blood cells was used as HPV negative control.

The standard plasmid DNA consisting of either HPV-6,11,16,18 or 33 DNA were obtained as bacterial cloned (*E. coli* strain HB 101), kindly provided by Dr. Viraphong Lulitanon, Khon Khen University. Preparation of plasmid DNA was followed the plasmid DNA "miniprep" technique [99]. In brief, the *E.coli* containing plasmid was grown in 2 ml of LB broth (see Appendix II) supplemented with ampicillin 50 μg/ml and shaked overnight at 37°C. Then, 1.5 ml of bacterial growth solution were transferred to 1.5 ml microcentrifuge tube. The bacterial cell pellet was harvested by microcentrifugation at 7,000 rpm for 2 minutes at room temperature. The cell pellet was resuspended with 100 μl of glucose solution (see Appendix II). Then the tube was placed on ice and 200 μl of Alkali SDS buffer (0.2 M NaOH, 1% SDS) was added, mixed by gentle inversion and incubated on ice for 5 minutes, followed by adding 150 μl of KAC solution (see Appendix II) and incubated an additional 5 minutes. The pellet cell debris and chromosomal DNA were precipitated by microcentrifugation for 5 minutes at room temperature. The supernatant was removed into a new tube, added 5 μl (10 mg/ml) of DNase-free RNase A and incubated at 37°C for 30 minutes. After

that, the supernatant was extracted with phenol : chloroform (1:1) 400 μl and followed by chloroform 400 μl. The DNA was precipitated with absolute ethanol and the pellet DNA was washed twice with 70% ethanol. Finally, the DNA was resuspended with sterile distilled water and stored at -20°C

The purified plasmid DNA concentration was determined by measurement of optical density (OD) at 260 nm and the purity of prepared DNA was determined by ratio of OD at 260 : OD 280. Good preparation should have the ratio equal to or higher than 1.8.

Polymerase chain reaction (PCR)

The PCR amplification of HPV-L1 region using consensus primers, MY11 and MY09, simultaneously with human β- globin gene using primers GH20 and PC04 were performed as described by Bauer, et al (1991)[98]. These two sets of oligonucleotide primers were synthesized and purified by BRL, USA (Table 4). The amplified HPV-L1 product was approximately 450 bp whereas, β– globin gene product was 268 bp.

Amplification of DNA was carried out in 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 β -globin primers, 1.25 unit of Taq polymerase (BRL; USA). After adding one μl of DNA sample, 30μl of mineral oil were overlaid to prevent evaporation. The DNA amplification was done using DNA thermal cycler (Perkin-Elmer Cetus, USA). The PCR was started at 95°C for 10 minutes, one cycle and followed by denatured at 95°C for one minute, annealing at 50°C for another one minute and followed by extension at 72°C for 2 minutes. The step was repeated for 40 cycles. In the last cycle, the extension period was allowed for 10 minutes. Successful amplification of β–globin fragment indicated that the

sample was adequate for HPV analysis and no inhibitors were present in the PCR reaction.

To prevent carry-over or any DNA contamination, all reagent mixture was prepared in area free of PCR product. Moreover, controls (HeLa cell, human DNA and distilled water) were included and run in parallel with the test system.

Table 4. Properties and Sequence of primers and probes

NAME	SEQUENCE (5'-3')	TARGET	PURPOSE
MY11	GCC CAG GGA CAT AAC AAT GG	L1	HPV-primer
MY09	CGT CCA AGG GGA AAC TGA TC	L1	HPV-primer
GH20	GAA GAG CCA AGG ACA GGT AC	β- globin	β-globin primer
PC04	CAA CTT CAT CCA GGT TCA CC	β- globin	β-globin primer
GP01	CTG TTG TTG ATA CTA CAC GCA GTA C	HPV	Generic probe
GP02	CTG TGG TAG ATA CCA CTC GCA GTA C	HPV	Generic probe
MY12	CAT CCG TAA CTA CAT CTT CCA	HPV-6	Specific probe
MY13	TCT GTG TCT AAA TCT GCT ACA	HPV-11	Specific probe
MY14	CAT ACA CCT CCA GGA CCT AA	HPV-16	Specific probe
WD74	GGA TGC TGC ACC GGC TGA	HPV-18	Specific probe
MY16	CAC ACA AGT AAC TAG TGA CAG	HPV-33	Specific probe

Gel electrophoresis (GE)

This is a standard method used for separation, identification and purification of DNA fragments based on the principle of different DNA fragment mobility. Ten microliters of amplified product were performed in horizontal gel electrophoresis by using 1.5 % agarose gel in 0.5 M Tris-borate

buffer (TBE) (see Appendix II) at 120 volts for one hour. After that, the gel was stained with 0.5 μg/ml of ethidium bromide and visualized by UV light.

Hybridization

Preparation of samples on to membrane

Dot hybridization (DH)

The remaining aqueous reaction mixture from PCR reaction was extracted with chloroform to remove the mineral oil. All amplified products were dotted on nylon membrane (Hybond ™ N^+ ; Amersham, England). The nylon membrane was pretreated by soaking in sterile water for 5 minutes, and 2X SSC (see Appendix II) for 10 minutes before use. Five microliters of amplified products were diluted into 20 μl of sterile water, denatured by heating at 95°C for 10 minutes, chilled rapidly on ice box and the denatured samples were loaded into the wells of Hybridot® (BRL, USA), and the vacuum was applied . The wells were washed with 10 X SSC see Appendix II)100 μl, and once with 2X SSC. Then, the membrane was baked at 80°C for one hour. Dried membrane can be stored at room temperature until use.

Southern blot hybridization (SH)

To perform Southern blot a nylon membrane (Hybond™ N^+ ,Amersham , England). Nylon membrane was cut and soaked in 10X SSC (see Appendix II) for 10 minutes before use. The agarose gel was pretreated with depurinate solution (0.025 M HCl) 20 minutes, denatured twice with denature solution (1.5 M NaCl, 0.5 M NaOH) 15 minutes each following with neutralized solution (0.1 M Tris, pH 7.5, 1.5 M NaOH) for 30

minutes. After that, the amplified products were transferred from gel to nylon membrane by diffusion blotting for 16-18 hours. The membrane was washed once with 5X SSC (see Appendix II) for 5 minutes and dried at 80°C for 2 hours Dried membrane can be stored at room temperature until use.

Probe labelling

The method of probe labelling was performed according to the ECL 3' oligolabelling and detection system manual. The labelling reaction catalyzed by Terminal transferase introduces a tail of fluorescein-11-dUTP on to the 3' end of an oligonucleotides probes (Figure 7). The reaction mixture of probes 10 pmole consists of fluorescein-11-dUTP one μl, Cacodylate buffer 1.6 μl, Terminal deoxynucleotidyl transferase 1.6 μl, and sterile distilled water to total volume of 16 μl. The reaction mixture was incubated in water bath at 37°C for 60 minutes and stored in -20°C until use.

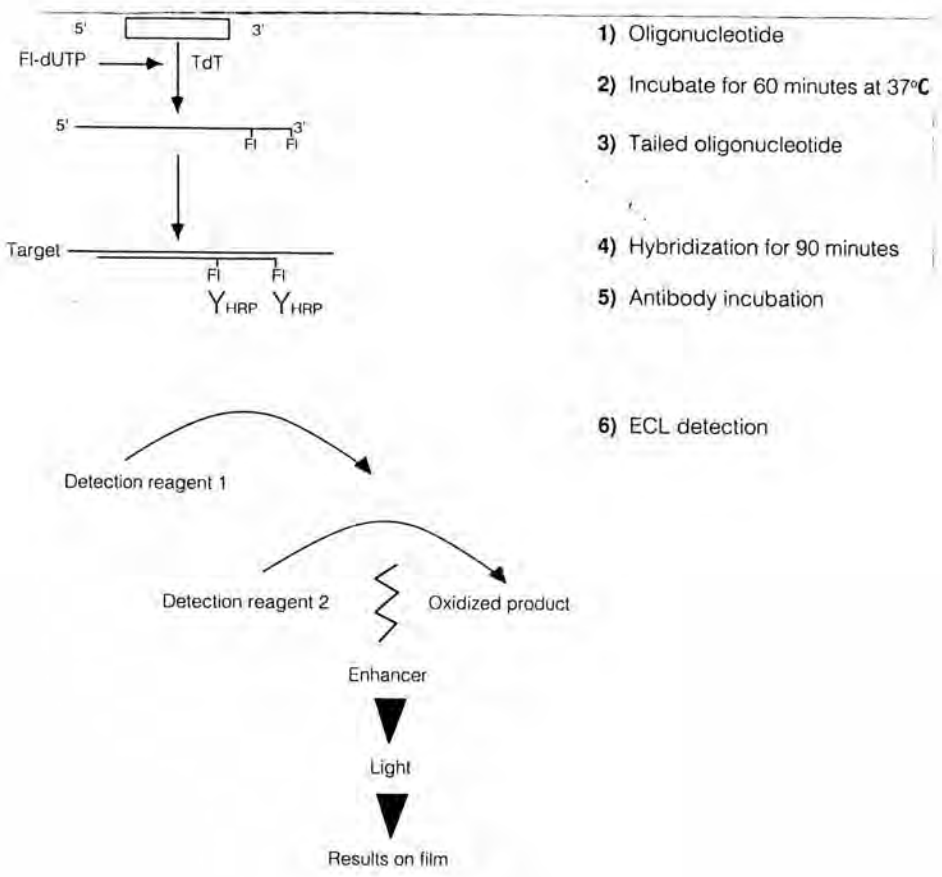

Figure 7. Principle of the ECL 3' oligolabelling and detection system.

Analysis of DNA on the nylon membrane

The membrane was prehybridized with hybridization buffer (see Appendix II), incubated at appropriated temperature of each probes for 30 minutes (Table 5). After prehybridization, appropriated concentration of each probes (Table 5) was added to the membrane, incubated at the same temperature for additional 90 minutes, the membrane was washed twice with 5X SSC containing 0.1% SDS at room temperature for 5 minutes and twice with 1X SSC containing 0.1% SDS at the appropriated temperature (Table 5) for 15 minutes each. Then, the membrane was soaked in buffer I (see Appendix II) for one minute and put in blocking solution (see Appendix II) for 30 minutes and rinsed in buffer I for one minute. After that, the hybridized DNA was detected using an ECL detection kit (Amersham; England). The procedure of detection was followed the manufacturer's instruction. The ECL labelling and detection system utilized the enhanced chemiluminescence associated with the horseradish peroxidase catalyzed oxidation of luminal to detect the oligonucleotide tailed at the 3' end with fluorescein-11dUTP hybridized to the target sequence on the membrane. The result was observed after the film was developed in developer and fixer solution (Kodak; USA)

Table 5. The optimized condition of hybridization for each probes.

Type specificity	Probe	Tm (^{O}C)	Conc.^{n} (pmole)	Pre and Hybridization condition (^{o}C)	Washing (^{O}C)
HPV-DNA	GP01	48.2	0.25	42	42
	GP02		0.25		
TS-6	MY12	46.9	5	40	40
TS-11	MY13	42.4	05	40	40
TS-16	MY14	4705	5	50	55
TS-18	WD74	57.7	5	59	65
TS-33	MY16	39.2	05	40	40

Statistic analysis [100]

The association between CIN-III and HPV-infection was examined by using Chi-square (χ^2) test. The strength of association was determined by odds ratio (OR)) and the significant difference of association between CIN-III patients and control group was calculated by 95% confidence interval (95% CI).