CHAPTER IV

MATERIALS AND METHODS

Part I. Samples

Twenty two patients were enrolled from women attending the Gynaecology Clinic at King Chulalongkorn Memorial Hospital, Bangkok, Thailand from August 2003 to June 2004. Eleven patients were diagnosed as having CIN: 2 patients with CIN I, 2 patients with CIN II and 7 patients with CIN III. Eleven patients were diagnosed as having CaCx: 6 patients with adenocarcinoma and 5 patients with squamous cell carcinoma. These patients were studied for their HPV-E7-specific CD8+ T cell responses. Five cord bloods were used as control group in this study. HPV typing in CIN and CaCx patients was performed on either fresh or paraffin-embedded tissues section.

Twenty known HPV-16 DNA samples obtained from HPV-16 infected patients were kindly provided by Associate Professor Dr. Parvapan Bhattarakosol of the Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. These samples were used for sequence variation analysis of HPV-16 E7.

Part II. HPV detection in tissue section

1. Extraction of DNA from tissue samples

DNA from fresh or paraffin-embedded tissue was extracted using QIAamp® DNA Mini Kit (Qiagen, Germany) following manufacturer's instruction. In brief, 25 mg of fresh tissue section was cut and placed in a 1.5 ml microcentrifuge tube. In case of paraffin-embedded tissue (20 μm, 30 pieces/case), paraffin was removed three times with xylene and washed twice with absolute ethanol. The pellet was dried at 37°C in the incubator until the ethanol has evaporated. The tissue pellet was resuspended in 180 μl of tissue lysis buffer (ATL) and was added with 20 μl of proteinase K. The suspensions were then incubated at 56°C until the tissues were completely lysed with occasionally mixed during incubation to disperse the sample. After that, 200 μl of AL buffer was

added to the suspension and mixed by vortex. After, the suspension was incubated at 70°C for 10 min. 200 µl of absolute ethanol was added. The suspension was applied to the QIAamp spin column and centrifuged at 8,000 rpm for 1 min. The QIAamp spin column was washed with 500 µl of wash buffer (AW1), centrifuged at 8,000 rpm for 1 min, washed again by adding 500 µl of wash buffer (AW2) and centrifuged at 14,000 rpm for 3 min. The QIAamp spin column was then placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl of elution buffer (AE) was added, incubated at room temperature for 5 min and centrifuged at 8,000 rpm for 1 min. This step was repeated twice and kept at -20°C until use.

2. Preparation of standard HPV-DNA

E. coli containing standard HPV-DNA (HPV-6, 11, 16, 18, 31, 33 and 35) plasmids used as controls in this study were generously provided by Associate Professor Dr. Parvapan Bhattarakosol of the Department of Microbiology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Standard HPV-DNA plasmid was prepared by using QIAprep® Miniprep (Qiagen, Germany) following manufacturer's instruction. Briefly, single colony of *E. coli*, the bacteria transformed with plasmids containing HPV-DNA insertion was grown in 2 ml LB broth (Luria-Bertani broth with Ampicillin 100 µg/µl) and incubated overnight at 37°C in shaking water bath. After that, the suspension was transferred to 1.5 ml microcentrifuge tube and followed by centrifugation at high speed for 2 min. The bacterial pallet was resuspended with digestion buffer (250 µl buffer P1, 250 µl buffer P2, 350 µl buffer N3) and mixed gently by inverting the tube. After centrifugation at high speed for 10 min, the supernatant was transferred to the QIAprep spin column followed by centrifugation at high speed for another 1 min. To wash DNA, 500 µl of PB buffer was added and centrifuged at high speed for 1 min. The supernatant was discarded and washed again by adding 750 µl of PE buffer followed by centrifugation for 1 min. The QIAprep column was placed in a clean 1.5 ml microcentrifuge tube. To elute DNA, 50 µl of EB buffer was added and incubated at room temperature for 5 min and then centrifuged at 8,000 rpm for 1 min. This process was repeated twice. Standard HPV-DNA was kept at -20°C until use.

3. Quantitative analysis of DNA extraction

The DNA was quantitated by ultraviolet spectrophotometer at 260 nm and 280 nm. The calculation was based on the fact that the optical density (OD) at 1 of 260 nm was equal to 50 μg/ml of double-stranded DNA. The purity of prepared DNA was confirmed when the ratio of OD at 260: OD at 280 was equal to or higher than 1.8.

4. HPV-DNA detection and typing

4.1 HPV-DNA detection by amplification of HPV L1 gene

HPV detection in CIN and CaCx patients was performed on fresh or paraffin-embedded tissues section by PCR amplification of HPV L1 region. In the present study, we have compared the sensitivity and efficiency of two detection systems using different set of primers. Whilst the first detection system used MY09 – MY11 primer set (MY09/11) (155) and produced approximately 450 bp amplified product, the second system used L1C1 – L1C2 primer set (L1C1/C2) (156) and produced approximately 250 bp amplified product (Table 6). As a control for DNA amplification, human β-globin gene was amplified by GH20 and PC04 primers and produced 268 bp amplified product (17) (Table 6).

Amplification of HPV L1 gene by MY09/11 primer set was performed as previously described by Bhattarakosol et al. (17). The 50 μl of PCR mixture contained 1 μl of DNA sample, 1X PCR buffer [100 mM KCl, 20 mM Tris (pH 8.0)], 4 mM $MgCl_2$, 200 μM dNTPs, 25 pmole of each MY09/11 primer and 1.25 unit of Taq polymerase (Promega, USA). One cycle of the PCR reaction was started at 95°C for 10 min, followed by denaturing at 95°C for 1min, annealing at 50°C for 1 min and extension at 72°C for 2 min. The step was repeated for 40 cycles and followed by an extension at 72°C for 10 min. In parallel, the human β-globin was amplified by primers GH20 and PC04. Successful amplification of β-globin was used to indicate that the sample DNA was adequate and no inhibitors were present in the PCR reaction. The standard HPV-DNA

was used as a HPV-DNA positive control. White blood cell (WBC) DNA from buffy coat and distilled water (DW) were used as a HPV-DNA negative control.

Another PCR primer set (L1C1/C2) (156) was performed in different condition. PCR was carried out in 50 µl total reaction mixture containing 1X PCR buffer [100 mM KCl, 20 mM Tris (pH 8.0)], 1.5 mM $MgCl_2$, 200 µM dNTPs, 25 pmole of each L1C1/C2 primer, 2 units of Taq polymerase (Promega, USA) and 1 µl of DNA sample. The PCR was started at 95°C for 10 min, one cycle and followed by denaturing at 95°C for 1.30 min, annealing at 40°C for 1.30 min and extension at 72°C for 2 min. The step was repeated for 40 cycles, followed by an extension at 72°C for 10 min. In parallel, the human β-globin gene was amplified by primers GH20 and PC04. The amplified HPV L1 (L1C1/C2) product was approximately 244 bp in HPV types 6 and 11, 253 bp in types 16 and 18 and 256 bp in types 31 and 33.

Size of the amplified product was determined by gel electrophoresis (GE). Ten microlitres of amplified product was run on a 1.5% agarose gel in 0.5X Tris borate EDTA (TBE) buffer. The DNA bands were stained with ethidium bromide and photographed under ultraviolet (UV) transillumination.

4.2 HPV typing by restriction fragment length polymorphism (RFLP)

Identification of HPV type was performed by the method described by Bernard et al (157) (MY09/11 primer set) and Yoshikawa et al (156) (L1C1/C2 primer set). The PCR product of MY09/11 primer set and L1C1/C2 primer set were analysed by RFLP using different restriction enzymes (RE). The RE *Hae* III and *Rsa* I were used for typing the PCR product of MY09/11 primer set as shown in Table 7 (157). The another PCR product of L1C1/C2 primer set was typed with RE *Hinf* I, *Hae* III and *Rsa* I (Table 8) (156). A volume of 10 µl of PCR product was cut with 10 units of each enzyme in a volume adjusted to 20 µl with 10X buffer and DW. The mixture was incubated at 37°C for 1 h. Digested products were then analysed by electrophoresis using 10% acrylamide gel. Gels were stained with ethidium bromide and patterns were visualised with UV transilluminator.

Table 6. Sequence of oligonucleotides used as primers for PCR amplification

Primers	Sequence (5'-3')	Target	PCR product (bp)	Reference
MY09	GCCCAAGGACATAACAATGG	L1	450	130
MY11	CGTCCAAGGGGAAACTGATC			
L1C1	CGTAAACGTTTTCCCTATTTTTTT	L1	250	131
L1C2	TACCCTAAATACTCTGTATTG			
GH20	GAAGAGCCAAGGACAGGTAC	β-globin	268	17
PC04	CAACTTCATCCACGTTCACC			

Table 7. RFLP patterns of HPV L1 genes amplified by MY09/11 primer set (155).

Enzyme	Digestion products (bp)						
	HPV-6	**HPV-11**	**HPV-16**	**HPV-18**	**HPV-31**	**HPV-33**	**HPV-35**
***Hae* III**	217 124 108	217 124 108	444 8	455	328 124	449	261 180 8 3
***Rsa* I**	161 149 72 67	216 135 72 26	310 72 70	135 125 85 72 38	380 72	236 102 72 39	177 161 72 42

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Table 8. RFLP patterns of HPV L1 genes amplified by L1C1/C2 primer set (157).

Enzyme	Digestion products (bp)					
	HPV-6	**HPV-11**	**HPV-16**	**HPV-18**	**HPV-31**	**HPV-33**
***Hinf* I**	244	147 97	253	141 112	256	256
***Hae* III**	207 37	207 37	200 53	210 43	122 91 43	112 91 43 10
***Rsa* I**	244	204 40	253	253	216 40	141 62 40 13

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Part III. HPV-E7 specific CD8+ T cell responses

1. Design of E7-overlapping peptides

We analysed HPV-E7 specific CD8+ T cell responses by ELISpot assays which the donor PBMC were stimulated by overlapping peptides spanning HPV-E7 protein. However, since the information of E7 sequence of HPV circulating in Thailand was not known. We sequenced HPV-16 E7 gene from stored HPV-16 DNA samples. The E7 nucleotide sequences were aligned and translated into an amino acid sequence for the overlapping peptide design.

1.1 HPV-16 E7 gene Sequencing

1.1.1 Amplification of HPV-16 E7 gene

Detection HPV-16-E7 gene was performed by PCR amplification of HPV-16 E7 region, using primer 5' GAA TTC ATG CAT GGA GAT ACA CCT AC 3' and 5' CAG GAT CCT GGT TTC TGA GAA CAG ATG G 3' (31). The amplification of DNA was carried out in 50 μl total reaction mixture containing 1X PCR buffer [50 mM KCl, 20 mM Tris (pH 8.5)], 1.5 mM $MgCl_2$, 200 μM dNTPs, 12.5 pmole of each HPV-16 E7 primers, 1.25 unit Taq polymerase (Promega, USA). The PCR was started at 95°C for 10 min, one cycle and followed by denaturing at 95°C for 1 min, annealing at 50°C for 1 min and followed by extension at 72°C for 2 min. The step was repeated for 40 cycles and followed by an extension at 72°C for 10 min. The amplified HPV-16 E7 gene product was approximately 295 bp.

Amplified E7 product was determined by GE on a 1.5% agarose gel in 0.5X TBE buffer. The DNA bands were stained with ethidium bromide and photographed under UV transillumination.

1.1.2 Purification of HPV-16 E7 gene PCR product

The PCR products were purified using QIAquick® PCR purification Kit (Qiagen, Germany) following manufacturer's instruction. Briefly, 40 μl of PCR product

was mixed with 200 µl of binding buffer (PB). The mixture was applied to the QIAquick column and centrifuged at 13,000 rpm for 1 min. The supernatant was discarded. DNA was washed by adding 750 µl of PE buffer and centrifugation for 1 min. The DNA was eluted by adding 20 µl of EB buffer, incubated at room temperature for 5 min, then centrifuged at 13,000 rpm for 1 min and kept at -20°C until use.

1.1.3 Preparation of purified DNA for sequencing

The purified PCR products were sequenced by ABI Prism 310 Genetic Analyser (Applied Biosystem, U.S.A.) following manufacturer's instruction. Briefly, the purified PCR product was carried out in 10 µl total reaction mixture and subjected to DNA sequencing by an ABI 310 automated sequencer using dideoxy terminator cycle sequencing kit (Applied Biosystem, U.S.A.). The purified PCR products were denatured at 96°C for 30 sec, annealing at 55°C for 10 sec and extension at 60°C for 4 min. The step was repeated for 25 cycles. The extension product was DNA precipitated with 3M sodium acetate and cold absolute ethanol followed by incubation at -70°C for overnight and centrifugation at 14,000 rpm for 30 min. The DNA was washed with cold 70% ethanol and centrifuged at 14,000 rpm for 10 min. The supernatant was discarded. The pellet was then dried at 90°C until the ethanol has evaporated.

1.1.4 Sequencing analysis

The DNA sequences were compared with standard HPV-16 DNA and the sequence database at Los Alamos (http://hpv-web.lanl.gov). The sequence homology was analysed by the Clustal X program.

1.2 Synthesis of overlapping peptide

We designed E7-overlapping peptide that based on HPV Thai sequence identified from known HPV-16 DNA samples of HPV-infected Thai women. The nucleotide sequences were aligned with Clustal X program and translated into amino acid sequences with Bioedit program for overlapping peptide design. Overlapping peptides with length of

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20 amino acid (aa) overlapping by 10 aa were designed by PeptGen program on Los Alamos server (http://hiv-web.lanl.gov).

2. Detection of HPV-E7-specific CD8+ T cell responses

2.1 Preparation of peripheral blood mononuclear cells (PBMC)

PBMC were separated from heparinised blood by ficoll-hypaque (Robbins Scientific, Norway) density gradient centrifugation. After centrifugation at 1,500 rpm for 30 min, the PBMC were washed with 10 ml of R10 [10% fetal bovine serum in RPMI 1640 (GIBCO BRL, U.S.A.)] twice at 1,800 rpm for 10 min. The cells were resuspended with R10. PBMC were counted and adjusted to desired concentration. The cells were either used immediately or stored at -180°C in liquid nitrogen with freezing medium [10% DMSO (Sigma, U.S.A.) in fetal bovine serum (GIBCO BRL, U.S.A.)].

2.2 ELISpot assay

2.2.1 *ex vivo* ELISpot assay

Nitrocellulose plates (Millipore, U.S.A.) were coated with 50 μl of monoclonal antibody to IFN-γ (anti-IFN-γ) (Mabtech, Sweden) at the concentration of 10 μg/ml in phosphate buffer saline (PBS) and were incubated at 37°C in 5% CO_2 for 3 h. The antibody-coated plate was washed six times with PBS and blocked with 200 μl of R10 at room temperature for 1 h. 100 μl of PBMC 5×10^6 cells/ml from patient was stimulated with 10 μl of HPV-16-E7 overlapping peptides (200 μg/ml) (Mimotope, Australia) at 37°C in 5% CO_2 for 16 h. The assays were performed in duplicate. A negative control was established by culturing PBMC with 10 μl of R10 and a positive was established by culturing PBMC with 2 μl of 1 μg/ml of Phytohemagglutinin (PHA) (Sigma, U.S.A.). The plate was washed six times with PBS/T [PBS containing 0.05% Tween 20 (USB, U.S.A.)] and once with PBS. The secondary antibody, 50 μl of biotinylated anti-IFN-γ (1 μg/ml) (Mabtech, Sweden), was added. Plate was washed six times with PBS/T and once with PBS after 3 h of incubation at room temperature. The 50 μl of streptavidin-conjugated alkaline phosphatase (1 μg/ml) (Mabtech, Sweden) was

added and incubated at room temperature for 1 h. The plate was washed six times with PBS/T and once with PBS. Finally, the substrate, 100 μl of 5-bromo-4-chloro-3-indolyphosphate/nitroblue tetrazolium (Bio-Rad, U.S.A.) was added and incubated for 30 min. The colour reaction was stopped by washing with water. After drying, the numbers of spots were counted under a dissecting microscope. Responses of the patient were considered positive if the number of spots exceeded the positive cut-off value (>1.25 time negative control well).

2.2.2 Cultured ELISpot assay (158)

We augmented HPV-specific CD8+ T cell responses by co-culturing PBMC 1×10^7 cells with 100 μl of HPV-E7 pooled overlapping peptides (200 μg/ml) (Mimotope, Australia) for 1 h at 37°C in 5% CO_2. Thereafter, the cells were washed twice with 5 ml of R10 followed by centrifugation at 1500 rpm for 5 min. The PBMC were diluted to 5×10^6 cells/ml with R10 and plated out at 1 ml/well into 24-well plate (IWAKI, Japan) with 12 μl of IL-7 (330 U/ml) (R&B, UK.). On day 3, R10 with 5 μl/ml of IL-2 (100 U/ml) (R&B, UK.) was added to the wells and every 3 days were also performed. On day 14, the CTL line was harvested and washed three times with 5 ml of R10. The T cells were counted and adjusted to desired concentration. 100 μl of T cell line 1×10^6 cells/ml from patient was stimulated with 10 μl of overlapping peptides (200 μg/ml) as previously described in 2.2.1.

2.3 CD8+ T cell depletion

We randomly selected patients whose T cells were positive for HPV-E7 by ELISpot assay. To confirm that the ELISpot positivity was mediated by CD8+ T cells, we performed CD8+ T cell depletion experiments. Briefly, PBMC 1×10^7 cells/ml from patient were cultured with 72 μl of anti-CD8-coated magnetic bead (Dynal beads CD8 [T-cytotoxic/suppressor cells], U.S.A.) and incubated at 4°C for 20 min, mixed during incubation every 5 min. After washing twice with 5 ml of RPMI 1640 at 1,500 rpm for 5 min, CD8+ T cell depletion were resuspended with R10 and tested in ELISpot assay. The detected response was considered being mediated by CD8+ T cells, if equal to or more than 50% of spots were decreased.