CHAPER III
RESEARCH METHODOLOGY

1. Materials

1.1 Clinical specimens

Specimens used in this study were FFPE cervical tissue biopsies histologically diagnosed as normal, LSIL , HSIL and SCC. They were collected from women in the 4 regions of Thailand including north, northeast, central and south. These samples belong to the project "Role of human papillomavirus in cervical carcinogenesis in Thai women" approved by the Ethics Committee of Rajavithi Hospital, Bangkok and the protocol of this study is supported by the Ethics Committee for Human Research at Khon Kaen University, Khon Kaen.

For clinical specimen preparation, 5 μm sections were cut using the so-called sandwich method [2]. Briefly, outer sections were stained with hematoxylin-eosin for histological analyses, whereas 5 inner sections were placed in a microcentrifuge tube and used for HPV detection and genotyping.

1.2 C33A cell line

The HPV-negative human cervical cancer cell line, C33A was kindly provided by Prof. Dr. Magnus von Knebel Doeberitz, Department of Applied Tumor Biology, Heidelberg University, Germany. The cell line were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Gaithersburg, MD), 100 U/ml penicillin, 100 μg/ml streptomycin, 40 μg/ml gentamicin and 2.5 μg/ml Fungizone. The C33A cells were cultured at 37^0C in a humidified atmosphere with 5% CO2 and were subpassaged twice a week.

1.3 Plasmid

1.3.1 HPV 16 Reference plasmid

HPV 16 Reference plasmid containing ampicillin resistance gene and the whole genome of HPV 16 prototype (7,906 bp) was kindly provided by Prof. Dr.

Ethel-.Michele. de Villiers, Division for the Characterization of Tumour Viruses, Heidelberg Germany.

1.3.2 The pDRIVE cloning vector

The pDRIVE cloning vector (Qiagen PCR Cloning Kit, Quigen) is a linear form vector with a U overhang at each end, hybridizes with high specificity to such PCR products using Taq and other non-proofreading DNA polymerases, which contain the single A overhang at each end of PCR products. This vector allows ampicillin and kanamycin selection, as well as blue/white colony screening. The vector contains several unique restriction endonuclease recognitionsites around the cloning site, allowing easy restriction analysis of recombinant plasmids. The vector contains a T7 and SP6 promoter on either side of the cloning site, allowing in vitro transcription of cloned PCR products.

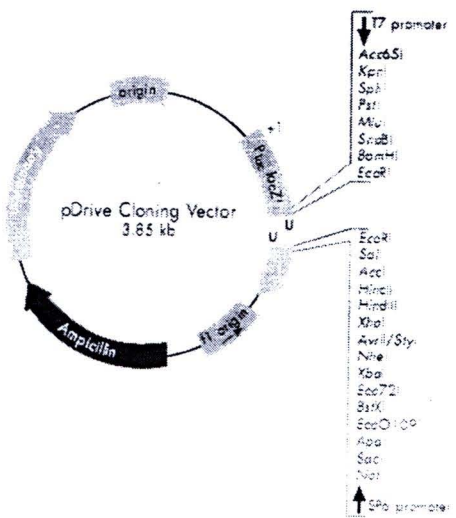

Figure 6 Restriction map and multiple cloning site (MCS) of pDRIVE cloning vector.

1.3.3 The pGL3 basic vector

The pGL3 basic vector (Promega) is a promoterless luciferase reporter vector providing maximum flexibility in cloning putative regulatory sequences. Expression of luciferase activity in cells transfected with this plasmid depends on insertion and proper orientation of a functional promoter upstream from luc+. Potential enhancer elements can also be inserted upstream of the multiple cloning sites downstream of

the luc+ gene. The backbone of the pGL3 Luciferase Reporter Vectors is designed for increased expression, and contains a modified coding region for firefly (Photinus pyralis) luciferase that has been optimized for monitoring transcriptional activity in transfected eukaryotic cells. A bacterial promoter upstream of an amplicilin-resistance cassette (Amp^r) confers amplicilin resistance in *E.coli*.

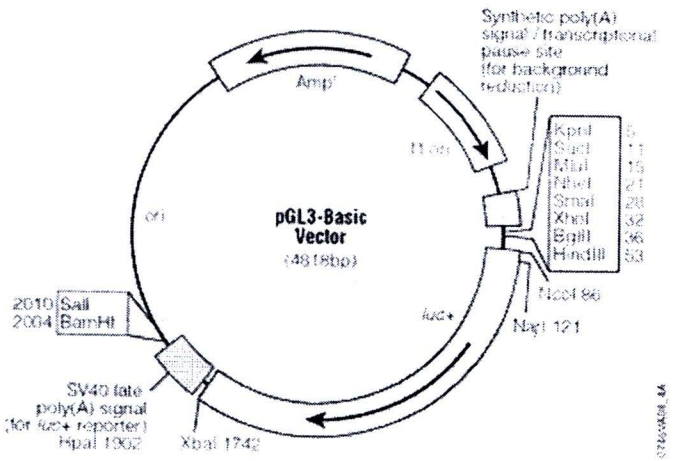

Figure 7 Restriction map and multiple cloning site (MCS) of pGL3 basic vector.

1.3.4 The pSV-β galactosidase control vector

The pSV-β-Galactosidase Control Vector is a positive control vector for monitoring transfection efficiencies of mammalian cells. This vector contains the SV40 early promoter and enhancer drive transcription of the *lacZ* gene, which encodes the β-galactosidase enzyme. The pSV-β-Galactosidase Control Vector can be transfected individually or co-transfected with DNA of interest. In this manner, the pSV-β-Galactosidase Vector acts as an internal control for transient expression assays. A negative control extract, prepared from mock-transfected cells, should also be assayed for the presence of endogenous β-galactosidase activity in cultured cells.

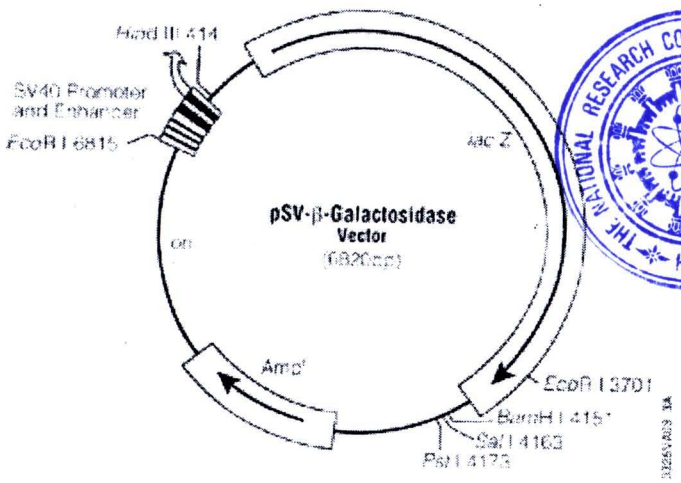

Figure 8 Restriction map and multiple cloning site (MCS) of pSV-β-Galactosidase control vector.

1.4 Transfection reagent

Lipofectamine ™ 2000 Transfection Reagent (Invitrogen Life Technologies) is formulated for the transfection of DNA into eukaryotic cells. It was used for transfection of a recombinant plasmid into C33A cells.

1.5 Luciferase assay reagent

Bright-Glo™ Luciferase assay reagent (Promega) is Bright-Glo™ Luciferase assay buffer and lyophilized Bright-Glo™ Substrate performed by Promega. For Bright-Glo™ Luciferase assay reagent preparation,the Bright-Glo™ Buffer was transferred to one bottle of Bright-Glo™ Substrate, mixed by inversion until the substrate is thoroughly dissolved. The Bright-Glo™ Luciferase assay reagent was stored at -20°C.

1.6 β-galactosidase assay reagent

The Beta-Glo® assay reagent (Promega) is Beta-Glo® assay buffer and Beta-Glo® assay substrate that are combined to form Beta-Glo® Reagent. For Beta-Glo® assay reagent preparation, the Beta-Glo® buffer was transferred to one bottle of Beta-Glo® substrate and mixed by inversion until the substrate is thoroughly dissolved. The reagent was stored at -20 °C and keeped away from the light.

2. Methods

2.1 DNA extraction from FFPE cervical tissue sections

DNA was extracted from the FFPE cervical tissue sections collected in the 1.5 ml microcentrifuge tube. The tissue sections were incubated in 1200 µl of xylene at 60o C for 30 minutes. Following deparaffinize twice, the tissue sections in microcentrifuge tube were mixed by a vortex mixer then centrifuged at 14,000 rpm at room temperature (RT) for 5 minutes to pellet the tissues and discard the xylene. The supernatant was removed. The pellets was washed twice by adding 1,200 µl of absolute ethanol and mixed by a vortex mixer then the tube was centrifuged at 14,000 rpm at RT for 5 minutes and the supernatant was removed. The open tube was incubated at 37oC until the ethanol evaporated. Then DNA extraction was performed according to the QIAamp® DNA Mini Kit (QIAGEN) as described in the manufacturer's instruction.

2.2 DNA qualification

For qualification of tissue DNA, the extracted DNA was tested by PCR amplification for a 268-bp fragment of the β-globin gene (the housekeeping gene) using PC04/GH20 consensus primers: PC04; 5'-CAACTTCATCCACGTTCACC-3' and GH20; 5'-GAAGAGCCAAGGACAGGTA-3' (Invitrogen Life Technologies, Carlsbad, CA, USA). A PCR master mix for β-globin gene amplifying in a total volume of 50 µl contained 1X PCR buffer, 0.2 mmole each deoxynucleoside triphosphate (dNTP), 3 mmole $MgCl_2$, 2.5 U of Taq DNA polymerase (Fermentas Life sciences), 50 pmole each of the primers and 5 µl of DNA samples. Amplifications were performed in a Gene Amp PCR System 2400 (Perkin-Elmer thermal circlers,) for 40 cycles following parameter: initial denaturing at 95°C for 5 min; each cycle at 95 °C for 1 min, 55°C for 1 min and 72 °C for 1 min; final extension at 72°C for 10 min. PCR product was verified by agarose gel electrophoresis. Five µl of the PCR product were mixed with 1 µl of loading dye solution and loaded into each well of 1.5% agarose gel prepared in 0.5X TAE. A 1 kb plus DNA ladder marker (Invitrogen Life Technologies, Carlsbad, CA, USA) was loaded into a well in order to estimate size of the amplicon. Gel was electrophoresed at 100 volts for 30 min, stained in 0.5 mg/ml ethidium bromide (EtBr) for 10 min and

destained in water for 20 min with gentle shaking. PCR products on the agarose gel were visualized by a UV transilluminator.

2.3 HPV DNA Detection

Qualified samples from positive β-globin gene PCR amplification were subjected to HPV DNA detection by PCR amplification using GP5+/GP6+ consensus primers for conserved L1 ORF of HPV (a 142-bp fragment) : GP5+; 5'-TTT GTT ACT GTG GTA GAT ACT AC-3' and GP6+; 5'-AAA AAT AAA CTG TAA ATC ATATTC-3' [61]. A 50 µl PCR master mix for L1 region amplifying contained 10x PCR buffer, 2 mM $MgCl_2$, 10 mM dNTP, 5U Taq DNA polymerase, 100 pM GP5+, 100 pM GP6+ and 3 µl DNA template. Amplification was performed for 40 cycles with following parameters: initial denature at 94 °C for 4 minutes , each cycles at 94 °C for 1 minute, 42 °C for 1 minute , 72 °C for 30 seconds, and the final extention step at 72 °C was prolonged for 4 minutes to ensure a complete extension of the amplified product. PCR products were visualized by ethidium bromide straining after electrophoresis in 1.5% agarose gel.

Negative controls for PCR analysis

Several precautions were taken to prevent false-positive results. Different steps such as sample preparation and amplification are performed in strictly separated room, and distilled water samples are included as negative PCR controls.

2.4 HPV Genotyping

The PCR products of all HPV DNA positive samples were examined for HPV genotype by RLBH using 37 HPV type–specific 5'-amino-linked oligonucleotide probe [62].

2.4.1 Biotin labeled PCR product preparation

The PCR products of HPV DNA positive samples were re-amplified in nested PCR using GP5+/biotin-labeled GP6+ primers;(biotin-labeled GP6+ primer 5′-GAAAAATAAACTGTAAATCATATTC-3′) (Invitrogen Life Technologies, Carlsbad, CA, USA). The amplification mixture and PCR condition were similar to HPV DNA detection condition. The amplified biotin-labeled PCR product was incubated with 37 HPV type-specific 5'-amino-linked oligonucleotide probe immobilized on a membrane by RLBH assay.

2.4.2 RLBH assay

The RLBH method was performed by using 37 HPV type–specific 5'-amino-linked oligonucleotide probes containing a 5'-amino group on a carboxyl end, which covalently bound to a negatively-charged membrane (Biodyne C; Pall Bio-Support). The biotin-labeled PCR product was hybridized to 37 HPV type-specific oligonucleotide probes immobilized on the membrane using miniblotter. Hybridized signals were visualized by chemiluminescence detection.

The membrane was activated by soaking in freshly prepared 16% (w/v) 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) (Sigma-Aldrich) in deionized water for 10 min with gentle shaking at RT. Then the activated membrane was rinsed with tap water and placed on a miniblotter. The oligonucleotide-amino labeled probes were diluted in 150 µl 500 mM $NaHCO_3$ (pH 8.4) to have a concentration at 300 pmole then pipetted in parallel lines on the miniblotter. After 5 min of incubation, oligonucleotide-amino labeled probes were removed by aspiration and the membrane was inactivated using 100 mM NaOH for 8 min, followed by washing in 2XSSPE/0.1%SDS for 8 min at 60°C.

For hybridization with biotin-labeled PCR product, the activated membrane was placed on a minibloter and hybridized with 150 µl of GP5+/ biotinylated GP6+ PCR product that was diluted in the hybridized solution containing 2XSSPE/0.1% SDS. The diluted PCR product was heat-denatured for 10 min at 99°C, then rapidly cooled on ice for 3 min. The PCR products were added into the slot of the membrane prepared on a minibloter (avoid air bubbles) and hybridized for 60 min at 42°C. The PCR product was removed from the minibloter by aspiration.Then the membrane was washed twice in 2XSSPE/0.5% SDS for 10 min at 52°C three times, subsequently, incubated with 1:6,000 diluted streptavidin-peroxidase conjugate (Zymed Labboratories Inc., S. San Francisco, CA, USA) in 2XSSPE/0.5% SDS for 45 min at 42°C. The membrane was washed for three times in 1X PBS/0.3% Tween 20 for 10 min at 45°C. For chemiluminescence detection, the membrane was incubated with chemiluminescence solution (LumiGLO; KPL Inc., Gaithersburg, MD, USA) in the dark room for 1 min and exposed to an X-ray film for 10 min in the cassettes. After film exposure, the film was developed in developer solution for 3 min, rinsed with tap

water, fixed in fixer solution for 3 min, and then washed with tap water for 30 s. The positive HPV DNA was determined by visualization of black spots on the X-ray film.

For repeated use, the membrane was stripped by washing twice in 1% SDS at 80°C for 30 min followed by incubation in 20 mM EDTA (pH 8) at RT for 20 min. The membrane was stored in a sealed plastic bag at 4°C until further used.

Table 4 37 HPV type–specific 5'-amino-linked oligonucleotide probes used for HPV genotyping by RLBH assay.

No.	Oligo Name*	Sequence (5'- 3')	Length
1	RLB-HPV 6	Amino-TCCGTAACTACATCTTCCA	19
2	RLB-HPV11	Amino-TCTGTGTCTAAATCTGCTAC	20
3	RLB-HPV 16	Amino-CATTATGTGCTGCCATATC	19
4	RLB-HPV18	Amino-TGCTTCTACACAGTCTCCT	19
5	RLB-HPV26	Amino-GTACATTATCTGCAGCATC	19
6	RLB-HPV31	Amino-GCAATTGCAAACAGTGATAC	20
7	RLB-HPV33	Amino-TGCACACAAGTAACTAGTGA	20
8	RLB-HPV34	Amino-TTTTCAGTTTGTGTAGGTACA	21
9	RLB-HPV35	Amino-CTGCTGTGTCTTCTAGTGA	19
10	RLB-HPV39	Amino-ATAGAGTCTTCCATACCTTC	20
11	RLB-HPV40	Amino-AGTCCCCCACACCAACC	17
12	RLB-HPV42	Amino-TGGTGATACATATACAGCTG	20
13	RLB-HPV43	Amino-TCTACTGACCCTACTGTG	18
14	RLB-HPV44	Amino-TACTAGTGAACAATATAAGCA	21
15	RLB-HPV45b	Amino-TAATTTAACATTATGTGCCTC	21
16	RLB-HPV51	Amino-TGCTGCGGTTTCCCCAA	17
17	RLB-HPV52	Amino-GAATACCTTCGTCATGGC	18
18	RLB-HPV53	Amino-TGTCTACATATAATTCAAAGC	21
19	RLB-HPV54	Amino-CACGCAGGATAGCTTTAAT	19
20	RLB-HPV55	Amino-TCAGTCTCCATCTACAACAT	20
21	RLB-HPV56	Amino-CAGAACAGTTAAGTAAATATG	21
22	RLB-HPV57	Amino-CCACAGAAACTAATTATAAAG	21
23	RLB-HPV58	Amino-TATGCACTGAAGTAACTAAG	20
24	RLB-HPV59	Amino-TCTACTACTGCTTCTATTCC	20
25	RLB-HPV61	Amino-CCCCCCCTGTATCTGAAT	18
26	RLB-HPV66	Amino-AGCTAAAAGCACATTAACTAA	21
27	RLB-HPV68	Amino-CTGAATCAGCTGTACCAAT	19
28	RLB-HPV70	Amino-GAAACGGCCATACCTGCT	18
29	RLB-HPV72	Amino-AGCGTCCTCTGTATCAGAA	19
30	RLB-HPV73	Amino-ACAGGCTAGTAGCTCTACT	19
31	RLB-HPV82-MM4	Amino-ACTGCTGTTACTCAATCTG	19
32	RLB-HPV83-MM7	Amino-TACACAGGCTAATGAATACA	20
33	RLB-HPV84-MM8	Amino-TGCTACCAACACCGAATCA	19
34	RLB-HPV82-IS39	Amino-TGCTACTCCATCAGTTGC	18
35	RLB-CP6108	Amino-CTTCCCAGTCTGCCACA	17
36	RLB-HPV71-CP8061	Amino-TGCTACCAAAACTGTTGAG	19
37	RLB-HPV81-CP8304	Amino-GCTACATCTGCTGCTGC	17

3. HPV 16 variants determination

3.1 HPV 16 E6 gene amplification

The extracted DNA of the HPV 16 positive samples were subjected for classification of HPV 16 variants by HPV 16 E6 gene PCR amplification. Three primer sets for HPV 16 E6 gene amplification were designed in conditions of ability to amplify the fragmented DNA in the FFPE cervical tissue sample, which should be less than 250 bps [63] as shown in Figure 9 and the Table 5. The amplification of E6 gene mixture contained 3 mmole $MgCl_2$, 2U Taq DNA polymerase, 1X PCR buffer, 0.2 mmole dNTP, 10 μM of each primer and 3μl of DNA template. Amplification was performed for 40 cycles with the following parameters: initial denaturing at 95°C for 5 min; each cycle at 95°C for 30 s, 55°C for 45 s and 72°C for 1 min; final extension at 72°C for 7 min. 5 μl of PCR product were mixed with 1 μl of loading dye solution and loaded into each well of 1.5% agarose gel prepared in 0.5XTAE. One kb plus DNA ladder was loaded into a well to estimate the size of amplicon. Gel was electrophoresed at 100 volts for 30 min, stained in 0.5 mg/ml EtBr for 10 min and destained for 20 min. PCR products on the agarose gel were visualized by a UV transilluminator. PCR products were purified and sequenced by Molecular Informatic Laboratory, Hong Kong.

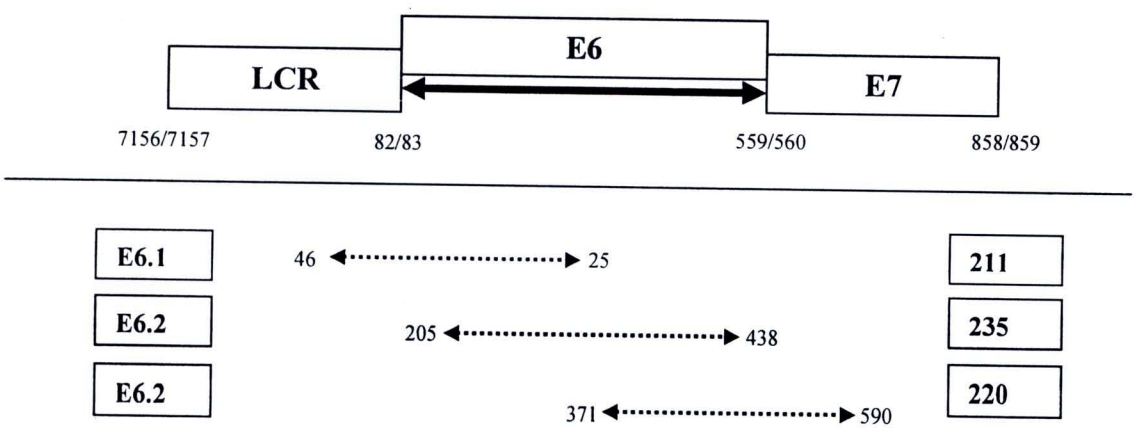

Figure 9 Position of three E6 primer sets overlapping used for HPV 16 E6 amplification. Nucleotide numbering is based on published HPV 16 reference sequence AY686584 that are available through the GeneBank database (NCBI, National Institute of health, Bethesda, MD, USA).

Table 5 The three E6 primer pairs used for HPV 16 E6 amplification by PCR.

Primer names	Nucleotide positions (nt)	Primer sequences (5'-3')	Product length (bp)	Reference
E6.1-F	46-65	TTGAACCGAAACCGGTTAGT		
E6.1-R	237-256	GCATAAATCCCGAAAAGCAA	211	[63]
E6.2-F	205-224	GCAACAGTTACTGCGACGTG		
E6.2-R	419-438	GGACACAGTGGCTTTTGACA	235	
E6.3-F	371-391	CAGCAATACAACAAACCGTTG		
E6.3-R	568-590	TCATGCAATGTAGGTGTATCTCC	220	

3.2 Analysis of E6 nucleotide sequence

HPV 16 variants are classified into phylogenetic classes and subclasses on the basis of their sequence variation at the E6 ORF nt 83-590, which is the most informative for HPV 16 variants identification. The prototype sequence (HPV 16 Ref), which belongs to the European lineage, was used for comparison and nucleotide position numbering. The classification of HPV 16 variants was followed by Yamada *et al.*

Table 6 Patterns of HPV 16 E6 nucleotide position changes used for HPV 16 variant classification modified from Yamada *et al* [4].

HPV 16 variant/ nucleotide position	109	131	132	143	145	178	183	286	289	335	350	403	532
European prototype (E)	T	A	G	C	G	T	T	T	A	C	T	A	A
European (E350G)	~	~	~	~	~	~	~	~	~	~	G	~	~
Asian (As)	~	~	~	~	~	G	~	~	~	~	~	~	~
African-1 (Af-1)	~	~	C	G	T	~	~	A	G	T	~	~	~
African-2 (Af-2)	C	~	C	G	T	~	~	A	G	T	~	~	~
North-American (NA)	~	~	~	~	T	~	~	A	G	T	G	~	~
Asian-American (AA)	~	~	~	~	T	~	~	A	G	T	G	~	G

4. HPV 16 As LCR polymorphism analysis

The LCR polymorphism of HPV 16 As variants was analyzed in HSIL and SCC cases using FFPE cervical tissue samples as well as in fresh cervical tissue samples of

SCC cases. The effect of formalin fixation on DNA was determined by comparison of FFPE and fresh cervical tissue samples.

4.1 HPV 16 LCR amplification and sequencing

Four primer sets were designed for HPV 16 LCR sequence amplification as previously described shown in the Figure 10 and Table 7. A 50 ul PCR mixture was consisted of 10x PCR buffer, 2.5 mM $MgCl_2$, 10 mM dNTP, 1U Taq DNA polymerase, 10 pmole each of specific primers and 3 ul DNA templates. Amplification was performed for 40 cycles in each primer set with following parameters: initial denature at 95 °C for 4 min , each cycles at 95°C for 45 sec, 52 °C for 1 min , 72 °C for 1 min and the final extension step at 72 °C was prolonged for 4 min to ensure a complete extension of the amplified product. PCR products were analyzed by 1.5% agarose gel electrophoresis. Positive LCR PCR products were purified and sequenced by the Molecular Informatic Laboratory, Hong Kong.

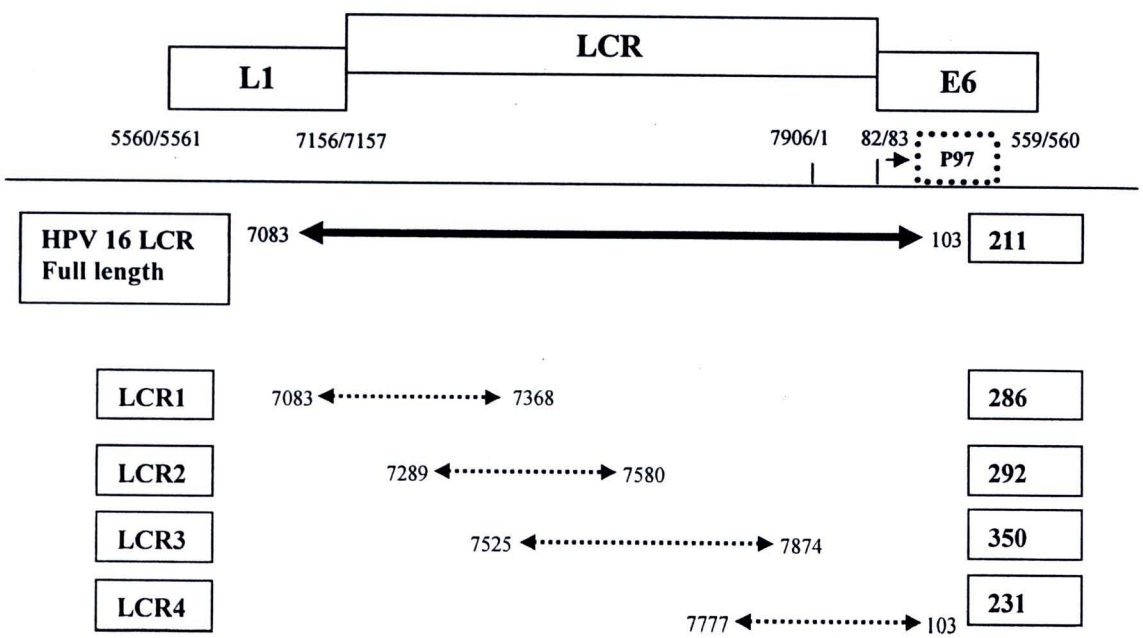

Figure 10 Position of four LCR primer sets overlapping used for HPV 16 LCR amplification. Nucleotide numbering is based on published HPV 16 reference sequence AY686584 that are available through the GeneBank database (NCBI, National Institute of health, Bethesda, MD, USA)

4.2 HPV 16 As LCR polymorphism analysis

The HPV 16 As LCR nucleic acid sequences were aligned and compared with HPV 16 reference sequence AY686584 by using the bioinformatic genomic tool Multalin sequence. HPV 16 reference plasmid was sequenced and used to confirm with reference sequence AY686584 that are available through the GeneBank database (NCBI, National Institute of health, Bethesda, MD, USA).

Table 7 Four LCR primer pairs used for HPV 16 LCR amplification.

HPV 16 LCR primers	Primer sequences (5'-3')	Genomic location (nts)	Length (bp)	PCR product size (bp)
LCR1-F	GAAAACGAAAAGCTACACCCA	7083-7104	21	286
LCR1-R	CAATGAATAACCACAACACAATTA	7345-7368	24	
LCR2-F	GCTTGTGTAACTATTGTGTCATG	7289-7311	23	292
LCR2-R	GTGCAGGTCAGGAAAACAG	7562-7580	19	
LCR3-F	ACTTGTACGTTTCCTGCTTG	7525-7544	20	350
LCR3-R	GTGTAACCCAAAATCGGTTTGC	7853-7874	22	
LCR4-F	GTCACCCTAGTTCATACATGA	7777-7797	21	231
LCR4-R	TGCAGTTCTCTTTTGGTGC	85-103	19	

5. HPV 16 As variant LCR transcriptional analysis

5.1 HPV 16 As variant LCR full length amplification

To determine the transcriptional activity of HPV 16As variant, the LCR of HPV 16 reference plasmid and 2 HPV 16 As variants with different nucleic acid sequence variation at position proximal to p97 promoter of E6/E7 oncogenes from fresh biopsy cases were used for expression vector construction.

The 927 bp full length of LCR (spanning nt 7083-103) was amplified using forward LCR1 primer and reverse LCR4 primer as shown in the Table 7. PCR master mix was prepared for a 50 ul total volume containing 10x PCR buffer, 2.5 mM $MgCl_2$, 10 mM dNTP, 1U Taq DNA polymerase, 10 pM of specific primers and 3 ul DNA templates. Amplification was performed for 40 cycles with following parameters: initial denature at 95 °C for 5 min, each cycle at 95°C for 1 min, 52 °C

for 90 sec, 72 °C for 2 min and the final extension step at 72 °C was prolonged for 10 min to ensure a complete extension of the amplified product. PCR products were visualized by ethidium bromide straining after electrophoresis in 2% agarose gel. After that the full length LCR PCR products, containing p97 HPV 16 E6 and E7 oncogene promoter, were purified with HiYield^{TM}Gel/PCR DNA Extraction Kit (RBC) according to the manufacturer's manuals.

5.2 Construction of pDRIVE cloning vector containing HPV 16 LCR

5.2.1 Preparation of competent cells

The *E.coli* XL-1 Blue was streaked on a lisogeny broth (LB) agar plate for isolated colonies. The plate was incubated at 37°C overnight. A single colony of the *E.coli* XL-1 Blue was grown in 3 ml of LB medium by shaking at 200 rpm, 37°C overnight. The cell suspension was diluted with LB medium in dilution 1:50 and the culture was shaken at 200 rpm, 37°C until the bacteria reached late log phase ($OD_{600} \simeq$ 0.5). The culture was centrifuged at 2,400 rpm, 4°C for 20 min and the supernatant was discarded. Then the pellet was resuspended in 3 ml of 50 mM $CaCl_2$-10 mM Tris-HCL (pH 8.0) solution, gently mixed and incubated on ice for 15 min. The mixture was centrifuged at 2,400 rpm, 4°C for 20 min and the supernatant was discarded. The pellets were resuspended in 200 µl of 50 mM $CaCl_2$-10 mM Tris-HCL (pH 8.0)-15% glycerol solution and gently mixed. The competent cells were stored at -70oC until used.

5.2.2 Preparation of pDRIVE cloning vector containing HPV 16 LCR

The purified PCR products of the full length LCR were ligated into the pDRIVE cloning vector (Qiagen PCR Cloning Kit, Quigen) as described in the manufacturer's instruction then the ligated products were transform in the competent E.coli XL-1 Blue to amplify the pDRIVE cloning vectors containing HPV 16 LCR.

The 3 µl from 10 µl of ligation mixture was added in 100 µl of competent cells. The mixture was chilled on ice for 30 min then incubated at 42°C for 90 sec to heat-shock. After that the mixture was chilled on ice for 20 min, added with 100 µl of SOC medium and incubated for 60 min, by shaking at 200 rpm, 37°C. 100 µl of the mixture was spread on a LB-agar plate containing 50 µg/ml of ampicillin (as a selection marker), and IPTG (50 µM) and X-gal (80 µg/ml) for blue/white screening of recombinant colonies. The plate was incubated at 37°C overnight. Ampicillin

resistant clones, which showed white colony, were selected to extract for plasmid. The positive clones were prepared for plasmid extraction and purification by Purelink™ Quick Plasmid Miniperp Kit (Invitrogen). The purified plasmid vector containing HPV 16 LCR was investigated by 0.7% agarose gel electrophoresis and confirmed the result of inserted full length LCR in the pDRIVE vector by PCR of HPV 16 LCR amplification as described above.

5.2.3 Digestion of pDRIVE cloning vector containing HPV 16 LCR using restriction enzyme

The pDRIVE cloning vector containing HPV 16 LCR was purified and digested with restriction endonuclease KpnI and Hind III (NEB) restriction enzymes. Ten μg of plasmid DNA was double-digested in solution of 1 U of KpnI restriction enzyme and 1 U of Hind III restriction enzyme by incubation at 37oC overnight. The result of restriction enzyme digestion was analyzed by 0.7% agarose gel electrophoresis. The full length LCR with KpnI and Hind III restriction sites was purified with HiYieldTMGel/PCR DNA Extraction Kit (RBC) and quantitated by spectrophotometer.

5.3 Construction of promoterless luciferase expression vector (pGL3 basic vector) containing HPV 16 LCR

5.3.1 pGL3 basic vector restriction enzyme digestion and pGL3 basic vector dephosphorylation

For pGL3 basic vector preparation, 1 μg of pGL3 basic vector was transformed in the competent E.coli XL-1 Blue to amplify the pGL3 basic vector and plasmid DNA was extracted as previously described.

Extracted DNA of the pGL3 basic vector was digested with restriction endonuclease KpnI and Hind III (NEB) restriction enzymes. Ten μg of plasmid DNA was double-digested in solution of 1 U of KpnI restriction enzyme and 1 U of Hind III restriction enzyme by incubation at 37oC overnight. The digestion patterns were analyzed by 0.7% agarose gel electrophoresis.

For dephosphorylation, 0.5 μg of linear pGL3 basic vector was dephosphorylated in 50 μl of dephosphorylation buffer containing 1 U of CIAP (Calf Intestine Alkaline Phosphatase) at 37°C for 30 min. Reaction of enzyme was stopped by incubation at 85°C for 15 min. The dephosphorylated pGL3 basic vector was

purified using HiYield™Gel/PCR DNA Extraction Kit (RBC) and quantitated by spectrophotometer.

5.3.2 Preparation of pGL3 basic vector containing HPV 16 LCR

The purified full length LCR with KpnI and Hind III restriction sites was ligated into the purified dephosphorylated pGL3 basic vector by using T4 ligase (Fermentus).

A 20 µl of reaction mixture was prepared with 1: 4 of plasmid and DNA for ligation, in 4 µl of 5x ligation buffer and sterile DW to a final volume of 20 µl. The mixture was added with 1 unit of T4 DNA ligase and incubated at 16°C for 16 h. The ligation solution was checked for the plasmid recombination by 0.7% agarose gel electrophoresis. Then, 3 µl of the ligated solution was transformed in the competent cells XL-1 Blue as previously described. The ampicillin resistant clones were selected and plasmid DNA was extracted by mini-Prep method. The extracted plasmid DNAs were analyzed by 0.7% agarose gel electrophoresis. The recombinant vector was confirmed by the PCR of HPV 16 LCR amplification as described above. The clone containing the recombinant plasmid was cultured for plasmid extraction and the pGL3 basic vector containing HPV 16 LCR was purified using Purelink™ Quick Plasmid Miniperp Kit (Invitrogen) for HPV 16 LCR transcriptional analysis.

5.3.3 Preparation of pSV-β galactosidase vector

One µg of pSV-β galactosidase vector stock was transformed in the competent E.coli XL-1 Blue to amplify the pSV-β galactosidase vector and plasmid vector extraction was performed as previously described. The extracted plasmids were analyzed by 0.7% agarose gel electrophoresis. The pSV-β galactosidase clone was cultured for plasmid extraction using PurelinkTM Quick Plasmid Miniperp Kit (Invitrogen). This vector was used to quantify the transfection efficiencies in next experimants.

5.3.4 C33A cell line co-transfection

The C33A cell line was transfected with the pGL3 containing LCR vector and co-transfected with control vector as pSV-β galactosidase vector using LipofectamineTM 2000 Reagent. One day before transfection, the C33A cell line was prepared in a 24-well tissue culture plate. The Cells were plated with 2 x 105 cells in 500 µl of 1x DMEM without antibiotics to prepare the cells with 90-95% confluent at

the time of transfection. For each transfection, plasmid DNA-LipofectamineTM 2000 complexes were performed as follows:

-Diluted the pGL3 vector containing LCR and pSV-β galactosidase vector with 1x serum-free DMEM (no antibiotic) to a final volume of 50 µl then mixed gently and incubated for 5 min at RT.

-Diluted 2 µl of LipofectamineTM 2000 Reagent in 50 µl of 1x serum-free DMEM (no antibiotic), gently mixed and incubated for 5 min at RT.

-Mixed the diluted plasmid and LipofectamineTM 2000 Reagent to a total volume of 100 µl, gently mixed and incubated at RT for 30 min to allow plasmid-lipofectamine complex formation,

-Added 100 µl of the plasmid DNA - LipofectamineTM 2000 complexes into each well of the C33A cells.

-Incubated the transfected cells at 37oC in CO2 incubator for 24 h.
Changed medium at 6 h after transfection.

5.3.5 Determination of HPV 16 As LCR transcriptional activity

The transcriptional activity of pGL3 vector containing HPV 16 As LCR was quantified by using Bright-GloTM Luciferase assay reagent (Promega) and compared with transcription activity of pGL3 containing HPV 16 reference LCR.
After 24 h post-transfection, the Bright-GloTM Luciferase reagent was added in an equal volume of culture medium in the well. After mixing and incubation for 2 min to allow complete cell lysis, the lysate was transferred to a thin wall 1.5 ml microcentrifuge tube and luciferase activity was measured using the Modulus Single tube Multimode reader (Turner Biosystem). For mornitoring of transfection efficiencies, the β galactosidase activity of pSV-β galactosidase control vector was evaluated using the Beta-Glo assay system. The Beta-Glo® assay reagent was added in an equal to the volume of culture medium in the well. The culture plate was put on a plate shaker for 30 sec. The complex was incubated for 30 min at RT to allow the signal stabilization, then transferred to a thin wall 1.5 ml microcentrifuge tube and measured the β galactosidase activity using Modulus Single tube Multimode reader (TurnerBiosystem).

6. Statistic analysis

The association between variants in normal cervical cells and in abnormal squamous cervical cells will be analyzed by χ^2 and 95% confidence intervals (CIs).