CHAPTER III

Material and Method

1. Sample

Cell lines and Patient samples

Normal cervical tissues and cancer tissues were collected at Chulalongkorn University Hospital, obtained by punch biopsy of lesions under direct visualization or under colposcopic examination. Specimens were divided in two. The first sample was submitted to routine histological examination, and the second was reserved for DNA and RNA analysis, and/or tissue culture. Blood samples were obtained by venipuncture from CC patients and healthy blood donors.

SiHa and two HeLa CC cell lines from different sources were grown in Dulbecco's modified Eagle's medium (DMEM,Gibco BRL) supplemented with 10% fetal bovine serum and antibiotics (50U/ml penicillin, 50mg/ml streptomycin) . All three cells were purchased from ATCC. SiHa, and HeLa (K) were grown and maintained in laboratories of Dr. Ponglikitmongkol M, Mahidol University, and Dr. Ruxrungthum K, Chulalongkorn University, respectively. HeLa (S) was a received from Dr. Gutkind JS, NIH, USA were grown in our laboratory with same media above, and incubated at 37° C, 5% CO_2 until the resulting cell layer had spread sufficiently were followed by DNA extraction step.

Cervical biopsy specimens and Papanicolaou smears were examined and reviewed by at least two gynecologic pathologists to ensure good quality control of the final pathology results. All CCs contained 20–95% malignant cells. The histological diagnoses distinguished between normal epithelium, low-grade SILs, high-grade SILs, microinvasive and invasive cancer. In case of invasive cancer, only those samples classified as squamous-cell lesions were used for further study carcinogenesis.

Six OTC-embedded frozen CCs and five normal cervices, obtained from hysterectomy specimens, were microdissected. Histologically normal epithelium, connective tissue and malignant cells were subjected to *CCNA1* methylation and expression studies.

An additional HPV-positive premalignant lesions were exfoliated cells, selected from routine cytological screening. In brief, cervical cells were collected with a cervical sampler (Digene Corporation) using the cervical cytobrush technique, and were divided into three parts. The first was reserved for routine cytological diagnosis. The second was tested for the presence of high-risk HPV DNA by Hybrid Capture 2 (Digene Corporation)(95) . In cases of positive high-risk HPV and complete histological tissue evaluation, the third part was subjected to *CCNA1* methylation analysis.

1. Materials

1.1 E. coli (DH5α)

1.2 pGEM-T easy vector

1.3 DNA purification kit (Promega, USA)

1.4 DNA clean up kit (Promega, USA)

1.5 MicroAmp PCR tube

1.6 Cryotube (Nunc, USA)

1.7 Counting chambers

1.8 Pipette tip : 10 µl, 100 µl,1,000 µl (Elkay, USA)

1.9 Microcentrifuge tube : 0.2 ml, 0.5ml, 1.5ml (Bio-rad Elkay, USA)

1.10 Beaker : 50 ml, 100 ml, 200 ml, 500 ml, 1,000 ml (Pyrex)

1.11 Flask : 250ml, 500ml, 1,000ml (Pyrex)

1.12 Reagent bottle : 100 ml, 250 ml, 500 ml, 1,000 ml (Duran, USA)

1.13 Cylinder : 25 ml, 50 ml, 100 ml, 250 ml, 500 ml, 1,000 ml (Witeg, Germany)

1.14 Glass Pipette : 5 ml, 10 ml (Witeg, Germany)

1.15 Microcentrifuge tube rack (Scientific plastics, USA)

1.16 Thermometer (Precision, Germany)

1.17 Plastic wrap

1.18 Stirring-magnetic bar

3. Equipments

3.1 Light microscope

3.2 Stereo microscope

3.3 Autoclave

3.4 Microwave oven

3.5 Hot air oven (Memmert, West Germany)

3.6 Pipette boy (Tecnomara, Switzerland)

3.7 Vortex (Scientific Industry, USA)

3.8 pH meter (Eutech Cybernatics)

3.9 Stirring hot plate (Bamstead/Thermolyne, USA)

3.10 Balance (Precisa, Switzerland)

3.11 Microcentrifuge (Fotodyne, USA)

3.12 DNA Thermal cycler 480 (Perkin Elmer, Cetus USA)

3.13 Thermal cycler (Touch Down, Hybraid USA)

3.14 Power supply model 250 (Gibco BRL, Scothland)

3.15 Power poc 3000 (Bio-Rad, USA)

3.16 Horizon 11-14 (Gibco BRL, Scothland)

3.17 Beta shield (C.B.S scientific. Co.)

3.18 Heat block (Bockel)

3.19 Incubator (Memmert, West Germany)

TOOHA5RHA

3.20 Thermostat shaking-water bath (Heto, Denmark)

3.21 Spectronic spectrophotometers (Genesys5, Milon Roy USA)

3.22 UV Transilluminator (Fotodyne USA)

3.23 UV-absorbing face shield (Spectronic, USA)

3.24 Gel doc 1000 (Bio-RAD)

3.25 Refrigerator 4 ^{0}C (Misubishi, Japan)

3.26 Deep freeze -20 ^{0}C, -80 ^{0}C (Revco)

3.27 Water purification equipment (Water pro Ps, Labconco USA)

3.28 Water bath (Memmert, West Germany)

3.29 Storm 840 and ImageQuaNT solfware (Molecular dynamics)

3.30 Gel star nucleic acid gel stain (Cambrex Bio Science)

4. Reagents

1 General reagent

1.1 Absolute ethanol (Merck)

1.2 Agarose gel (FMC Bioproducts)

1.3 Ammonium acetate (Merck)

1.4 Bisulfite (Merck)

1.5 Bromphenol blue (Pharmacia)

1.6 Chloroform (Merck)

1.7 Disodium ethylenediamine tetracetic acid : EDTA (Merck)

1.8 Dimethyl sulfoxide (DMSO) (Sigma)

1.9 Dnasel (Gibco BRL)

1.10 Diethylpyrocarbonate(DEPC) (Sigma)

1.11 Ethidium bromide (Gibco BRL)

1.12 Fetal bovine serum (Gibco BRL)

1.13 Guanidium thiocyanate (USB)

1.14 Glycogen (Sigma)

1.15 Hydrochloric acid (Merck)

1.16 Hydroquinone (Merck)

1.17 IPTG (Promega)

1.18 Isoamyl alcohol (Merck)

1.19 Isopropanol (Merck)

1.20 LB medium (Gibco BRL)

1.21 Mineral oil (Sigma)

1.22 Phenol (Sigma)

1.23 Penicillin/Streptomycin (Gibco BRL)

1.24 Sodium acetate (Sigma)

1.25 Sodium bisulfite (Sigma)

1.26 Sodium chloride (Merck)

1.27 Sodium hydroxide (Merck)

1.28 Trypan blue (Gibco BRL)

1.29 Triton X-100 (Pharmacia)

1.30 TrizolL reagent (Invitrogen)

1.31 X-gal (Promega)

1.32 Xylene (Merck)

1.33 10 base pair DNA ladder (Biolabs)

1.34 40%acrylamide/bis solution 19:1 (Bio-Rad)

1.35 Wizard DNA Clean-up System(Promega)

1.36 QIAamp DNA blood mini kit(QIAGEN)

1.37 2-Mercaptoethanol (2-ME) (Sigma)

2.Reagent of PCR and RTPCR

2.1 10X PCR buffer (500 mM KCl, 200 mM Tris-HCl pH 8.4) (GibcoBRL, Perkin Elmer)

2.2 Magnesium chloride (GibcoBRL, Perkin Elmer)

2.3 Deoxynucleotide triphosphates (dNTPs) (Promega)

2.4 Oligonucleotide primers (BSU, GENSET) in appendix B

2.5 Hotstart*Taq* DNA polymerase (Qiagen)

2.6 MMLV reverse transcriptase (Fermentas)

2.7 cDNA synthesis kit (Fermentas)

2.8 Genomic DNA sample

5. Methods

Frozen tissue preparation and microdissection

Fresh tissue was snapped frozen in liquid nitrogen as close as possible to the time of surgical removal. The tissue then was embedded in OTC, and frozen sections of 10 microns in thickness were cut in a Leica-Jung Frigocut 2800N cryostats under strict RNase free condition. The unstained frozen tissues are microdissected under guidance of sandwich H&E stained mirror image. Mirror sections were comfirmed tumor and normal areas by pathologist and at least 40 mm^2 of 10-20 sections were done microdissected by 24-guage needle under microscope on RNase free glass slide. The microdissected tissue were immediately placed in a RNase free microcentrifuge tube and kept on ice until processing. To eliminate the OTC, the microdissected frozen tissue specimens were wash with RNase free absolute ethanol 2 times , RNase free 75% ethanol 2 times, RNase free 50% ethanol 2 times and DEPC water 2 times respectively. After washing step, the microdissected of tumor and surrounding tissue were ready for RNA and DNA extraction.

DNA extraction

DNA sample was extracted by a standard technique with a lysis buffer containing proteinase K followed by phenol, chloroform, and isoamyl alcohol organic extraction.

Cell lines and Tissue samples

Briefly, Five to ten x 10^6 cells or 100 mg of tissue sample was wash with 1.0 ml of PBS buffer twice time for remove the culture mediun or residual blood. The sample was lysed with 1.0 ml of digestion buffer containing Lysis buffer2 and 1/10 volume of proteinase K solution; 20 mg Proteinase K in 1.0 ml of 1% SDS-2mM EDTA, should be prepare 30 min before use. The sample was incubated in water bath at 37 °C for overnight (16-24 h) for complete digestion. Adding 1 volume of phenol-chloroform isoamyl alcohol (25:24:1) shake vigorously for 15 seconds and then centrifuge at 10,000g for 5 min. The supernatant was volume 7.5 M Amonium acetate (CH_3COONH_4) and 1 volume of cold (100%) absolute ethanol and then mix by inversion. The DNA should immediately from a stringy precipitate and then mix by inversion. The DNA was recovered by centrifugation at 10,000 g for 15 min. The supernatant was removed and then the pellet was washed with 1 volume of 70% ethanol and centrifuge at 7,500 g for 5 min. The ethanol was decanted and the pellet was air dried. The digested DNA was re-suspended in 100 µl of the distilled water at 65 °C until dissolved. The final preparation had a ratio of absorbance at 260 nm to absorbance at 280 nm of> 1.8. The purified DNA was stored at -70 °C until the DNA methylation assay.

White blood cells (WBC)

Five to ten ml of whole blood was centrifuged for 10 min at 1500 g. The supernatant was removed and the buffy coat was collected to an new polypropylene tube. The buffy coat was added with 10 volumes of cold lysis buffer 1 (or 10 ml), then was mixed thoroughly and incubated at -20°C for 5 min . The tube was centrifuged for 10 min at 1000 g, then remove supernatant. The pellet was added with 3 ml of cold lysis buffer 1, then was mixed thoroughly and was centrifuged at 1000 g for 5 min. The supernatant was discard and added 900 µl of lysis buffer 2, 10 µl of Proteinase K solution (20 mg of Proteinase K in 1.0 ml of 1% SDS-mM EDTA, Should be prepare 30 min before use), and 50 of 10% SDS, then mix vigorously for 15 seconds. The sample was incubated in water bath at 37°C for overnight (16-24 h) for complete digestion. Adding 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) shake vigorously for 15 seconds and then centrifuge at 10,000 g for 5 min. The supernatant was transferred to a new microcentrifuge tube. The DNA was precipitated with 0.5 volume 7.5 M Amonium acetate (CH_3COONH_4) and 1 volume of cold (100%) absolute ethanol and then mix by inversion. The DNA should immediately from a stringy precipitate and then the DNA was recovered by centrifugation at 10,000 g for 15 min. The supernatant was removed and then the pellet was washed with 1 volume of 70% ethanol and centrifuge at 7,500 g for 5 min. The ethanol was decanted and the pellet was air dried. The digested DNA was re-suspended in 50 µl of the double distilled water at 65°C until dissolved. The DNA was stored at -70°C until the DNA methylation assay.

HPV Detection, Quantitation and Typing

For analysis of the HPV DNA, amplification human papillomavirus (HPV) L1 and E6 region in expected size 460 and 250 bps respectively was done. The PCR reaction was in a total volume of 20 µl containing 200 µM of each dNTP , 10 µM Tris-HCl (pH8.4), 50mM potassium chloride, 4.0 mM magnesium chloride, 0.5 units of AmpliTaq Gold (Perkin Elmer Cetus),

Each L1 amplification reaction contained 25 pmol each of the L1 degenerate primers MY11 and MY09 , 2.5 pmol of primers specific for β-globin gene, (a housekeeping gene, were performed to test the quality of DNA).The E6 reactions contained 5 pmol of WD72, WD66, and WD154, 20 pmol of WD67and WD76. Both reactions were subjected to 40 amplification cycles. Each cycle was performed at 95°C for 1 minute, at 55°C for 1 min and 72°C for 2 minutes. An additional 5 minutes final elongation cycle at 72°C was included .

For HPV typing. 50 µl of PCR product was prepared for dot blot experiments. L1 and E6 type – specific probes were used for HPV typing. Positive controls of HPV type 6, 11, 16, 18, 31 and 33 from each PCR amplification were included (96) . Products obtained form L1 and E6 PCR reaction were heated to 95 °c and thereafter 1 volume of 20 X SSC was added . Aliquots of 40 µl were applied to a Hybond-N$^+$ nylon membrane (Amersham Pharmacia Biotech) under vacuum, prewetted in denaturing solution (1.5 M NaCl, 0.5 M Tris – HCl pH 7.2, 0.001M EDTA) for 1 min . The membranes were air dried at room temperature, soaked in 0.4 M NaOH for 20 min for

fixastion and washed with 5 X SSC. Prehybridization at 65°c for 1 h was carried out using 6 X SSC , 5 X Denhardt's solution , 0.5% SDS and 100 μg of single – stranded sheared salmon sperm DNA per ml. Replicate membranes were separately hybridized with denatured ^{32}P –labelled, type-specific probes in prehybridizing solution for 1 hr at 55 °c . Probes WD170 required hybridization at 45 °c . Filters were rinsed briefly in 2 X SCC and 0.1% SDS at room temperature and then twice for 10 min at 45 °c (WD170), 50-52 °c (WD132 ,RR1 and RR2), 55-56 °c (WD103, WD165 and WD166), 56-57 °c (MY12, MY13, WD126, WD128, MY16, MY59, MY18, MY46, MY57, WD 147, WD133 and WD134). or 58-59 °c (MY14 and WD74) The membranes were exposed to a phosphorus screen and the signals were visualized on a PhosphoImager using ImageQunNT software (Molecular Dynamics). Results for L1 and E6 dot blots were scored independently. Duplicate filters were prepared for all specimens. All oligonucleotide sequence of primers and probes were shown in appendix C.

Some of premalignant lesions collected from routine cytological screening which using cervical sampler (Digene Corporation) were tested for the presence of high-risk HPV (types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) DNA by Hybrid Capture 2 (Digene Corporation) according manufacturer's instruction.

Detection of HPV Integration by PCR

All DNA samples that were positive for HPV type 16 and 18 were analyzed in PCR reactions covered *E2* region . Using 0.5 μM of each primer (5' E2 consensus primer HPV -16/18, 3' E2 primer HPV-16 and HPV-18 to detect 1026 and 1028 bp PCR products from HPV 16 and 18 to episomal forms, respectively (97). The PCR reactions contain 200 μM dNTP of each , 10 μM Tris-HCl (pH 8.4), 50 mM potassium chloride, 1.5 mM magnesium chloride, 0.5 units of *Thermus aquaticus* DNA polymerase (Perkin Elmer Cetus), and the PCR amplifications were performed as follows: initial denaturation at 95 °c for 5 min, followed by 40 cycles of denaturation at 95 °c for 1 min,annealing at 45 °c, extension at 72 °c for 7 min . Upon electrophoresis in a 2% agarose gel stained with ethidium bromide on preparation the amplified products were visualized under UV light as distinct bands.

Bisulfite treatment of DNA

After extraction, all DNA samples were treated with sodium bisulfite as previously described (98) . Briefly, 1μg / 50 μl of genomic DNA was denatured in 0.22 M NaOH (5.5 μl of 2M NaOH) at 37 °C for 10 min. Thirty micorliters of 10mM hydroquinone and 520 μl of 3M sodium bisulfite were added for 16-20 hrs at 50 °C . The DNA was desalted with the DNA clean-up kit (Promage, Medison, WI) , and then was desulfonated by incubation in 0.33 M NaOH (5.5 μl of 3M NaOH) at 25 °C for 3 min. The DNA was ethanol precipitated by adding 1 μl of 20ng/ml glycogen, 23 μl of 7.5 M

Amonium acetate (CH_3COONH_4), 240 µl cold (100%) absolute ethanol and then mix by inversion. The DNA should precipitate by incubation at -20 °C for 2 hrs, and then the DNA was recovered by centrifugation at 23,000 g (14,000 rpm) for 15 min. The supernatant was removed and then the pellet was washed with 1.0 ml of 70% ethanol and centrifuge at 7,500 g for 5 min. The ethanol was decanted and the pellet was air dried. The degested DNA was re-suspended in 50 µl of the double distilled water at 65 °C until dissolved. The DNA was stored at -70 °C until the DNA methylation assay.

Primer design for *CCNA1*

Primers were designed to amplify the methylated and unmethylated allele equally . The primer design mention about the difference between methylated allele and non-methylated allele after standard sodium bisulfite treatment and PCR amplification results in the conversion of unmethylated cytosine residues to thymine and methylated cytosine residues to cytosine. This sequence conversion lead to the methylation-dependent creation of specific PCR amplification between methylated and non-methylated allelle. Here general strategy (Appendix B) :

- Identify the region 5′UTR of CCNA1 / CpG island. This is the unconverted map.
- Copy sequence and paste in a text editor.

- Convert all C to T except for CG. First convert all CG to XG. Then convert all C to T. Then convert all X to C. Make a map of this converted sequence(Methylated map)
- Convert all remaining C to T. Make a map of this converted sequence(Non-methylated map)
- Design 2 paired primers of methylated and unmethylated map covered the CG rich region. In this thesis, we design primer about 681 To 604 bp before start size. All primers sequence were shown in the Appendix D and figure 7

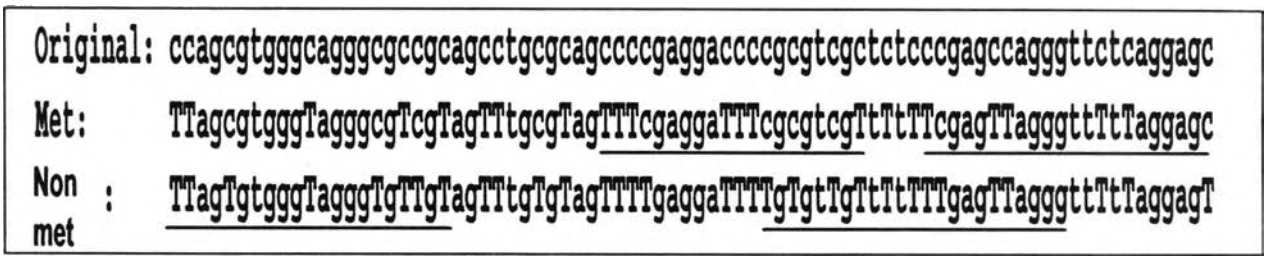

Figure 7. Bisulfite modification sequence and MSP primers. diagram of methylated and non-methylated sequences after bisulfite modification covering the area of both primers (underlined) in the promoter region of *CCNA1*

Duplex methylation-specific PCR (MSP)

Duplex MSPs were performed to identify the *CCNA1* methylation status of all samples. The duplex PCR mixtures contained 10X PCR buffer (Qiagen), deoxynucleotide triphosphates (0.2 mM), primers CCNA1metF, CCNA1metR, CCNA1unmetF and CCNA1unmetR (final concentration 0.4 μM each per reaction)

(Appendix D), 1 U of HotStarTaq (Qiagen) and bisulfited DNA (80 ng). The amplification reaction was carried out for 30 cycles in a 2400 Perkin Elmer thermal cycler. Then 10-µl aliquots of the PCR products were stained with cyber green, run on an 8% non-denaturing polyacrylamide gel, visualized and measured band intensity using a phosphoimager.

RNA preparation and analysis

Total RNA was extracted from cell lines as well as frozen section biopsies using the Trizol reagent (Invitrogen, Singapore) according to the manufacturer's instructions. Six to ten x 10^6 cells from each cell line sample or 50-100 mg from microdissected frozen section was mixed with 1 ml Trizol reagent. After vortexing, RNA was extracted by mix of 200 µl chroloform. The sample was shaked vigorously for 15 seconds and incubated at room temperature for 10 minutes. The sample was centrifuged at 12,000 g for 15 minutes at 4 °C. The aqueous phase containing RNA was transferred to new microcentrifuge tube, adding 0.5 ml. Isopropyl alcohol and incubation at room temperature for 10 min. RNA was precipitated by centrifugation at 12,000 g for 10 min. RNA was precipitated by centrifugation at 12,000 g for 10 min at 4 °C . RNA pellet was washed twice time with 1 ml of cooled 75% ethanol, then dried at room temperature for 5 min and resuspend the RNA pellet in 50 µl DEPC treated sterile water. RNA was kept at -70 °C for used as template for RT-PCR.

After that 5 μg of each sample was subjected to cDNA synthesis using MMLV reverse transcriptase (Fermentas). PCR mixtures contained 10× PCR buffer, 0.2 mM dNTPs, 0.4 μM each of primers CCNA1cDNAF and CCNA1cDNAR, 1 U of HotStartaq and 80 ng cDNA. *GAPDH* served as the internal control (Appendix D). Aliquots of 10 μl of the PCR products were subjected to electrophoresis on a 2% agarose gel stained with ethidium bromide on preparation, and were visualized by a UV trans-illuminator.

Bisulfite genome sequence analysis

Some *CCNA1* methylation-positive CCs were selected for sequence analysis. The bisulfited DNAs were amplified using CCNA1cloningF and CCNA1cloningR (Appendix C). The amplified fragments were cloned using the pGem-T easy vector (Promega) according to manufacture's protocol. The ligated products were transformed into *E. coli* DH5α. The colnes were selected by X-gal/IPTG and amplicillin resistant. Recombinat plasmid were purified by DNA purified PCR kit (Promega) according to manufacturer's protocol. For sequencing analysis, the purified recombinant plasmid (pGem-T easy containing 196 bp . PCR product of *CCNA1* promoter region) were used for the sequencing reaction using Prism Ready Reaction DyeDeoxy Terminator FS cycle Sequencing Kit (Applied Biosystem) according manufacturer's instruction. The DNA template was mixed with 8 μl of Prism Terminator Mix, 3.2 pmol of primer M13 (sense) and distilled water was added to bring the final volume to 20μl reaction. The

sequencing reaction was subjected to 25 PCR cycles, each consisted of 96 °C for 30 seconds, 50 °C for 15 seconds and 60 °C for 4 min in a thermalcycler (GeneAmp PCR system 2400)

The sequencing reaction was purified using simplified ethanol precipitation. Ten microliters of sequencing reaction was added with 2 µl of 3M Sodium acetate (NaOAC), 50 µl of 95% ethanol, vortexed briefly, and stood at room temperature in the dark of 15 min. The precipitate of sequencing reaction was centrifuged at 13,000 g at room temperature for 20 min, and the pellet was washed with 250 µl of 70% ethanol. The suspension was centrifuged at 13,000 g at room temperature for 5 min, and the pellet was dried for 1 min at 90 °C in a Dri-bath . The pellet could be kept at -20 °C for 1 week.

The pellet of was subjected to sequencing analysis by ABI Prism 310 Genetic Analyzer (PE-Applied Biosystems). Regarding the rest of subsequent steps, we referred to the ABI Prism 310 Genetic Analyzer user's manual. The nucleotide sequences were analyzed with Sequence Analysis Software which analyzed the electrophroregram pattern in comparision with the de matrix file.

Data analysis

Data regarding methylation, histology, tumor staging, clinical phenotypes, treatment outcome, HPV PCR and typing were collected in a double-blind fashion until analyzed. Statistical significance of promoter methylation of cyclin A1 and clinical parameter assessed by χ^2 test or fisher exact test as appropriate. P values of <0.05 were taken as statistically significance.

Quantitation is performed with a Molecular Dynamics Phosphorimager. Levels of CCNA1 promoter methylation and L1/Hat exon 4 correlation were determined by Linear Regression and Spearman's rho test. Differentiation of methylation in HPV episomal and integrated group were evaluated with two tail sample T-test by the SPSS software for windows 10.0 (SPSS Inc.,Chicago, IL).