CHAPTER III

MATERIALS AND METHODS

Cell culture

HeLa (cervical cancer) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM). Daudi (B lymphoblast) and Jurkat (T cell leukemia) cell lines were cultured in RPMI 1640. The both media were supplemented with 10% fetal bovine serum (FBS) and 100 units/ml of penicillin-streptomycin. All cells were grown in a humidified atmosphere at 37°C with 5% CO_2.

Cell synchronization

The day before HeLa cells were synchronized at G0 G1/S and S phases, this cell lines were cultured at a density of 10^6 cells per 25 cm^2 tissue culture flask for 24 hours. Then, HeLa cells were synchronized at G0 phase by culture in serum deprivation medium, DMEM with 0.2% FBS, for 48 h. In G1/S and S phase, HeLa cells were synchronized by the thymidine block method. Cell lines were cultured with 2 mM thymidine for 18 hours to obtain cells at G1/S phase (79). To release cells into S phase, HeLa cells were washed three times with Phosphate buffered saline (PBS) then replaced with fresh medium and incubated for 3 and 5 hours. After synchronization, cells were washed with PBS, incubated with 0.5 ml of 0.25% trypsin for 5 min and collected in 15 ml tubes. Cells were stained with propidium iodide and DNA content was measured by flow cytometry to determine the percentage of cells at different stages of the cell cycle as well as the percentage of fragmented and apoptotic cells (80).

siRNA

For genetically inhibit Ku86, RAD51, ATM and DNA-PKcs the oligonucleotide sequences of siRNA targeting RAD51 is as previously described by Zhang *et al* (81). The hairpin siRNA template oligonucleotides were dissolved in approximately 100 µl of nuclease-free water. Dilute 1 µl of each oligonucleotide 1:100 in TE buffer and determine the absorbance at 260 nm. Calculate the concentration for hairpin siRNA

oligonucleotides by multiplying the A260 by the dilution factor and then by the extinction coefficient (~33 µg/ml). The hairpin siRNA template oligonucleotides were diluted to approximately 1 µg/µl. and then assemble the 50 µl annealing mixture as follows: 2 µl of sense and antisense siRNA template oligonucleotide and 46 µl 1X DNA Annealing Solution (Ambion). The mixture were heated to 90°C for 3 minutes, then placed in a 37°C incubator and incubated for 1 hour. 5 µl of the annealed siRNA templates were diluted with 45 µl nuclease-free water for a final concentration of 8 ng/µl. Set up 10 µl ligation reaction following: 1 diluted annealed siRNA insert, 6 µl nuclease-free water, 1 µl 10X T4 DNA ligase buffer, 1 µl p*Silencer* 3.1 hygro vector (Ambion), 1 µl T4 DNA ligase (5U/µl), mixed and then incubated overnight at 16°C. Ligated plasmids were transformed into *E. coli* DH5α and plated the transformed cells on LB plates containing 100 µg/ml ampicillin and grown overnight at 37°C. Bacterial clones were selected and cultured for plasmid extraction. Plasmids were performed DNA sequencing to confirm the siRNA sequence without any mutation.

For DNA transfection step, 2 X10^5 of HeLa cells were plated in DMEM with 10% FBS to achieve 30% - 60% confluent after approximately 24 hours. The complex reagents for transfection of HeLa in 6 well plates were prepared as describe. 3 µl of siPORT *XP-1* were diluted in Opti-MEM I medium (Gibco BRL) in polystyrene tube for a final volume of 100 µl and vortex thoroughly then incubate at room temperature for 5-20 minutes. Add 1 µg of plasmid DNA to the diluted siPORT *XP-1*, mix by gently flicking the tube and incubate at room temperature for 5-20 minutes. HeLa cells were washed with PBS and rinsed briefly with serum-free DMEM. Adjust the volume of serum-free medium in 6 well plates containing HeLa cells to 2 ml then overlaid the siPORT *XP-1*/DNA complex dropwise onto the cells, gently rock the plates back and forth to evenly distribute the complexes. Incubate 2-8 hours in 37°C with 5% CO_2 then removed the medium containing siPORT XP-1/DNA complex and replaced with DMEM with 10% FBS, After 24 hours, change a medium to DMEM with 10% FBS and 250 µg/ml of hygromycin (Roche). Culture the cells in medium containing hygromycin until all of the cells in non-transfected control culture were killed. Culture the cells until have grown to confluency in

6 well plates, split them, and grown them with 150 μg/ml hygromycin to prevent the accumulation of cells that no longer express hygromycin resistance. HeLa cells with siRNA expression were harvested to perform western blot for checking knock-down gene expression and prepared HMW DNA.

High-molecular-weight (HMW) DNA preparation

To prepare HMW DNA, cell lines were embedded in 1% low-melting point agarose (LMP) at a density of 5 x 10^5 cells per plug. The plug was lysed and digested in 400 μl of digestion buffer (1 mg/ml proteinase K, 50 mM Tris, pH 8.0, 20 mM EDTA, 1% sodium lauryl sarcosine) at 37°C overnight. After, the plug was rinsed four times in TE buffer for 20 minutes. To polish cohesive-end EDSBs, T4 DNA polymerase (New England Biolabs) was added and later inactivated by adding EDTA (disodium) to a concentration of 20 mM for 5 minutes followed by rinsing four times in TE buffer for 20 minutes. The modified LMPCR linkers were prepared from the two oligonucleotides 5'-AGGTAACGAGTCAGACCACCGATCGCTCGGAAGCTTACCTCGTGGACGT-3' and 5′-ACGTCCACGAG-3′. Linker (50 pmol) was ligated to HMW DNA using T4 DNA ligase (New England Biolabs) at 25°C overnight. DNA was extracted from agarose plugs using a QIAquick gel extraction kit (QIAGEN).

L1-EDSB ligation-mediated realtime PCR (L1-EDSB-LMPCR)

The quantity of L1-EDSB was measured by realtime PCR using a Lighcycler™ instrument (Roche Applied Science) with L1 primers 5′-CTCCCAGCGTGAGCGAC -3′ (outward) and 5′-AAGCCGGTCTGAAAAGCGCAA-3′ (inward), the linker primer, and the Taqman probe homologous to the 3′ linker sequence (6-fam) ACGTCCACGA GGTAAGCTTCCGAGCGA (tamra) (phosphate). Amplification was performed in 20 μl reactions with 0.2 mM dNTPs, 4 mM $MgCl_2$, 0.5 μM of each primer, 0.4 μM Taqman probe, 2 unit of HotStarTaq (QIAGEN), 1 μg/μl BSA, 1x PCR buffer and 10 ng of ligated DNA. Initial denaturation was at 95°C for 15 minutes, followed by denaturation at 95°C for 5 second, annealing at 58°C for 5 second, extension for 2 minutes at 69°C for up to

40 cycles, with quantification after the extension steps. Two types of control DNA were used. The first was a 100-bp oligonucleotide sequence with the 5′ linker sequence and 3′ homology to L1 oligonucleotide sequences. The second was DNA digested with *Eco*RV and *Alu*I and ligated to the LMPCR linkers. Ligated control DNA was subjected to realtime PCR for comparison with control oligonucleotide sequences to calculate the amount of control DNA DSBs. Because we have experienced the control oligonucleotide degradation in working solution after preparation for several weeks, for most experiments, the amounts of EDSBs were compared with the ligated control digested DNA and reported as L1-EDSB-LMPCR templates per nanogrum of DNA.

Bisulfite treatment

Ligated HMW DNA was modified by sodium bisulfite using standard protocol (82). Dilute 1 µg of DNA into 50 µl with dH_2O, 5.5 µl of 2 M NaOH were added then incubate for 10 minutes at 37°C to create single-stranded DNA. Then, 30 µl of 10 mM hydroquinone and freshly prepared 520 µl of 3 M sodium bisulfite at pH 5.0 were added and mixed. The sample was incubated at 50°C for 16 hours. The bisulfite-treated DNA was isolated using Wizard DNA Clean-Up System (Promega). The DNA was eluted by 50 µl of water at 95°C and 5.5 µl of 3 M NaOH were added and incubate at room temperature for 5 minutes. The DNA was precipitated by adding 17 µl of 10 M NH_4OAc, 3 volumes of ethanol and 1 µl of 20 mg/ml glycogen as a carrier then incubated at -20°C for 2 hours. After incubation, DNA was centrifuged at 14,000 rpm for 15 minutes. The DNA pellet was washed with 70% ethanol then centrifuged at 14,000 rpm for 5 minutes. and air dry. The DNA pellet was resuspended in 20 µl of water. Bisulfite-treated DNA was stored at -20°C until ready for used.

COBRA-L1 and COBRA-L1-EDSB

For COBRA-L1 (56), a 20 µl PCR was carried out in 1X PCR buffer contained with 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1 unit of HotStarTaq (QIAGEN), 0.3 µM of B-L1-inward 5′-CGTAAGGGGTTAGGGAGTTTTT-3′, 0.3 µM of B-L1-outward 5′-

RTAAAACCCTCCRAACCAAATATAAA-3′, and 2 µl of bisulfite-modified DNA. PCR was performed under the following conditions, initial denaturation at 95°C for 15 minutes, followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 50°C for 1 minute, extension at 72°C for 1 minute, and final extension at 72°C for 7 minutes.

For COBRA-L1-EDSB, the B-L1-inward oligonucleotide was replaced with B-LMPCR oligonucleotide, 5′-GTTTGGAAGTTTATTTTGTGGAT-3′. A 20 µl PCR was carried out in 1X PCR buffer contained with 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix, 1 unit of HotStarTaq, 0.3 µM of B-LMPCR, 0.3 µM of B-L1-outward, and 2 µl of bisulfite-treated DNA. PCR cycling conditions are initial denaturation at 95°C for 15 minutes followed by 40 cycles of denaturation at 95°C for 1 minute, annealing at 48°C for 1 minute, extension at 72°C for 2 minutes, and final extension at 72°C for 7 minutes. Bisulfite-treated Daudi, Jurkat and HeLa DNA were digested with *Eco*RV and *Alu*I and ligated LMPCR linker were used as positive controls to normalize the inter-assay variation of all COBRA experiments. HeLa DNA without ligation was used as a negative control.

To prevent heteroduplex amplicons, hot-stop technique was applied in COBRA L1 and COBRA-L1-EDSB assay. ^{32}P-labeled-bisulfite-L1-outward oligonucleotides were added in the last PCR cycle. The amplicons were double-digested in a 10 µl reaction volume with 2 unit of *Taq*I and 8 unit of *Tas*I in 1x *Taq*I buffer (MBI Fermentas) then incubate at 65°C for 4 hours. This was designed to detect unmethylated and methylated sequences of 98 and 80 bp, respectively. Digested products were then electrophoresed in 6% denaturing polyacrylamide gel. The intensity of DNA fragments was measured with a PhosphorImager using Image Quant software (Molecular Dynamic). The LINE-1 methylation level was calculated as the percentage of *Taq*I intensity divided by the sum of *Taq*I- and *Tas*I-positive amplicons. HeLa DNA without linker was used as a negative control.

Western blotting

Cellular proteins are extracted with lysis buffer and then sonicated with an Ultrasonics sonicator at 70% power output for three 10 seconds intervals on ice. Total protein was electrophoresed on 8% SDS-polyacrylamide gel at 200 V for 40 minutes and transferred to nitrocellulose at 100 V for 40 minutes for detecting Rad51. To detect RAD51, the nitrocellulose was blocked with 5% nonfat dry milk and 0.2% Tween 20 in Tris buffered saline for 1 hour at room temperature with constant agitation, and the incubated with anti-Rad51, diluted in freshly prepared TBST-MLK (1:1000) for overnight at 4°C with constant agitation. Then, the nitrocellulose was incubated with goat anti-rabbit HRP conjugated IgG in TBST-MLK (1:2000) for 1 h at room temperature with agitation and visualized by chemiluminescence. To detect ATM and DNA-PKcs, total proteins were electrophoresed on 5% SDS polyacrylamide gel electrophoresis (SDS-PAGE) at 120 V for 1 hour 30 minutes then transferred to nitrocellulose membrane at 100 V for 1 hour to detect DNA-PKcs and ATM proteins. Blotted nitrocellulose membrane was blocked in freshly prepared Tris buffer saline (TBS) with 0.1 % Tween 20™ and 5% nonfat dry milk (TBST-M) for 1 hour at room temperature with constant agitation. To detect DNA-PKcs or ATM, the blocked membranes were incubated with either antibody to anti-DNA-PKcs (G-4) (Santa Cruz Biotechnology) or anti-ATM (2C1) (GeneTex) (1:1000 in TBST-M) overnight at 4°C with constant agitation. G3PDH protein levels were used as a control for equal protein loading. Total protein of G3PDH on 8% SDS-polyacrylamide gel at 200 V for 30 min and transferred to nitrocellulose at 100 V for 1 h After blocking with 5% nonfat dry milk and 0.1% Tween 20 in Tris buffered saline, membranes are incubated with anti- G3PDH, diluted in freshly prepared TBST-MLK (1:1000). Subsequently, the nitrocellulose was incubated with goat anti-rabbit HRP conjugated IgG in PBS-MLK (1:2000) and visualized by SuperSignal West Pico Chemiluminascent Substrate™ (Pierce). In brief, the two substrate components were mixed at a 1:1 ratio to prepare the substrate working solution then added to nitrocellulose blot and incubated for 5 minutes at room temperature. The blot was remove from working solution and covered with plastic wrap. The protected nitrocellulose membrane was placed in a film cassette with the protein side facing up

and exposed to X-ray film (Kodak). Exposure time may be varied to achieve optimal results.

Statistical Analyses

Statistical significance was determined according to an independent sample t-test and a paired sample *t*-test using the SPSS program version 16 as specified.