CHAPTER III

MATERIALS AND METHODS

Cell culture

HeLa (cervical cancer), K-562 (erythroleukemia), and SW480 (colorectal adenocarcinoma) cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM). Daudi (B lymphoblast), Jurkat (T cell leukemia) and Molt4 (T lymphoblast) 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

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 (h). Then, HeLa cells were synchronized at G0 phase by culture in serum deprivation medium, DMEM plus 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 h to obtain cells at G1/S phase (82). To release cells into S phase, HeLa cells were washed with PBS, replaced with fresh medium and incubated for 3 and 5 h. After synchronization, cells were washed with Phosphate buffered saline (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 (83).

5-aza-2-deoxycytidine treatment

A 10 mM stock solution of 5-aza-2-deoxycytidine was prepared in sterile water and stored at -80 °C in aliquots. Cells were counted, split and seeded at the initial concentration of 2.5-3 x 10^5cells/ml in a total volume of 30 ml per flask. Immediately before use, 3 µl of the 10 mM 5-aza-2-deoxycytidine stock solution was thawed and

added to the flasks and thoroughly resuspended (final concentration 1 μM). The medium was changed every 48 h (84).

Trichostatin (TSA) treatment

Before treated with TSA, HeLa cells were seeded at a density of 10^6 cells in 25 cm^2 tissue culture flask, cultured in serum deprivation medium and incubated for 24 h. These cells were grown in serum deprivation medium with TSA (100 ng/ml) as an inhibitor of histone deacetylase (HDAC) for 2, 4 and 8 h. After treatment, cells were harvested as same as synchronized cells.

Radiation procedure

HeLa cells were grown in tissue culture flask for 24 after the medium of HeLa cells was replaced with 5 ml of ice-cold medium. Then, cells were exposed to 0.01, 0.1, 1.0, 2.0, 10, 20, 40 and 60 Gy γ-rays at a rate of 6.22 cGy/min with a ^{60}Co source (Eldorado78).

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 (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) for 20 min. To polish cohesive-end EDSBs, T4 DNA polymerase was added and later inactivated by adding EDTA to a concentration of 20 mM for 5 min followed by rinsing four times in TE buffer for 20 min. 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 at 25 °C overnight. DNA was extracted from agarose plugs using a QIAquick gel extraction kit. Then, 100 ng of DNA was tested for fragmentation using the LMPCR ladder technique (66). After primary polymerization at 72 °C for 1 min, 35 cycles of PCR

were carried out using the linker primer 5′-AGGTAACGAGTCAGACCACCGA-3′ at 58 °C. The amplicons were electrophoresed in agarose gel to visualize apoptotic fragmented DNA ladders.

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

The quantity of L1-EDSB was measured by realtime PCR with the L1 primers 5′-CTCCCAGCGTGAGCGAC-3′ (outward) and 5′-AAGCCGGTCTGAAAAGCGCAA-3′ (inward), the linker primer, and a Taqman probe homologous to the 3′ linker sequence (6-fam) ACGTCCACGAGGTAAGCTTCCGAGCGA (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, 1 μg/μl BSA, 1x PCR buffer and 10 ng of ligated DNA. Initial denaturation was at 95 °C for 15 min, followed by denaturation at 95 °C for 5 s, annealing at 58 °C for 5 s, extension for 2 min 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 ng of DNA.

Bisulfite treatment

Ligated HMW DNA was modified by sodium bisulfite using standard protocol (26). In brief, 2 μg of DNA was denatured in 50 μl of 2 M NaOH for 10 minutes at 37 °C. Then, 30 μl of freshly prepared 10 mM hydroquinone and 520 μl of 3 M sodium bisulfite at pH 5.0 were added and mixed. The sample incubated at 50 °C for 16 hours. The bisulfite-treated DNA was isolated using Wizard DNA Clean-Up System. The DNA was

eluted by 50 µl of warm water at 95 °C and 5.5 µl of 3 M NaOH are added for 5 min. The DNA was precipitated with ethanol and glycogen as a carrier and then 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 (3), 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, 0.3 µM of B-L1-inward 5′-CGTAAGGGGTTAGGGAGTTTTT-3′, 0.3 µM of B-L1-outward 5′-RTAAAACCCTCCR AACCAAATATAAA-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 is carried out in 1X PCR buffer contained with 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1 unit of HotStarTaq, 0.3 µM of B-LMPCR oligo, 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 DNAs 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- DSB assay. α^{32}P-labeled-bisulfite-L1-outward oligo was added in the last PCR cycle. The amplicons were doubly digested in a 10 µl reaction volume with 2 unit of *Taq*I and 8 unit of *Tas*I in 1x *Taq*I buffer at 65 °C for 4 h. 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. 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.

L1-EDSB-LM methylation specific PCR (L1-EDSB-LM-MSP)

Methylated and unmethylated band patterns of EDSBs were analyzed by this assay. First PCR, bisulfite-treated DNA was amplified with B-LMPCR 1st primer (5'-GGTAATGAGTTAGATTATTGATTGT-3') and specific primers including methylated primer (Met-L1-inward: 5'-CTCCCAACGTAAACGACG-3') or unmethylated primer (Unmet-L1-inward: 5'-ACAACTCCCAACATAAACAACA -3'). A 20 µl PCR was carried out in 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1 unit of HotStarTaq, 0.3 µM each primer, and 2 µl of bisulfite-converted genomic DNA. PCR cycling conditions were 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 2 minutes, and final extension at 72 °C for 7 minutes. Second PCR, the amplicons were amplified with α^{32}P labelled B-LMPCR primer and L1-Bihot primer (5'-CATCTCACTAAAAAATACCAAAC-3'). A 20 µl PCR was carried out in 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1 unit of HotStarTaq, 0.6 µM each primer, and 1 µl of 1st PCR products. PCR cycling conditions were initial denaturation at 95 °C for 15 minutes followed by 18 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. The PCR products were separated on 6% denaturing PAGE and analyzed by Phosphoimager as described above to compare band patterns.

Chromatin immunoprecipitation (ChIP)

The ChIP assay was performed essentially as previously described with some modifications (24, 85). Histone cross-linking to DNA was induced by adding formaldehyde directly to culture medium at a final concentration of 1%, with incubation at 37 °C for 10 min. After stopping the reaction with glycine (0.125 M final concentration)

and incubation for 5 min at room temperature, adherent cells were washed twice with ice-cold PBS, and then scraped into ice-cold PBS containing protease Inhibitor. Non-adherent cells were collected by centrifugation for 4 min at 510 g at 4 °C and washed as above. Nuclei were isolated by resuspending the cell pellet in cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, containing protease Inhibitor) and incubated on ice for 20 min. Intact nuclei were collected by centrifugation at 3,210 g for 5 min at 4 °C, resuspended in nuclear lysis buffer (1% SDS, 50 mM Tris-HCl pH 8.1, 10 mM EDTA, containing protease Inhibitor), and incubated on ice for 10 min. Chromatin was sheared with an Ultrasonics sonicator at 30% power output for four 30 s intervals on ice to an average size of 500–1000 bp. After centrifugation at 21,720 g for 10 min at 4 °C, the chromatin solution was diluted 10-fold with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl, containing protease Inhibitor) and then precleared for 30 min at 4 °C with protein G Plus-Agarose with rotation. The agarose beads were pelleted for 1 min at 180 g and the chromatin fragments were immunoprecipitated overnight at 4 °C with Anti-phospho-Histone H2AX monoclonal antibody or normal mouse IgG antibody as a negative control on a rotator. Protein-DNA-antibody complexes were isolated by the addition of protein G Plus-Agarose. After 2 h, agarose beads were collected by centrifugation at 120 g for 1 min, washed once each in 500 mM, 550 mM and 600 mM high-salt wash buffers (0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl pH 8, 2 mM EDTA, 500–600 mM NaCl), and twice in wash buffer (100 mM Tris-HCl pH 8, 500 mM LiCl, 1% Nonidet P-40, 1% deoxycholic acid). Complexes were eluted with elution buffer (50 mM $NaHCO_3$, 1% SDS) for 15 min at room temperature. Cross-links were reversed by adding NaCl (200 mM final concentration) and RNA was removed by adding 10 mg/ml of RNase A, followed by incubation for 4 h at 65°C, and then precipitated overnight with ethanol. Samples were deproteinized with proteinase K. After phenol/chloroform extraction, the DNA was precipitated with ethanol. The precipitated DNA was subjected to realtime 5′L1PCR and COBRA-L1.

Realtime 5′ L1 PCR

Quantification of the amount of immunoprecipitated DNA was carried out by realtime PCR using SYBR Green according to the manufacturer's instructions. Briefly, 1x QuantiTect SYBR Green PCR Master Mix, 0.2 μM forward primer (L1.2HpaIIRFLPF: 5′-CTCCCAGCGTGAGCGAC-3′), and 0.2 μM reverse primer (5′LIDSIP1st: 5′-ACTCCCTAGTGAGATGAACCCG-3′) were used for each PCR assay. The PCR program was initiated at 95 °C for 15 min to activate the HotStarTaq DNA polymerase, followed by 50 thermal cycles of 15 s at 95 °C, 20 s at 57 °C and 20 s at 72 °C. A melting curve test (68 °C) was always carried out after the final reaction step to confirm that appropriate amplification products were obtained. Each sample was analyzed in triplicate PCR reactions. DNA precipitated by Anti-phospho-Histone H2AX was 2 to 20-fold greater (average 10-fold) than DNA precipitated by normal mouse IgG antibody. One sample with less than double the amount of the mock control was excluded. The precise amount and methylation level of γ-H2AX-bound DNA were calculated from the quantity of anti-phospho-Histone H2AX minus the DNA bound in mock control IgG antibody experiments. The amount of γ-H2AX-bound DNA was estimated by relating the L1 quantity to positive control HeLa genomic DNA.

Western blotting

Cellular proteins are extracted with lysis buffer and then sonicated with an Ultrasonics sonicator at 70% power output for three 10 s intervals on ice. Total protein was electrophoresed on 15% SDS-polyacrylamide gel at 200 V for 40 min and transferred to nitrocellulose at 100 V for 40 min for detecting γ-H2AX and acetyl-histone H4 . To detect γ-H2AX, the nitrocellulose was blocked with 5% nonfat dry milk and 0.2% Tween 20 in Tris buffered saline for 1 h at room temperature with constant agitation, and the incubated with anti-phospho-Histone H2A.X (Ser139), diluted in freshly prepared TBST-MLK (1:1000) for overnight at 4 °C with constant agitation. Then, the nitrocellulose was incubated with goat anti-mouse HRP conjugated IgG in TBST-MLK (1:2000) for 1 h at room temperature with agitation and visualized by chemiluminescence. For acetyl-histone H4, after blocking with 3% nonfat dry milk in PBS buffer for 1 h at room temperature with constant agitation, membrane was incubated with anti-acetyl-histone

H4, diluted in.freshly prepared PBS-MLK (1:1000) for overnight at 4 ^{o}C with constant agitation. Then, the nitrocellulose was incubated with goat anti-rabbit HRP conjugated IgG in PBS-MLK (1:2000) for 1.5 hour at room temperature with agitation and visualized by chemiluminescence. 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 chemiluminescence.

Statistical Analyses

Statistical significance was determined according to an independent sample t-test, a paired sample *t*-test, or ANOVA using the SPSS program version 11.5 as specified.