LINE-1 ในมะเร็งปากมดลูก

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LINE-1 IN CERVICAL CANCERS

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ระดับเมทิลเลชันที่ลดลงทั่วทั้งจีโนมทำให้เกิดมะเร็งได้โดยกลไกหลายอย่าง คือ 1) กระตุ้นให้ยีนก่อมะเร็งทำงาน 2) กระตุ้นให้ยีนที่สามารถเคลื่อนที่ได้ในจีโนมทำงาน 3) ทำให้โครโมโซมเกิดความไม่เสถียร วัตถุประสงค์ของวิทยานิพนธ์นี้ต้องการศึกษากลไกการเกิดความไม่เสถียรของจีโนมที่เกิดจากการเหนี่ยวนำของระดับเมทิลเลชันที่ลดลงทั่วทั้งจีโนม เราสมมติฐานว่าระดับเมทิลเลชันที่ลดลงทั่วทั้งจีโนมเหนี่ยวนำให้เกิดความไม่เสถียรของจีโนมโดยทำให้เกิดการแทรกตัวของไลน์วัน เราได้ทำการพัฒนาเทคนิคที่เรียกว่า "LIDSIP" ใช้ในการตรวจหาไลน์วันที่แทรกใหม่ในจีโนมเปรียบเทียบระหว่างเซลล์มะเร็งปากมดลูกและเซลล์ปกติของคนไข้คนเดียวกัน จากการตรวจหาพบว่าการแทรกตัวของไลน์วันพบได้ยากและไม่ควรเป็นกลไกหลักของการเกิดการกลายพันธุ์ในมะเร็งปากมดลูก ดังนั้นเราจึงเสนอสมมติฐานใหม่ว่า ความไม่เสถียรของจีโนมเกี่ยวข้องกับดีเอนเอเมทิลเลชันบนสายดีเอนเอง โดยกลไกการเกิดความไม่เสถียรของจีโนมขึ้นอยู่กับการที่ดีเอนเอเมทิลเลชันเกี่ยวข้องกับการเกิดหรือการซ่อมสายดีเอนเอฉีกขาดที่เกิดขึ้นเองในเซลล์ เราได้พัฒนาเทคนิคในการวัดปริมาณสายดีเอนเอฉีกขาดที่เกิดขึ้นเองในเซลล์และวัดระดับเมทิลเลชันของจีโนมและของสายดีเอนเอฉีกขาดที่เกิดขึ้นเองในเซลล์ เทคนิคเหล่านี้มีวัตถุประสงค์เพื่อศึกษาว่าระดับเมทิลเลชันที่ลดลงทั่วทั้งจีโนมเหนี่ยวนำให้เกิดความไม่เสถียรของจีโนมโดยตรง โดยเกิดจากสายดีเอนเอฉีกขาดที่เกิดขึ้นเองในเซลล์ ดังนั้นระดับเมทิลเลชันของสายดีเอนเอฉีกขาดที่เกิดขึ้นเองในเซลล์ควรจะสัมพันธ์กับกลไกการเกิดความไม่เสถียรของจีโนม

ผลการศึกษาพบว่าการฉีกขาดของสายดีเอนเอสามารถพบได้ในสภาวะปกติของเซลล์และปริมาณของดีเอนเอฉีกขาดที่เกิดขึ้นเองในเซลล์เกี่ยวข้องกับชนิดของเซลล์ที่ศึกษา โดยสายดีเอนเอฉีกขาดที่เกิดขึ้นเองที่พบส่วนใหญ่เป็นสายดีเอนเอในบริเวณที่มีหมู่เมทิลและการฉีกขาดที่เกิดขึ้นจำเป็นที่ต้องมีการซ่อม จากการศึกษาพบว่าความผิดปกติของโปรตีนเอทีเอ็มซึ่งเป็นโปรตีนที่เกี่ยวข้องกับการซ่อมสายดีเอนเอที่ฉีกขาด ทำให้ปริมาณสายดีเอนเอฉีกขาดที่เกิดขึ้นเองในบริเวณที่มีหมู่เมทิลในเซลล์เพิ่มขึ้น ดังนั้นการซ่อมสายดีเอนเอฉีกขาดที่เกิดขึ้นเองในบริเวณที่มีหมู่เมทิลขึ้นอยู่กับโปรตีนเอทีเอ็มซึ่งกลไกการซ่อมมีความแม่นยำ โดยสรุปการซ่อมสายดีเอนเอฉีกขาดที่เกิดขึ้นเองในบริเวณที่มีหรือไม่มีหมู่เมทิลเกิดผ่านกลไกที่ต่างกันโดยขึ้นอยู่กับสถานะของเมทิลเลชันบนสายดีเอนเอ ดังนั้นอัตราการกลายพันธุ์ที่เพิ่มขึ้นเองเนื่องจากระดับเมทิลเลชันที่ลดลงอาจเกี่ยวข้องกับกลไกที่ต่างกันของการซ่อมสายดีเอนเอฉีกขาดที่เกิดขึ้นเองในบริเวณที่มีหรือไม่มีหมู่เมทิล

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Global hypomethylation may potentially promote carcinogenesis via at least three possible mechanisms: activation of oncogenes, reactivation of transposable elements and genomic instability. The thesis aims to study the mechanism of global hypomethylation induces genomic instability. We hypothesized whether global hypomethylation induces instability *in trans* by reactivated LINE-1 retrotranspositional activity. We established a new technique to map and screen for new LINE-1 insertions, called "LIDSIP". The PCR was applied to compare between cervical cancer and normal cells from the same patients. However, we discovered that LINE-1 retrotransposition is rare and should not be the major mechanism of cervical cancer mutations. Therefore, we evaluated our second hypothesis that genomic instability is related to DNA methylation *in cis*. This mechanism may depend on how DNA methylation is related to how endogenous DNA double-stranded breaks (EDSBs) are produced or repaired. We developed a set of new techniques for quantification of EDSBs and methylation levels of genome and EDSBs. The aims of these techniques were to investigate whether global hypomethylation induces genomic instability *in cis* via EDSBs. Therefore, EDBS methylation statuses should be association with this mechanism. Our study discovered that DNA breakages are commonly retained event under normal physiologic circumstance and the quantity of retained EDSBs is cell types specific. The majority of retained EDSBs are methylated. Nonetheless, methylated EDSBs are eventually repaired. A defect in Ataxia Telangiectasia Mutated (ATM) repair raised the EDSB methylation level. Therefore, methylated EDSB repair is dependent on ATM dependent precise repair pathways. In conclusion, unmethylated and methylated EDSBs preferentially undergo different repair pathways. Consequently, increase of spontaneous mutation rate due to the genomic hypomethylation level may be related to how methylated and unmethylated EDSBs are differentially processed.

Field of study Medical microbiology Student's signature Wichai Pornthanakasem

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TABLE OF CONTENTS

FIGURE PAGE

FIGURE PAGE

FIGURE PAGE

TABLE PAGE

LIST OF ABBREVIATIONS

ATM	Ataxia telangiectasia mutated protein
CIN	Chromosomal instability
COBRA	Combined with bisulfite restriction analysis
COBRA-L1	COBRA of L1s
COBRA-L1-EDSB	COBRA of L1-EDSB
DNA-PKcs	DNA-dependent protein kinase
DNMTs	DNA methyltransferases
DSBs	DNA double-strand breaks
EDSBs	Endogenous DNA double-strand breaks
HMW	High-molecular-weight
HPV	Human papilloma virus
HR	Homologous recombination
IRSPCR	Interspersed repetitive sequence PCR
L1-EDSB-LMPCR	LINE-1 (L1) human retrotransposons
LINE-1 or L1	Long Interspersed Nuclear Element type 1
LMPCR	Ligation-mediated polymerase chain reaction
MIN	Microsatellite instability
MMR	Mismatch repair
MRN	RAD50–MRE11–NBS1
NHEJ	Non-homologous end-joining
ORF	Open reading frame
RPA	Replication protein A
ssDNA	Single-strand DNA
SSBs	Single-strand breaks
SSLs	Single-strand lesions

TPRT	Target-primed reverse transcription
WBCs	White blood cells

CHAPTER I

INTRODUCTION

Background and Rationale

Cancer is a disease characterized by a population of cells that grow and divide without respect to normal limits, invade and destroy adjacent tissues, and may spread to distant anatomic sites through a process called metastasis. Most cancers appear to be the result of somatic mutations and some cases are caused by an inherited predisposition (1). The process which normal cells are transformed into cancer cells called carcinogenesis. Global hypomethylation is one of the most common molecular events in the multistep carcinogenesis (2-4). This process leads to activation of oncogenes, reactivation of retrotransposons, and chromosome instability (5). Long interspersed nuclear elements 1 (LINE-1) is the most abundant human retrotransposon. Several studies indicated that there were a high level of expression and hypomethylation of LINE-1 in several cancers (4, 6-9). Interestingly, DNA double-strand breaks (DSBs) are intermediate products of the spontaneous global hypomethylation-related mutations (10-12). In 2003, Vilenchik and Knudson estimated the existence of endogenous DSBs (EDSBs) and concluded that EDSBs could account for a substantial fraction of oncogenic events in human carcinomas (13). If EDSBs do not arise uniformly or are not processed at equal rates across the genome, mutation hot spots should be present(13). This thesis tried to explore the mechanism how LINE-1 hypomethylation involves in cancer genome mutations by evaluating the link with EDSBs.

1. LINE-1 Hypomethylation and Cancer

1.1 LINE-1 and genome

The human genome sequence analysis revealed approximately 45% of 3 X 10^9 bp consists of transposable elements. LINE-1 family is estimated to contain 600,000 copies, accounting approximately 17% of human genome with 3,000 - 5,000 full-lengths. Most LINE-1 elements in human genome have lost the ability to retrotranspose because of 5' truncation, rearrangements and point mutations. Full-length LINE-1s are 6 kb with two open reading frames, ORF1 and ORF2, and a terminal poly-A sequence. ORF1

encodes p40, an RNA-binding protein. ORF2 encodes a protein with endonuclease and reverse transcriptase activities, allowing their mobilization in genomes through an RNA intermediate (14-16). LINE-1s influence genomic structure and function through an insertion, transduction of 3' flanking sequences or recombination mechanisms (17). The mechanism of LINE-1 retrotransposition is similar to the target-primed reverse transcription (TPRT) mechanism established for the insect R2Bm element (18). LINE-1 insertions are derived from a minor subfamily of expressed LINE-1s denoted the Ta (19). The Ta subset is characterized by substitution of ACA for GAG 92-94 bp upstream of the poly A tail (20). Blot hybridization estimates placed the number of full-length Ta elements in the diploid human genome at about 200 copies (20). Sassaman *et al.* isolated a number of full-length Ta elements from a genomic library and found that 50% had two intact ORFs and roughly one half of these, or about one quarter of the total, was retrotranspositionally active in the cell culture assay. This led to an estimate of 30-60 active LINE-1s in the human genome remain retrotransposition competent (RC-L1s) (20).

1.2 Global hypomethylation

DNA methylation involves with the addition of a methyl group to 5' carbon of deoxycytosine in DNA. It is one of epigenetic modification found in DNA that does not alter the coding sequence. DNA methylation is inheritance and involves in regulation of gene transcription. Hypomethylation of regulatory sequences usually correlates with gene expression, while methylation results in transcriptional suppression (21). Hypomethylation and hypermethylation of DNA are often used to describe relative state denote less or more methylation than normal degree of methylation. Global hypomethylation has been used to describe the overall downregulation of methylated CpG dinucleotides, which disperse throughout the whole genome both in noncoding repetitive sequences and genes (22, 23). Genome-wide losses of DNA methylation have been recognized as a common epigenetic event in malignancies and may play crucial roles during cancer development (2-4, 24). Global hypomethylation potentially promotes carcinogenesis via three possible mechanisms: activation of oncogenes, reactivation of transposable elements and chromosomal instability (17).

1.3 LINE-1 hypomethylation

Cytosine methylation of LINE-1 elements is known to play important roles in transcriptional repression of these retrotransposons suggesting that LINE-1 elements with hypomethylated at 5' ends are transcriptionally active (25). Previous studies have described the hypomethylation of LINE-1 elements in several cancers (4, 7-9, 26, 27). One particular study from Chalitchagorn *et al.* evaluated the methylation status of LINE-1 repetitive sequences in genomic DNA derived form microdissected samples from several human normal and neoplastic tissues. They found that most carcinomas including breast, colon, lung, head and neck, bladder, esophagus, liver, prostate, and stomach, revealed a greater percentage of hypomethylation than their normal tissue counterparts. Additionally, DNA derived from sera of patients with carcinoma displayed more LINE-1 hypomethylation than those of noncarcinoma individuals. They also detected significantly greater hypomethylation in carcinoma than those of dysplastic polyp and histological normal colonic epithelium (4). LINE-1 hypomethylation leads to movement of LINE-1 and reintegration at new sites in the genome, leading to insertional mutagenesis and disturbance of transcriptional activity and gene regulation (28-30). Furthermore, expression of LINE-1 also involves in RNA editing process (31).

2. Global Hypomethylation and Genomic Instability

Cancer cells develop through the accumulation of genetic alterations of oncogenes and tumor suppressor genes. Mutations in these genes increase the net reproductive rate of cells and might lead to clonal expansion. It has been proposed that genomic instability can be divided into two type including chromosomal instability (CIN) and microsatellite instability (MIN). CIN phenotype is characterized by the gross rearrangement of chromosomes. Common chromosomal aberrations include the losses and gains of complete chromosomes or chromosomal fragments, and the breakage-fusion-bridge of chromosomal segments (32). MIN is recognized as alterations at the nucleotide levels because of a deletion or insertion of DNA sequences, particularly in repetitive sequences. This instability is most easily detected at short sequences of DNA repeats scattering throughout the genome, called microsatellite (33, 34).

Genome-wide demethylation has been suggested to be a step in carcinogenesis (35). Evidence for this notion come from the frequency observed global hypomethylation in tumor cells (4, 7-9, 26-28, 36), and from a previous study suggesting that defects in DNA methylation might contribute to the genomic instability of some colorectal tumor cell lines (10). DNA hypomethylation has also been associated with abnormal chromosomal structures, as observed in cells form patients with ICF (Immunodeficiency, Centromeric instability and Facial abnormalities) syndrome (37, 38) and in cell treated with the demethylating agent 5-aza-2-deoxycytidine (39). Several studies from mutation experiments also support the evidence that DNA methylation maintains the stability of the genome. The first study showed that murine embryonic stem cells carrying a defect in DNA methyltransferase 1 (*DNMT1)* gene exhibited a significantly increase mutation rates (12). In the second study, mice carrying mutant *DNMT1* allele were shown to develop aggressive T cell lymphomas and their cancer cells displayed chromosomal aberrations (11). Similarity to the second study, mice with highly reduced levels of *DNMT1* expression developed aggressive T cell lymphomas with a high frequency of chromosome 15 trisomy (24). These studies imply an important role of mammalian DNA methylation in maintaining genomic stability.

3. Hypotheses Mechanism

3.1 Genomic instability associated with LINE-1 retrotransposition

This thesis aimed to identify the role of LINE-1 hypomethylation and genomic instability. There are several lines of evidences that expressions of these elements are controlled by DNA methylation (4, 25, 40). Therefore, it is possible that hypomethylation increases LINE-1 transcription leading to retrotransposition as intermediate of mutations. We also look another possibility that LINE-1 retrotransposition is not the genomic instability in cancer. We proposed another hypothesis that endogenous DNA double strand breaks are intermediate of mutations due to global hypomethylation. To prove this hypothesis LINE-1 sequences has been a great tools for EDSB identification. EDSBs occur rarely and scatter throughout human genome. Since we have enormous copies of

LINE-1 interspersed throughout, we modified LINE-1 insertion dimorphisms identification by PCR (LIDSIP) for EDSB identification.

LINE-1 activity, retrotransposition, influences genomic structure and function through an insertion mutagenesis, transduction, rearrangement and deletion of genomic sequence (41, 42). LINE-1 retrotransposition mechanism is TPRT. The mechanism followed by the LINE-1 endonuclease cleaves between A and T on the bottom strand of DNA, exposing 3' hydroxyl group. The target DNA functions as a primer for reverse transcription with the LINE-1 RNA as template. Newly inserted copies may be full-length and potentially active. However, most of them have 5' truncations or 5' truncation with inversions and incapable of further retrotransposition (16). This mechanism can cause genome instability via an insertion of LINE-1 into gene that involved in cell transformation. Sometimes, LINE-1 proteins act *in trans* to mobilize non-LINE-1 RNAs. LINE-1s have likely been responsible for the genome-wide dispersal of processed pseudogenes (roughly 1% of the genome) and of Alu nonautonomous retrotransposons. A processed pseudogene forms can be occurred when a spliced mRNA is reverse transcribed by LINE-1 reverse transcriptase and the resulting DNA is inserted into the genome. Rarely, LINE-1s are able to integrate into preformed DSBs generated independently of the LINE-1 endonuclease (41). For transduction, LINE-1s are able to mobilize both 3' and, less commonly, 5' flaking DNA upon retrotransposition and carry these flanks to new genomic locations. In case of 3' transduction, the weak LINE-1 polyadenylation signal is occasionally bypassed by the RNA 3' processing machinery in favor of a second downstream polyadenylation signal. Sequence 5' of an LINE-1 may be transduced if the LINE-1 promoter initiates transcription at an abnormal upstream site, or if the presence of a non-LINE-1 upstream promoter generates an elongated LINE-1 transcript. Thus, 3' transduction of LINE-1 is a potential mechanism for exon shuffling (43). LINE-1 residing in the intron of a gene might transduce upstream or downstream exonic sequence to a new site, possibly into another gene. LINE-1 insertion also associated with large-scale deletions and inversions resulting to chromosomal rearrangements. The mechanism is still not known but one possible mechanism is that

RNA has provided a template used to patch double-stranded and degraded DNA breaks within cells hosting the LINE-1 insertions (42, 44).

A major form of genetic instability attributed to LINE-1 is direct insertional mutagenesis. LINE-1 has resulted in diseases including muscular dystrophy, haemophilia, colon cancer and breast cancer (16). Previous reports showed, insertions of LINE-1 into exon 14 of the factor VIII gene of patients with haemophilia (45) and insertion of 5' truncated LINE-1 into the 3' end of exon 44 of the dystrophin gene resulted in skipping of the exon during splicing in Duchenne muscular dystrophy (DMD) patient (46). LINE-1 retrotransposition can lead to tumorigenesis from the evidences of LINE-1 insertions into the adenomatous polyposis coli (APC) gene of adenocarcinoma cells from a colon cancer patient and the *c-myc* proto-oncogene in breast cancer, but not in the surrounding normal tissue (47, 48). For LINE-1 mediated transduction, Moran *et al.* demonstrated 3' transduction experimentally in cell culture assay. They placed the retrotransposition marker cassette 3' of the LINE-1 poly A signal, and showed that LINE-1s are able to retrotranspose sequences from their 3' flanks to new genomic sites. Further, LINE-1 could retrotranspose a promoterless marker cassette into a transcribed gene, leading to formation of new fusion proteins (43). From the characterization of new insertion of LINE-1 in vitro, Symer *et al.* showed that from 42 de novo integrants, numerous LINE-1 inversions, extra nucleotide insertions, exon deletions, a chromosomal inversion, and 5' transduction were identified (44). Gilbert et al. showed that new LINE-1 retrotransposition in HeLa cells can result in a variety of large target site alterations of genomic sequence. Large sequence deletion results from the variable position of top strand cleavage in TPRT mechanism (42).

3.2 Genomic instability associated with EDSBs and DNA repair

If global hypomethylation induces genomic instability is not associated with LINE retrotransposition, production or repair of EDSBs may involve with instability. DSBs are type of DNA lesion that both DNA strands of the double helix are damaged. DSBs can be induced by either exogenous or endogenous factors. DSBs occur spontaneously at background levels, are termed EDSBs. The cause of EDSB come from activity of cell

such as free radical from metabolism, single-strand breaks (SSBs) in DNA, collapsed replication and special mechanisms in cell (e.g. V(D)J recombination) (5, 13). DSBs are regarded as one of the particularly dangerous form of DNA damage. DSBs can easily lead to gross chromosomal aberrations if not rejoined quickly (5). EDSBs are particularly dangerous lesions if they occur during the replication of the genome and during the segregation of duplicated chromosomes into daughter cells. The fidelity of EDSBs repair is important to cell fate. The failure to repair EDSBs or an inaccurate repair can lead to CIN that contributes to carcinogenesis (13). Eukaryotic cells repair DSBs by at least two pathways, non-homologous end joining (NHEJ) and homologous recombination (HR) (49). NHEJ joins two DNA ends directly but often prone to error, and small sequence deletions. HR repairs precisely using complementary sister chromatid as a template. Numerous studies employing mouse models and cellular models have demonstrated the correlation between the formation of DSBs and the generation of chromosomal aberrations. The chicken B-cell line DT40 cells without functional Ku70 and/or Rad54 were generated as a cellular model for NHEJ and HR respectively. Disruption of a component of the HR pathway, the *RAD54* gene, caused radiosensitivity, whereas inactivation of a component of the NHEJ pathway, the *Ku70* gene, had no detectable effect on survival after γ-irradiation. Disruption of *RAD54* also increased the rates of chromosomal aberration, mainly in the form of chromatid type breaks. A low dose of γ-irradiation markedly increased the number of breaks in this mutant. The *Ku70* mutation did not significantly affect chromosomal instability in DT40 cells, which suggested that HR is the main pathway to repair DSBs in these cells (50). Other evidence for the involvement of DSBs in chromosomal aberrations came from the studies in mouse model. The DSB-repair-defective mouse mutant was the SCID (severe combined immunodeficiency) mouse, which carried a spontaneous mutation that prevents the production of mature B and T cells, owning to a defect in joining the DSB intermediate in V(D)J recombination. These mice not only had a defect in the development of their immune system, but also were hypersensitive to ionizing radiation (51).

4. Research Methodology

To prove the hypothesis that global hypomethylation induces instability *in trans* by LINE-1 retrotransposition, we developed a new technique to map the LINE-1 insertion sites, called L1-AM-PCR, to compare between cervical cancer and normal cells form the same patients. We choose cervical cancer because of clonal in origin, and a cervical cancer cell line, HeLa cell, shows LINE-1 retrotransposition *in vitro*. To prove the hypothesis that genomic instability involves with EDSBs and global hypomethylation, we developed a novel technique, called L1-EDSB-LMPCR. Locus-specific EDSBs can be detected using ligation-mediated polymerase chain reaction (LMPCR) (52) combined with interspersed repetitive sequence PCR (IRSPCR) (53) using LINE-1 human retrotransposons (L1-EDSB-LMPCR) to measure the EDSB quantity. Therefore, LMPCR Linker oligonucleotides are ligated to EDSBs in high molecular weight DNA preparation and quantitatively analyzed by realtime PCR using an L1 primer and a Taqman probe complementary to the linker. Additionally, methylation status of L1s have been extensively studied in several cancers and normal tissues by PCR combined with bisulfite restriction analysis (COBRA) of L1s (COBRA-L1) (4). Methylation level of EDSBs can be measured by combination of L1-EDSB-LMPCR and COBRA-L1 through the treatment of linker-ligated DNA with bisulfite prior to PCR with L1 and linker primers and restriction analysis (COBRA-L1-EDSB).

From our study results, LINE-1 insertion involved with carcinogenesis can not be observed, this may result from the clonal expansion during cancer development. Then, we study the EDSBs in associated with global hypomethylation and genomic instability. We found that most of EDSBs were methylated. Hypermethylated EDSBs may arise from two possibility, either cells have a higher EDSB production rate in methylated sequences or a lower repairing rate in methylated EDSBs. To characterize these hypotheses, we first examined EDSB production rate. We hypothesized that EDSBs were preferentially produced in S phase from the conversion of single-stranded lesions. To observe EDSBs repairing rate, we inhibited proteins involve in DNA repair pathway. DNA-PKcs and ATM,

components of HR and NHEJ, respectively, were chemically or genetically inhibited then examined methylation of EDSBs.

Research Questions

1. Is there any LINE-1 movements involve in cervical carcinogenesis?
2. Is there any significant amount of EDSBs exist and associated with DNA methylation?
3. How EDSBs are in association with methylation?
4. Is the association due to the generation or repair of EDSBs?

Objectives

1. To establish techniques for mapping the location of LINE-1 elements between cervical cancers and normal tissues.
2. To compare the location of LINE-1 element between cervical cancers and normal tissues.
3. To establish techniques for detecting the quantity, methylation level of EDSBs.
4. To compare percentage of methylation level between EDSBs and genomic DNA.
5. To detect the amount and methylation status of EDSBs which are generated differently during cell cycle.
6. To prove whether DNA repair pathways involve with the methylation statuses of EDSBs.

Hypotheses

1. Global hypomethylation induces instability *in trans* by LINE-1 retrotransposition.

2. Genomic instability is related to DNA methylation *in cis* and relate to how EDSBs are occurred or repaired.

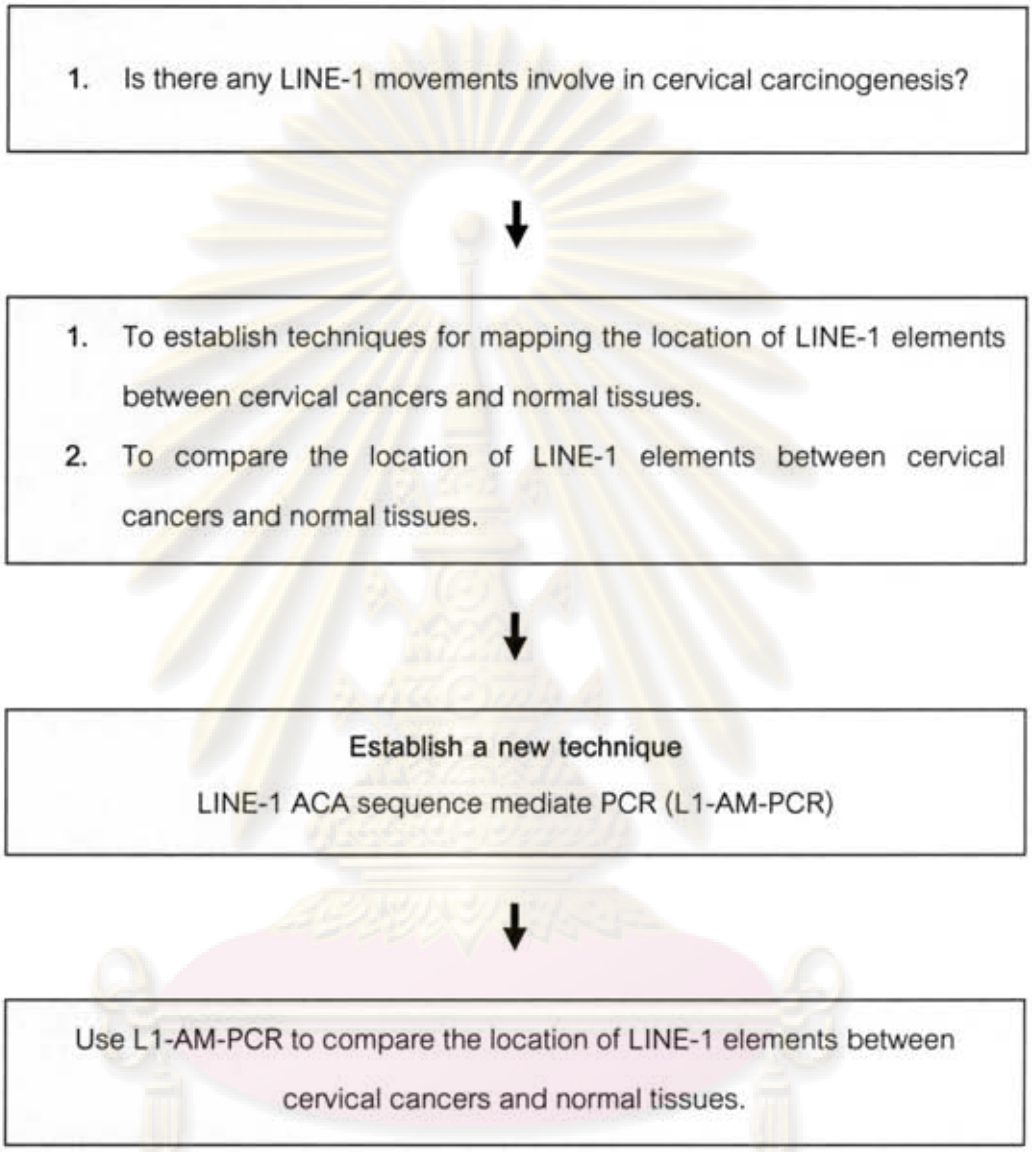
Key Words

LINE-1, Cervical cancer, Global hypomethylation, Endogenous DNA double-stranded breaks, Genomic instability, DNA methylation, Mutations

Expected Benefit

The results of this study will help us to understand the mechanisms of mutations in cancer.

Conceptual Framework



2. Is there any significant amount of EDSBs exist and associated with DNA methylation?

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3. To establish techniques for detecting the quantity, methylation level of EDSBs.
4. To compare percentage of methylation level between EDSBs and genomic DNA.

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Establish new techniques

- **L1-EDSB-LMPCR**
  To measure the quantity of EDSBs in cell.
- **COBRA-L1**
  To measure the level of DNA methylation in cell.
- **COBRA-L1-EDSB**
  To measure the unmethylated and methylated EDSBs in cell.

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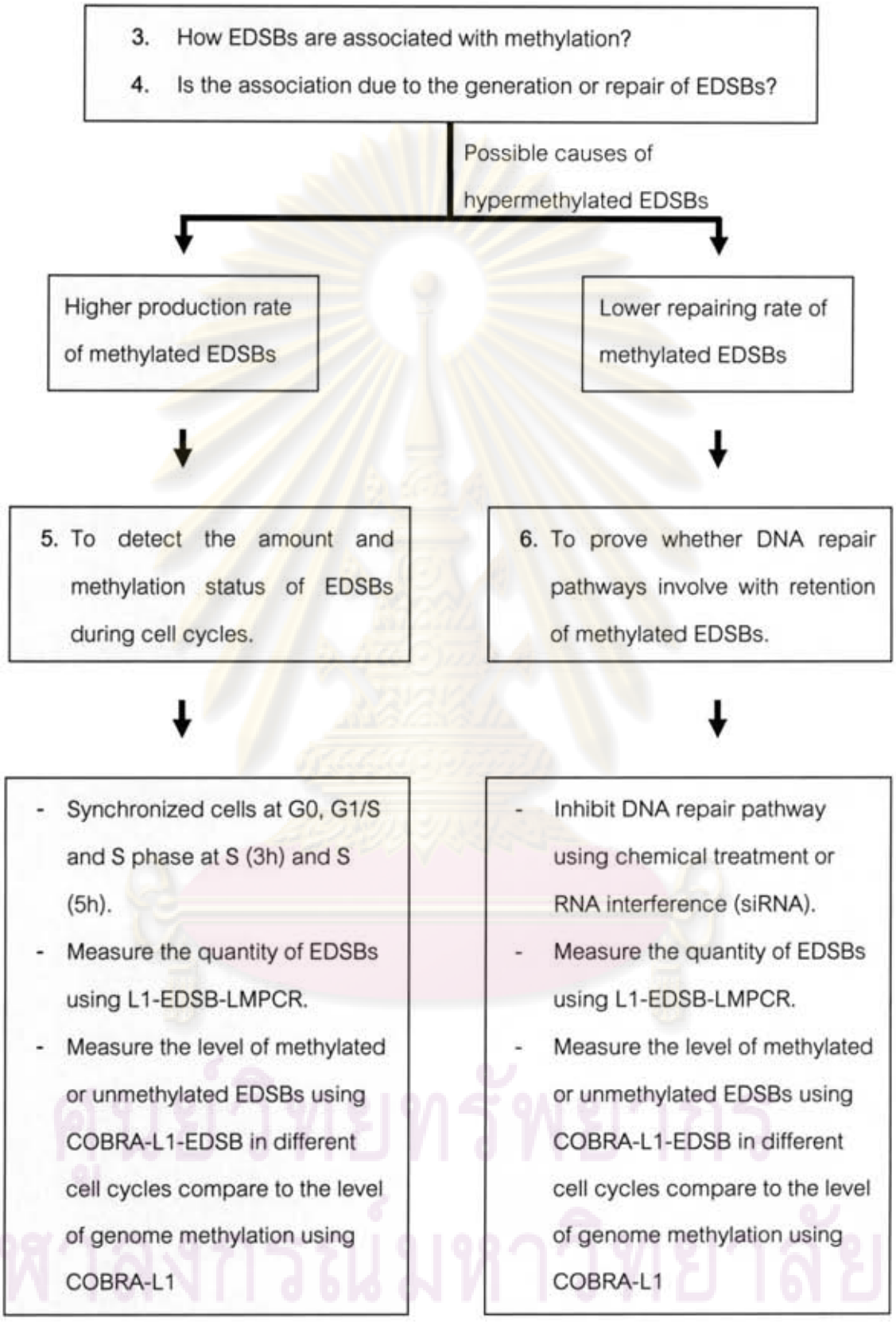
Use new techniques to detect the quantity, methylation level of EDSBs and compare percentage of methylation level between EDSBs and genomic DNA


3. How EDSBs are associated with methylation?
4. Is the association due to the generation or repair of EDSBs?
Possible causes of hypermethylated EDSBs
Higher production rate of methylated EDSBs
Lower repairing rate of methylated EDSBs
5. To detect the amount and methylation status of EDSBs during cell cycles.
6. To prove whether DNA repair pathways involve with retention of methylated EDSBs.
- Synchronized cells at G0, G1/S and S phase at S (3h) and S (5h).
- Measure the quantity of EDSBs using L1-EDSB-LMPCR.
- Measure the level of methylated or unmethylated EDSBs using COBRA-L1-EDSB in different cell cycles compare to the level of genome methylation using COBRA-L1
- Inhibit DNA repair pathway using chemical treatment or RNA interference (siRNA).
- Measure the quantity of EDSBs using L1-EDSB-LMPCR.
- Measure the level of methylated or unmethylated EDSBs using COBRA-L1-EDSB in different cell cycles compare to the level of genome methylation using COBRA-L1

CHAPTER II
REVIEW OF RELATED LITERATURE

CARCINOGENESIS

Cancer cells have a unique characteristics differ from normal cells. Normal cells have a number of important characteristics. They can reproduce themselves exactly, stop reproducing at the right time, stick together in the right place, self destruct if they are damages, and become specialized or mature. Cancer cells are different to normal cells in several ways. They acquired capability of limitless replicative potential, self-sufficiency in growth signals, insensitivity to anti-growth signals, evading programmed cell death, sustained angiogenesis, and tissue invasion and metastasis (54). In order to acquire these properties, normal cells are transformed into cancer cells by a process called carcinogenesis which is multistep and complicated process. Almost all cancers are caused by abnormalities in the genetic material of normal cells. These abnormalities may come from chemicals, infectious agents, radiation or spontaneous mutations. New aspects of genetics of cancer pathogenesis such as DNA methylation and microRNAs are increasingly being recognized as important factors in carcinogenesis (2, 10-12, 24, 27, 29, 30, 40, 55, 56).

Carcinogens

The mutation in genes involving in carcinogenesis can be caused by many factors. Carcinogens are substances that can cause changes and lead to cancer. Carcinogens do not cause cancer in every case all the time. Substances classified as carcinogens may have different levels of cancer-causing potential. The risk of cancer development depends on many factors, including the length and intensity of exposure to the carcinogen and genetics background or each person. International Agency for Research on Cancer (IARC) which is part of the World Health Organization (WHO) classify carcinogens in to the following categories (57).

Group 1: Carcinogenic to humans. e.g. Arsenic, gamma radiation, asbestos, Benzene, Epstein-Barr virus, Human papilloma virus types 16, 18, Hepatitis B virus, Coal-tars, Salted fish etc.

Group 2A: Probably carcinogenic to humans. e.g. Acrylamide, Etoposide, Diesel engine exhaust, Kaposi's sarcoma herpes virus etc.

Group 2B: Possibly carcinogenic to humans. e.g. Hexachlorobenzene, Lead, Naphthalene, Pickled vegetables etc.

Group 3: Unclassifiable as to carcinogenicity in humans. e.g. Diazepam, Eugenol, Hepatitis D virus, Nitrotoluenes, Xylenes etc.

Group 4: Probably not carcinogenic to humans. e.g. Carprolactam.

The first carcinogen reported from Percivall Pott observed a high rate of skin cancer of the scrotum among chimney sweeps in England 1775. In 1917, Yamagiwa K. and Ichikawa K. were able to induce skin cancer in rabbits by repeated application a solution of coal tar (58). Payton Rous was the first who gave the idea that carcinogenesis involved a two-step process: initiation and promotion. At present, carcinogenesis can be divided into four steps: tumor initiation, tumor promotion, malignant conversion, and tumor progression (Figure 1).The concept of tumor initiation indicated that the initial changes and irreversible damage in genetic material. For mutation to accumulate, they must arise in cells that proliferate and survive the lifetime of the organism. A chemical carcinogen causes a genetic error by modifying the molecular structure of DNA that can lead to a mutation during DNA synthesis. Carcinogen-DNA adduct formation is central to theories of chemical carcinogenesis, and it may be a necessary, but not a sufficient, prerequisite for tumor initiation (59).

Multistage Carcinogenesis

At present, carcinogenesis can be divided into four steps: tumor initiation, tumor promotion, malignant conversion, and tumor progression (Figure 1).The concept of

tumor initiation indicated that the initial changes and irreversible damage in genetic material. For mutation to accumulate, they must arise in cells that proliferate and survival the lifetime of the organism. A chemical carcinogen causes a genetic error by modifying the molecular structure of DNA that can lead to a mutation during DNA synthesis. Carcinogen-DNA adduct formation is central to theories of chemical carcinogenesis, and it may be a necessary, but not a sufficient, prerequisite for tumor initiation (35, 59). Tumor promotion includes the selective clonal expansion of initiated cells (Figure 2). Tumor promoters are generally nonmutagenic, are not carcinogenic alone and often are able to mediate their biologic effects without metabolic activation. Chemical or agents capable of both tumor initiation and promotion are known as complete carcinogens, e.g. benzo[α]pyrene and 4-aminobiphenyl. Chemicals, complex mixtures of chemicals, or other agents that have been shown to have tumor-promoting properties include dioxin, phenol, saccharin, ultraviolet light, estrogens and other hormones, dichlorodiphenyl trichloroethane (DDT), wounding, and other chronic irritation (60). Malignant Conversion is the transformation of a preneoplastic cell into the cell with malignant phenotype. This process requires further genetic changes. If tumor promoter is discontinued before malignant conversion has occurred, premalignant or benign lesions may regress. The low chances of malignant conversion can be increased substantially by the exposure of preneoplastic cell to DNA-damaging agents (60). Tumor progression comprises the expression of the malignant phenotype and the tendency of malignant cells to acquire more aggressive characteristics. An obvious characteristic of the malignant phenotype is the tendency for genomic instability and uncontrolled growth(32). Viral carcinogenesis is a process by which normal cells are transformed into cancer cell by action of virus. Rous sarcoma virus (RSV) is the first virus being know that can cause cancer. RSV contains a gene called *v-src*, it was found that the *v-src* gene is required for the formation of cancer (61). Some Human papilloma virus (HPV) is associated with cervical cancer. The HPV *E6* and *E7* genes prevent cell-cycle arrest, the cell cycle division by interact with host cell proteins, *p53* and *pRb* respectively. Another well known virus causing cancer is hepatitis B virus (HBV) that associated with hepatocellular carcinoma (HCC). One possible mechanism of HBV infection leads to HCC is an integration of HBV

DNA to host genome may release the growth control for hepatocytes by coding for a factor like X-protein, which activates protooncogenes, or silences tumor-suppressor genes. An insertion of HBV DNA sequences can also activate and influence the transcription of cellular genes by causing chronic inflammation with cell death and hepatocytes regeneration and with fibrosis and by activation of immune system to secrete cytokines at the wrong time and place (59). Radiation exposure leads to enhancement in the rate of genetic changes after many generations of DNA replications. DNA structural analyses show that most of radiation-induced mutations in human cell result form large-scale genetic events involving loss of entire active gene and often extending to other loci on the same chromosome (62). Radiation may disrupt a regulation of cellular processes, leading to a state that disturbs the normal regulatory and signaling pathways so that disrupting cellular homeostasis (63). The balance between cell proliferation and programmed cell death is maintained by tightly regulating both processes to ensure the integrity of organs and tissues, termed homeostasis. Cell proliferation was considered to be important, especially as an integral part of the process of converting DNA adducts to mutation, an enhancing factor for the mutation frequency by inducing errors in DNA replication, and an important factor in determining dose-response relationships for some carcinogens (64). Programmed cell death prevents the cells from proliferation after initiated by carcinogens. These initiated cells are eliminated from the tissue population without increasing the risk of tumor formation (65).

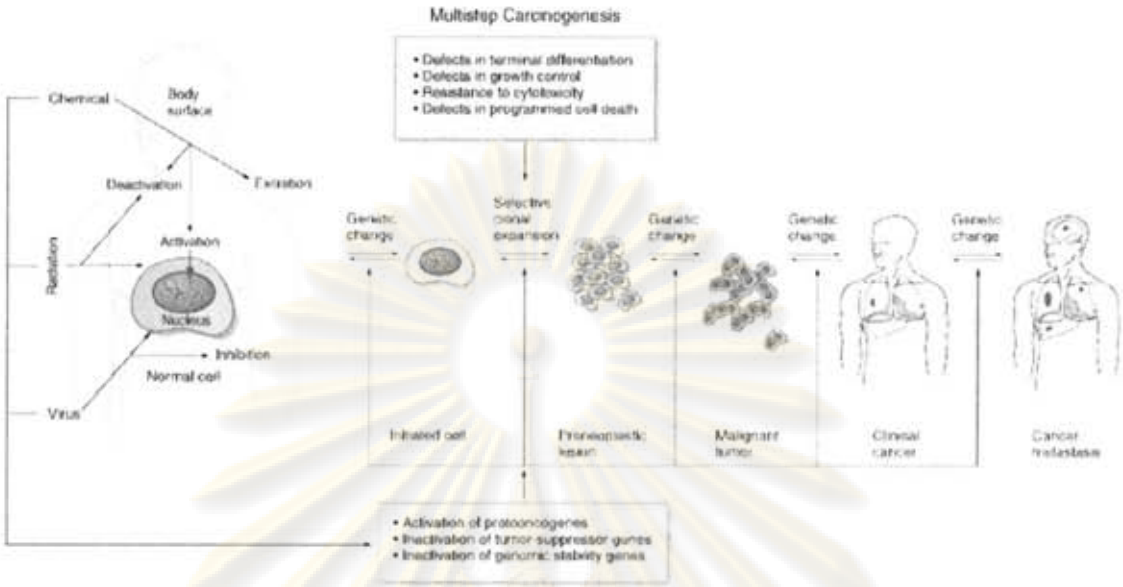


Figure 1. **Multistage Carcinogenesis.** The activation of protooncogenes and inactivation of tumor suppressor genes are mutational evens that result from carcinogens. The accumulation of mutations, and not necessarily the order in which they occur, constitutes multistage carcinogenesis (59).

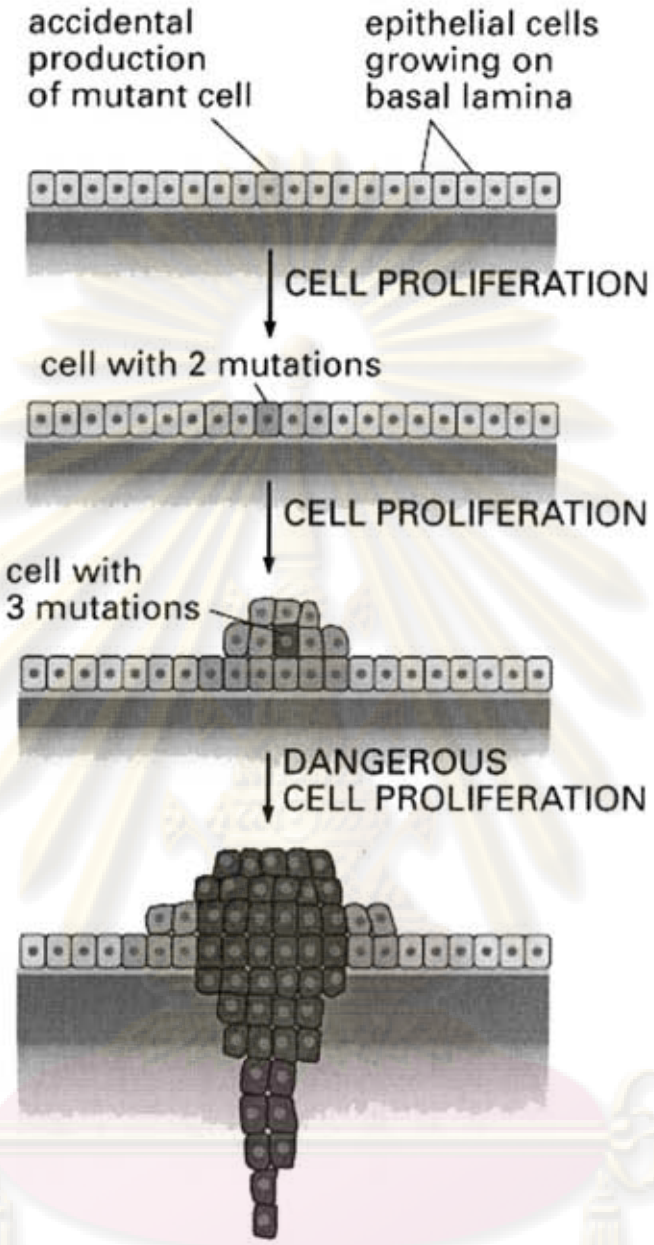


Figure 2. **Clonal Evolution.** A tumor develops through repeated rounds of mutation and proliferation, giving rise eventually to a clone of fully malignant cancer cells. At each step, a single cell undergoes a mutation that enhances cell proliferation, so that its progeny become the dominant clone in the tumor. Proliferation of this clone then hastens occurrence of the next step of tumor progression by increasing the size of the cell population at risk of undergoing an additional mutation (49).

Mutation in Protooncogenes and Tumor Suppressor Genes

There are different types of genes, oncogenes, tumor suppressor genes and DNA damage repair genes that are important in carcinogenesis. There are many evidences show that loss of tumor suppressor genes or gain of oncogenes can lead to cancer (66). Oncogenes are genes involving promote cell growth and cell division which usually called protooncogenes in normal cells. After activation, protooncogenes can become oncogenes by a relatively small modification of its original function. There are at least three mechanisms which protooncogenes can become oncogenes via deletion or point mutation in coding sequence, gene amplification and chromosomal rearrangement. This may include retroviral transduction or retroviral integration, and protein-protein interactions (49, 61). The activation caused an increase in protein activity, expression, stability, and quantity. Some protein that normally express in some cell can be oncogenes in other cell. Protooncogenes can be classified into 7 groups.

1. Growth Factors. e.g. *c-sis* that encodes the PDGF B chain growth factor, *int-2* that encodes an FGF-related growth factor.

2. Receptor Tyrosine Kinases. e.g. c-kit that encodes the mast cell growth factor receptor.

3. Membrane Associated Non-Receptor Tyrosine Kinases. e.g. *c-src* that encodes protein tyrosine kinase.

4. G-protein Coupled Receptors. e.g. *mas* that encodes angiotensin receptor.

5. Membrane Associated G-Proteins. e.g. *Ras*

6. Serine/Threonine Kinases. e.g. *raf* that involved in the signaling of receptor tyrosine kinases pathway.

7. Nuclear DNA-Binding/Transcription Factors. e.g. *c-myc* that encodes protein causing retroviral integration and transduction as well as chromosomal rearrangements.

For example, the Neu gene was identified as an EGF receptor-related gene in an ethylnitrosourea-induced neuroblastoma. The conversion of proto-oncogenic to oncogenic Neu requires only a single amino acid change in the transmembrane domain (67). Tumor suppressor genes are gene that reduce or repress effect on the regulation of the cell cycle or promote apoptosis, and sometimes do both. The functions of tumor suppressor proteins can be classified into several categories (68). a) Repression of genes that is essential for the continuing of the cell cycle. If these genes are not expressed, the cell cycle will not continue, effectively inhibiting cell division. b) Coupling the cell cycle to DNA damage. If there is damaged DNA in the cell, it will not divide. If the damage can be repaired, the cell cycle can continue. c) If the damage can not be repaired, the cell will initiate programmed cell death. d) Some proteins involved in cell adhesion prevent cancer cells from dispersing, block loss of contact inhibition, and inhibit metastasis (69). Unlike oncogenes, tumor suppressor gene need the "two hit hypothesis" which means both alleles of a particular gene must damage. Tumor suppressor genes are usually not haploinsufficient, although there are an exception e.g. *p53*. If only one allele of the gene is damaged, the second allele can still produce the correct protein. The first discovered tumor suppressor protein is the *pRb* protein in human retinoblastoma (70). The activation of a protooncogene or the inactivation of a tumor suppressor gene can be categorized as a tumor-initiating event. In summary, cancer is caused by both external (chemical, radiation, and viruses) and internal (hormones, immune conditions, and inherited mutations) factors. These factors may act together, or in sequence, to initiate or promote carcinogenesis.

LONG INTERSPERSED NUCLEAR ELEMENTS (LINE-1s)

Almost all of the interspersed repetitive noncoding DNA in the human genome is derived from transposable elements (also called transposons). Transposable elements are repetitive mobile sequences that can disperse throughout the genome. Transposable elements are divided into two main classes.

1. Those, which transpose directly through DNA intermediate, are generally referred to transposons (this term has a more specific meaning in reference to bacterial mobile elements) e.g. Ac element of maize and the P element of *Drosophila*.

2. Those, which transpose via RNA intermediate transcribed from the mobile element by RNA polymerase and then converted back into double-stranded DNA by a reverse transcriptase, are the retrotransposons or retroposons.

These in turn can be divided into two major categories.

a) LTR (Long Terminal Repeat) retrotransposons: are analogous to retroviruses, except that they cannot move from cell to cell e.g. Ty elements in yeast.

b) Non-LTR retrotransposons: are the most abundant mobile elements in mammal e.g. LINE family in human.

Transposable elements can also classify according to their degree of mechanistic self-sufficiency. Autonomous transposable elements, such as LINEs, are thought to encode essentially all of the machinery that are required for their movement. In contrast, the movement of non-autonomous transposable elements, such as short interspersed nuclear elements (SINEs), entirely depends on other transposons. In some cases, the distinction between autonomous and non-autonomous element becomes blurred. For example, it is unknown whether human endogenous retroviruses (HERVs), if mobilized, require the help of other element or viruses in *trans*. Even autonomous elements probably rely on the cellular machinery of their hosts. Transposable elements

comprise a surprisingly large fraction of eukaryotic genomes. For example, it was estimate that around 36% of the human genome composed of transposable elements of one sort or another, though most of them are defective of the full-length elements (17, 61, 71).

Human has viral retrotransposons that lack LTRs, know as LINE elements. LINE was first identified as a class intermediate repeats DNA dispersed throughout the genome of various mammals, including humans. Subsequently, LINE-like elements have been found in a wide range of other species. The major LINE element in humans is called LINE-1 retrotransposon. LINE-1 family is estimated to contain 600,000 copies, accounting for at least 15 % of the human genomic DNA, only about 3,000 – 5,000 represent full-length elements (72, 73). The majority of LINE-1 elements within the human genome have lost the ability to retrotranspose because of 5' truncation, rearrangements or point mutations. Some full-length LINE-1s retain the ability to retrotranspose. The general structure of LINE-1 elements, based on a consensus direct repeats, the hallmark of mobile elements. A full-length (~6.1 kilobase, kb) LINE-1 element consists of a 5' untranslated region (5'-UTR) with internal promoter activity. The consensus sequence contains two open reading frames (ORF1 and ORF2) that are separated by an intergenic spacer; followed by a 3'-UTR that ends in an AATAAA polyadenylation signal and a poly A tail (Figure 3). ORF1 is ~1 kb in length and has homology with viral gag sequences. ORF1 encodes a 40 kDa RNA-binding protein. ORF2 is ~4 kb in length encodes a ~150 kDa protein, ORF2p, with conserved endonuclease (EN) and reverse transcriptase (RT) that is similar in sequence to the reverse transcriptases of retroviruses and viral retrotransposons, an endonuclease domain and a cysteine-rich region (16, 17, 61, 74-77).

LINE-1 elements are reverse transcribed and integrated into the genome by a coupled reverse transcription/integration process called target-primed reverse transcription (TPRT) (Figure 4). The LINE-1 RT sequence and TPRT mechanism are similar to those of group II introns found in bacteria, chloroplasts and mitochondria (78-81). It has also been suggested that the TPRT mechanism of non-LTR retrotransposons

may have originated with telomerase (82). After transcription of LINE-1, TPRT mechanisms initiate with endonuclease of LINE-1 cleaves between an A and a T on the bottom strand of target DNA. The 3' hydroxy group of target DNA exposes and functions as a primer for its complementary LINE-1 RNA to be a template for the reverse transcription reaction. Reverse transcription of the RNA is followed by cleavage of the coding strand from LINE-1 endonuclease and then integrate by repairing the gap between LINE-1 cDNA and host chromosomal DNA leaving target-site duplication which flank the new inserted LINE-1 (17). The majority of LINE-1 insertions in vivo are truncated at the 5' end such that the average insertion length is only about 1 kb (73).

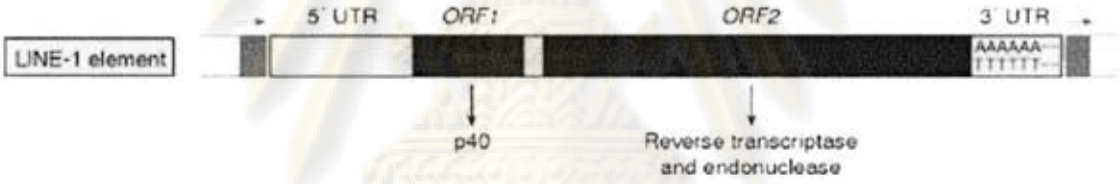


Figure 3. **Structure of Full-Length LINE-1 Repeats.** The consensus full-length LINE-1 element is 6.1 kb long but most LINE-1 elements are truncated and the average sized is very much smaller. (ORF1, ORE2, open reading frames 1 and 2) (83).

Figure 4. **Target-Primed Reverse Transcription (TPRT) Mechanism.** The mechanism of LINE-1 movement starting with endonuclease of LINE-1 cleave bottom strand of host DNA. LINE-1 RNA binds to 3' of host DNA and reverse transcribe for LINE-1 cDNA. The second cleavage occur then repair or filling the gap. (TSD, target-site duplication) (17).

LINE-1s also have the ability to transduce non-LINE-1 DNA flanking their 3' ends to new genomic location. The first evidence of 3' transduction comes from Holmes *et al.* experiment, showing the importance of a 3' transduction event seen in an LINE-1 insertion into the human dystrophin gene on chromosome 1q of a muscular dystrophy patient (84). Secondly, a novel feature of LINE-1 propagation and function was described recently by Moran *et al.* 1999, who showed that LINE-1s were able to retrotranspose sequences from their 3' flanks to new genomic locations in a cultured cell including HeLa cells, and can be driven by either the cytomegalovirus promoter or the native LINE-1 promoter. Transposition efficiency is higher when a strong polyadenylation signal is introduced downstream form the LINE-1. This indicates that exons downstream of active LINE-1s could be shuffled into new sites to create new genes (43). Finally, 3' transduction by LINE-1s has been analyzed by LINE-1 sequences present in the GenBank database and found that among relatively recent LINE-1 retrotransposition events, transduction of 3'-flanking sequence was a common occurrence, 15-23 % of human LINE-1 insertion contain 3' transduced sequences that accounted for 0.5-1.0 % of the human genome (85, 86). These data indicated that active LINE-1 elements exist in the human genome and were capable of retrotransposition activity.

LINE-1 and Human Diseases

The majority of LINE-1 movements insert in intergenic and intronic sequences with little or no effect to host. Occasionally LINE-1 insertions can cause human disease by several mechanisms including promoting unequal homologous recombination, direct LINE-1 insertion into genes, and providing the machinery for insertion other retrotransposons into genes. Mispairing of repeated sequences caused unequal homologous recombination. This mechanism was commonly found in Alu elements not in LINE-1 (87). There are reports of diseases caused by LINE-1 mispairing and unequal crossing over. Unequal homologous recombination between two LINE-1 elements flanking exon 8 of the phosphorylase kinase β subunit gene (*PHKB*), causing the deletion of a sequence of 7,574 nucleotides including exon 8 and giving rise to phosphorylase kinase glycogen storage disease. This is the first example of a mutation

due to unequal homologous recombination between LINE-1 elements (88). Other disease is Alport syndrome and associated diffuse leiomyomatosis, causing by unequal homologous recombination between two LINE-1 in intron 2 of *COL4A6* and resulting in a >40 kb deletion (89). There are several genetic disorders caused by direct insertion of LINE-1 into gene, including hemophilia A, Duchenne muscular dystrophy, type 2 retinitis pigmentosa, thalassaemia and chronic granulomatous disease (45, 46, 84, 90-93). Because of the density of LINE-1 retrotransposons on the X chromosome is approximately twice as those on autosomes (26% in X chromosome and 13% in autosomes), then the insertion of LINE-1 that involved in these genetic disorders are X-chromosome linked (71, 94). Interestingly, nearly all of human LINE-1 insertions come from a subset of human LINE-1 called the Ta subset. This LINE-1 Ta subset is distinguished by substitution of ACA for GAG 92-94 bp upstream of the poly A tail (14, 20). The study from blot hybridization estimates the number of full-length Ta elements in human genome at about 200 copies and 30 – 60 still retrotranspositionally active (20).

LINE-1 and Cancers

The direct evidences of LINE-1 retrotransposition involving cancer development came from the insertions of LINE-1 into *c-myc* protooncogene in breast cancer and *APC* disruption in adenocarcinoma cells from a colon cancer patient (47, 48). Gilbert et al. performed LINE-1 retrotransposition in transformed human cells and showed that substantial deletions, a genome instability, is associated with about 10% of LINE-1 insertions (42). Symer et al. demonstrated small target site deletions along with two types of gross chromosomal rearrangements at LINE-1 target site (large deletions and a chromosome inversion) (44). Bratthauer et al. raised the possibility that a proteins from LINE-1 expression may function as oncoproteins in some cancer (95). The experiment in urothelial carcinomas showed that hypomethylation of LINE-1 sequences was highly prevalent but not in normal tissues. Thus it was speculated that decreased methylation of LINE-1 elements, in particular, contributed to genomic instability in specific human tumors by rendering normal repressed sequences competent for transcription and recombination (8). Active retrotransposition of LINE-1 can lead to inactivation of tumor

suppressor genes or to activation of oncogenes when an LINE-1 was inserted next to an oncogenes and the promoter of the LINE-1 enhanced the expression of the oncogenes (27). LINE-1 elements, which are hypomethylated in numerous cell lines, and that elements that are hypomethylated in malignant germ cells, comprise a different subset of elements from those that are hypomethylated in non-germ cell malignancies (96). Furthermore, the hypomethylation could also lead to increased incidence of retrotransposition and resultant genomic instability in hepatocellular carcinomas (95).

GENOMIC INSTABILITY AND CARCINOGENESIS

In multistage carcinogenesis, cells that are transformed to become malignant contain mutations in cellular oncogenes and tumor suppressor genes, as well as chromosomal abnormalities. Genomic instability appears early in carcinogenesis and is believed to play a critical role in the malignant process. Many somatic changes accumulate in the genome during tumor progression (1, 32, 97). Cancer cells exhibit genomic instability either at sequence level, called microsatellite instability (MIN), or at chromosomal level, called chromosomal instability (CIN), but not generally at both levels. It seems that one form of instability is sufficient to drive carcinogenesis because both instabilities are rarely found together in tumors (32).

Microsatellite Instability and Carcinogenesis

Microsatellites are repetitive DNA sequences dispersed throughout the human genome. The most common microsatellite in humans is a dinucleotide repeat of cytosine and adenine $(CA)_n$. Somatic alteration in microsatellite sequences due the loss or gain of one or more repeat units is termed MIN (32, 33). MIN is a direct consequence of defects in nucleotide mismatch repair (MMR). The MMR machinery removes misincorporated nucleotides from the DNA molecule and has been shown to be involved in genomic instability. The MIN is found in subsets of several cancer types, and germ-line defects in MMR underlie a cancer syndrome(34). The first clue to the role of MMR cancer came with the discovery of a group of sporadic (non-familial) colorectal cancers that exhibited widespread alterations of poly(A) tracts in their genomes. MIN occurs in most cancer in

patients with hereditary nonpolyposis colon cancer (HNPCC) (98). Studies of HNPCCs revealed mutation in MMR genes such as *MSH1* and *MLH2*, which encode proteins that repair nucleotide mismatches (99, 100). Moreover, targeted mutations in mouse homologues cause a spectrum of MIN and cancer susceptibility phenotypes. *Msh2*$^{-/-}$ and *Mlh1*$^{-/-}$ mice have high levels of MIN in all somatic tissues tested and highly penetrant colon cancer susceptibility (101). MIN has been observed in bacteria with defects in the MMR genes *mutS* or *mutL*, and showed that *Saccharomyces cerevisiae* with defects in the yeast homologues of either *mutS* or *mutL* exhibited a similar MIN phenotype. Six human *mutS* or *mutL* homologues genes are known that, when recessively inactivated lead to a MIN phenotype in cancer patients (102).

Chromosomal Instability and carcinogenesis

CIN cancers are usually aneuploid and exhibit increased rates of chromosomal changes but have normal point mutation rates. Common chromosomal aberrations include the loss or gain of whole chromosomes or chromosome fragments, and the amplification of chromosome segments. Loss of large regions of a chromosome can lead to the inactivation of tumor suppressor gene (for example, by loss of heterozygosity), whereas amplification of chromosomal regions might promote carcinogenesis by the activation of protooncogenes. Cancer cells that do not show MIN usually have CIN. Genes that can lead to CIN when mutated including those involved in chromosome condensation, sister-chromatid cohesion, functioning of the centromere and kinetochore, or checkpoints such as a spindle checkpoint that prevents chromatids separating until the chromosome is correctly aligned on the spindle (32). A large number of genetic alterations can trigger CIN in yeast, but only a few genes that cause CIN have been identified in humans. For examples, genes involved in the DNA-damage checkpoint have been implicated in human tumorigenesis, including *ATM*, the *ATM*-related gene (*ATR*), the *BRCA1* and *BRCA2* genes, which interact with the human *RAD51* homologues, and *p53* (103). Murine lymphocytes carrying homozygous mutation of *Brca2*, frequently developed multiple cytogenetic lesion including chromosomal fragmentation, multi-radial formations and random translocations. *MAD2*$^{+/-}$ cells showed

premature entry into anaphase and increased percentage of chromosome loss. Mutations in the kinetochore binding proteins MAS, BUB, and the securins, key components of large multi-protein cascade known as the anaphase-promoting complex (APC) in eukaryotes, increased chromosomal instability by different mechanisms (104, 105).

Translocations can directly activate oncogenes by different mechanisms. First, oncogenes can be activated by transposition to an active chromatin domain resulting in increased expression of translocated gene. Activation of the *c-myc* protooncogene is a central event of Burkitt's lymphoma (BL). A characteristic chromosomal translocation, t(8;14)(q24;q32) is seen in 75-85% of patients. The reciprocal translocation puts *c-myc* close to an immunoglobulin locus, *IGH*, on chromosome 14. This translocation results in potent transcriptional activation of *c-myc* in antibody-producing B-cells, leading to overexpression and cellular transformation (106, 107). Second, Translocation can result in the creation of a fusion gene that encodes a novel oncogenic protein. The best known tumor specific rearrangement produces the Philadelphia (Ph) chromosome, a very small acrocentric chromosome, associated with chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (ALL). This Ph chromosome turns out to be produced by a balanced reciprocal 9;22 translocation. The breakpoint on chromosome 9 is within and intron of the *ABL* protooncogene. The translocation joins most of the *ABL* genomic sequence onto a gene called *BCR* (breakpoint cluster region) on chromosome 22, creating a novel BCR–ABL fusion protein with non-receptor tyrosine kinase hyperactivity that functions in the RAS-signaling pathway which drives cellular proliferation (108). Besides translocation, Amplification of proto-oncogenes is another common means of oncogene activation in numerous tumor types. Gene amplification is generally defined as an increase in the cellular copy number of a gene or genomic region relative to the rest of the genome. However, the mechanisms leading to amplification within a tumor have remained obscure (109). Breast cancers often amplify *ERBB2* and sometimes *MYC*, a related gene *n-myc* is usually amplified in late-stage neuroblastomas (110). In addition to translocations and gene amplifications, cancer cells may also have chromosomal deletions. Deletions can potentially result in oncogene activation by

removal of negative regulatory elements or elimination of regulatory protein domains. It is more likely that deletion will cause the loss of a tumor suppressor. Massive chromosomal deletion is frequently seen in myelodysplastic syndrome (MDS), a precursor of acute myeloid leukemia (AML), frequently affecting in chromosomes 5, 7 and 20. One candidate tumor suppressor is the ETF1 translation factor on chromosome 5, while candidate genes on chromosomes 7 and 20 remain to be elucidated (111, 112). Another region commonly deleted in human is 13q14 deletion at this position have been observed in B-cell chronic lymphocytic leukemia (B-CLL) (113), non-Hodgkin's lymphoma (NHL), and ALL (114).

DNA METHYLATION

DNA methylation is a change in DNA by chemical modification, involving the addition of a methyl group to the carbon-5 position of cytosine in a CpG dinucleotide in human which disperse throughout the whole genomes both in noncoding repetitive sequences and genes (Figure 5). DNA methylation does not change in DNA coding sequence because both cytosine and 5-methylcytosine base pair with guanine. DNA methylation is associated with condensation of chromatin, stabilization of chromosomes, transcriptional silencing of X chromosome, genomic imprinting and tissue-specific silencing of gene expression (21, 115). The Enzymes responsible for the process of DNA methylation are DNA methyltransferases (DNMTs) that can be divided in to maintenance and *de novo* DNMTs. DNMT1 is the maintenance methyltransferase responsible for reproducing the parental DNA methylation pattern into daughter cells during DNA replication. The newly synthesized DNA contains hemimethylated sites that provide the signal for DNMT1 to transfer a methyl group to a cytosine residue from its cofactor, S-adenosylmethionine (SAM). If maintenance methylation does not occur either by a decrease in capacity or fidelity of DNMT1 activity and/or decreased levels of SAM, the daughter DNA will lose a pattern of DNA methylation. DNMT3A and DNMT3B are *de novo* methyltransferases responsible for adding methyl groups to CpG dinucleotide of unmethylated DNA (Figure 6) (115). Both enzymes in *de novo* methylation are necessary for proper development of mammalian embryos by establishing new methylation

patterns, especially DNMT3B for methylation of specific genomic regions such as pericentromeric repetitive sequences and CpG islands on the inactive X chromosome (116, 117).

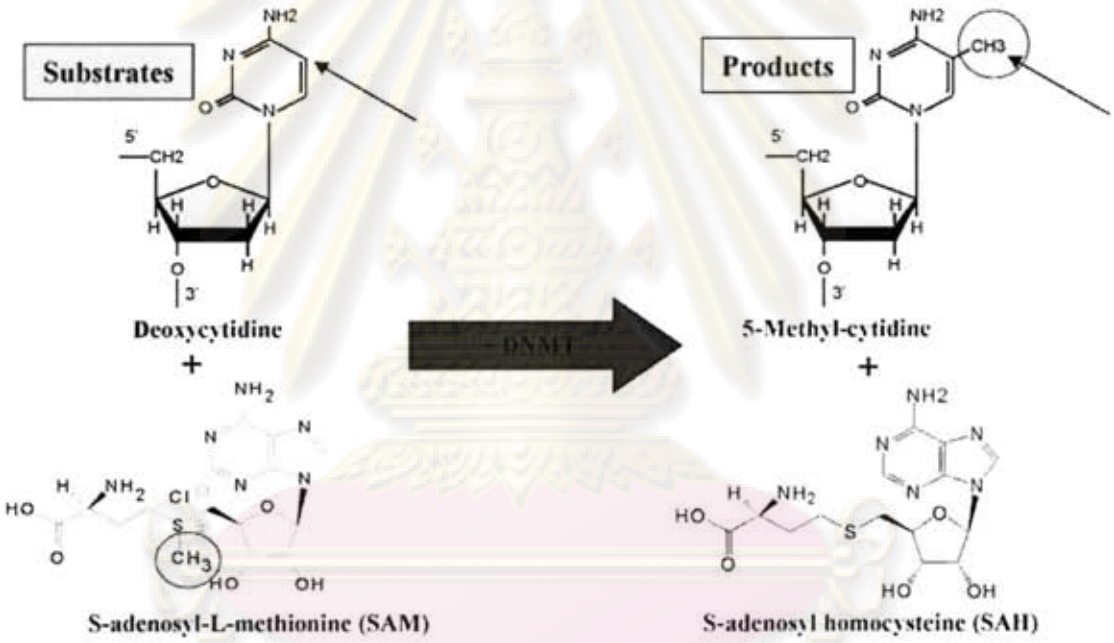


Figure 5. **Methylation of Cytosine.** The DNA methyltransferase catalyze the transfer of the methyl group from S-adenosylmethionine to cytosine, producing 5-methylcytosine and S-adenosylhomocysteine(From http://www.med.ufl.edu/biochem/keithr/fig1pt1.html).

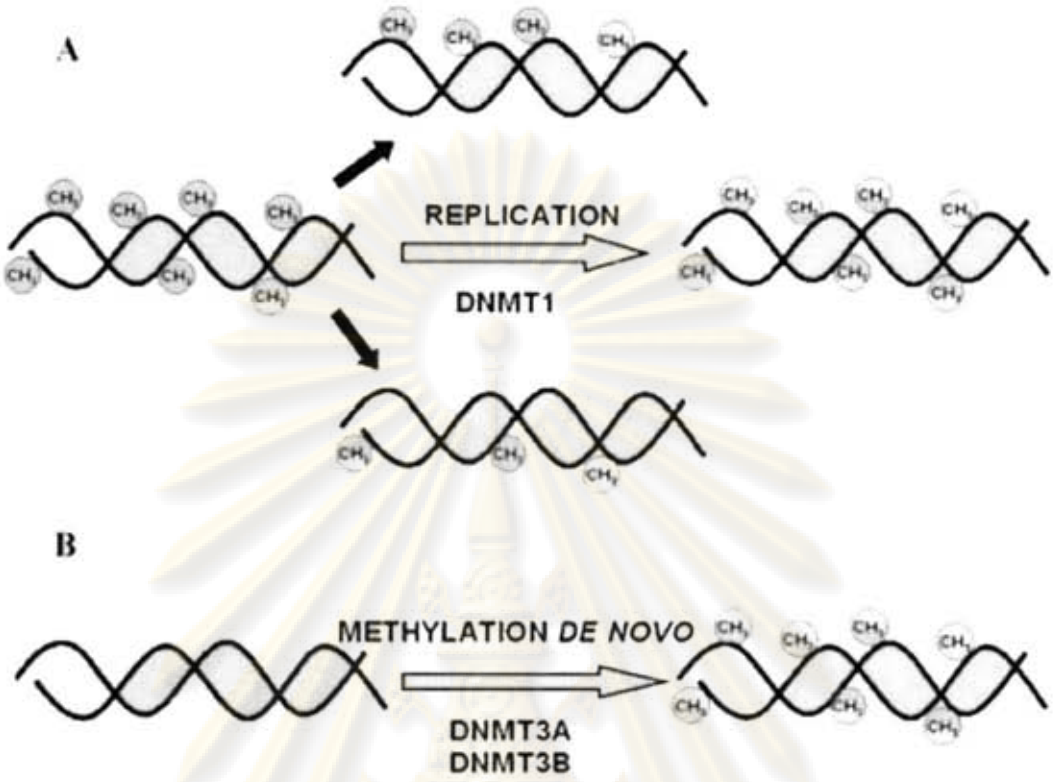


Figure 6. **Maintenance and *de novo* DNA Methylation.** A) Maintenance DNA methylation, DNMT1 add methyl groups to the hemimethylated DNA during replication, whereas, *de novo* DNA methylation, DNMT3A and DNMT3B can add methyl groups to CpG dinucleotides of unmethylated DNA (115).

Global Hypomethylation

Most cancers show downregulation of methylated CpG dinucleotides throughout their genome, termed global hypomethylation, as a common epigenetic change during cancer development (2, 3, 11, 118). Hyper- and hypomethylation in cancers imply aberrant levels of methylation in relation to normal state, which may be associated with functional changes in the genome that have the potential to serve as oncogenic mechanisms. Global losses of methylation in cancer may lead to alteration in the expression of protooncogenes critical to carcinogenesis and may facilitate chromosomal instability (11, 30). Previous studies to qualitatively evaluate the methylation status of LINE-1 in DNA from whole tissue samples used southern blotting and/or distinguishing PCR products by methylation sensitive enzyme in several malignancies including carcinoma of urinary bladder, liver, prostate and colon (7, 9, 26-28). The study from Chalitchagorn *et al.* (4) developed an improved quantitative COBRA technique to evaluate the methylation status of LINE-1 in microdissected DNA samples from several human normal and neoplastic tissues. The results from leukocyte showed that there was no difference in the level of LINE-1 methylation among age and gender. In contrast, normal tissues from different organs showed tissue-specific levels of methylated LINE-1. Most carcinomas including breast, colon, lung, head and neck, bladder, esophagus, liver, prostate, and stomach, revealed a greater percentage of hypomethylation than their normal tissue counterparts. Furthermore, DNA derived from sera of patients with carcinoma displayed more LINE-1 hypomethylation than those of noncarcinoma individuals. Finally, in a colonic carcinogenesis model, they detected significantly greater hypomethylation in carcinoma than those of dysplastic polyp and histological normal colonic epithelium. Thus, the methylation status is a unique feature of a specific tissue type and the global hypomethylation is a common epigenetic process in cancer, which may progressively evolve during multistage carcinogenesis.

Global Hypomethylation and Carcinogenesis

Since genome-wide losses of DNA methylation has been found in many types of human cancer and associated with the degree of malignancy. Global hypomethylation may play crucial roles in carcinogenesis. Global hypomethylation potentially promotes carcinogenesis via three possible mechanisms: activation of oncogenes, reactivation of transposable elements and chromosomal instability. These mechanisms contribute to carcinogenesis by overlapping properties, including aberrant recombination and deregulated gene expression (29, 30).

1) Activation of Oncogenes

In 1975, Holliday and Pugh (119) proposed that hypomethylation could provide a selective advantage for the tumor cells if hypomethylation leads to inappropriate activation of genes important in neoplastic growth. There was an observation support this notion from the studies of hypomethylation of the H-*ras* oncogenes in bladder cancer (120) and *c-myc* oncogene in non-small cell lung cancer (NSCLC) (121). Another example comes from the study in urokinase-type plasminogen activator (uPA), a member of serine protease family and can break down various components of the extracellular matrix to promote growth, invasion, and metastasis of several malignancies including breast cancer. They showed that 90% of CpG dinucleotides in the *uPA* promoter are methylated in non-metastatic breast cancer cell lines, whereas fully demethylated CpGs were detected metastatic breast cancer cell lines. The expression of *uPA* gene can be restored when non-metastatic breast cancer cell lines were treated with 5-aza-2-deoxycytidine, a DNA methyltransferase inhibitor (122). These results support the possibility that global hypomethylation involved in the activation of oncogenes.

2) Reactivation of Transposable Elements

The human genome contains numerous mobile genetic elements, termed retrotransposons, and expression from these elements is usually suppressed by DNA methylation, thereby inhibiting its ability to mobilize. The most abundant

retrotransposons in the human genome are LINE-1. Loss of promoter methylation and transcriptional activation of LINE-1 elements have been reported in a variety of sporadic cancer types (6, 8, 15, 96). This could potentially lead to movement of the retrotransposons and reintegration at new sites in the genome, leading to insertional mutagenesis and disturbance of transcriptional activity and gene regulation. LINE-1 mutational insertions in sporadic cancers have been found that disrupt the *APC* gene and *c-myc* gene in a sporadic tumor of the colon and breast, respectively, suggesting that certain LINE-1 are active in human cells (47, 48). Mutational insertion of non-autonomous retrotransposons such as Alu elements may also occur in the germline (28). Such Alu mediated mutations have been observed in BRCA1 and BRCA2 in families with hereditary predisposition to breast and ovarian cancer (123, 124) and in the *MLH1* gene in families predisposed to colon cancer (125). Furthermore, it has been suggested that because of the typically strong activity of the 5'LTRs or promoters of LINE-1s, hypomethylation mediated transcriptional activation of LINE-1s could also disrupt expression of nearby genes. While the promoters of most LINE-1s have been deleted, other abundant retrotransposons such as human endogenous retroviruses (HERVs) retain the 5'LTR(28).

3) Chromosomal Instability

Chromosome aberrations are common in cancer, and it has been suggested that DNA methylation could be involved in the control of chromosome stability. DNA hypomethylation has been associated with abnormal chromosomal structures, as observed in cell form patients with ICF (immunodeficiency, centromeric instability and facial abnormalities) syndrome (37, 126) and in cells treated with 5-aza-2-deoxycytidine (39). The ICF syndrome is caused by inherited mutations in the DNMT3B. In normal somatic cells, pericentromeric heterochromatin regions on chromosome 1 and 16 are heavily methylated but are abnormally hypomethylated in all somatic cells of ICF patients. Mitogen stimulation of lymphocytes from ICF patients resulted in a high frequency of abnormalities involving chromosomes 1 and 16. In breast adenocarcinomas, ovarian epithelial tumors, and sporadic Wilms tumors, these

chromosomes are significantly hypomethylated and frequently unstable (127-129). Similar rearrangements involving chromosomes 1 and 16 are induced in mitogen stimulated normal cells treated with 5-aza-2-deoxycytidine (39). In 1998, Chen *et al.* reported that murine embryonic stem (ES) cells with *DNMT1* knockout exhibited a significantly increase in mutation rates (about 10-fold), primarily involving genomic deletion (12). Thus, all of these data implied that genomic hypomethylation may predispose to chromosome abnormalities, possibly facilitated by additional growth stimulating factors or inappropriate cell division.

DNA DOUBLE-STRAND BREAKS (DSBs) AND DNA REPAIR

DNA double-strand breaks (DSBs) are type of DNA lesion that both DNA strands of the double helix are damaged, which prevent the use of complementary DNA strand as a template for repair so that they are more difficult to repair than other type of DNA damage. DSBs can be induced by extrinsic insult including ionizing radiation and chemicals and intrinsic insult such as free radicals, single-strand breaks (SSBs) in DNA, collapsed replication and special mechanisms in cell (V(D)J recombination, class switching, somatic hypermutation and meiosis) (5). DSBs occur by intrinsic insult are called endogenous double-strand breaks (EDSBs). DSBs are particularly dangerous lesions if they occur during DNA replication of the genome and during the segregation of duplicated chromosomes into daughter cells. Eukaryotic cells have several cell cycle checkpoints at G1/S to prevent cells from starting DNA replication, at intra S to prevent cells from replication and G2/M to prevent from going into mitosis when cells have DNA damage (130). These cell cycle checkpoints give a time for cell to either repair DNA damage or drive cell to apoptosis. DSBs involve in carcinogenesis e.g. in chromosomal rearrangements and deletions, and in mitotic recombination in somatic cells if they are not repaired properly (109). Two main DNA repair pathways are homologous recombination (HR) and non-homologous end-joining (NHEJ) which differ in their requirement of a homologous template DNA and in the accuracy of DSB repair. NHEJ can be correctly repaired only if the free ends of the DNA rejoin exactly while HR ensure accurate DSB repair using their undamaged sister chromatid as a template. HR

functions efficiently in S/G2 checkpoint when cell has sister chromatid but NHEJ can operate in all cell cycle (131).

Homologous Recombination (HR)

HR can repair DNA damage with high fidelity which is important in proliferating cells. The basic mechanism of HR is conserved from yeast to human. There are many proteins involve in HR pathway including RAD50, RAD51, RAD52, RAD54, the RAD 51 paralogues (RAD51B, RAD51C, RAD51D, XRCC2, XRCC3 and DMC1), meiotic recombination (MRE11), ataxia telangiectasia mutated (ATM), replication protein A (RPA), NBS1, BRCA1 and BRCA2. A schematic of HR pathway and its related proteins are shown in Figure 7. An early event in HR is the resection of the broken ends in the 5' to 3' direction to yield 3' single-strand overhang by the MRN complex (MRE11-RAD50-NBS1) after ATM activation (NBS1 in MRN complex is phosphorylated by ATM). The MRN complex has been linked to many DNA metabolic events that involve in sensing, processing and repair of DSBs (132). The single-stand overhang is bound by the RAD51 protein then searching for a homologous DNA template, resulting in strand inversion, stand exchange and joint molecule formation between the broken DNA ends and the repair template. This step require many proteins to facilitate the action of RAD51 including the RPA, which binds single-stranded DNA (ssDNA), RAD52, which can bind DNA ends and anneal complementary ssDNA molecules, RAD54, which can unwind the DNA at the DSB because it has ATPase activity and is related to DNA helicase, and RAD51 paralogues (133). The joint molecules provide the substrate for DNA synthesis to fill the gap by DNA polymerase and seal by DNA ligase. The DNA polymerase and DNA ligase involve in this step are still not known (131, 134).

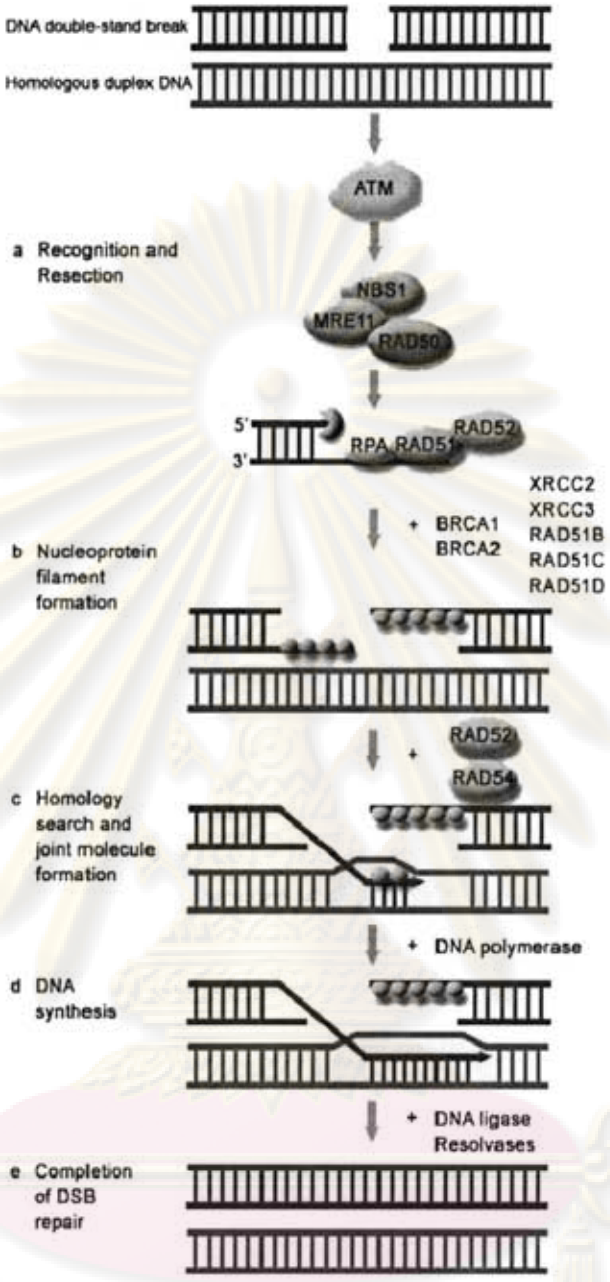


Figure 7. **Schematic of Homologous Recombination Repair Pathway.** (a) DSB triggers cell cycle checkpoints and activate ATM to phosphorylate NBS1 in MRN complex. The result of this process generates a 3' single stranded (ss) DNA overhang. (b) RPA bind to ssDNA overhang then recruit RAD51 and RAD52 to form ssDNA-RAD51 nucleoprotein filaments. (c) The nucleoprotein filament searches for the homologous duplex DNA in the undamaged sister chromatid. This results in strand inversion, strand exchange and joint molecule formation which stimulate by RAD52 and RAD54. (d) DNA synthesis fills the gap by DNA polymerase and (c) seals by DNA ligase.

Non-Homologous End Joining (NHEJ)

In contrast to HR, NHEJ does not use a complementary template for repair therefore this DSB-repair pathway is intrinsically error prone. NHEJ can repair DSBs generated by exogenous DNA-damaging agents, such as ionizing radiation, but also required to process the DSB intermediates that are generated during V(D)J recombination. A key component of the NHEJ pathway is the DNA-dependent protein kinase (DNA-PK). DNA-PK consists of a heterodimeric DNA targeting subunit (KU) and DNA-dependent protein kinase (DNA-PKcs). The KU heterodimer consisting of KU70 and KU80 has a high affinity for DNA ends which indicates that it has an early role in the NHEJ process. Inactivation of KU70 or KU80 leads to multiple defects including growth retardation, hypersensitivity to IR and severe combined immune deficiency (SCID) due to impaired V(D)J recombination (51). DNA-PKcs is a 470 kDa polypeptide with a protein kinase domain near its carboxyl terminus. DNA-PKcs mutant mice have a high incidence of T-cell lymphomas (51). NHEJ repair pathway can be divided into three steps: (1) end binding, (2) end processing and (3) ligation (Figure 8). In the end binding step, KU heterodimer binds to DNA ends then attracts the DNA-PKcs. The second step, end processing is particularly important for repairing of more complex non-complementary ends. They process their terminals via nucleolysis and polymerization resulting in the removal or addition of a few base pairs. Several enzymes have been linked to operate in this step including MRN complex, Artemis and Werner syndrome helicase (WRN). MRN complex might be involved in the unwinding and/or nucleolytic processing of the DNA ends. Artemis is a 5'-3' exonuclease which can be phosphorylated by DNA-PKcs. WRN, which is stimulated by KU but inhibited by DNA-PKcs, possesses 3'-5' exonuclease activity with a preference for recessed 3' DNA ends. In the ligation step, the complex of DNA-PK recruits DNA ligase IV and XRCC4 complex to accomplishes the rejoining step. DNA ligase IV can be stabilized by forming a complex with XRCC4 then stimulates the ligation activity. This complex presents in all eukaryotes including yeast which lacks DNA-PKcs but not KU (5, 134-136).

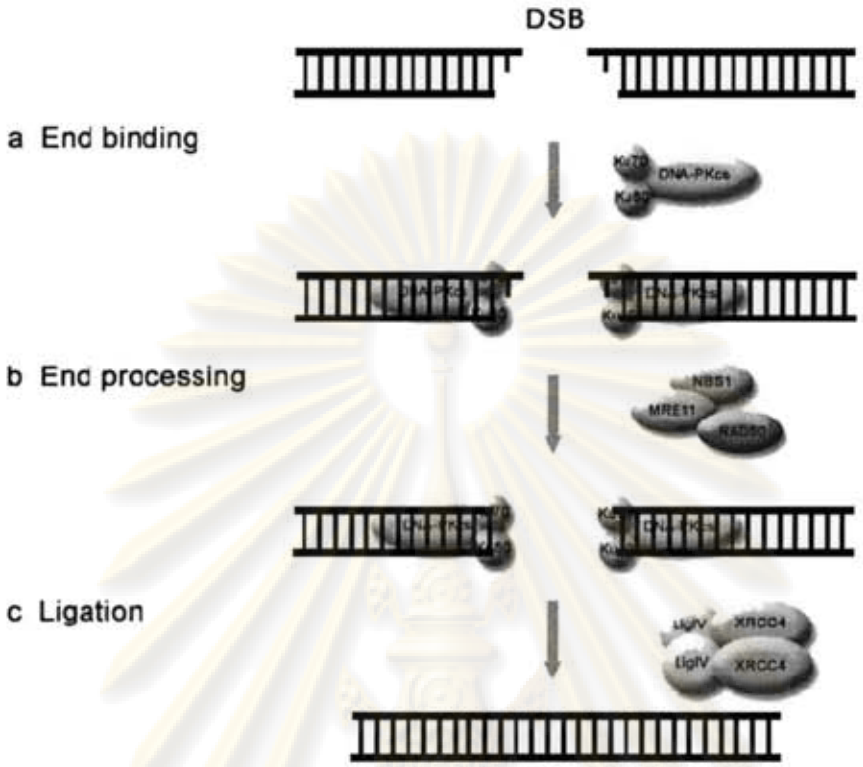


Figure 8. **Schematic of Non-Homologous End Joining Repair Pathway.** (a) After DSB formation, the KU-DNA-PKcs complex recognizes the DSB at the ends. (b) The ends are processed by removal or addition of a few base pair. (c) The DNA ligase IV-XRCC4 complex is responsible for rejoining the ends.

CERVICAL CANCER

Cervical cancer is a malignant cancer of the cervix. It is the seventh in frequency overall, but the second most common among women worldwide, with an estimated 493,000 new cases and 274,000 deaths in the year 2002. It is much more common in developing countries, where 83% of cases occur and where cervical cancer accounts for 15% of female cancers, with a risk before age 65 of 1.5%. In developed countries, cervical cancer accounts for only 3.6% of new cancers, with a cumulative risk (0 to 64) of 0.8% (137). Squamous cell carcinoma of the cervix accounts for about 85% of cervical cancers. The terminology for squamous epithelial lesions includes the follow categories: 1) atypical squamous cells of undetermined significance; 2) squamous intraepithelial lesion (SIL), which encompasses the spectrum of squamous cell carcinoma precursors, divided into low grade SIL (human papilloma virus (HPV)-associated cellular changes, mild dysplasia, and cervical intraepithelial neoplasia (CIN) I) and high grade SIL (moderate dysplasia, severe dysplasia, and carcinoma in situ and CIN II and CIN III); and 3) squamous cell carcinoma (Figure 9) (138).

The prominent risk factor of cervical cancer is sexual history especially the number of sexual partners. Human papilloma virus (HPV) has long been established as a necessary but not sufficient cause for cervical carcinoma development. HPV is detected in 99% of invasive disease, 94% of CIN lesions and 46% of normal cervical epithelium (139). Over 100 different HPV types have been characterized. The HPVs are classified into high-risk and low-risk groups, referring to their association with cervical cancer. Some sexually transmitted low-risk HPVs, such as types HPV 6 and 11, can cause genital warts. Sexually transmitted, high-risk HPVs include types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 69, and possible a few others may leads to the development of cervical cancer (140). HPV-induced cancers often have viral sequences integrated into the cellular DNA. Some of the HPV early genes, such as E6 and E7, are known to act as oncogenes that promote tumor growth and malignant transformation. The proteins form high-risk HPV types, E6 was shown to target the p53 tumor suppression protein for degradation and the E7 was shown to bind to the active form of

the retinoblastoma (Rb) protein(141). The E6 protein from the low-risk HPV types binds p53 with much lower affinity and does not target p53 degradation (142). The HPV E7 proteins bind to pRb and inhibit its function; however, the binding affinity of the low-risk E7 proteins much lower than the high-risk E7 proteins (143). Most of HPV DNA is in an episomal form in low-grade lesion, but in an integrated form in high-grade lesions and cancer (144). Integration of HPV DNA into the host genome can disrupt the E2 ORF, results to eliminate the transcriptional regulatory proteins and leading to an overexpression of the E6 and E7 oncoproteins (145).

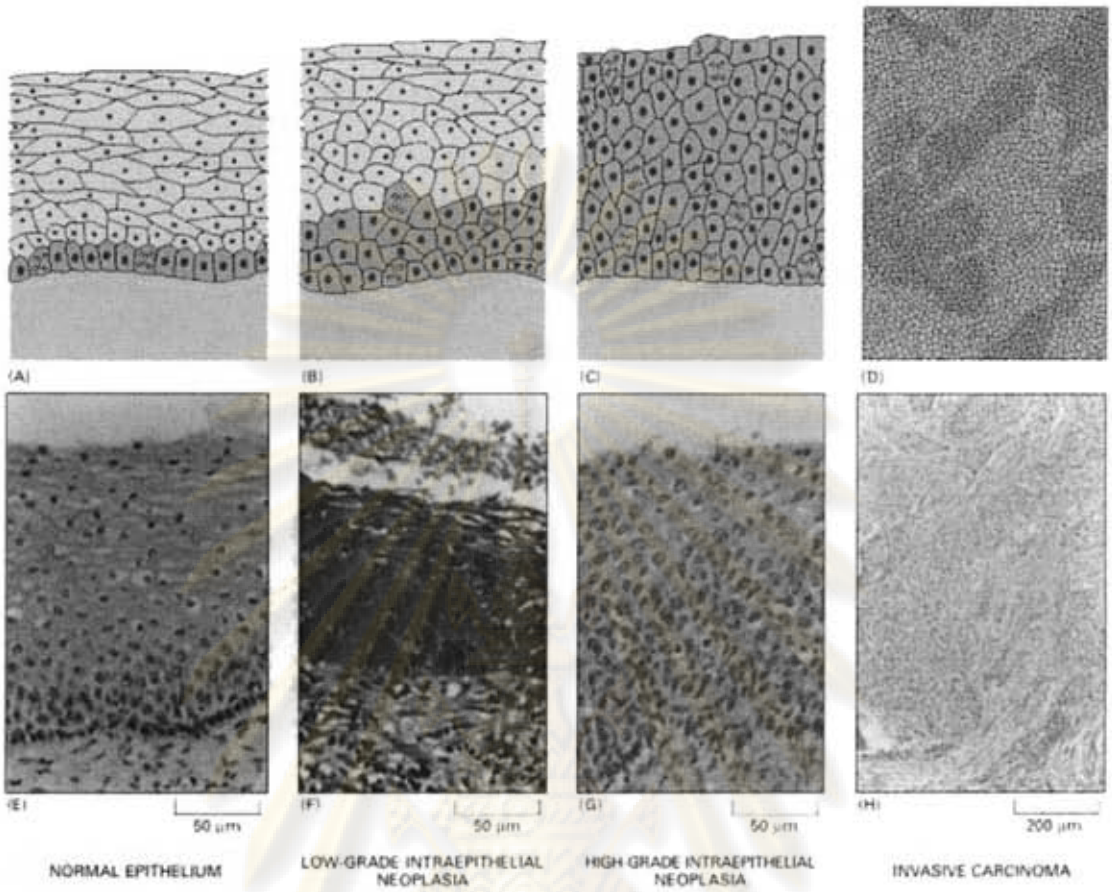


Figure 9. **The Stages of Progression in the Development of Cancer of Epithelium of the Uterine Cervix.** Schematic diagrams are shown in A-D; sections of cervical epithelium corresponding to these changes are shown in E-H. (A, E) In the normal stratified squamous epithelium, dividing cells are confined to the basal layer. (B, F) In low-grade intraepithelial neoplasia, dividing cells can be found throughout the lower third of the epithelium; the superficial cells are still flattened and show signs of differentiation, but this is incomplete. (C, G) In high-grade intraepithelial neoplasia, cells in all the epithelial layers are proliferating and show no sign of differentiation. (D, H) True malignancy begins when the cells move through or destroy the basal lamina and invade the underlying connective tissue (49).

CHAPTER III

MATERIALS AND METHODS

Population and Sample Selection

Primary cervical cancer tissues were collected from patients at King Chulalongkorn Memorial Hospital. The tissues were divided into two parts. The first part was sent for routine histological examination. The second part was preceded for frozen as described. Pour iso-Pentane into a small container that is embedded in liquid nitrogen. Place a lid on the bucket and let it chill. Then, place the fresh specimen immediately into the chilled iso-Pentane. After freezing, take the specimen out quickly from the liquid nitrogen, write the identification on the corner of the mold. Place the specimen into center of the mold and put the tissue freezing medium on the top of the specimen up to the first rim and then place on dry-ice. Turn it over with a long forceps or handles of a scissors and give several sharp strikes to the back and at the same time run a thumb or finger across the back of the mold to warm enough to allow the sample drop out. Do not let any thawing to occur. Place the specimen into a small vial with its identification written on the outside and then place it back into liquid nitrogen. Continue with this procedure until all samples are frozen and ready to store in -70°C.

Normal controls were obtained from normal tissues surround tumor or blood samples. DNA extraction was performed directly for normal tissues. For blood samples, they were centrifuged at 1,000 g for 10 minutes to obtained peripheral blood mononuclear cells (PBMCs) and then performed DNA extraction.

DNA Extraction

Frozen tissues will be separated for DNA extraction using microdissection techniques previously describe(146). The area of tumor tissues was dissected from each 5 µm sections. Tissues were resuspended and incubated at 65°C overnight in 200µl of lysis II buffer and 200 µg/ml of proteinase K. For blood samples, buffy coat was separated by centrifugation at 1,000 g for 10 minute and resuspended with lysis I buffer and centrifuge at 1,000 g for 5 min discard supernatant then repeat this step once. The

pellet was resuspended and incubated at 65°C overnight in 1ml of lysis II buffer and 200 µg/ml proteinase K. The DNA was purified by phenol/chloroform extraction and ethanol precipitation upon addition of glycogen to help small amount of DNA to precipitate. The DNA was washed with 70% ethanol then spin down and air dry. The DNA was resuspended in TE buffer.

LINE-1 ACA Sequence Mediate PCR (L1-AM-PCR)

Genomic DNA from tumors and blood were digested with *BstY* I under a condition; 500 ng of DNA was dissolved in NEB buffer 2 (New England Biolabs), 5 units of restriction enzyme was added then incubated at 60°C for 4 hours then inactivated enzyme at 80°C for 20 minutes. Digested DNA was purified with phenol/chloroform extraction and ethanol precipitation. Ligation reaction was performed by T4 DNA ligase (New England Biolabs) with 20 pmol of linkers at 16°C overnight. Linker with *BstY* I overhang were generated using L1-AM-PCRLINK anneal with BstY I-LINK. The PCR reaction was performed in a total volume for 20 µl reaction mixture in 200 µM dNTPs, 1X Qiagen PCR buffer(contains 1.5 mM $MgCl_2$), 0.1 unit HotStarTaq (QIAGEN), 0.4 µM of L1-ACA and linker primer and 50 ng of ligated DNA. The PCR amplification were performed as follows: initial denaturation at 95°C for 15 minute, followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 52°C for 45 seconds, extension at 72°C for 2 minute and a final extension at 72°C for 7 minute. One microliter of the amplicons was used as a template for second PCR. The second PCR was performed in 32 reactions with chimeric primers and L-1 nested primers with an appropriate anneal temperature for each chimeric primers for 20 cycles. The PCR products were then analyzed using 2% agarose gel electrophoresis.

Cloning and DNA Sequencing

The different band of amplicons was cut and extracted from agarose gel using QIAquick gel extraction kit (QIAGEN) then ligated to pGEM-T easy cloning vector (Promega) and transformed into *E. coli* DH5α. Plate the transformation reaction onto ampicillin LB agar plate containing X-Gal and IPTG, incubated at 37°C overnight. The

white bacterial colonies were selected and colonies were then cultured in LB broth containing antibiotic overnight and extracted plasmid by miniprep. DNA sequencing was performed on an Applied Biosystems DNA sequencer using M13 primers.

DNA Sequencing Analysis

DNA sequencing results were analyzed by nucleotide BLAST program from www.ncbi.nlm.nih.gov/BLAST. Sequences that located differently form normal DNA was then proved back by PCR using primers within analyzed sequence and the primer in the flanking region of analyzed sequence.

LINE-1 Insertion Dimorphisms (LIDs) PCR

Genomic sequence data from GenBank were used to design LIDs PCR primers from unique sequence 5' and 3' without LINE-1 ACA sequence. PCR were performed from DNA samples to confirm a dimorphic status.

Cell Culture

HeLa (cervical cancer), K-562 (erythroleukemia), and SW480 (colorectal adenocarcinoma) cell lines were cultured 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. 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.

Single Colony LINE-1 Mapping

HeLa cells were serial diluted and plated on 6 well culture dish. The cells must be plated at low enough density to grow into colonies without growing into one another. To pick clones, dip sterilized cloning ring into sterile grease and then place one on top of each colony. Cells within the cloning ring were removed by trypsinization and transferred them in to a fresh 96 well culture dish, when the cells have grown to confluency in a well of a 96 well culture dish, move them to a well in a 24 well culture

dish. Single colony HeLa cells were continuously culture until the amount of HeLa cells reached for DNA extraction. HeLa clones were assayed individually for a LINE-1 mapping

Cell Synchronization

The day before HeLa cells were synchronized at G0 G1/S and S phases, HeLa cells were cultured at a density of 10^6 cells in 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 with 0.2% FBS, for 48 h. For G1/S and S phase, HeLa cells were synchronized by the thymidine block method. HeLa cells were cultured with 2 mM thymidine for 18 h to obtain cells at G1/S phase(147). 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 h. 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(148).

High-Molecular-Weight (HMW) DNA Preparation

To prepare HMW DNA, 5 X 10^5 cells were embedded in 1% low-melting-point agarose, lysed and digested in 400 µl of 1 mg/ml proteinase K and HMW digestion buffer. The plugs were 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 min followed by rinsing four times in TE buffer for 20 min. The linkers (50 pmol) were 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). After extraction, 100 ng of DNA was tested for fragmentation using the LMPCR ladder technique(149). After primary polymerization at 72 °C for 1 min, 35 cycles of PCR were

carried out using the Linker primer at 58 °C. The amplicons were electrophoresed in agarose gel to visualize apoptotic fragmented DNA ladders.

L1-EDSB-LMPCR

The quantity of L1-EDSB was measured by real-time PCR using a Lightcycler™ instrument (Roche Applied Science) with the intersperse repetitive sequence (IRS) primers including L1 primers (outward and inward), Alu primers (Alu-CL2 or Tigger1), the linker primer, and the Taqman probe homologous to the 3′ linker sequence. Amplification was performed with 0.5 μM of each primer, 0.4 μM Taqman probe, 2 units of HotStarTaq (QIAGEN), 1× PCR buffer and 10 ng of ligated DNA 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 (DSBcontrol) with the 5′ linker sequence and 3′ homology to L1 oligonucleotide sequences. The second was more stable DNA digested with *Eco*RV and *Alu*I and ligated to the LMPCR linkers. The amounts of EDSBs were compared with the ligated control digested DNA and reported as L1-EDSB-LMPCR templates per nanogram of DNA.

Bisulfite Treatment

Ligated HMW DNA was modified by sodium bisulfite using standard protocol(150). 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 min. 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 min. The DNA pellet was washed with 70% ethanol then centrifuged at 14,000 rpm for 5 min. 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 (4), a 20 µl PCR was carried out in 1X PCR buffer contained with 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit of HotStarTaq (QIAGEN), 0.3 µM of B-L1-inward, 0.3 µM of B-L1-outward, 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. 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 with 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. ^{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 units of *Taq*I and 8 unit of *Tas*I in 1x *Taq*I buffer (MBI Fermentas) then incubate 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 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.

Vanillin Treatment and siRNA

For chemically inhibit DNA-PKcs, HeLa cells were treated for 24 hours with 2.5 mM vanillin (Sigma-Aldrich) and then harvested the cells.

For genetically inhibit DNA-PKcs and ATM, the oligonucleotide sequences of siRNA targeting ATM and DNA-PKcs are as previously described by Zhang *et al*(151) and An *et al(152)*, respectively. 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 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. Five microliters of the annealed siRNA templates were diluted with 45 µl nuclease-free water for a final concentration of 8 ng/ µl. Set up10 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.

Western Blotting

HeLa cells were washed twice with PBS and then added ice-cold RIPA lysis buffer with Halt protease inhibitor cocktail (Pierce) directly to cells (1 ml/ 10^7 cells/100 mm dish). Cells were scraped off the dish with a plastic cell scraper and transferred the cell suspension into a 1.5 ml centrifuge tube. Lysed cells were incubated on ice for 10 minutes then sonicated with an ultrasonic sonicator at 70% power output for 10 seconds three-times on ice. Sonicated cell lysate was then centrifuged at max speed for 10 minutes at 4°C. Total proteins were electrophoresed on 12% 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. GAPDH was determined as a control using anti-GAPDH antibody (Trevigen). The nitrocellulose membrane was washed three times with agitation in TBST for 5 minutes and then incubated with secondary antibody, horseradish peroxidase (HRP)-goat anti-rabbit IgG (H+L) conjugate (Zymed® Laboratories) for GAPDH or goat anti-mouse IgG-HRP sc-2005 HRP conjugated (Santa Cruz Biotechnology) for ATM and DNA-PKcs, in TBST-M (1:5000) for 1 hour at room temperature with agitation and washed the nitrocellulose membrane three times for 5 minutes each time with TBST. The DNA-PKcs or ATM proteins were detected by using 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 Analysis

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.

Table1. **List of Primers in Experiments**

primer name	sequence (5' - 3')
L1-AM-PCRLINK F	AGGTAACGAGTCAGACCACCGATCGCTCGGAA-GCTTACCTCGTGGACGT
L1-AM-PCRLINK R	ACGTCCACGAG
L1-LINK F	AGGTAACGAGTCAGACCACCGACTCGTGGACGT
BstYI-LINK R	GATCACGTCCACGAG
TaqI-LINK R	CGACGTCCACGAG
L1-ACA	GAGATCTACCTAATGCTAGATGACACA
L-1 nested primer	GCGCACCAGCATGGCACA
Linker primer	AGGTAACGAGTCAGACCACCGA
DSBcontrol	ACCGTTCGAACGAGAATCGCACTGTCGACGTCG-ACGATTCCCACTGTGACGTCCACGAGGTAAGCT-TCCGAGCGATCGGTGGTCTGACTCGTTACCTA
Taqman Probe	(6-Fam)ACGTCCACGAGGTAAGCTTCCGAGCG-A(Tamra)(phosphate)
L1 primer outward	CTCCCAGCGTGAGCGAC
L1 primer inward	AAGCCGGTCTGAAAAGCGCAA
Alu-CL2	ACTGCACTCCAGCCTGGGC
Tigger1	CTCGCTGAAGGCTCAGATGATC
B-LMPCR	GTTTGGAAGTTTATTTTGTGGAT
B-L1-outward	RTAAAACCCTCCRAACCAAATATAAA
B-L1-inward	CCGTAAGGGGTTAGGGAGTTTTT
Chimeic primers	GACTCGTGGACGTGATC(C/T)XX where (C/T) is C or T, and XX is 2 bp of randomly selected sequence; e.g. TGA chimeric primer is GACTCGTGGACGTGATCTGA and CGA chimeric primer is GACTCGTGGACGTGATCCGA so that there will be 32 chimeric primers.

CHAPTER IV
RESULTS

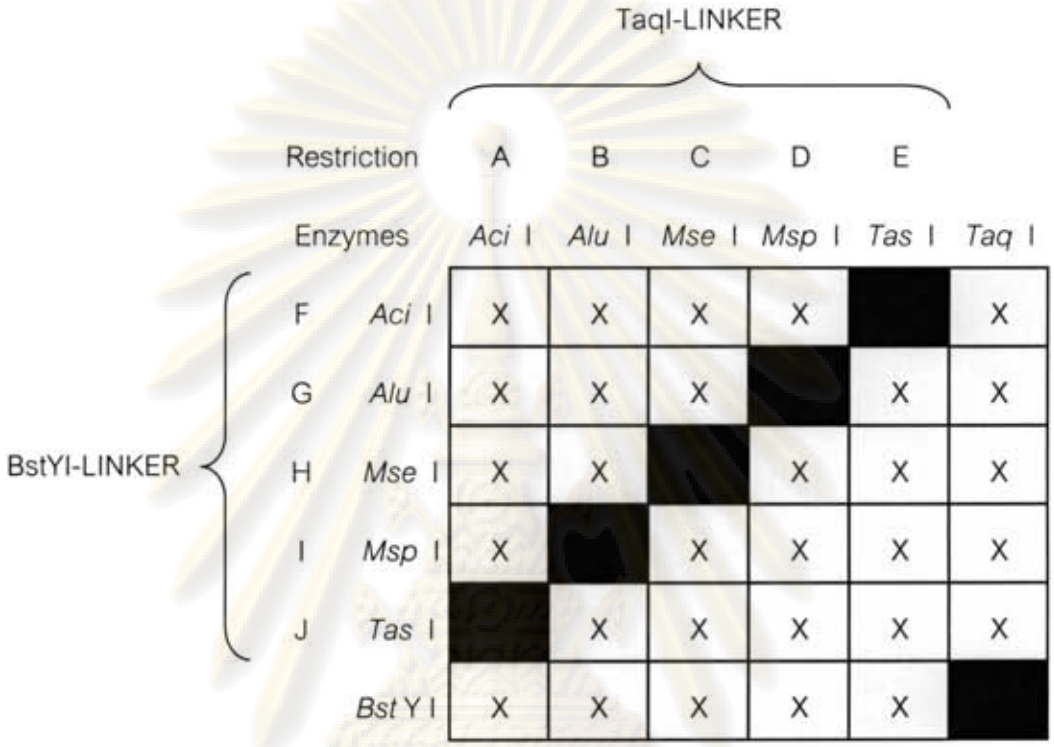
Loss of DNA methylation is a common molecular event in cancers and can cause CIN by three possible mechanisms: activation of oncogenes, reactivation of retrotransposons, and chromosome instability. We hypothesized whether instability of cancer is induced *in trans* by LINE-1 retrotransposition or *in cis* by DNA methylation levels related to how EDSBs are occurred or repaired.

LINE-1 Mapping in Cervical Cancer

In order to determine whether global hypomethylation induced instability *in trans* by LINE-1 retrotransposition, we aim to detect LINE-1 movements from hypomethylation state of cancer genome during cervical cancer development, which a different pattern may be found in cervical cancers when compare with normal cells. Cervical cancer was selected as a model because of its clonal in origin and a cervical cancer cell line, HeLa, was proved to yield transfected recombinant LINE-1 vector movement *in vitro* (153). Since, it remains difficult to estimate the fraction of human disease-associated mutation due to the insertion of retrotransposons because the most mutation-detection strategies utilize PCR are fail to detect large insertions (154). First, we generate a PCR to amplify from LINE-1 Ta subset to a specific restriction site because there are approximately 200 copies in the diploid human genome, LINE-1 that frequently moves in human genome (20), which are too concentrate to be differentiated by gel electrophoresis. The strategy started from digesting DNA from tumor and PBMC with restriction enzyme that does not cut the LINE-1 sequence from the position of designed primers, either *Taq*I or *Bst*YI, then ligate with TaqI-LINKER (L1-LINK F + TaqI-LINK R) or BstYI-LINKER (L1-LINK F + BstYI-LINK R), respectively. The ligated DNA were then cut with a combinations of restriction enzyme as show in Table 2, for example F combination, DNA were ligated with BstYI LINKER and then digested with *Aci*I, *Alu*I, *Mse*I, *Msp*I, *Tas*I and *Taq*I. As showed in figure the amplicons from several combination groups were separated by 2% and 3% agarose gel electrophoresis (Figure 10) but they were too difficult to discriminated pattern between each combination. 5% polyacrylamide gel

electrophoresis (PAGE) was used to solve this problem (Figure 11). Band patterns can be discriminated among difference combination so that cervical cancer and normal sample from the same patient was applied to map LINE-1 (Figure 12). We expected band pattern of amplicons from cancer cells that differ from normal cells and then searched sequences for identification of a disrupted gene involve with an insertion. This LINE-1 mapping technique gave some problems due to a rapid degradation of digested DNA with linker which make it unable to reproduce the same patterns when we tried to repeat the experiment and its difficulty to purify interested bands from polyacrylamide gel to subclone into plasmid for further DNA sequencing. We changed a strategy of LINE-1 mapping technique to avoid these problems. DNA were digested with *Bst*YI, ligated with BstYI-LINKER, and then performed nested PCR. Amplicons from 3' UTR of LINE-1 Ta subset and linker primer were subjected to second PCR by using 32 different chimeric primers (see materials and methods) and L-1 nested primers (Figure 13). The nested step using chimeric primers helps fractionate the repetitive products similar to *Mse*I 3+ primers in sequence specific amplification polymorphism (S-SAP), which was developed to display plant retrotransposons (155). Each PCR yields a limited number of products by dividing LINE-1 products into several subsets with nested PCR using chimeric primers so that it is possible to size differentiation by agarose gel electrophoresis. Furthermore, this method used small amount of original DNA, can be reproduced by the nested PCR when reproducing LINE-1 mapping recovery is needed, and can be recovered interested bands with normal gel recovery. Our strategy can globally map LINE-1 Ta subset in genome which is different form the previous study because the finding that disruption of *APC* gene in colon cancer caused by somatic insertion of LINE-1 was performed by DNA sequence analysis in *APC* gene from 150 colon cancer patients (47). By our strategy, we mapped LINE-1 in 23 cervical cancer patients, the example pictures of LINE-1 mapping has been shown in Figure 14. No different LINE-1 pattern was found among 23 cervical cancer patients.

Table 2. **List of Linkers and Restriction Enzymes Use for Establish LINE-1 Mapping.**



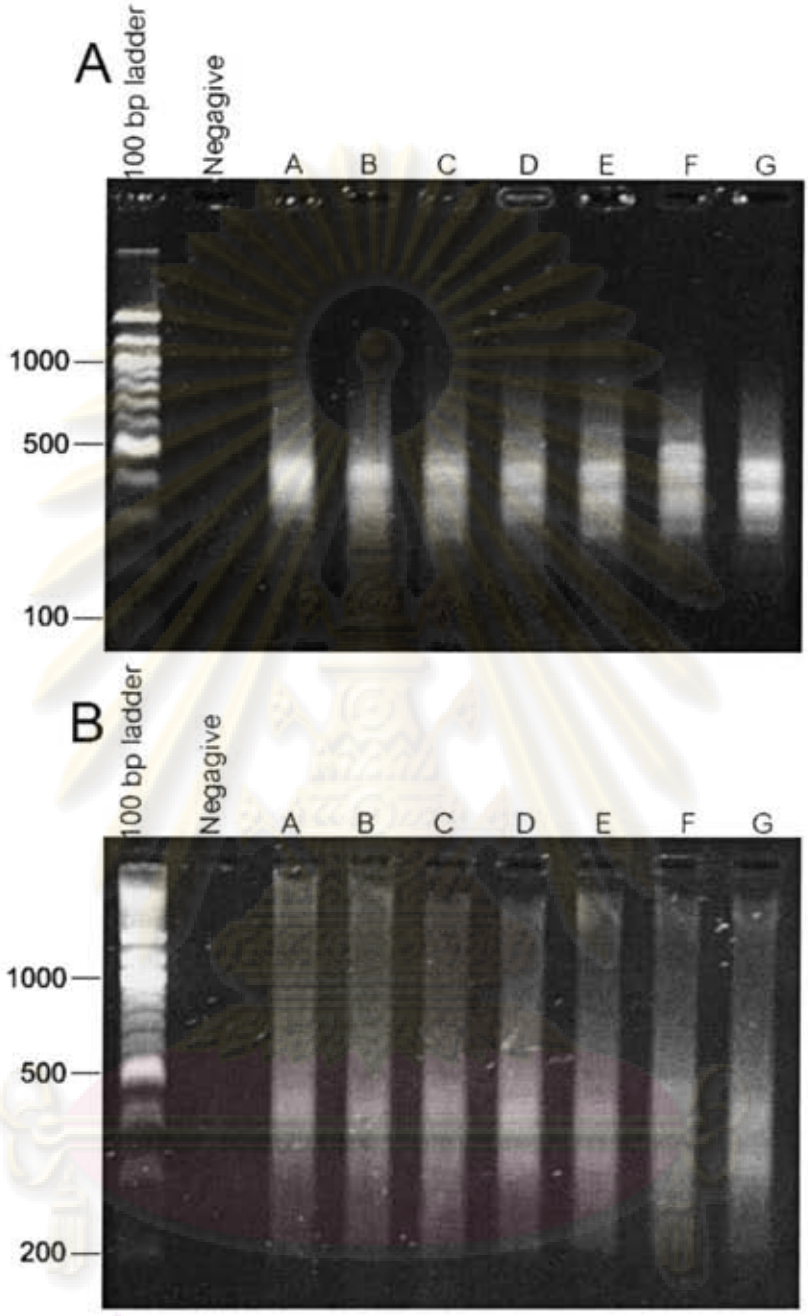


Figure10. **LINE-1 Mapping PCR Separated on Agarose Gel.** LINE-1 mapping PCR generated from PBMC DNA with difference combinations of restriction enzyme. a) Amplicons were run on 2% agarose gel electrophoresis. b) Amplicons were run on 3% agarose gel electrophoresis. A to G are combinations of restriction enzyme that showed in Table 2.

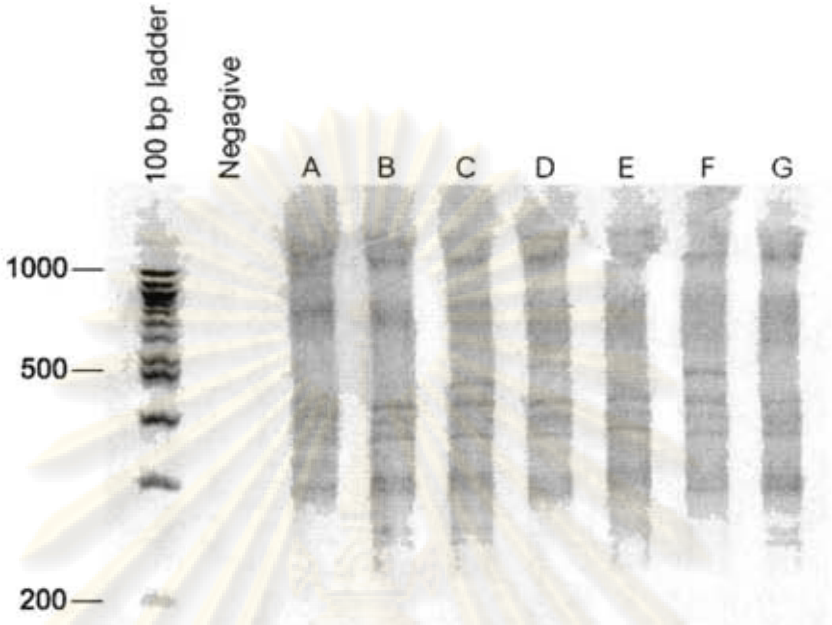


Figure 11. **LINE-1 Mapping PCR Separated on Polyacrylamide Gel.** LINE-1 mapping PCR generated from PBMC DNA with difference combinations of restriction enzyme. Amplicons were run on 5% acrylamide gel electrophoresis. A to G are combinations of restriction enzyme that showed in Table 2.

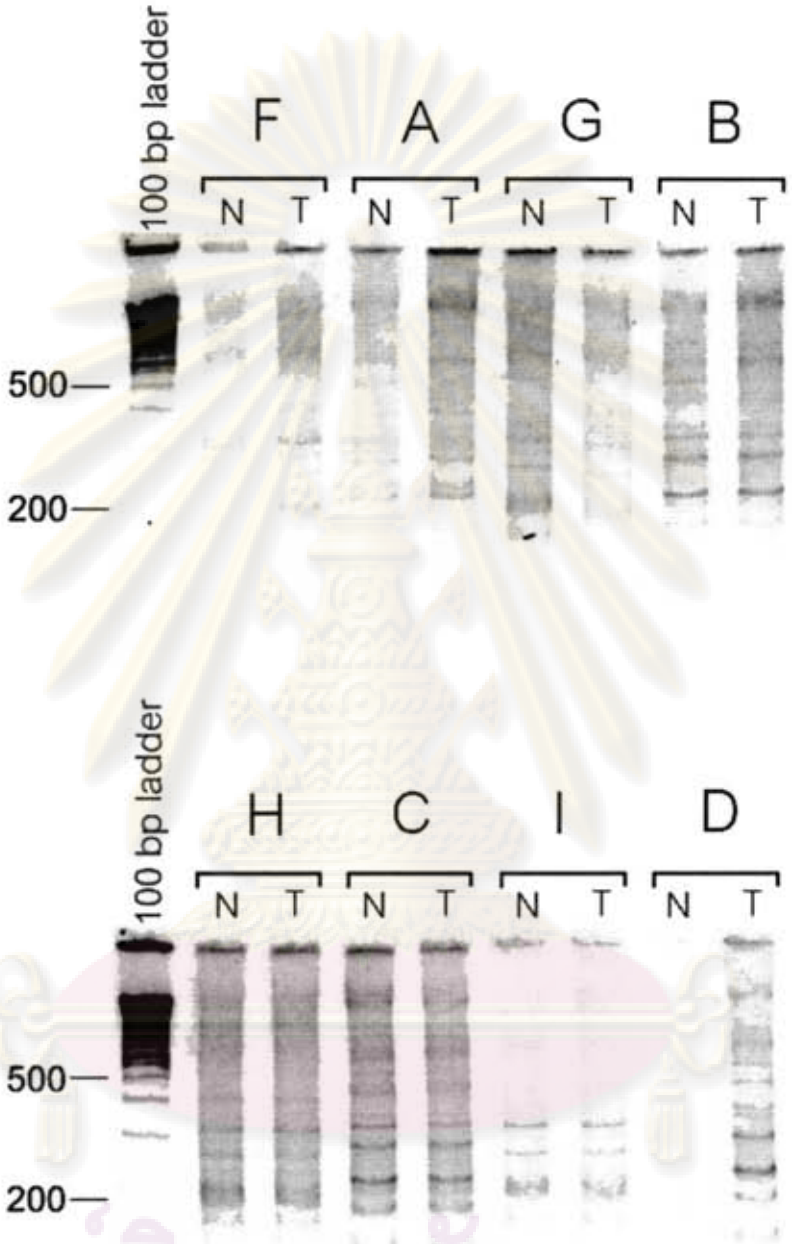


Figure 12. **LINE-1 Mapping Compare Between Cervical Cancer and Normal Sample from Patients.** LINE-1 mapping PCR generated from cervical cancer (T) and normal sample (N) from the same patient. A to G are combinations of restriction enzyme that showed in Table 2.

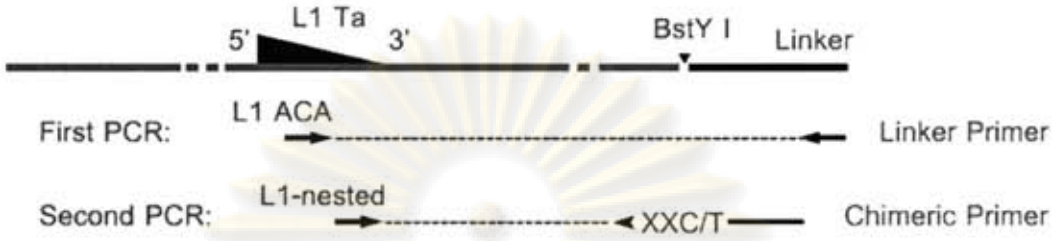


Figure 13. **LINE-1 Mapping Using Chimeric Primer Diagram.** A modified ligation-mediated PCR (LMPCR) linker (black line) was ligated to genomic DNA (grey line) at the *BstY* I site. The L1-Ta subset is illustrated as a black triangle. The dashed lines represent the first and nested products. The L1-ACA and L1-nested primers are 3' sequences of LINE-1. The linker primer is the 5' sequence of the linker. The chimeric primer is the 3'sequence of the linker and the restriction site plus a randomly unique sequence of two nucleotides, illustrated as X.

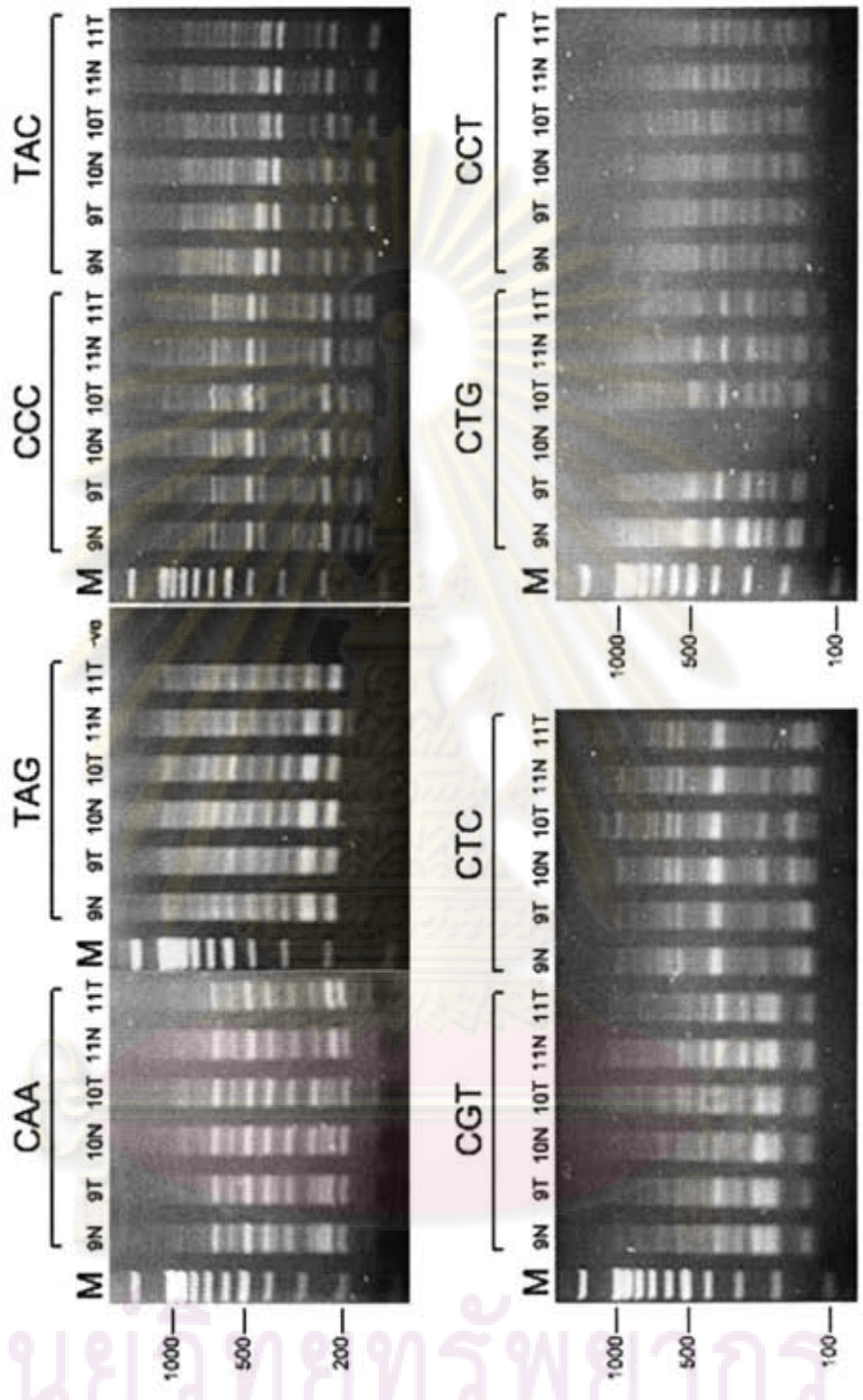


Figure 14. **LINE-1 Mapping in Cervical Cancer and Normal Using 32 Different Chimeric Primers.** DNA from tumor (T) and normal (N) from cervical cancer patients number 9, 10 and 11 were amplified using linker primer and L1-ACA then nested with L1-nested primer and chimeric primer. Different chimeric primers are indicated (e.g. CAA, TAG, CCC etc.). M: 100 bp ladder.

63

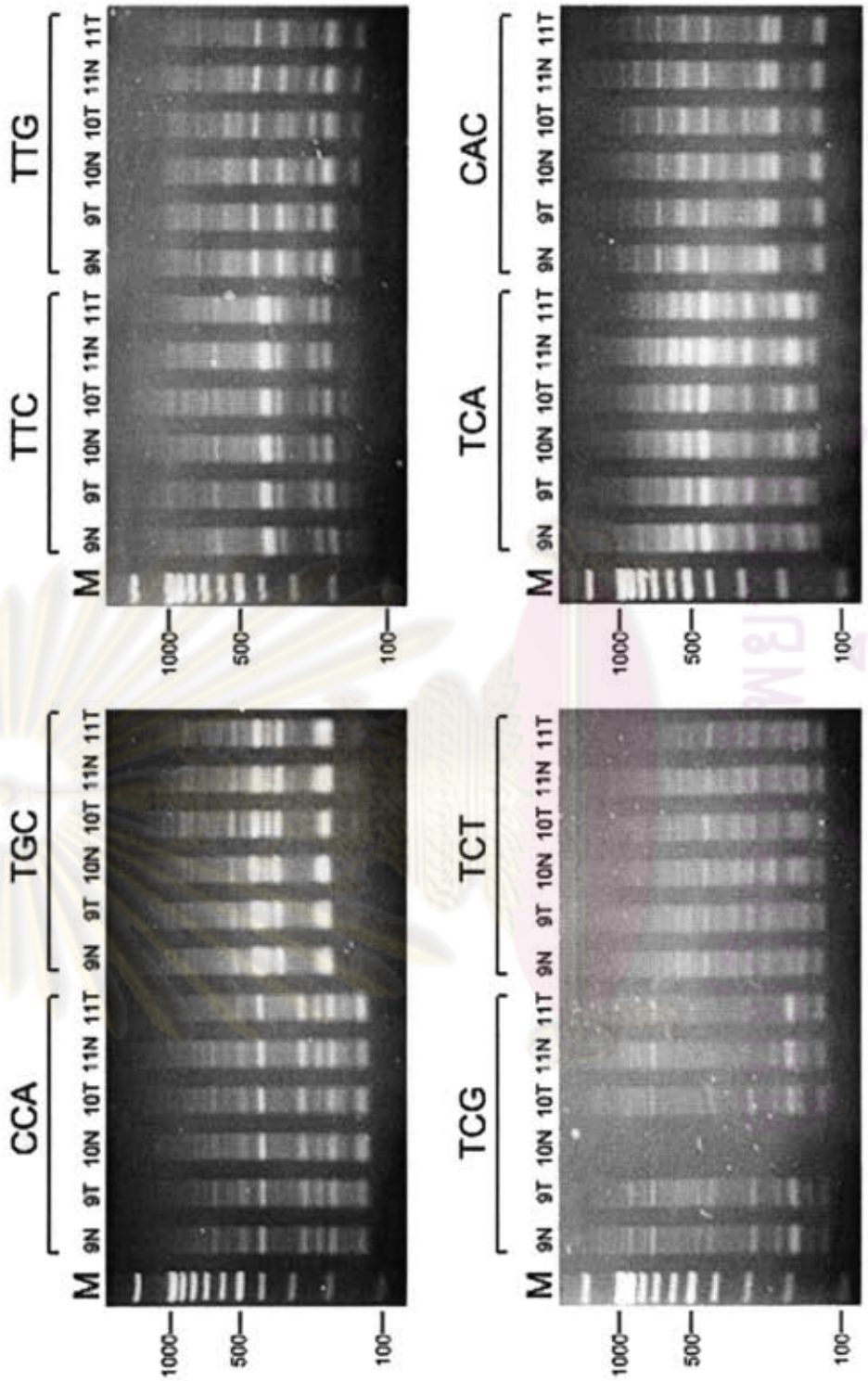


Figure 14. LINE-1 Mapping in Cervical Cancer and Normal Using 32 Different Chimeric Primers. (Continue)

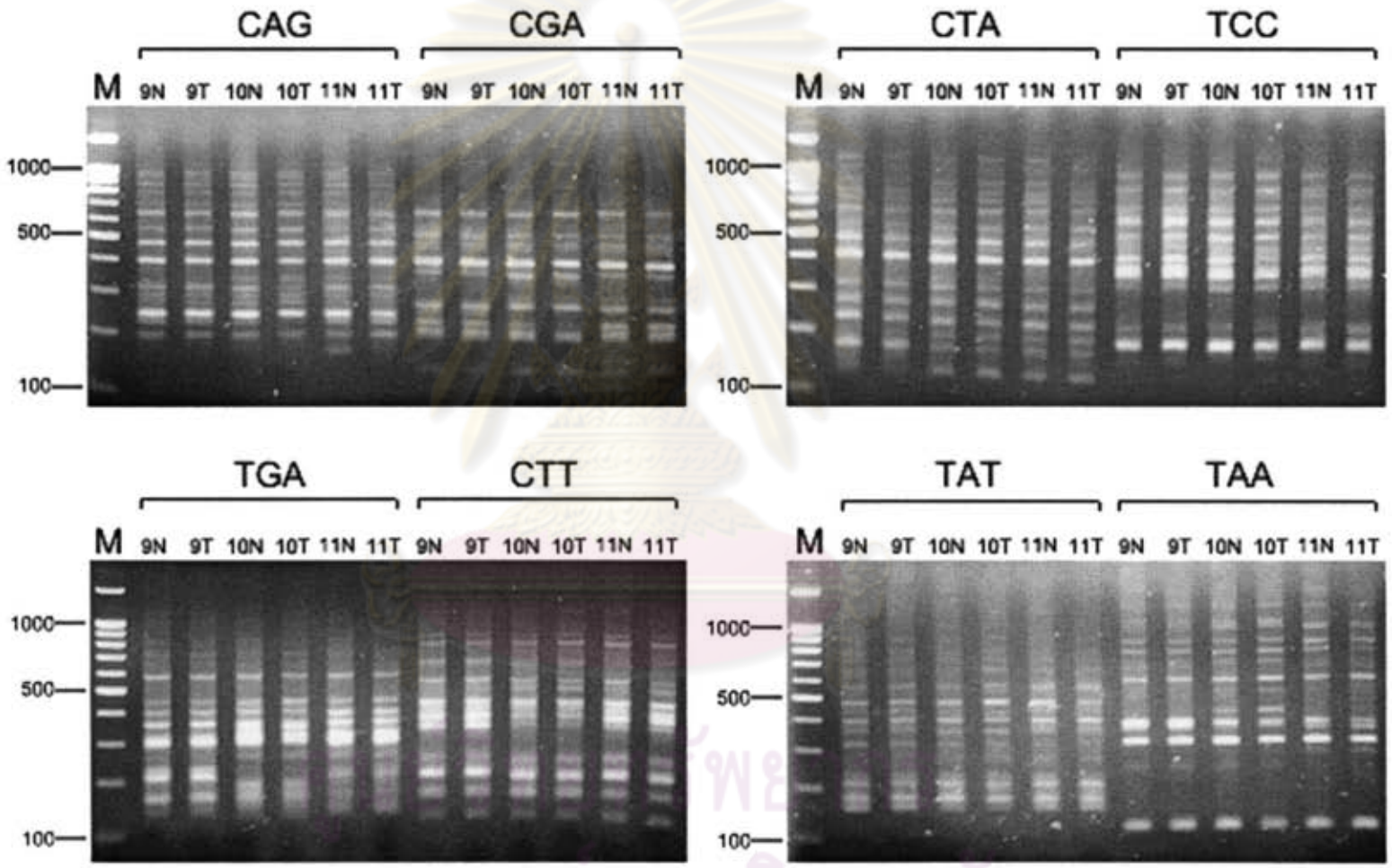


Figure 14. LINE-1 Mapping in Cervical Cancer and Normal Using 32 Different Chimeric Primers. (Continue)

65

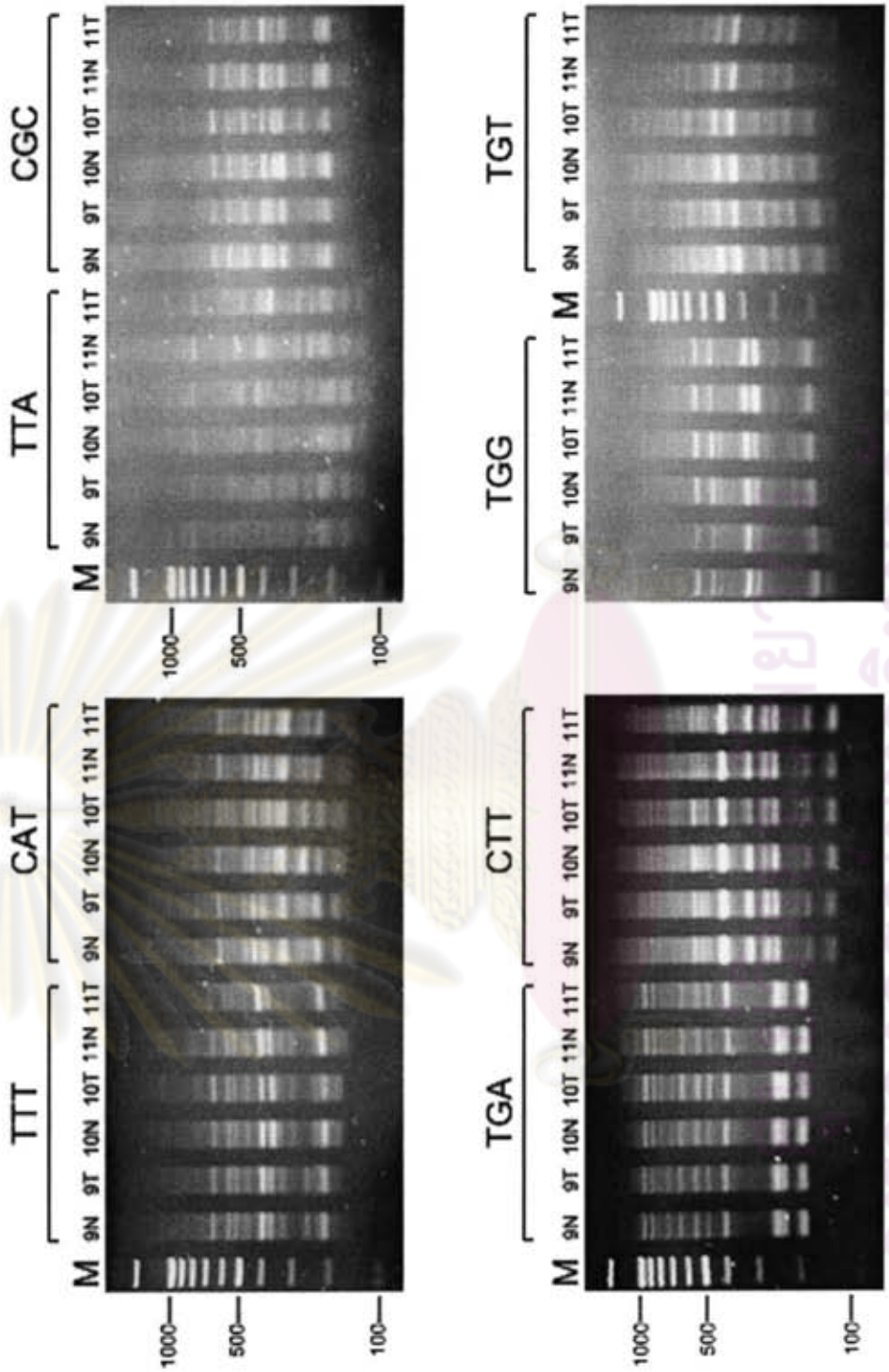


Figure 14. LINE-1 Mapping in Cervical Cancer and Normal Using 32 Different Chimeric Primers. (Continue)

Since cervical cancer is clonal in origin, these results may come from the clonal selection in multistep process during carcinogenesis because cell that LINE-1 inserted into a gene that not important for transformation will not be naturally selected from a mixed population. The other possibility is that cervical cancer may have LINE-1 movement after cells have already been transformed then different LINE-1 patterns can not be seen when we amplified LINE-1 in multiple cells. To prove this, we cultured HeLa cell line into six single colonies and then performed a single colony LINE-1 mapping with 32 chimeric primers (Figure 15). The result was LINE-1 movement can not be observed in 6 individuals HeLa single colony. We concluded that instability in cervical cancer does not involve *in trans* mechanism by LINE-1 movements from hypomethylation state of cancer genome during cervical cancer development.

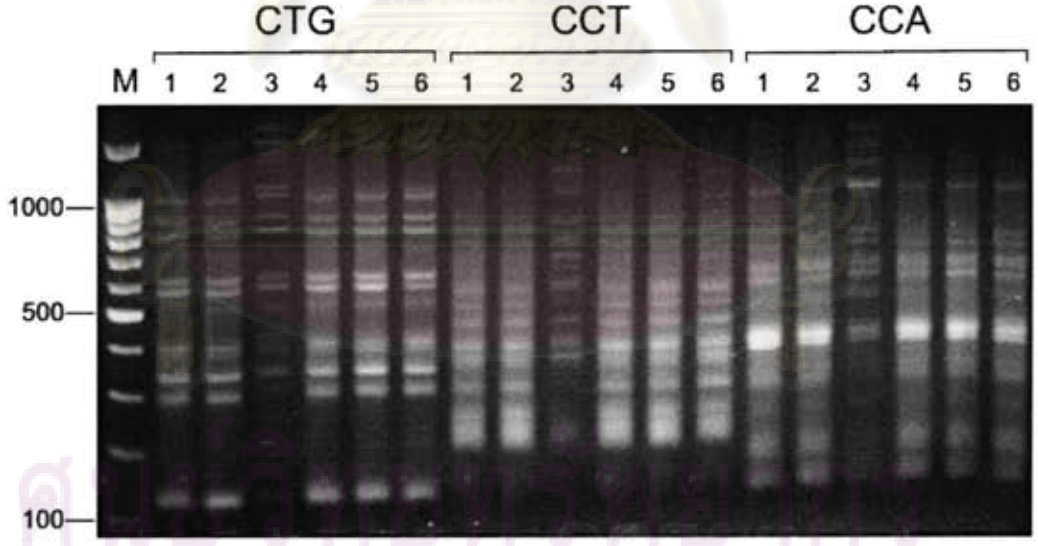


Figure 15. **The Examples of LINE-1 Single Colony Mapping.** HeLa cells were cultured into six single colony and then perform LINE-1 single colony mapping. 1 to 6 are single colony of HeLa clone 1 to 6 respectively. Different chimeric primers are indicated as CTG, CCT, and CCA. M: 100 bp ladder.

Although we did not find any LINE-1 movements between normal and tumor from LINE-1 mapping study in cervical cancers but LINE-1 insertion dimorphisms (LIDs), among individual samples were found by coincidence during LINE-1 mapping (Figure 16). LIDs are a polymorphism that can be distinguished among populations by the present or absent of LINE-1 at the same position in genome. Differentiating between LIDs is predominantly useful for studying in the retrotransposition and population genetics or evolution based on polymorphic markers where the ancestral state is known (i.e., absence of the insertion) (20, 28, 71, 156, 157). From this experiment, 37 candidate LIDs could be identified, and five of them were selected, cloned to the pGEM-T easy cloning vector, and transformed into *E. coli* DH5α. DNA sequencing was performed and results were analyzed using BLAST program. BLAST results from all clones showed no LINE-1 upstream of the flanking sequence and only the preintegration sites were actually present in the human genome database (GenBank® accession nos. AC015547 at 86,101 bp, AC087307 at 13,330 bp, AL392087 at 11,869 bp, AL583842 at 37,981 bp, and AP001996 at 37,981 bp), suggesting recent retrotranspositions. All LIDs were confirmed by PCR across insertion sites among DNA from normal samples. LIDPCR was revealed as two bands because of diploid genome with one allele insertion or one band if the insertion size is too large and when the amplification can not be achieved (Figure 17). The finding of LIDs with LINE-1 mapping technique overcomes the previous PCR technique from its simplicity and efficacy (156). We published the method to identify LIDs by PCR (LIDSIP) in *Biotechniques* (158).

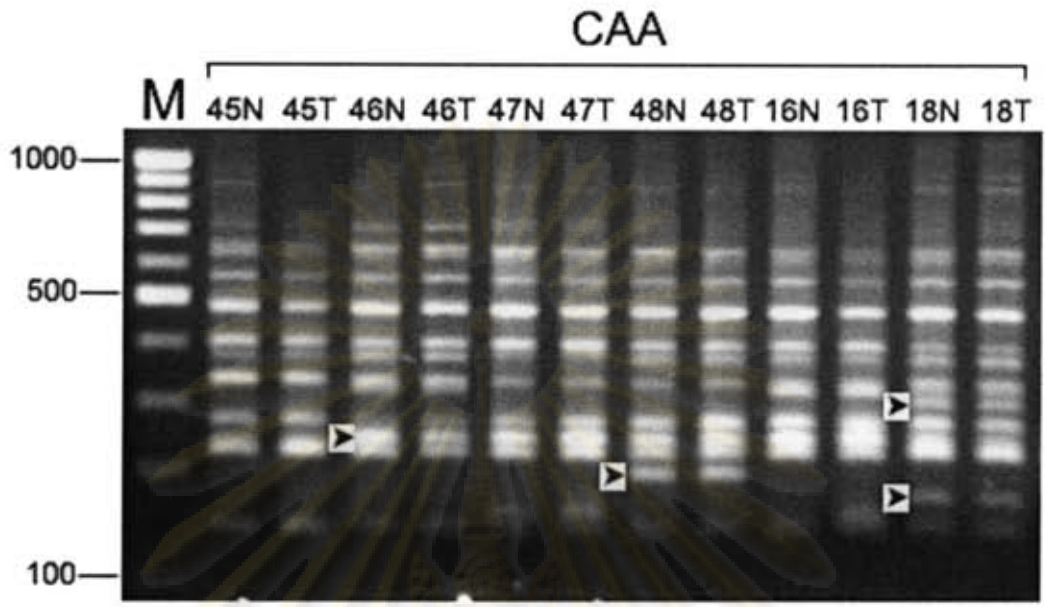


Figure 16. **LINE-1 Insertion Dimorphisms (LIDs) from LINE-1 Mapping in Cervical Cancer Patients.** LINE-1 mapping amplicons from six cervical cancer patients, each was identified by CCA chimeric primers. Candidate LIDs were indicated at the arrows. M is 100 bp ladder, N is normal and T is tumor.

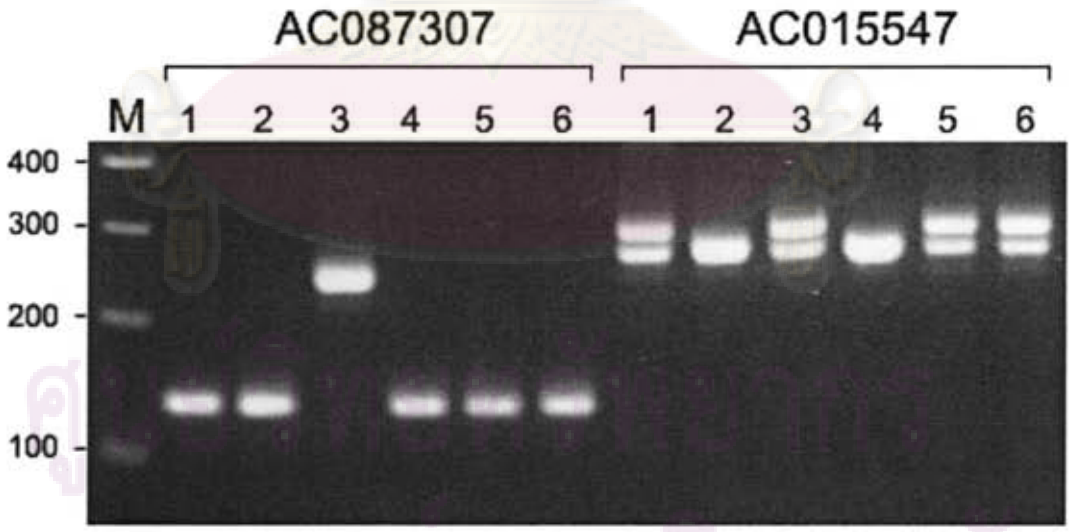


Figure 17. Confirmation of LINE-1 Insertion Dimorphisms (LIDs) by PCR. Two LID PCRs demonstrated polymorphisms among six different individuals, lane 1-6. The GenBank ® numbers were indicated. M is 100 bp ladder.

Cells Possess Different Amount of EDSB

Previous result of LINE-1 mapping show that global hypomethylation induces genomic instability is not associated with LINE-1 retrotransposition, we prove further whether genomic instability involves with EDSBs. Since both EDSBs and global hypomethylation have been proposed to cause CIN, which is crucial for cancer development process and LINE-1 can cause DNA recombination due its endonuclease activity during retrotransposition (5). In order to determine quantity of EDSBs, we developed a novel assay for the detection of EDSBs, called L1-EDSB-LMPCR. Locus-specific EDSBs can be detected using ligation-mediated polymerase chain reaction (LMPCR) (52), a commonly used PCR technique designed for the analysis of EDSBs during lymphoid development, such as V(D)J recombination and somatic hypermutation (159). Since general EDSBs are believed to occur rarely and randomly throughout the genome, repetitive sequences that widely intersperse in the human genome can be applied in a similar assay for the detection of EDSBs in their proximity, which would represent genome-wide EDSBs. Therefore, we combined LMPCR with interspersed repetitive sequence PCR (IRSPCR) (53) using LINE-1 (L1) retrotransposons (28) (L1-EDSB-LMPCR). In this assay, high molecular weight DNA were prepared and ligated with linker oligonucleotides then quantitatively analyzed by realtime PCR using an L1 primer, Linker primer and a Taqman probe complementary to the linker (Fig. 18). Figure 19 showed an example of results of L1-EDSB-LMPCR by using realtime PCR. It indicated that EDSBs could be quantitated by this technique. This novel technique had minimal intra-assay variations, but a larger range of inter-assay variations (Fig. 20). The quantity of EDSBs was not related to the proportion of fragmented cells (Fig. 21). While there were positive amplifications, we were unable to detect any apoptotic fragmented DNA or cells, as determined by LMPCR ladder (149) and flow cytometry (148), respectively. Furthermore, L1-EDSB levels did not differ relative to the directions of L1 primers used in experiment (Fig. 22). We evaluated EDSBs quantity whether which types of cells possess EDSBs and if the quantity of EDSBs reflects carcinogenic potency. Using L1-EDSB-LMPCR, significant amounts of EDSBs were determined in all samples from several cancer cell lines, including Daudi, Jurkat, Molt4, K562, SW480, and HeLa

cells, as well as in normal cells, including sperm and white blood cells (WBCs), from several individuals (Fig. 23). Our study from different cell types showed significantly different quantities of EDSBs but epithelial cells such as SW480 and HeLa cells possessed less EDSBs than hematopoietic cells including Daudi, Jurkat, Molt4 and K562 (Fig. 23).

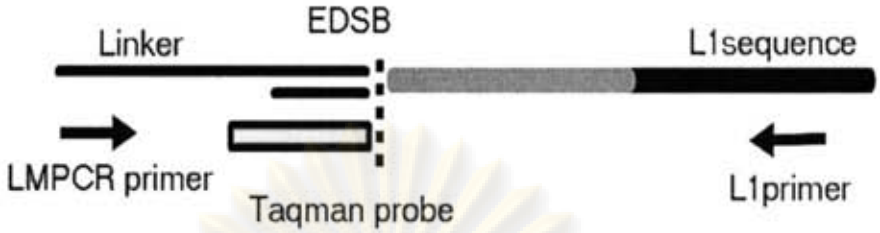


Figure 18. **Schematic Illustration of L1-EDSB-LMLPCR.** L1 sequence ligated by linker at EDSB. The white rectangle is Taqman probe complementary to LMPCR linker. Arrows are PCR primers.

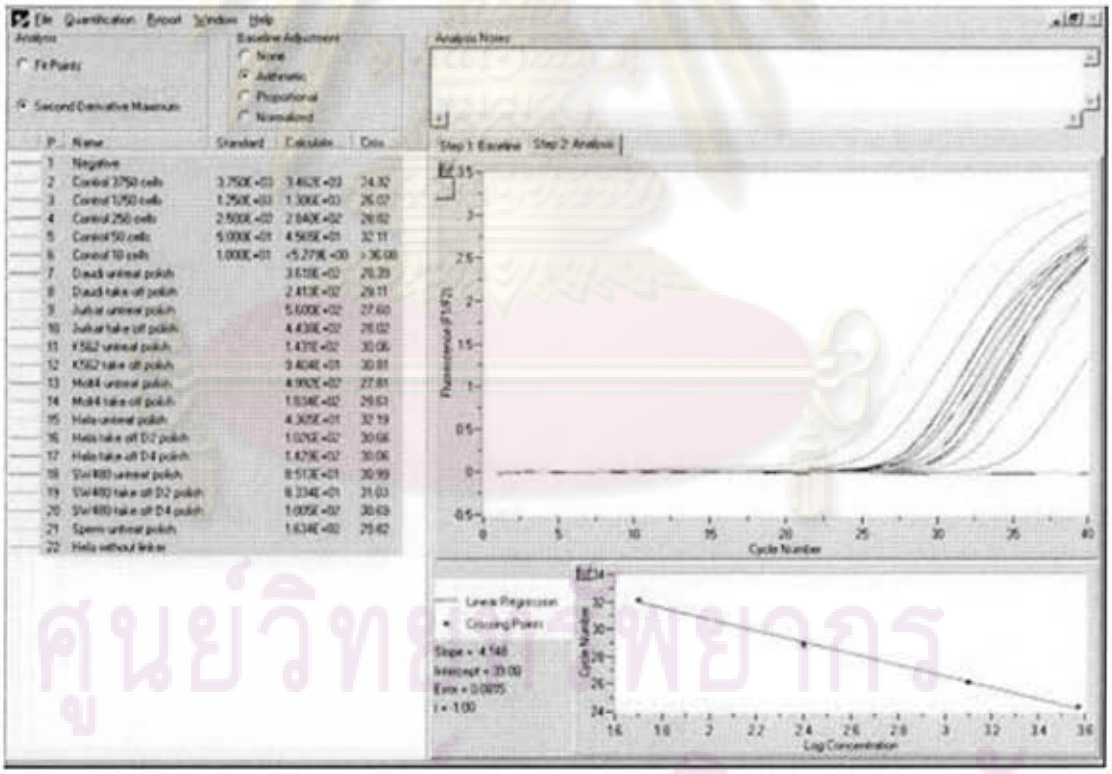


Figure 19. **The Example Results of L1-EDSB-LMPCR Using Realtime PCR.**

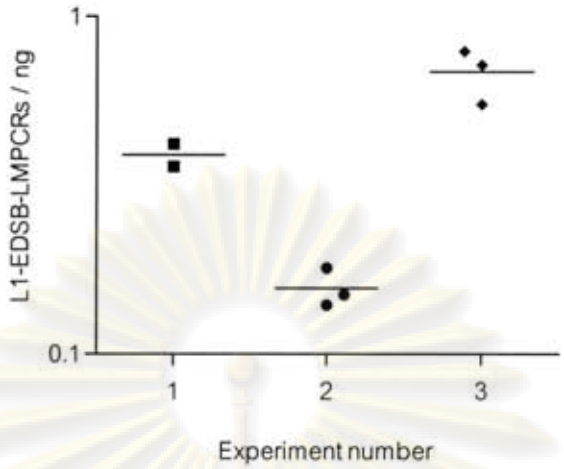


Figure 20. **Intra- and Inter-Assay Variation of L1-EDSB-LMPCR.** Experiment number 1, 2 and 3 indicates independent assays and each spot represents a different culture flask.

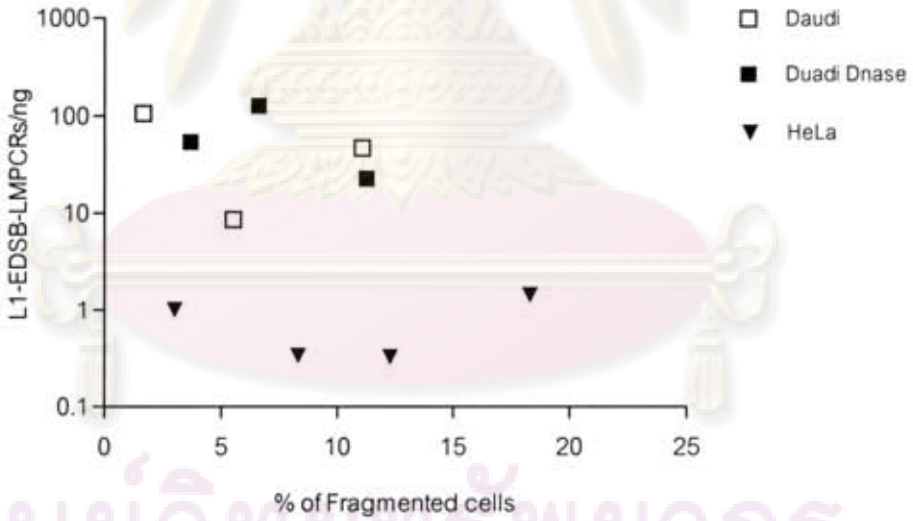


Figure 21. **L1-EDSB-LMPCR Quantities in Relation to % Fragmented Cells.** Each cell types were evaluated % fragmented cells represent cell death by flow cytometry. Daudi DNase represents Daudi cells that were treated with DNase I.

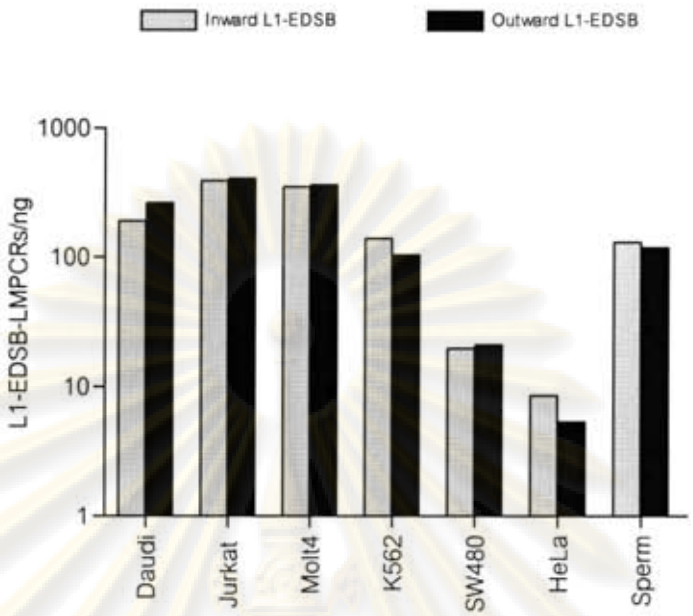


Figure 22. **L1-EDSB-LMPCR Quantities in Several Cell Types by Using Different Direction of L1 Primers.** L1 inward primer amplified inside L1 sequence while L1 outward primer amplifies outside. Inward L1-EDSB-LMPCR was normalized by the proportion of number of L1 oligonucleotide sequence copies in the human genome (www.ncbi.nih.gov).

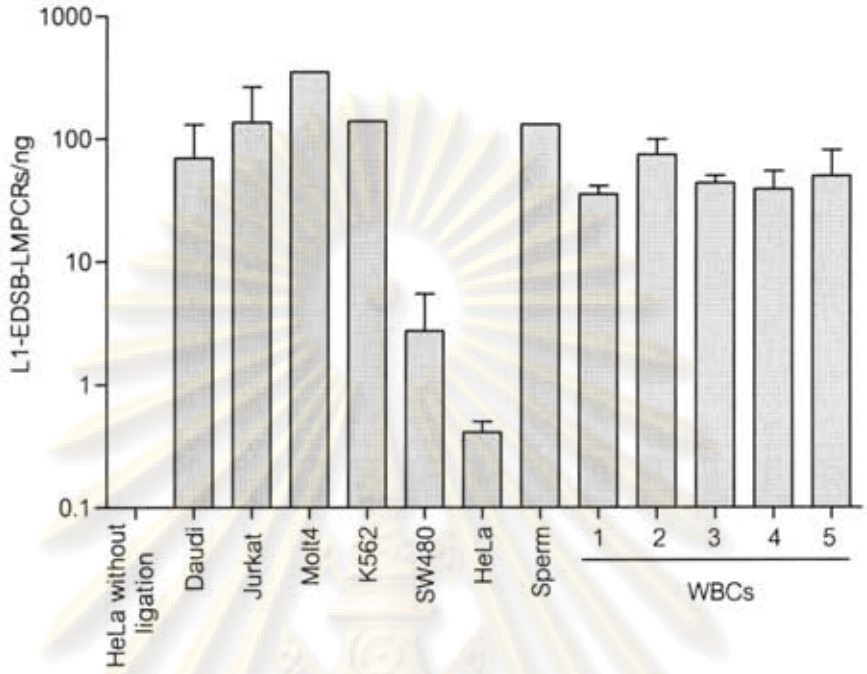


Figure 23. **L1-EDSB-LMPCR Quantities in Several Cell Types.** L1-EDSB-LMPCR was performed in cancer cell lines and normal cells including sperm cell and white blood cells (WBCs) from several individuals. HeLa without linker ligation was used as a negative control.

Methylated EDSB are Predominant

Methylation status of LINE-1s have been extensively studied in several cancers and normal tissues by PCR combined with bisulfite restriction analysis (COBRA) of LINE-1s (COBRA-L1) (4). To prove the hypothesis that genomic instability involves with EDSBs and global hypomethylation, we developed new techniques, called COBRA-L1-EDSB, to analyze the extent and methylation level of genomic EDSB to determine how DNA methylation affects EDSBs. By using COBRA-L1 and COBRA-L1-EDSB, we compared the percentage of methylation level between genomic DNA and EDSBs from matched-pair level of methylation between LINE-1 and LINE-1-EDSB sequences, COBRA-L1 was performed as previously described to quantify genome-wide methylation status (4). For COBRA-L1-EDSB, all ligated HMW DNA samples were chemically modified by bisulfite. Bisulfite treatment generates detectable methylation-dependent changes in the restriction pattern of PCR-amplified LINE-1 sequences from the conversion of unmethylated cytosines, but not methylated cytosines, to uracils and then thymines after PCR. After amplification, the amplicons of bisulfite 5'LINE-1 sequences were digested with restriction enzymes, *Taq*I and *Tas*I. Methylated LINE-1 sequences can be detected by digestion amplicons with *Taq*I while nonmethylated LINE-1 sequences can be detected by digestion amplicons with *Tas*I. The percentage of amplicons digested with *Taq*I was measured as COBRA-L1 methylation level by radiation intensity using ^{32}P 5'-labeled-bisulfite-L1-outward, B-L1-outward. For COBRA-L1-EDSB, the same protocol was adopted but the B-L1-inward primer was replaced by a linker primer, B-LMPCR (Fig. 24). Figure 25 showed an example results from COBRA-L1 and COBRA-L1-EDSB measuring radiation intensity. Similar to L1-EDSB-LMPCR, this technique had minimal intra-assay variations, but a larger range of inter-assay variations (Fig. 26). Although fluctuation of EDSB methylation status was observed among different experiments, EDSBs were hypermethylated across all tested cells, including several cancer cell lines, sperm and WBCs (Fig. 27). This result indicated that EDSBs were associated with DNA methylation.

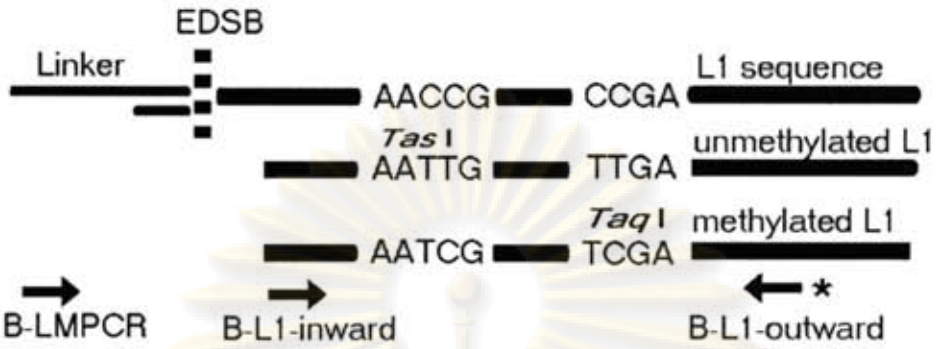


Figure 24. **Schematic Illustration of COBRA-L1 and COBRA-L1-EDSB.** L1 sequence ligated by linker at EDSB. Arrows are PCR primers, with star indicating 5' labeled primer with ^{32}P for COBRA. AACCG and CCGA are L1 sequences; when treated with bisulfite and PCR, unmethylated AACCG will be converted to AATTG (*Tas*I site) and methylated CCGA to TCGA (*Taq*I site).

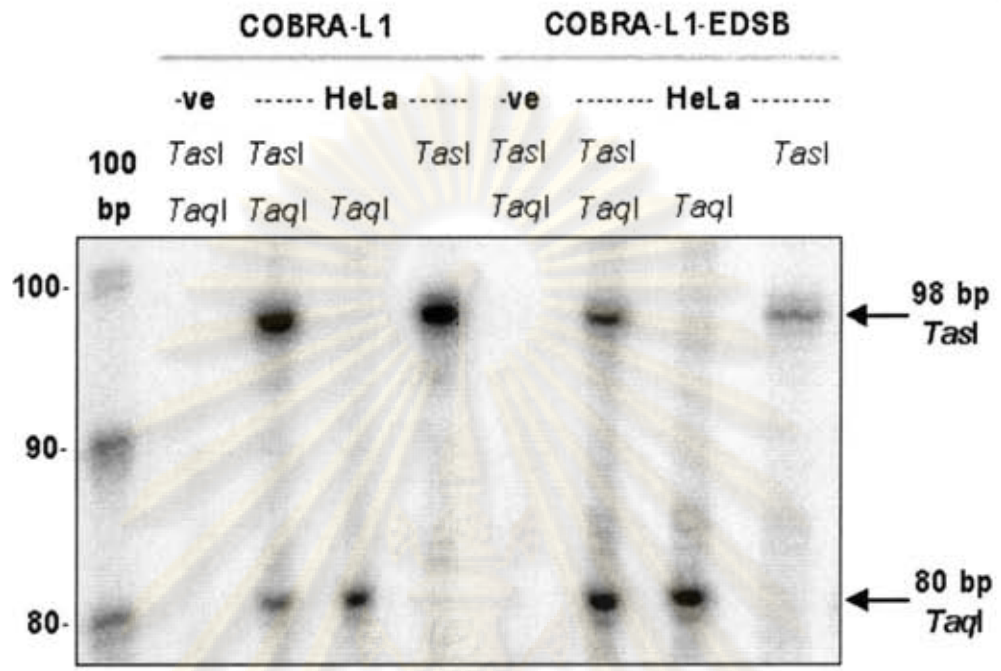


Figure 25. **A Typical Example of Results form COBRA-L1 and COBRA-L1-EDSB Experiments.** The arrow at 98 bp indicates *Tas*I digested unmethylated L1 sequences and the arrow at 80 bp indicates *Taq*I digested methylated L1 sequences –ve is dH_2O for COBRA-L1 and nonligated HMW DNA for COBRA-L1-EDSB. *Tas*I and *Taq*I are enzymes added in each experiment.

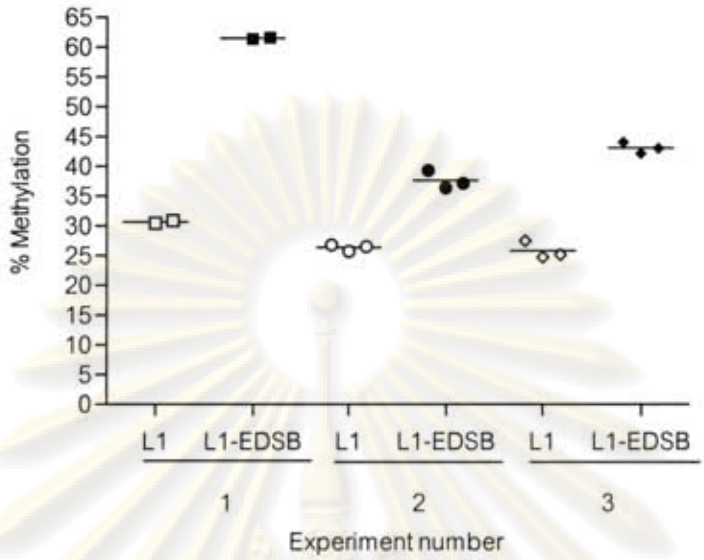


Figure 26. **Intra-and inter-assay variation of COBRA-L1 (L1) and COBRA-L1-EDSB (L1-EDSB).** Experiment number 1, 2 and 3 indicated independent assays and each spot represents a different culture flask.

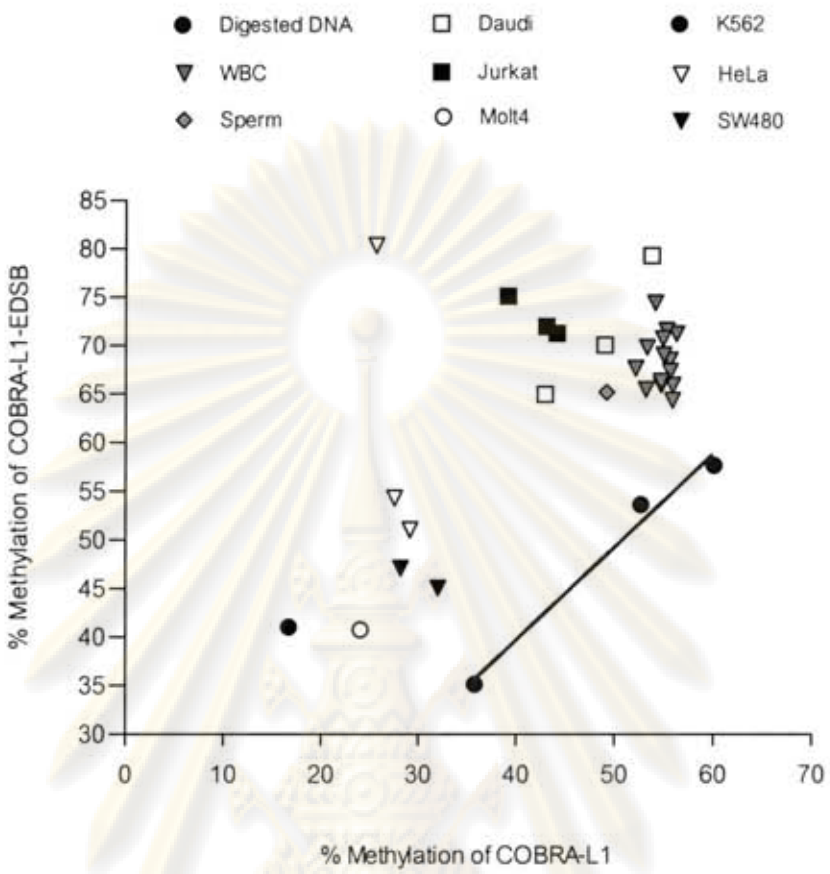


Figure 27. **COBRA-L1 and COBRA-L1-EDSB Comparison among Cell Types.** Digested HeLa, Jurkat and Daudi DNA were digested with AluI and EcoRV as controls.

Methylated EDSBs were DNA Replication-Independent

Interestingly, our study from COBRA-L1-EDSB found the majority of EDSBs were methylated when compare with methylation level in genome. Therefore, we hypothesized that hypermethylated EDSBs may arise from two possible causes; either a higher rate of EDSB production from methylated DNA or a lower rate of repair in methylated EDSB. To distinguish between these two hypotheses, we first examined EDSB production. Since EDSBs were proposed to be preferentially produced in S phase from the conversion of single strand lesions (13), we assessed the amount of EDSBs and their methylation status in several cell cycle phases, G0, G1/S and S, in HeLa cells. Before HeLa cells were measured EDSB level by L1-EDSB-LMPCR and methylation status by COBRA-L1 and COBRA-L1-EDSB, they were synchronized in G1/S and S phase by thymidine block and in G0 phase by serum deprivation. For S phase, we observed EDSB quantity and methylation status in both early (S 3h) and late (S 5h) S phase. Our results demonstrated that HeLa cells in G0 phase possess the least EDSBs when compare with active replications and showed statistical significance (Fig. 28). In addition, we observed that EDSBs were hypermethylated in most examined cell phases. Especially, G0 phase illustrated the most statistical significance (Fig. 29). These data suggested that hypermethylation of EDSBs was not associated with DNA replication and the level of methylated EDSB should not be positively influenced by the production of EDSBs during DNA replication.

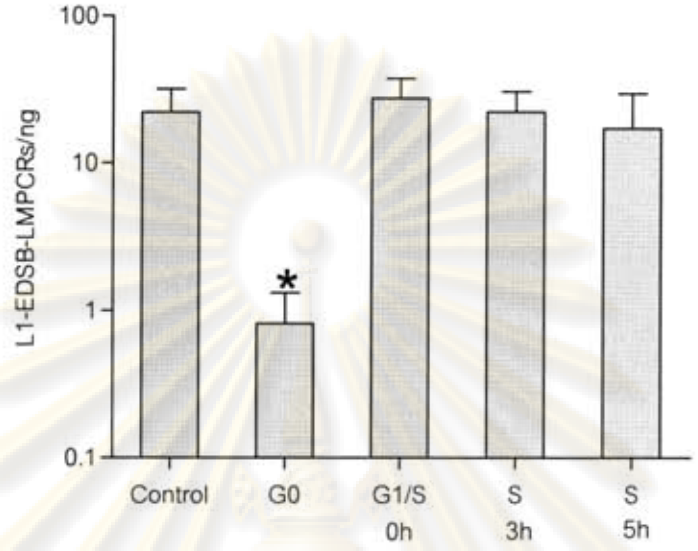


Figure 28. **EDSBs Quantities from HeLa with Cell Synchronization.** L1-EDSB-LMPCR quantities from HeLa cells at G0, G1/S at 0h, and S phases at 3 and 5 h after the release into S phase form thymidine block. Control is HeLa cells without cell synchronization. Data represents means ±SEM. * indicates $P<0.05$ (independent 1-taliled *t*-test).

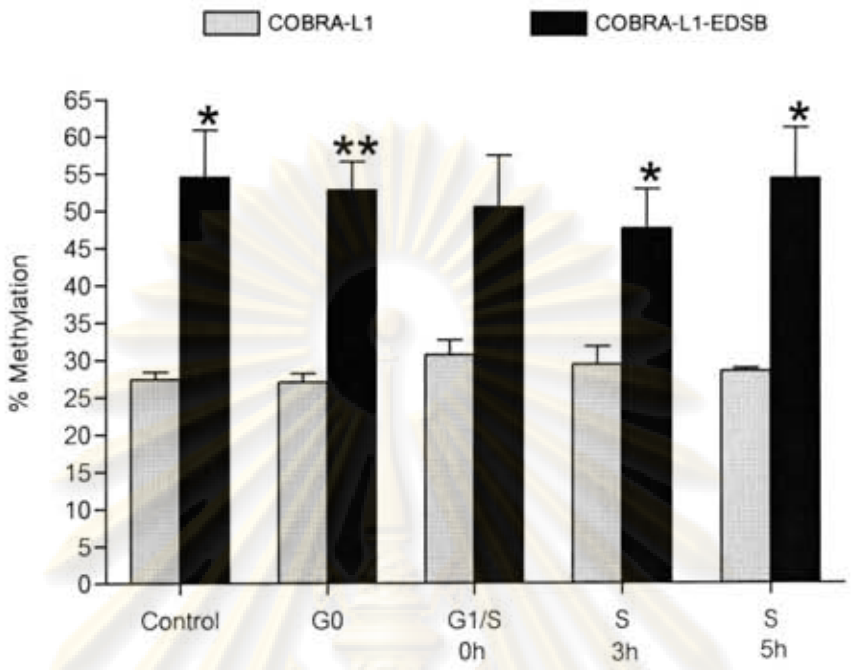


Figure 29. **Comparation of Methylation Levels Between Genome and EDSB.** COBRA-L1 and COBRA-L1-EDSB was performed using HeLa cells in several cell cycles. Control is HeLa cells without cell synchronization. Data represents means ±SEM. * indicates $P<0.05$ (independent 1-taliled *t*-test).

NHEJ Preferentially Repair Nonmethylated EDSBs

Our previous experiment showed that most EDSBs are methylated under normal physiologic circumstances, and not associated with EDSB production so that we hypothesized whether methylated EDSBs may be repaired at lower rate when compare to unmethylated EDSBs. NHEJ is thought to repair the majority of DSBs and involves error-prone relegation of the two broken DNA ends (135). Recently, a sub-pathway of precise NHEJ that can repair DSBs with high fidelity has been proposed (160, 161). While the DNA-PKcs, a phosphatidylinositol-3-kinase, is required for general NHEJ, ATM acts jointly with checkpoint kinase 2 (CDK2) and BRCA1 in controlling the fidelity of DNA end-joining by precise NHEJ (160). We analyzed EDSB methylation level in cells chemically or genetically deprived of DNA-PKcs and ATM. First, HeLa cells were incubated for 24 hours with 2.5 mM Vanillin, an inhibitor of phosphatidylinositol-3-kinase selective for DNA-PKcs. We observed sporadic accumulation of hypomethylated EDSBs when the DNA-PKcs mediated repair of *de novo* EDSBs is inhibited (Figure 30). In contrast, EDSB methylation levels remarkably increased when ATM was genetically deprived by stably transfecting siRNA (Figure 31). The mechanism decreasing EDSB levels of control siRNA is not known. Nevertheless, this interference is not lesser our conclusion that there is the significant influence of decreased ATM in increasing methylation level of EDSBs. Stably transfection of DNA-PKcs siRNA in HeLa cells causes down-regulation not only DNA-PKcs but also ATM (Figure 32) as has previous been observed (162). EDSB methylation levels of DNA-PKcs siRNA cells were significantly lower than that of ATM siRNA cells, especially in G0 phase, as if the loss of DNA-PKcs compensated the influence of ATM deficiency on the methylation level of accumulated EDSBs (Figure 31). These results suggest that DNA-PKcs is more important in the repair of unmethylated EDSBs whereas ATM is more important for methylated DNA.

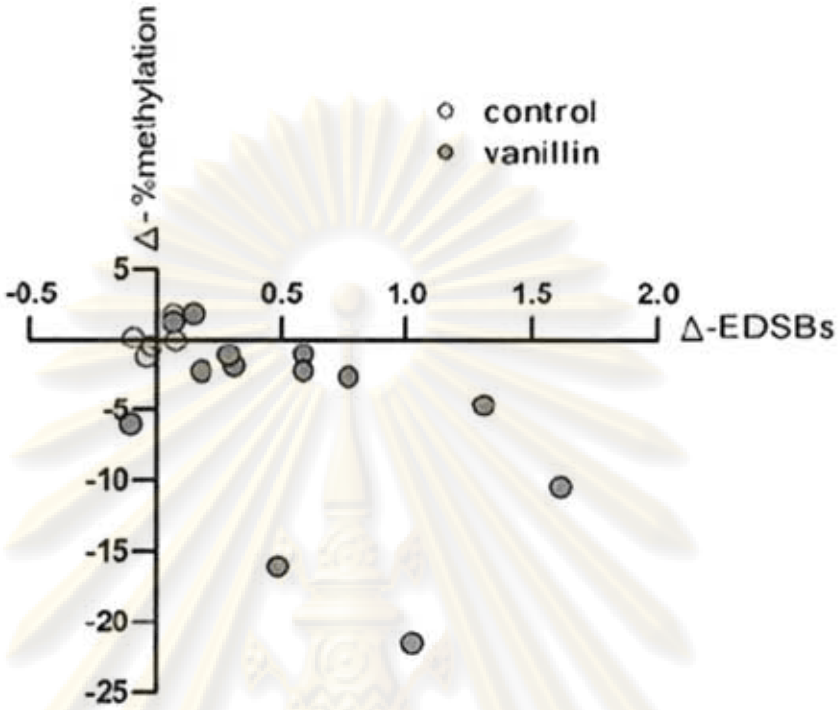


Figure 30. **Changes in the Quantity and Methylation Level of EDSBs after Incubation with Vanillin.** L1-EDSB-LMPCR and COBRA-L1-EDSB from HeLa with chemically inhibit DNA-PKcs. The Δ-EDSBs axis and the Δ-%methylation axis are values of EDSB quantity and methylation level of vanillin treated cells subtracted by the means of mock experiments, respectively.

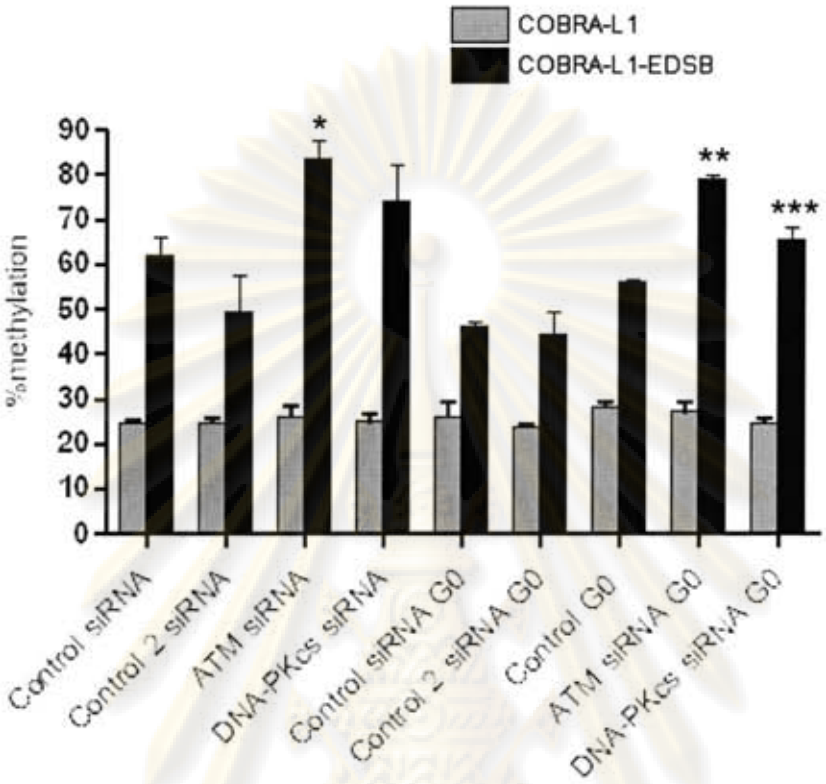


Figure 31. **Inhibition of DNA-PKcs Leads to Accumulation of Unmethylated EDSBs while Downregulation of ATM Increases Methylation Level of EDSBs.** COBRA-L1 and COBRA-L1-EDSB of stable DNA-PKcs and ATM siRNA transfected HeLa cells. Control siRNA and control2 siRNA are nonspecific siRNA transfected in 2 different experiments. Control is HeLa without transfection. Data represent means ±SEM, with statistical significance determined by paired 2-tailed t-test, *P<0.05, **P<0.001 when compared with control, and ***P<0.01 with ATM siRNA G0.

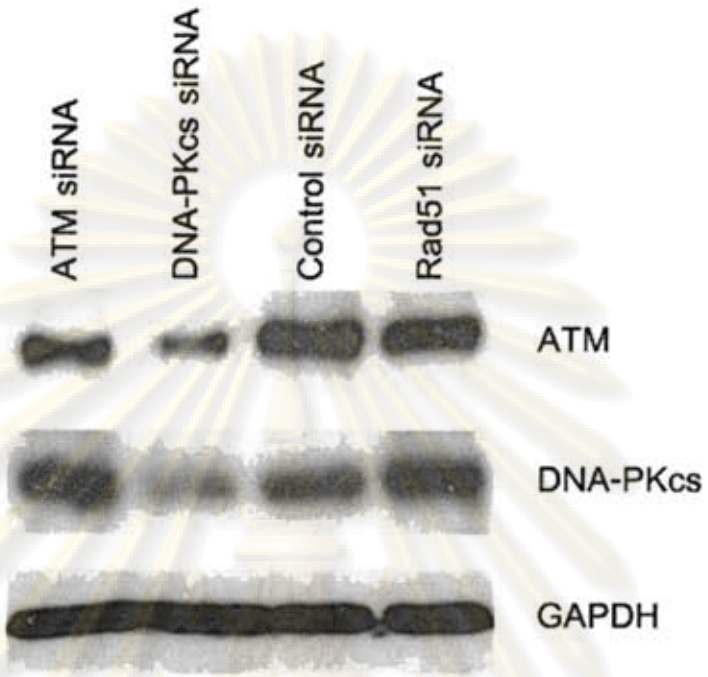


Figure 32. **Western Blotting of DNA-PKcs and ATM in HeLa Cells Transfected with siRNA.** The analyses of DNA-PKcs and ATM expression in HeLa cells transfected with DNA-PKcs-, ATM-specific siRNAs constructs or nonspecific siRNA (control and Rad51) vector.

CHAPTER V
DISCUSSION

Global hypomethylation leads to chromosome instability, characterized by a higher rate of chromosome mutations and DNA deletion (10-12). This study determined whether the global hypomethylation and genomic instability are associated by activation of LINE-1 or how EDSBs are occurred or repaired. Our study discovered, 1) retrotransposition of LINE-1 from global hypomethylation may not be a major cause of instability in cancer. 2) DNA breakages are common event under normal physiologic condition and the quantity of EDSBs is cell types specific. 3) the majority of EDSBs are methylated and differentially repaired depending on DNA methylation status. Furthermore, we established a technique to identify LIDs.

LINE-1 retrotransposition is not a major cause of instability

Reactivation of retrotransposon is proposed to be one of the genomic instability consequences from global hypomethylation. We developed a technique to characterize active LINE-1 genomic distribution patterns and aimed to characterize if there are new LINE-1 insertion in cervical cancer. It is possible that there are LINE-1 movement during cancer development since previous studies of LINE-1 disruption gene in colon and breast cancers (47, 48). However, there is no information how extensive LINE-1 retrotransposition would occur in cancer. My Thesis demonstrated no different LINE-1 patterns were found among 23 cervical cancer patients. Therefore, LINE-1 retrotransposition may not be the important event in genes that crucial for tumor transformation, especially mutations tumor suppressor genes or oncogenes. Consequently, there is no selective clonal expansion as a consequence of LINE-1 retrotransposition. This study, however, can not conclude that there is no LINE-1 retrotransposition in cancer. If retrotransposition occur randomly without initiating crucial mutation in cancer, the mutations will not be remained. Consequently study LIDs in cervical should still give negative result. Since previous experiment showed genetic engineer LINE-1 can retrotransposition in a cervical cancer cell line (153), there are still a possibility that natural LINE-1 movement occurs in cancer. From these reasons, we

mapped LINE-1 in HeLa single colonies. However, we found that distinguished LINE-1 patterns can not be observed from 6 individuals HeLa single colony. Our results may imply that LINE-1 retrotransposition in cancer is an infrequent process and instability of cancer may not directly involve *in trans* by LINE-1 retrotransposition from hypomethylation state during carcinogenesis.

Even though our LIDSIP did not identify LINE-1 movement in cancer, the technique can be applied to population genetic study. Differentiating between recent LINE-1 (L1) insertion dimorphisms (LIDs) is predominantly useful for studying not only the transposition mechanism but also population genetics or evolution based on polymorphic markers where the ancestral state is known (i.e., absence of the insertion) (20, 28, 71, 156, 157). Previous techniques such as L1 display (156) or ATLAS (157) have identified new LIDs from distinct populations. To improve both efficiency and simplicity, we designed a PCR-based approach, LID identification by PCR (LIDSIP), by combining and modifying LMPCR (52) and IRSPCR (53). This method requires only a small amount of DNA, a limited and specific number of PCRs to provide a genome-wide scan within a PCR range of an appropriate restriction site, conventional molecular genetic techniques such as agarose gel electrophoresis devoid of radioactive label, and in addition, this method yields clear, easily distinguishable, specific results.

Applying LIDSIP has improved both simplicity and efficacy compared with the two previous PCR techniques. The first, L1 display (156), screens for LIDs by PCR using arbitary primers, Southern blot analysis, and hybridization for identification. Each PCR yields a limited number of products. Therefore, to cover the whole human genome, the technique requires both a significant amount of DNA and number of PCRs. Both ATLAS and LIDSIP apply the same principle of single-site PCR and yield more sequences depending on restriction enzyme sites next to L1. Because of the large number of size-variable templates, with ATLAS, those products have to be labeled with radioactive [γ-^{33}P]ATP and separated by denaturing long-range polyacrylamide gel electrophoresis. With LIDSIP, radioactive labeling is not required, and gel recovery is greatly facilitated. Hence, by dividing L1 products into several subsets with nested PCR using chimeric

primers, size differentiation by agarose gel electrophoresis becomes feasible. Additionally, LIDSIP is able to limit utilization of the original DNA by the nested PCR when reproducing LID recovery is needed. The simplicity of LIDSIP will not only globally expand the discovery rate, but also, by adjusting restriction enzymes and nested chimeric primers, the methodological principle of S-SAP can be modified to identify polymorphic genome insertions of other mobile elements (155).

EDSBs are commonly methylated and their levels are cell type specific

We showed that EDSBs can be measured both quantity and methylation status from cells by our novel techniques, L1-EDSB-LMPCR and COBRA-L1-EDSB respectively. EDSB quantities are different among cell types, in which epithelial cells possessed significantly less amount than those of hematopoietic cells. This suggests that the quantity of EDSBs is not directly associated with carcinogenesis but is influenced by cellular physiologic process. These physiologic EDSBs are important to maintain dynamic chromosome integrity, increment or decrement of EDSBs may affect chromosome integrity and result to abnormal chromosomal rearrangement. We evaluated methylation status of EDSB and found that methylated EDSBs are predominant in all tested cells including normal. Although DSBs are hazardous to cells, leading to a complete loss of function of the broken genes and faulty DNA recombination, it is not surprising that the majority of EDSBs are methylated because DSBs in methylated DNA should be less harmful due to methylated genome is usually brace with heterochromatin and consequently prevented from recombination errors (163).

Cell repaired EDSBs depending on methylation status

Our experiments provide a new explanation for the common finding that in many cancers there is a direct correlation between loss of DNA methylation, global hypomethylation, and increasing rate of de novo mutations, genetic instability, *in cis* (12, 129, 164). This study concludes that methylated and unmethylated EDSBs are not processed at equal efficiency. Therefore mutation hot spots should be present in relation

to genomic methylation level. Methylated EDSBs are retained in heterochromatin, concealed from the early DSB repair response, and repaired by ATM dependent NHEJ pathway. On the contrary, unmethylated EDSBs are not retained and may less depend on ATM. Interestingly, in contrast to general NHEJ and B-NHEJ pathways (135, 165), ATM dependent repair pathway has been proposed to be more precise (160). Therefore, methylated EDSBs may be able to limit DNA repair errors. Consequently, the rate of spontaneous mutations may be limited. This, therefore, could be a reason why more spontaneous mutations arise in hypomethylated genomes.

The discovery of methylated EDSBs in all cell phases is not surprising. Although DSBs are hazardous to cells, leading to faulty DNA recombination, DSBs in methylated DNA should be less harmful. This is because DNA methylation usually associates with heterochromatin (150), whose tightly packed structure may brace the broken chromosome and hide EDSB ends from random recombination. In S phase, EDSBs are still hypermethylated albeit with less significance than G0. Since origins of replication in euchromatin usually fire earlier than those in heterochromatin (166), the majority of new EDSBs from the conversion of SSLs during early S phase may locate in euchromatin and thus are less methylated. This when combined with the methylation level of EDSBs retained in heterochromatin, could result in the observed lower methylation of total EDSBs in S phase. Furthermore, because DNA replication does not occur simultaneously throughout the genome, heterochromatin may still capture the methylated EDSBs that locate away from replication forks.

Genomic instability is a cardinal feature of cancer (32). Understanding the molecular mechanism involved in how it arises would be essential for the development of effective approaches in cancer prevention (167) and treatment to prevent cancer progression (32). This study proposes how genomic hypomethylation causes instability could be linked to differential biological processes of EDSBs depending on the methylation statuses. These finding may be crucial for the identification of molecular targets that can prevent hypomethylation-related spontaneous mutations and cancer.

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APPENDICES

APPENDIX A

BUFFER AND REAGENT

1. Lysis I buffer

Sucrose	109.54	g
1 M Tris-HCl (pH 7.5)	10	ml
1M $MgCl_2$	5	ml
Triton X-100	10	ml
Distilled water to volume	1,000	ml

Sterilize the solution by autoclaving and store at 4°C.

2. Lysis II buffer

5 M NaCl	15	ml
0.5 M EDTA (pH 8.0)	48	ml
Distilled water to volume	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

3. 10% SDS solution

Sodium dodecyl sulfate	10	g
Distilled water to volume	100	ml

Mix the solution and store at room temperature.

4. Proteinase K

Proteinase K	20	mg
Distilled water to volume	1	ml

Mix the solution and store at -20°C.

5. 1 M Tris-HCl (pH 7.5)

Tris base	12.11	g

Dissolve in distilled water and adjusted pH to 7.5 with HCl (conc)

Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

6. 0.5 M EDTA (pH 8.0)

Disodium ethyleneidamine tetraacetate	18.66	g

Dissolve in distilled water and adjusted pH to 8.0 with NaOH

Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

7. 1 M $MgCl_2$

$MgCl_2.6H_2O$	20.33	g
Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

8. 5 M NaCl

NaCl	29.25	g
Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

9. 10X TBE buffer

Tris-base	108	g
Boric acid	55	g
0.5 M EDTA (pH 8.0)	40	ml
Distilled water to volume	1,000	ml

Mix the solution and store at room temperature.

10. 6X loading dye

Ficoll 400	15	g
Bromphenol blue	0.25	g
Xylene cyanol	0.25	g
1 M Tris (pH 8.0)	1	ml
Distilled water to volume	100	ml

Mix well and store at room temperature.

11. 10 M NH4OAc

NH4OAc	77.08	g
Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

12. 25:24:1 (v/v) phenol : chloroform : isoamyl alcohol

Saturated phenol	50	ml
Chloroform	48	ml
Isoamyl alcohol	2	ml

Mix the reagent vigorously, cover with TE buffer and store at 4°C.

13. TE buffer (pH 8.0)

1 M Tris-HCl (pH 8.0)	10	ml
0.5 M EDTA (pH 8.0)	2	ml
Distilled water to volume	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

14. HMW digestion buffer

1 M Tris-HCl (pH 8.0)	50	µl
20 mg/ml proteinase K	50	µl
10 % Sodium lauryl sarcosine	100	µl
0.2 M EDTA (pH 8.0)	100	µl
Distilled water	500	µl

Mix the solution and use 400 µl per agarose plug.

15. 10% Sodium lauryl sarcosine

Sodium lauryl sarcosine	10	g
Distilled water to volume	100	ml

Mix the solution and store at room temperature.

16. 20mg/ml glycogen

Glycogen	200	mg
Distilled water to volume	10	ml

Sterilize the solution by filter through 0.2 μm membrane, aliquot and store at -20°C.

17. 200 mM Thymidine

Thymidine	48.44	mg
Distilled water to volume	1	ml

Sterilize the solution by filter through 0.2 μm membrane and store at 4°C.

18. 250 mM Vanillin

Vanillin	15.21	mg
Distilled water to volume	1	ml

Sterilize the solution by filter through 0.2 μm membrane and store at 4°C.

19. 10X PBS

NaCl	80	g
Na2HPO4	2	g
KCl	14.4	g
KH2PO4	2.4	g
Distilled water to volume	1,000	ml

Mix to dissolve and adjust pH to 7.4

Sterilize the solution by autoclaving and store at room temperature.

20. 10X TBS

Tris base	61	g
NaCl	90	g
Distilled water to volume	1,000	ml

Mix to dissolve and adjust pH to 7.6

Sterilize the solution by autoclaving and store at room temperature.

21. 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Resolving gel (10 ml)

40% Acrylamide:Bis (37.5:1)	3	ml
1 M Tris-HCl (pH 8.8)	2.5	ml
10% SDS	0.1	ml
10% $(NH_4)_2S_2O_8$	0.1	ml
TEMED	0.01	ml
Distilled water	4.29	ml

Stacking gel (10 ml)

40% Acrylamide:Bis (37.5:1)	1	ml
0.5 M Tris-HCl (pH 6.8)	2.5	ml
10% SDS	0.1	ml
10% $(NH_4)_2S_2O_8$	0.1	ml
TEMED	0.01	ml
Distilled water	4.29	ml

22. 1 M Tris-HCl (pH 8.8)

Tris base	12.11	g

Dissolve in distilled water and adjusted pH to 8.8 with HCl (conc)

Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

23. 0.5 M Tris-HCl (pH 6.8)

Tris base	6.055	g

Dissolve in distilled water and adjusted pH to 6.8 with HCl (conc)

Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

24. 10X Tris-glycine (pH 8.3)

Tris base	6.055	g
Glycine	147.1372	g

Dissolve in distilled water and adjusted pH to 8.3

Distilled water to volume	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

25. Running Buffer

10X Tris-glycine (pH 8.3)	100	ml
10% SDS	10	ml
Distilled water	890	ml

Mix the solution and store at room temperature.

26. Transfer buffer

10X Tris-glycine (pH 8.3)	100	ml
Methanol	200	ml
Distilled water	800	ml

Mix the solution and store at room temperature.

27. 3X Sample loading buffer

1 M Tris-HCl pH 6.8	2.4	ml
20% SDS	3	ml
Glycerol (100%)	3	ml
β-mercaptoethanol	1.6	ml
Bromphenol blue	6	mg

Mix well and store at 4°C.

APPENDIX B

SEQUENCE OF LINE-1

>gi|339773|gb|M80343.1|HUMTNL22 Human transposon L1.2

GGGGGGAGGAGCCAAGATGGCCGAATAGGAACAGCTCCGGTCTACAGCTCCCAGCGTGAGCGACGCAGAA
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APPENDIX C

LIDs DNA SEQUENCING AND BLAST RESULTS

AC087307 at 13,330 bp

DNA sequencing from L1-AM-PCR with chimeric primer TGA

Cloning Site ↓ L1 ACA primer

GCGGGAATTCGATTGCGCACCAGCATGGCACATGTATACATATGTAACTAACCTGCAC

AATGTGCACATGTACCCTAAAACTTAGAGTATAATAAAAAAAAAAAAAAAAAAAAAAA

AAAATTAATTTGGAAACTTTTACTTAACATTCAATCTGGTAGCATCTATAGAAATATCAGA

AAATCAGACCACATTAGACCAGAATTCCTACATAGTAACTAACAATCATCTAGAGCAAA

Cloning Site ↓ Chimeric primer TGA

ATATATGTTCAGATCACGTCCACGAGTCAATCACTAGTGAATTCGCGGCCGCCTGCA

BLAST result

```
>gi|12331589|gb|AC087307.3|AC087307  Homo sapiens chromosome RPCI-11 clone RP11-312N22, WORKING DRAFT
          SEQUENCE, 5 unordered pieces
        Length = 172073

 Score =  238 bits (120), Expect = 2e-60
 Identities = 120/120 (100%)
 Strand = Plus / Minus

Query: 1     tttggaaacttttacttaacattcaatctggtagcatctatagaaatatcagaaaatcag 60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 13318 tttggaaacttttacttaacattcaatctggtagcatctatagaaatatcagaaaatcag 13259

Query: 61    accacattagaccagaattcctacatagtaactaacaatcatctagagcaaaatatatgt 120
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 13258 accacattagaccagaattcctacatagtaactaacaatcatctagagcaaaatatatgt 13199
```

```
13081 acatttgaac aatgaaaaag accctgaaaa ctgtttttgc gctaaagagg actacttaaa
13141 atttaaagcc aaacctctgt cctttattga ttaaccattc cagtatgtag agatctgaac
13201 atatattttg ctctagatga ttgttagtta ctatgtagga attctggtct aatgtggtct
13261 gattttctga tatttctata gatgctacca gattgaatgt taagtaaaag tttccaaatt
13321 aatttttttt attaaagtaa aaaaaaggta gggataagag aactttcaca gtactttagg
13381 ttatttatga gagggaaagt gctactttgt aatgttccta ttacccttaa cactgttata
13441 catttttag tattggcttg tagctaagca tttttaagtc tggtgaaata tggtaacttt
```

The sequence underlines are complementary to the clone sequence.

LIDsAC087307 primer

Forward	5'-GTAGGAATTCTGGTCTAATGTG-3'
Reverse	5'-GTGAAAGTTCTCTTATCCCTAC-3'

AC015547 at 86,101 bp

DNA sequencing from L1-AM-PCR with chimeric primer TCG

Cloning Site ↓ L1 ACA primer

GCATGCTCCCGGCCGCCATGGCGGCCGCGGGAATTCGATTGCGCACCAGCATGGC

ACATGTATACATATGTAACTAACCTGCACAATGTGCACATGTACCCTAAAACTTAGAGT

ATAATAAAAAAAAAAAAAAAAAAAGAAAGAAAGAAAGAAAGAATAAGCCAGAAGAACA

CGACCTACATATAGAACATAGCTTTTCTTTCAAACCCAGAGATCCTGGTGGGAAGTGA

TTGAATTATGGGGGCGGGTCTTTCCCGTGCTGTTCTCGTGATAGTAAGTCTCACGAGA

Chimeric primer TCG ↓ Cloning Site

TCACGTCCACGAGTCAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATAT

BLAST result

```
>gi|20279340|gb|AC015547.11|  Homo sapiens chromosome 3 clone RP11-195J23, WORKING DRAFT SEQUENCE, 32
          unordered pieces
        Length = 134443

 Score =  123 bits (62), Expect = 9e-26
 Identities = 62/62 (100%)
 Strand = Plus / Plus

Query: 1     gaataagccagaagaacacgacctacatatagaacatagcttttctttcaaacccagaga 60
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct: 86116 gaataagccagaagaacacgacctacatatagaacatagcttttctttcaaacccagaga 86175

Query: 61    tc 62
              ||
Sbjct: 86176 tc 86177
```

```
85981 gctactcagg aggttgaggt gagaggatca cttgaatcca gtagtccaag gctgcagtga
86041 gctatgattg tgtcactata ctccagcctg agtgataggg tgagaccctg tctcttaaaa
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86281 gaaagaattt gccatagaga ggcataagga gaaaccagat gaactcacag aaaactttta
86341 tggtttgatg tgggctagaa taatggatca gaatcccaaa gagtcacagc caaagagtaa
86401 gtctgtaaaa ctaatacttt atctagacta gatttaaaat agggaaaaag agatctgtgg
```

The sequence underlines are complementary to the clone sequence.

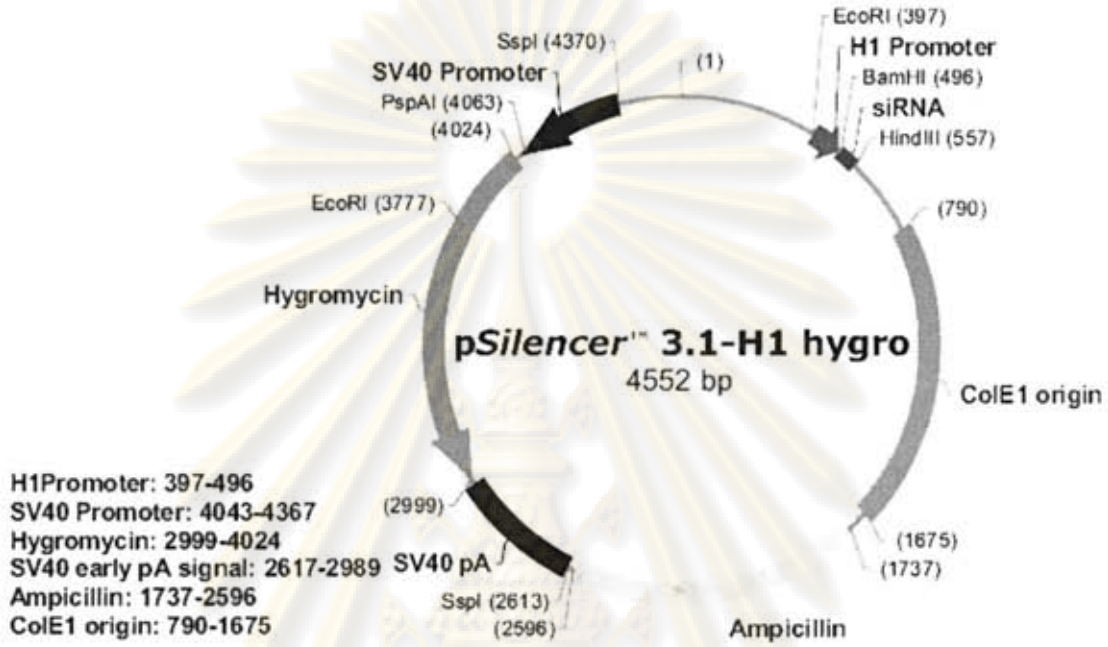
LIDsAC015547 primer

Forward	5'-GAAGGAAGTTTTAGCCAGGACT-3'
Reverse	5'-GAAGGGCTTACCGCTTTATGG-3'

APPENDIX D

PLASMID AND siRNA SEQUENCES

1. p*Silencer*™ 3.1-H1 hygro



2. siRNA sequences

Negative siRNA from p*Silencer*™ kit (Ambion, Cat # 5760) was used as control	
Rad51 siRNA	5'-GAGCUUGACAAACUACUUC-3'
DNA-PKcs siRNA	5'-GGGCGCUAAUCGUACUGAA-3'
ATM siRNA	5'-GCACCAGUCCAGUAUUGGC-3'

BIOGRAPHY

Mr. Wichai Pornthanakasem was born on September 3rd, 1974 in Bangkok. He received his bachelor degree in Biology from Faculty of Science, Ramkhamhaeng University in 1996 and then worked as a scientist at Department of Anatomy, Faculty of Medicine, Chulalongkorn University. He got a Royal Golden Jubilee (RGJ) Ph.D. Scholarship from the Thailand Research Fund (TRF) and participated in Medical Microbiology program, Graduate School, Chulalongkorn University in 1999.