การศึกษาความสัมพันธ์ระหว่างหมู่เมทิลของดีเอนเอ, การฉีกขาดของดีเอนเอสายคู่ที่เกิดขึ้นเองและความไม่เสถียรของจีโนม

นางสาวอารยา ทองนาค

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์การแพทย์
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2554

THE RELATIONSHIP AMONG DNA METHYLATION, ENDOGENOUS DNA DOUBLE STRAND BREAKS AND GENOMIC INSTABILITY

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A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Medical Science

Faculty of Medicine

Chulalongkorn University

Academic Year 2011

Thesis Title	THE RELATIONSHIP AMONG DNA METHYLATION, ENDOGENOUS DNA DOUBLE STRAND BREAKS AND GENOMIC INSTABILITY
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อารยา ทองนาค : ความสัมพันธ์ระหว่างหมู่เมทิลของดีเอนเอ, การฉีกขาดของดีเอนเอสายคู่ที่เกิดขึ้นเอง และความไม่เสถียรของจีโนม (The relationship among DNA methylation, endogenous DNA double strand breaks and genomic instability) อ. ที่ปรึกษาหลัก : ศ.นพ. อภิวัฒน์ มุทิรางกูร, 55 หน้า.

การยับยั้งโปรตีน DNA-PKcs ซึ่งเป็นโปรตีนที่มีหน้าที่สำคัญในกระบวนการซ่อมแซมดีเอนเอที่ฉีกขาดทั้งสองสายด้วยวิธี non-homologous end joining (NHEJ) ภายใต้การเหนี่ยวนำให้เกิดกระบวนการเติมหมู่อะเซติลที่ฮิสโตน (histone acetylation) จะทำให้การรอดชีวิตของเซลล์ลดลง อย่างไรก็ตามยังไม่ทราบกระบวนการที่แน่ชัด เร็วๆ นี้ได้มีการคิดค้นวิธีที่จะวัดปริมาณดีเอนเอที่ฉีกขาดแบบสายคู่ที่เกิดขึ้นเองโดยอาศัยเทคนิค PCR ซึ่งทำให้พบว่ามีดีเอนเอแบบสายคู่ฉีกขาดที่เกิดขึ้นเองในระยะพักของเซลล์ (G_0) ด้วย จึงเรียกดีเอนเอฉีกขาดแบบสายคู่ที่เกิดขึ้นเองนี้ว่า replication independent EDSBs หรือ RIND-EDSBs ซึ่งหมายถึงดีเอนเอฉีกขาดแบบสายคู่ที่เกิดขึ้นเองที่ไม่ได้เป็นผลมาจากการเกิดการจำลองตัวเองของดีเอนเอ โดยที่ RIND-EDSBs นั้นสามารถจำแนกออกได้เป็นสองชนิด ชนิดแรกปรากฏทั่วไปในเซลล์จำนวนหนึ่งภายใต้สภาวะทางสรีรวิทยา เรียกชื่อว่า NRIND-EDSBs (normal physiological RIND-EDSBs) ซึ่งมักจะอยู่ในบริเวณที่มีหมู่เมทิลสูงในจีโนม หรือบริเวณที่สามารถเปลี่ยนแปลงได้ของเฮเทโรโครมาติน (facultative heterochromatin) ซึ่งไม่สามารถตรวจพบการเกิดฟอสโฟรีเลชันที่ γ-H2AX ได้ และมักเกิดการซ่อมแซมได้ช้ากว่าด้วยกระบวนการที่ช้ากว่าโดยอาศัยโปรตีน ATM ซึ่งทำหน้าที่ในกระบวนการซ่อมแซมดีเอนเอที่ฉีกขาดทั้งสองสายด้วยวิธี NHEJ อีกตัวหนึ่ง RIND-EDSBs ชนิดที่สองเรียกชื่อว่า PRIND-EDSBs (pathogenic RIND-EDSBs) มักพบในบริเวณที่มีหมู่เมทิลต่ำในจีโนมและสามารถเกิด γ-H2AX ได้ ซึ่ง RIND-EDSBs ชนิดนี้จะสามารถพบได้เมื่อมีการยับยั้งกระบวนการซ่อมแซมดีเอนเอที่ฉีกขาดทั้งสองสาย เนื่องจาก RIND-EDSBs ชนิดแรกสามารถตรวจพบได้ในเซลล์ทุกชนิด จึงคาดว่า RIND-EDSBs ชนิดนี้น่าจะมีบทบาทในทางสรีรวิทยาของเซลล์และในขณะเดียวกันเซลล์จำแนกและจดจำ RIND-EDSBs ชนิดที่สองเช่นเดียวกับเมื่อมีการเกิดดีเอนเอฉีกขาดแบบสายคู่ที่เกิดจากการเหนี่ยวนำของรังสี ดังนั้น RIND-EDSBs ชนิดที่สองจึงน่าจะเป็นชนิดที่ทำให้เกิดพยาธิสภาพบางอย่างในเซลล์ได้ ปริมาณของ RIND-EDSBs ชนิดที่หนึ่งสามารถถูกทำให้ลดลงได้เมื่อโครมาตินถูกเหนี่ยวนำด้วยสารเคมี Trichostatin A (TSA) ให้มีสภาพเป็นไฮเปอร์อะซิติลเลชั่น แต่ RIND-EDSBs ชนิดที่หนึ่งจะกลับเพิ่มปริมาณขึ้นได้เมื่อเซลล์ถูกเหนี่ยวนำด้วย TSA พร้อมๆ กับการใส่วานิลลิน (Vanillin) ซึ่งเป็นตัวยับยั้งโปรตีน DNA-PKcs และคาเฟอีน (Caffeine) ซึ่งเป็นตัวยับยั้งโปรตีน ATM ดังนั้นจึงตั้งสมมติฐานว่า RIND-EDSBs ที่สามารถพบได้ซึ่งเป็น RIND-EDSBs ที่มีบทบาทในทางสรีรวิทยาของเซลล์น่าจะช่วยป้องกันการเกิด EDSBs ได้ เนื่องจากการลดลงของ RIND-EDSBs โดยการเหนี่ยวนำด้วย TSA เป็นการเพิ่ม RIND-EDSBs ที่ส่งผลต่อพยาธิสภาพบางอย่างของเซลล์และทำให้เซลล์ตายเมื่อการซ่อมแซมถูกยับยั้ง จากการตั้งข้อสังเกตการลดลงของ RIND-EDSBs เกิดโดย 3 กระบวนการ ได้แก่ การมีหมู่เมทิลต่ำ, การเปลี่ยนสภาพโครมาตินด้วย TSA และการลดการทำงานของโปรตีน High-mobility group protein B1 (HMGB1) ที่มีบทบาทสำคัญเกี่ยวข้องกับโครมาติน และยังพบอีกว่าเกี่ยวกับความผันแปรของปริมาณ RIND-EDSBs ในสภาวะปกติของเซลล์ด้วย โดยที่เมื่อใส่วานิลลินให้กับเซลล์ที่ถูกใส่ TSA มาก่อนหรือเซลล์ที่ถูกลดการทำงานของโปรตีน HMGB1 มาก่อน จะพบการเพิ่มขึ้นของ EDSBs ที่ 2 ชั่วโมง เป็นผลให้การรอดชีวิตต่ำลงในเซลล์ทั้งสองชนิดตามมา นอกจากนี้ยังพบว่าปริมาณของ RIND-EDSBs ที่มีบทบาทในทางสรีรวิทยาของเซลล์มีความสัมพันธ์ไปในทางเดียวกับการรอดชีวิตของเซลล์ต่อวานิลลิน โดยสรุป RIND-EDSBs จะพบค้างอยู่ในเซลล์ปกติในปริมาณหนึ่งเนื่องด้วยบทบาทของ ดีเอนเอเมทิลเลชัน, ฮิสโตนอะเซติลเลชัน และโปรตีน HMGB1 ซึ่งการลดลงจะทำให้เกิด RIND-EDSBs ที่ส่งผลต่อพยาธิสภาพบางอย่างของเซลล์และทำให้เซลล์ตายเมื่อถูกยับยั้งโดยวานิลลิน และยังสันนิษฐานว่าหากไม่มีการยับยั้งกระบวนการซ่อมแซมดีเอนเอแล้ว RIND-EDSBs ที่ส่งผลต่อพยาธิสภาพบางอย่างของเซลล์อาจจะนำไปสู่การซ่อมแซมดีเอนเอที่ฉีกขาดทั้งสองสายแบบผิดพลาดได้ นอกจากนี้การลดลงของ RIND-EDSBs ที่มีบทบาทในทางสรีรวิทยาของเซลล์ที่เกิดจากการลดลงของระดับหมู่เมทิลในจีโนมอาจจะเป็นสาเหตุของการเกิดความไม่เสถียรในจีโนมได้จากกระบวนการที่กล่าวมาข้างต้น

สาขาวิชา.....วิทยาศาสตร์การแพทย์..... ลายมือชื่อนิสิต.....

ปีการศึกษา.....2554..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

517 48543 30: MAJOR MEDICAL SCIENCE
KEYWORDS: ENDOGENOUS DNA DOUBLE STRAND BREAKS / METHYLATION / GENOMIC INSTABILITY

ARAYA THONGNAK: THE RELATIONSHIP AMONG DNA METHYLATION, ENDOGENOUS DNA DOUBLE STRAND BREAKS AND GENOMIC INSTABILITY. ADVISOR: PROF. APIWAT MUTIRANGURA, M.D., Ph.D., 55 pp.

Inhibition of DNA-PKcs-mediated non-homologous end joining (NHEJs) under drug- induced histone acetylation reduces cell survival. However the mechanism is not well understood. Recently, we developed a new PCR-based technique to measure the amounts and types of epigenetic modification of endogenous DNA double strand breaks (EDSBs). We found that EDSBs in cells at G_0 stage, known as replication-independent EDSBs (RIND-EDSBs) exist in two classes, namely, those present at a given level in all cell types under normal physiological conditions (named NRIND-EDSBs), which are hypermethylated, retained in facultative heterochromatin, devoid of γ-H2AX phosphorylation, and are repaired by a slow but more precise ATM-mediated NHEJs; and those (known as PRIND-EDSBs) that are hypomethylated and bound by γ-H2AX are only detected when DSB repair is inhibited. As NRIND-EDSBs are found in all cell types, we propose that they may possess normal physiological function. On the other hand, cells recognize PRIND-EDSBs as equivalent to radiation-induced DSBs and may be pathogenic. NRIND-EDSBs were reduced when chromatin became hyperacetylated by treatment with 100 ng/ml trichostatin A (TSA) (histone deacetylase inhibitor), but were increased when cells were treated with a combination of 100 ng/ml TSA, 2.5 mM vanillin (DNA-PKcs inhibitor) and 5 mM caffeine (ATM inhibitor). Therefore, we hypothesize that NRIND-EDSBs prevent PRIND-EDSB production, and that reduction of NRIND-EDSBs should increase PRIND-EDSBs, leading to cell death when repair of EDSBs are inhibited. We observed a reduction in NRIND-EDSBs under three situations: DNA hypomethylation, histone hyperacetylation and down regulation of high-mobility group protein B1 (HMGB1). We also found variations in NRIND-EDSB levels under normal culture conditions. When TSA-treated cells or those containing reduced levels of HMGB1 were exposed to vanillin, there was an increase in PRIND-EDSBs after 2 hours. Consequently, lower survivals were observed in both types of test cells. There is also a direct correlation between NRIND-EDSB level and resistance to vanillin treatment. In summary, NRIND-EDSBs are maintained in normal cells at a given level by DNA methylation, histone acetylation and an appropriate amount of HMGB1. Reduction in NRIND-EDSBs results in an increase in PRIND-EDSBs, thereby causing cell death when cells are treated with inhibitor of DSB repair (viz. vanillin). We postulate that in the absence of DNA repair inhibitors, PRIND-EDSBs should lead to DSB repair errors. Thus, a reduction of NRIND-EDSBs as a result of global hypomethylation, histone hyperacetylation or reduction of HMGB1 may underlie genomic instability.

Field of Study : Medical Science ..... Student's Signature.....

Academic Year : 2011 ..... Advisor's Signature.....

ACKNOWLEDGEMENTS

I would like to express my gratitude to all these people who gave me the possibility to complete this thesis. I want to thank the committee of Medical Science Program for giving me permission to commence this thesis in the first instance, to do the necessary research work. I am deeply indebted to my advisor Professor Dr.Apiwat Mutirangura, for his stimulating guidance and support. I thank my other committee and special members, Associate Professor Wilai Anomasiri, Associate Professor Kanya Suphapeetiporn, Assistant Professor Oranart Matangkasombut and Professor Prapon Wilairat for their helpful suggestions, edit thesis and insightful comments during my study.

I am so appreciate to these persons, Miss Chutipa, Miss Narisorn, Miss Jirapan, Miss Wachiraporn, Mr. Prakasit for their assistance and their help and encouragement. If I lacked them, this work would not be accomplished. Finally, I would like to express my deepest gratitude to my family for their love and understanding all the time.

This thesis was partially supported by Affairs Thesis grants for graduate students in Public Universities, Graduate school, Chulalongkorn University.

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LIST OF ABBREVIATIONS

COBRA	=	Combined bisulfite restriction analysis
DNA-PKcs	=	DNA-dependent protein kinase catalytic subunit (PRKDC gene)
EDSBs	=	Endogenous DNA Double Strand Breaks
HMGB1	=	High Mobility Group Box 1
PCR	=	Polymerase chain reaction
RIND-EDSBs	=	Replication Independent Endogenous DNA Double Strand Breaks
RT-PCR	=	Reverse transcriptase polymerase chain reaction
S.D.	=	Standard deviation

CHAPTER I

INTRODUCTION

Background and Rationale

Recently, we discovered a new class of endogenous DNA double strand breaks (EDSBs) that does no harm to cells. In this study, we will prove that these EDSBs may benefit human genomes. We invent EDSB-PCR method to measure quantity and methylation of EDSBs. We chose to extensively evaluate the long interspersed element-1 (L1 or LINE-1) because the methylation status of these retrotransposable elements has been extensively studied [1-3]. The number and methylation state of EDSBs were analyzed at the L1 sequences near EDSBs of L1-EDSB-LMPCR templates [4]. Interestingly, we found L1-EDSBs of almost all tested cells were hypermethylated, having higher methylation levels in L1s at the sites nearest EDSBs than at other sites in the genome [4]. Surprisingly, the hypermethylation is most significant in G_0. Therefore, the EDSB hypermethylation is replication independent. Recently, we further studied replication independent EDSBs (RIND-EDSBs). There are two classes of RIND-EDSBs. The first presents at a certain level in all cells underphysiologic condition. These RIND-EDSBs are hypermethylated, retained in facultative heterochromatin, devoid of γ-H2AX phosphorylation, and repaired by a slower but more precise ATM mediated NHEJ. The second class of RIND-EDSBs are hypomethylated and bound by γ-H2AX. These RIND-EDSBs are not usually detectable, and only are found when DSB repair is inhibited.

Because genomic hypomethylation induces genomic instability, we speculate here that retained RIND-EDSBs may influence genomic integrity. Furthermore, cell survival under DNA-PKcs inhibitor show that if cells possess higher level of RIND-EDSBs prior to vanillin treatment; these cells survived more than the others. We postulate a hypothesis here that retained RIND-EDSBs in high level of methylation chromatin may

prevent the spontaneous generation of pathogenic RIND-EDSBs. Therefore, hypomethylated genome may produce more pathogenic RIND-EDSBs. Moreover pathogenic RIND-EDSBs should have the same influences as radiation induced DSBs or replication dependent EDSBs that promote fast error prone repair leading to mutation.

Objective

- To determine the relationship between DNA methylation and retained RIND-EDSBs.

- To examine the relationship between chromatin modification, histone acetylation, and RIND-EDSBs.

- To determine the relationship between retained RIND-EDSBs and cell survival when DNA-PKcs was inhibited.

Question

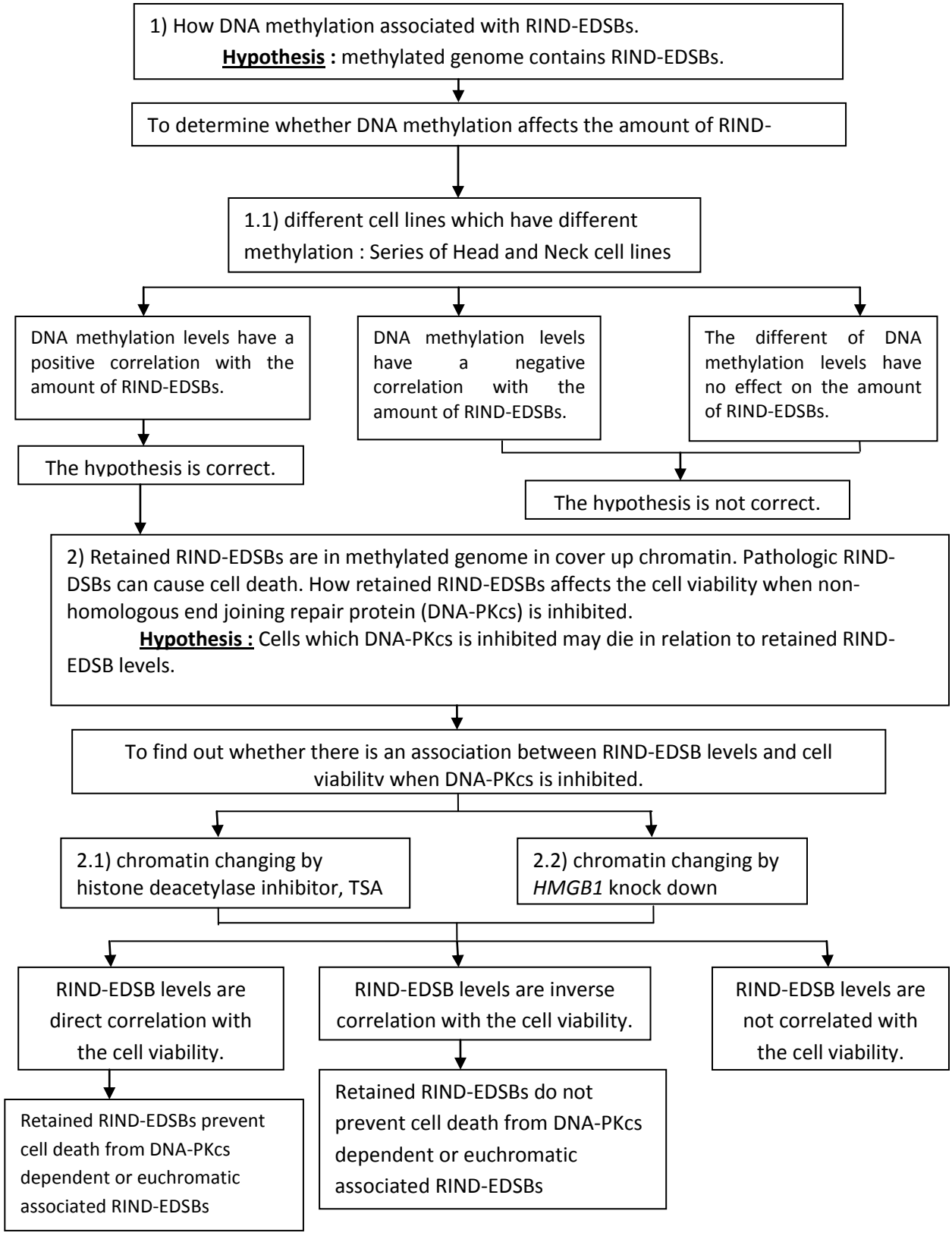
Do retained RIND-EDSBs play a role in DNA methylation and chromatin maintaining genomic integrity?

Hypotheses

- DNA methylation, histone acetylation and HMGB1 form heterochromatin complex to retain RIND-EDSBs.

- One of the biological roles of retain RIND-EDSBs is to maintain genomic integrity which is to prevent unwanted DNA break.

Conceptual Framework



Operation definition

-

Expected benefit

Benefit is a new knowledge to prove if retained RIND-EDSBs possess physiological roles that one of them is to maintain genome integrity by prevent unwanted DNA break. This knowledge when adds up with previous evidences that retained RIND-EDSBs are always present by a function of certain genes suggests that RIND-EDSBs provide selective advantage for evolution process and should be considered as another epigenetic mark.

Research Methodology

1. Cell culture and cell collection
2. DNA extraction
3. Sodium bisulfite treatment and desalted with DNA clean-up system.
4. Cloned and sequenced
5. Primer design for *HMGB1.*
6. RT-PCR
7. RNAi transfection
8. qPCR : standard curve study and quantitative - comparative CT ($\Delta\Delta$CT) study

CHAPTER II

REVIEW OF RELATED LITERATURES

1. Replication independent endogenous DNA double strand breaks (RIND-EDSBs)

Hazardous chemical agents and ionizing radiation produce large numbers of DNA double strand breaks (DSBs), which can be observed as fragmented DNA [5, 6]. This breakage can trigger apoptosis and error repair may lead to mutation[7]. Nevertheless, low levels of DSBs can occur spontaneously and are known as endogenous DSBs (EDSBs). Several mechanisms, including replicative DNA polymerase encountering single-strand DNA breaks, temperature, osmolarity, oxidative DNA damage and endonucleases [8-16], produce γ-H2AX foci. γ-H2AX, the serine 139 phosphorylated form of histone H2AX, is one of the earliest DSB repair responses [17, 18]. The roles of EDSBs induced by different causes are different. In 2003, Vilenchik and Knudson proposed that the number of EDSBs [8] should be enough to play a key role in genomic instability in cancer, as they can be intermediates in DNA or chromosomal rearrangements [8]. EDSBs, however, do not play a role in heat or hypertonicity-induced cell killing [13, 19]. V(D)J recombination is important in lymphocyte development [20], and topoisomerase II helps maintaining genomic integrity [21].

2. Epigenetics

Hypomethylation and hypermethylation are most often used to describe relative states, best understood in relative to an expected, “normal” setting or degree of methylation (i.e. the level of methylation that in seen in nonmalignant cells). Hypomethylation and hypermethylation of DNA are relative terms and indicate less or more methylation than in some standard DNA. However, there are substantial

differences in the number and distribution of DNA methylation among different vertebrate tissues because DNA methylation is not only species specific but also tissue-specifc [22].

The discovery of vast cancer associated with DNA hypomethylation in the human genome [23] preceded that of cancer-linked DNA hypermethylation. DNA hypomethylation in cancer often affects more of the genome than does hypermethylation so that net losses of genomic 5-methylcytosine are seen in many human cancers. The role in carcinogenesis of cancer linked hypermethylation of transcription control regions is clear because of the consequent transcriptional silencing of genes important for prevention of cancer (tumor suppressor gene) .The biological significance of DNA hypomethylation in cancer is less understood.

3. DNA methylation and Genomic stability

Due to the methylation levels, several evidences indicated that alteration of DNA methylation in genome appears to be related to cell death and cancer progression. Since global DNA hypomethylation tends to become more potent as precancerous progress via genetic instability [24-34]. Mutations which are the recombination products between different loci, including chromosomal rearrangements, loss of heterozygosity, deletion, telomere insertion, are DSBs-mediated and are the consequences of Global hypomethylation also. It is interesting whether genome-wide hypomethylation promoting genetic instability might be as a result of EDSBs existing in the cell. Genomic instability is a cardinal feature of cancer [35]. Understanding the molecular mechanisms involved in this instability is essential for the development of effective approaches in cancer prevention [36] and treatment to prevent cancer progression [35]. RIND-EDSBs may mediate mutations that are produced by genomic hypomethylation. Hypomethylation-induced mutations are the result of recombination between different loci, and methylated

and unmethylated EDSBs are processed differently. Remarkably increased in RIND-EDSBs when chromatin became hyperacetylated and NHEJ repair was prevented. Therefore, euchromatin-associated DNA is prone to be broken; however, unmethylated L1-EDSBs may be immediately repaired. Under normal condition, RIND-EDSBs are hypermethylated; therefore, the mechanical DNA repair processes for methylated and unmethylated L1-EDSBs should be different. In general, DSB repair pathways are redundant and interchangeable [37], but reduced ATM expression leads to increased methylation of L1-EDSBs. Retained RIND-EDSBs may be similar to radiation-induced DSBs in heterochromatin that are slowly repaired by ATM [38]. In contrast to other NHEJ pathways, the ATM-dependent repair pathway has been proposed to be more precise [35-56]. Therefore, methylated L1-EDSBs, but not unmethylated forms, may be able to escape error-prone NHEJ repair [57-61]. Consequently, the rate of spontaneous mutations in methylated DNA may be less than in hypomethylated genomic regions.

L1-EDSBs are detectable and hypermethylated because RIND-EDSBs surrounded by methylated genomic regions are inertly retained in heterochromatin. Unlike radiation-induced DSBs and euchromatin-associated RIND-EDSBs, retained methylated RIND-EDSBs do not initiate an immediate cellular response, which can lead to fast but more error-prone repair or to cell death. Moreover, retained RIND-EDSBs are slowly repaired by more precise repair pathways. This process may help prevent spontaneous mutations within methylated genomic regions.

4. DNA-PKcs and Histone deacetylase inhibitors and cell survival

The DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine protein kinase that is activated upon the association with DNA. DNA-PK complex is composed of the DNA-PK catalytic subunit (DNA-PKcs) and the Ku70/Ku80 heterodimer. It plays a crucial role in nonhomologous end joining (NHEJ) pathway of DNA double strand break repair. Moreover, DNA-PKcs might have a possible role in

processing of oxidatively-induced DNA damage together with BRCA1 [62]. In mouse and human cells, deficiency of DNA-PKcs leads to hypersensitive to ionizing radiation and defective in V(D)J recombination [63].

DNA-PK has been reported to phosphorylate a several protein in NHEJ pathway. but only phosphorylation of Artemis and autophosphorylation of DNA-PKcs has, importantly, been demonstrated to alter biological activity and NHEJ catalyzed repair [64, 65]. DNA-PKkcs is also activated in an early step of damage checkpoint-induced cell cycle arrest [63]. Notably, both DNA-PKcs and ATM are required for normal levels of p53 phosphorylation in B and T cells and p53 dependent apoptosis. These demonstrate that DNA-PKcs dependent pathway regulates DNA repair and activates p53 in the lack of ATM [66].

DNA-PKcs independent DSB repair initiates by loading of the Ku70/Ku80 heterodimer onto DNA ends. DNA-PKcs, then, is recruited to form complex with DNA at DSB sites to protect DNA ends from exonuclease enzymes [67]. Its serine/threonine kinase activity is required for DNA end processing and ligation subsequently.

Apart from the heterodimerization between Ku70 and Ku80, regulation of DNA-PK activity and function seem to be regulated by several mechanisms. Perhaps chromatin context possibly influence over the DNA-PK activation in vivo by high mobility group (HMG) proteins 1 and 2 [68].

The recent report shows that the DNA structure and orientation influence to DNA-PK activation and provide a molecular mechanism of activation resulting from compatible termini [64]. Kongrattanachok et al. communication showed a remarkably increase in RIND-EDSB methylation levels was observed when cells were depleted of ATM but when depleted of not Ku86. However, RIND-EDSBs surrounded by methylated genomic regions are inertly retained in nonacetylated heterochromatin. The compact histones may block rapid cellular responses to DNA breaks but allow these breaks to be repaired by another slower but more precise pathway. Consequently, spontaneous

mutations in methylated DNA may be prevented by avoiding repair by the more error-prone NHEJ mechanisms which DNA-PKcs lays at the center of signal transduction to trigger other proteins to the DSBs [69].

5. HMGB1

Extensively, previous papers known that HMGB1 proteins were involved in diverse biological processes such as transcription, DNA repair, assembly of nucleoprotein complexes, and V(D)J recombination [70]. Mutants of the high mobility group box (HMG) reduced level of RIND-EDSBs. HMGB1 is an important group of proteins that function as protein carriers in chromatin structure arrangement. For example, HMGB1 bind to the DNA lesion after DNA damage to effect on DNA repair and chromatin remodeling [71]. In addition, *HMGB1* was important function for V(D)J cleavage [72]. Interestingly, in 2003, Brezniceanu *et al*. reported the new function of *HMGB1* was inhibited cells death in yeast and human cells. The data supported that *HMGB1* might suppress cell death [73].

6. hypomethylation and genomic instability

DNA methylation is one of the necessity processes for normal development, cellular differentiation , X-chromosome inactivation genomic imprinting , and repression of retrotransposons and endogenous retroviruses . Alterations of DNA methylation are commonly recognized as an important constituent lead to cancer development [74]. In mouse and human, abnormalities of genomic methylation patterns are also known to involve Immunodeficiency-centromeric instability-facial anomalies (ICF) syndrome [75, 76] by almost completely unmethylated in satellite DNA in all tissues [77].

Aberrant methylation patterns presented in cancer cells have two types, gene-specific hypermethylation and genome wide hypomethylation. Focusing on global

hypomethylation, most of which occurs in repetitive DNA elements, is often reduced methylation aberrantly and investigated to be correlated with genetic instability [78, 79],a cellular state, that can be characterized by which genetic changes rate are increased, including DNA sequence changes, gene amplifications, chromosome translocations and/or aneuploidy [27]. Global hypomethylation has been reported on having a significant associated with chromosomal instability in various types of cancers, colorectal cancers [80, 81], breast cancers, cancer-associated stromal myofibroblasts [82] and head and neck squamous cell carcinoma (HNSCC) which LOH analysis was significantly correlated with LINE-1 hypomethylation [78]. In addition, DNA demethylation is also preceding significant age-dependent genomic alterations in subset of gastrointestinal cancers [83]. Noteworthy, the type of chromosomal alteration was investigated whether it was differentially associated with DNA hypomethylation. The result showed that DNA hypomethylation affects the types of chromosomal instability differently, not preferentially with one specific kind and it tends to affect the stability of all chromosomes[81].

DNA methyltransferase (*Dnmt*) genes are one of the causation that can exert global methylation. Several reports indicated that Dnmt1 deficiency affects the global genomic hypomethylation and mutation rate, lacking of *Dnmt1* gene had shown significantly increasing mutation rates of hypoxanthine phosphoribosyltransferase (*Hprt*) gene and tymidine kinase [30] transgene in murine embryonic stem cells [31], and promoted tumors in mice somatic cells [84, 85]. It had also been reported that Dnmt1 deficiency results in constitutive genomic instability particularly by chromosomal translocations in a human colon cancer cell line [27]. In addition, Dnmt1 deficiency was founded that led to enhance the microsatellite slippage rate in mouse embryonic stem cells [86]. Besides it had been reported that Dnmt3b-deficiency MEFs may engaged genomic instability which leads to spontaneous immortalization via a p53-independent mechanism [87].

DNA methylation inhibition experiments, with DNA methyltransferase inhibitors 5'-aza-cytidine and/or 5'-aza-2'-deoxycytidine, support an association between DNA

hypomethylation and carcinogenesis. Recent study in mammalian cells which are treated by 5'-aza-2'-deoxycytidine showed that triplet repeat instability has increased dramatically [88], and has increased the rate of nucleotide repeat contraction [89] These imply a crucial role of DNA methylation in maintaining genome stability.

CHAPTER III

MATERIALS AND METHODS

Cell culture

HeLa (Cervical cancer), HN8, HN12, HN17, HN19, HN30, HN31 (Head and Neck cancer) and HCT116 (Colorectal adenocarcinoma) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich). The medium was supplemented with 10% fetal bovine serum (FBS) and 1% of 100 units/ml penicillin-streptomycin (Gibco). All cell lines were grown in humidified atmosphere at 37°C with 5% CO_2. In this study, cell lines were synchronized at G_0 phase. All cell lines were cultured in 25 cm^2 tissue culture flask at the density of $1x10^6$ cells for 24 hrs. Cells were cultured in serum deprivation medium, DMEM without FBS for 24 hrs, to synchronize cells to G_0 phase. Harvesting cells, cells were washed by Phosphate Buffered Saline (PBS), detached from the flask bottom by incubate in 0.25% trypsin for 5 min and collected to 15 ml tubes.

High-Molecular-Weight (HMW) DNA Preparation

To prepare HMW DNA, cell lines were embedded in 1% low-melting-point agarose (LMP) at density of $5x10^5$ cells per plug. The plugs were lysed and digested in 400 µl of digestion buffer, consists of 1 mg/ml proteinase K, 50 mM Tris pH 8.0, 20 mM EDTA, 1% sodium lauryl sacrosine, at 37°C overnight. The plugs were rinsed six times in TE buffer for 40 min. To polish cohesive-end EDSBs, T4 DNA polymerase (New England Biolabs) was added for 1 h, followed by rinsing six times in TE buffer for 40 min. The modified LMPCR linkers were prepared from the oligonucleotides 5'-AGGTAACGAGTCAGACCACCGATCGCTCGGAAGCTTACCTCGTGGACGT-3' and

5´-ACGTCCACGAG-3´(Sigma-Aldrich). The linkers (50 pmol) were ligated to HMW DNA using T4 DNA ligase (New England Biolabs) at 25°C overnight. DNA was extracted from the agarose plugs using a QIAquick gel extraction kit (QIAGEN).

The detection of L1-EDSBs

After the LMPCR linkers, 5'-AGGTAACGAGTCAGACCACCGATCGCTCGGAAG CTTACCTCGTGGACGT-3' and 5´-ACGTCCACGAG-3´, were ligated to HMW DNA, the number of L1-EDSBs was measured as previously described for L1-EDSB-LMPCR with some modifications [4]. A duplicate or triplicate number of L1-EDSBs were measured by realtime PCR using a ABI PRISM® 7500 instrument (Applied Biosystems) with the IRS primers, including the L1 primers 5´-CTCCCAGCGTGAGCGAC-3´ (outward), the linker primer 5´-AGGTAACGAGTCAGACCACCGA-3´, and the Taqman probe homologous to the 3´ linker sequence (6-fam)-ACGTCCACGAGGTAAGCTTCCGAGCGA-(tamra) (phosphate). Amplification was performed with 0.5 µM of each primer, 0.3 µM Taqman probe, 0.025 U of HotStarTaq (QIAGEN), 1x TaqMan® Universal PCR Master Mix (Applied Biosystem) and 40 ng of ligated DNA for up to 60 cycles, with quantification after the extension steps. Control HeLa DNA was digested with *Eco*RV and *Alu*I and ligated to the LMPCR linkers. The numbers of EDSBs were compared with the ligated control digested DNA and reported as L1 ligated *Eco*RV and *Alu*I digested genome per cell and L1-EDSBs genome per control genome.

COBRA-L1 and COBRA-L1-EDSB

L1-EDSB, COBRA-L1, and COBRA-L1-EDSB were measured by combined

bisulfate restriction analysis as previously described [4]. Ligated HMW DNA was modified with bisulfite. Bisulfite-modified DNA was recovered using a Wizard DNA clean-up kit (Promega) and desulfonated before PCR amplification. For COBRA-L-1, bisulfite-treated DNA was subjected to 35 cycles of PCR with two primers, B-L1-inward 5´-CGTAAGGGGTTAGGGAGTTTTT-3´ and B-L1-outward 5´-RTAAAACCCTCCRAACCAAATATAAA-3´. The amplicons were doubly digested in a 10 µl reaction volume with 2 U of *Taq*I and 8 U of *Tas*I in 1x *Taq*I buffer (Fermentas) at 65°C overnight. This method was designed to detect unmethylated and methylated sequences of 98 and 80 bp, respectively. The intensity of DNA fragments was measured with a PhosphorImager using Image Quant software (Molecular Dynamics, GE Healthcare). 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. For COBRA-L1-EDSB, the B-L1-inward oligo was replaced with the B-LMPCR oligo, 5´-GTTTGGAAGTTTATTTTGTGGAT-3´, and 40 PCR cycles were carried out according to the same protocol. Bisulfite-treated Daudi, Jurkat, and HeLa DNA digested with *Eco*RV and *Alu*I and ligated LMPCR linker were used as positive controls to normalize the inter-assay variation of all COBRA experiments. HeLa DNA without ligation was used as a negative control.

RNA interference

The oligonucleotide sequences of siRNA targeting High Mobility Group Box 1 (HMGB1) provided by the pre-designed Stealth RNAi (HSS142453, HSS142454, HSS142455; Invitrogen). Nontargeting negative control siRNA, Stealth RNAi™ siRNA Negative Control (Invitrogen), which lacks significant sequence homology to the genome was used to control the experiments. Before transfection, HeLa cells were cultured in DMEM without antibiotic supplement at the density of 7.5×10^4 cells per well in 6-well plate for 24 hrs. siRNA oligonucleotides were transfected into HeLa by

Lipofectamine2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. After 72 hrs, two wells of transfected cells were collected to determine the level of HMGB1 mRNA and the amount of EDSBs whereas the rest were treated with DNA-PKcs inhibitor reagent, vanillin, for 24 hrs to observe cell viability.

The stable gene-knocked down cell lines were established by short double strand DNA oligonucleotides coding for proteins. The sequences of targeting oligonucleotides used in this study are; DNA-PKcs 5'-GGGCGCTAATCGTACTGAA-3' [90]; HMGB1 5'-GGAGAACATCCTGGCCTGT-3' [91]. The DNA-PKcs and HMGB1 oligonucleotide sequences were inserted into *BamH*I and *Hind*III sites of the vector p*Silencer*™ 3.1-H1 hygro kit and p*Silencer*™ 3.1-H1 neo kit (Applied Biosystems) by T4 ligase to generate p*Silencer* siRNA vectors. Ligated plasmids were transformed into *E.coli* DH5α and plated on LB plates containing 100 µg/ml ampicillin and grown overnight at 37°C. Transformants were selected and cultured for plasmid extraction. Selected clones were sequenced before use to confirm legitimately inserted sequences. The negative control whose sequence is not found in the mouse, human, or rat genome databases supplied with pSilencer kit was used as control. HeLa cells which were plated at the density of 7.5×10^4 cells per well in 6-well plate 24 hrs before were transfected by the same method as siRNA transfection. Twenty- four hours after transfection, cells were added 250 µg/ml Hygromycin B and 500 µg/ml G418 (Roche) for DNA-PKcs-transfection and HMGB1-transfection, respectively. Cells were maintained in the medium supplemented with selective antibiotics for 2–3 weeks to screen stably transfected clones.

Gene expression

Quantitative PCR (qPCR) was used to determine whether siRNA sequence down-regulated the target gene. Cells which knocked down by RNAi methods were harvested after 24 hrs synchronization as described above. RNA extraction was

performed manually and five micrograms of the RNA was used for changing to cDNA by RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). Total cDNA of each sample was analyzed in triplicates using SyBr by quantitative - comparative CT ($\Delta\Delta$CT) study with HMGB1 forward primer 5'-ATATGGCAAAAGCGGACAAG-3' and HMGB1 reverse primer 5'GCAACATCACCAATGGACAG-3' (Sigma) [92]. To detect HMGB1 amplification using an ABI PRISM® 7500 instrument (Applied Biosystems). The relative expression of HMGB1 was quantified as a ratio of GAPDH expression.

Trichostatin A (TSA) and Vanillin treatment

Before TSA treatment, cells were cultured at the density of $1x10^6$ cells in 25 cm^2 tissue culture flask and were synchronized to G_0 by culture in serum deprivation medium for 24 hrs. cells which grew in serum deprivation were added a dose of 100 ng/ml TSA (Sigma-Aldrich), an inhibitor of histone deacetylase (HDAC), into the flasks for 4 and 24 hrs. For vanillin treatment, cells were treated for 24 hrs with 2.5 mM vanillin (Sigma-Aldrich) to inhibit DNA-PKcs activities.

Cell survival

To analyze survival, cells were trypsinized and suspended in 1 ml of medium before testing their viability with a trypan blue stain. Viable cells that excluded the trypan blue stain were counted.

Statistical analyses

Statistical analysis was determined by a paired sample t-test, ANOVA or Pearson's rank correlation statistics, when appropriated.

CHAPTER IV

RESULTS

DNA methylation and chromatin were associated to maintain and organize the genome by epigenetic roles. Retained RIND-EDSBs in cells that can be detected by the novel technique based on PCR have been proposed to find out at the vicinity of high level of L1 methylation. However, global hypomethylation have been reported that relate with genomic instability. Thus, this thesis was aimed to study whether DNA methylation and chromatin are related with RIND-EDSBs. Moreover, the amount of retained RIND-EDSBs can affect cell viability pathologic EDSB production when non-homologous end joining (NHEJ) repair protein was inhibited.

RIND-EDSB levels and DNA methylation

According to the previous study, we showed that DNA close to RIND-EDSBs typically exhibits a higher methylation level than γ-H2AX-bound DNA. To observe whether RIND-EDSBs were involved with higher methylation genome, COBRA-L1 and L1-EDSB-LMPCR were used to measure the L1 methylation level and the quantity of RIND-EDSBs respectively by using the series of Head and Neck cell lines; HN8, HN12, HN17, HN19, HN30, HN31. The result showed that the higher number of RIND-EDSBs was observed in cells which have high level of L1 methylation (HN8, HN12, HN17) and cells which have lower number of RIND-EDSBs were found in low level of L1 methylation genome (HN19, HN30, HN31) (Figure 1). The correlation between the number of RIND-EDSBs and the level of L1 methylation in genome was not significant (two-tailed spearman, $n = 36$, $r = 0.7143$, $p = 0.1361$).

but the data can separate into two groups obviously.In addition, we found that the higher number of RIND-EDSBs which have high level of L1 methylation group has a broad range of RIND-EDSBs distribution contrarily with the lower number of RIND-EDSBs which have low level of L1 methylation group.

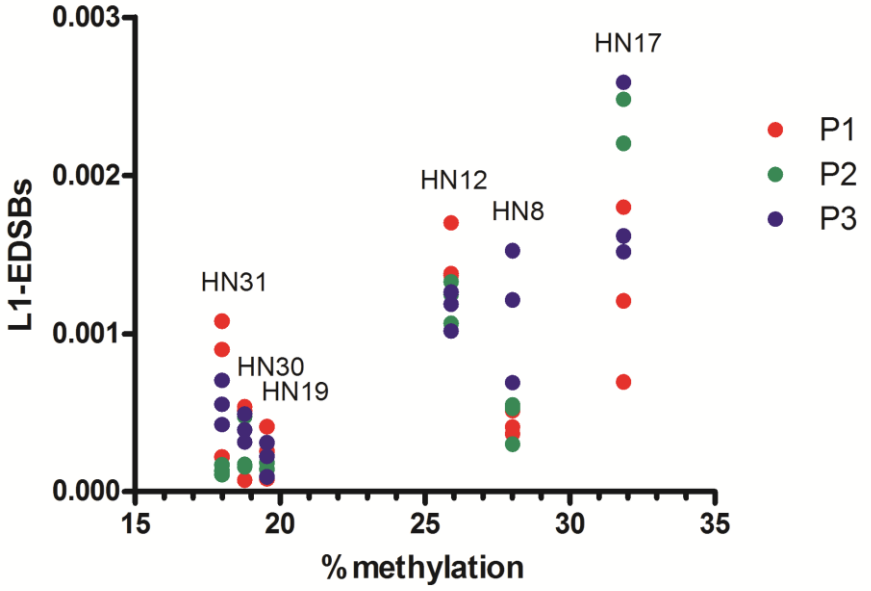


Figure 1 The correlation between L1 methylation and the amount of RIND-EDSBs in Head and Neck cell lines (HN8, HN12, HN17, HN19, HN30, HN31). P1, P2, P3 represented passages of each cell line with triplicate.

Reduction of RIND-EDSBs by Histone deacetylase inhibitor causes DNA breaks production and decreases cell survival when DNA-PKcs was inhibited.

To examine how the role of chromatin dynamics by epigenetic mechanism, histone acetylation, affects the amount of RIND-EDSBs. Cells were treated with a histone deacetylase inhibitor, Trichostatin (TSA), to hyperacetylate histones and consequently decondensethe chromatin. At 4 hrs, histones were hyperacetylated (data not shown). The number of RIND-EDSBs in the control and in cells after 4 hrs of TSA treatment was compared. TSA treatment of serum deprived cells significantly reduced the number of RIND-EDSBs (two-tailed paired t-test, n = 3, p = 0.0398)(Figure 2). Contrarily, RIND-EDSBs were increased when 4 hrs TSA treated cells was combined with inhibitors of critical NHEJ proteins; vanillin, inhibitors of DNA-PKcs for 2 hrs (two-tailed paired t-test, n = 3, p = 0.0498) (Figure 3). Moreover, TSA treated cells for 4hrs combined with vanillin treatment for 24hrs can cause cell survival reducing less than cells which were treated by TSA treatment alone but no significant (two-tailed paired t-test, n = 5, p = 0.0563)(Figure 4).

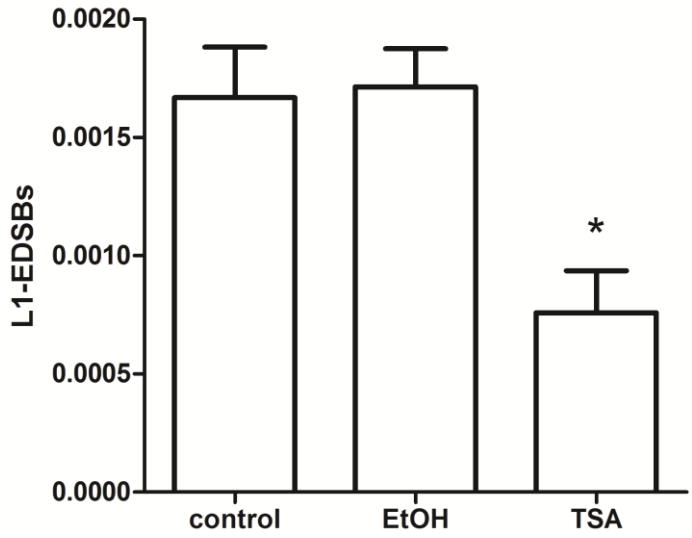


Figure 2 L1-EDSB-LMPCR quantities in cell line when histone deacetylase was inhibited by TSA. RIND-EDSBs were decreased when histone deacetylase was inhibited by TSA compare with control and ethanol which is using to be the solvent.

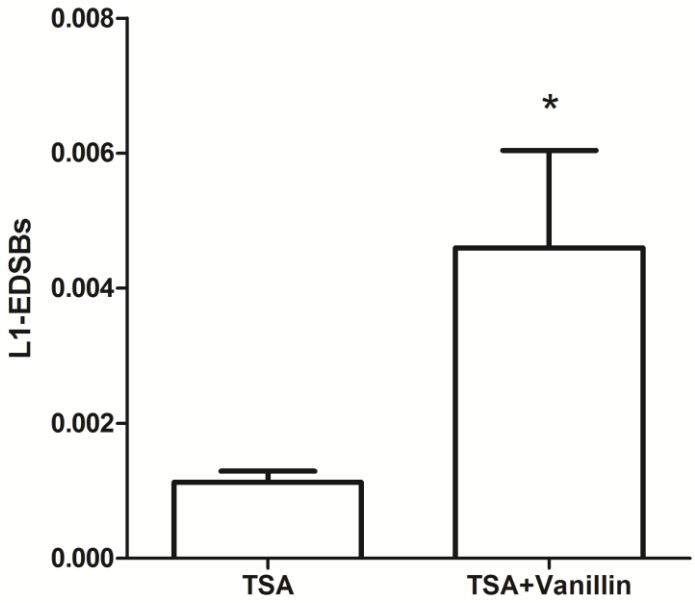


Figure 3 L1-EDSB-LMPCR quantities in cell line when DNA-PKcs was inhibited after histone deacetylase was inhibited by TSA. RIND-EDSBs was increasing when non-homologous end joining repair protein, DNA-PKcs, was inhibited by vanillin for 2 hrs after treated with TSA for 4 hrs

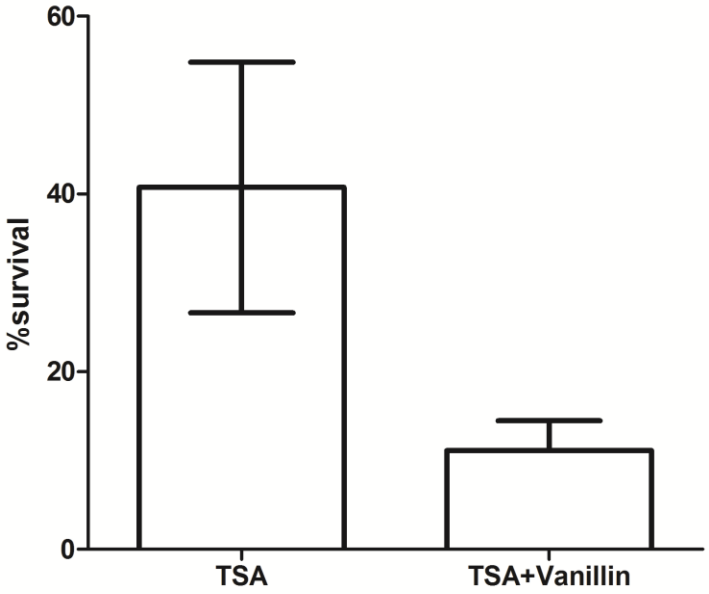


Figure 4 The percentage of cell survival when combine between histone deacetylase inhibitor, TSA, and DNA-PKcs inhibitor, vanillin. Cell survival was reduced in TSA-vanillin treated cells compare with TSA treated cell.

The lower RIND-EDSBs produces DSBs is not the effects of TSA

RIND-EDSBs would be lower in yeast strains lacking any genes involved in the production or the retention of RIND-EDSBs. Remarkably, the levels of RIND-EDSBs were significantly decreased in nhp6aΔ, rox1Δ, ixr1Δ, and hmo1Δ strains. Nhp6A and Nhp6B are highly homologous proteins. They contain an HMGB domain that binds to the minor groove of the DNA and bends it sharply. They also function in association with several chromatin-remodeling complexes [(93)]. HMGB domain was also found in human genome which has several functions connect to chromatin. HMGB1 is an intracellular protein that can translocate to the nucleus where it binds DNA and regulates gene expression. As evidence in yeast, *HMGB1* was selected to confirm if down regulation of HMGB1 protein by RNA interferences can decrease RIND-EDSBs. After HMGB1 transient transfection for 72 hrs, RIND-EDSBs was found decreasing in cells which were transfected by HMGB1si compare with negative control, the non-homology oligonucleotides to human genome (two-tailed unpaired t-test, n = 6, p = 0.0371)(Figure 5). In addition, the methylation status at L1 near RIND-EDSBs in HMGB1 was lower than negative control significantly (two-tailed unpaired t-test, n = 6, p = 0.0056)(Figure 6). However, L1 methylation level in genome compared between HMGB1 knock down cells and negative control cell were not different (Figure 7). As a result of HMGB1 knock down can decrease RIND-EDSBs, DNA-PKcs inhibitor (vanillin) was added for 2 hrs to examine how important of RIND-EDSBs when they are lower level in genome. The result show that RIND-EDSBs in *HMGB1* knock down cells were increasing than negative control when vanillin was added for 2 hrs (two-tailed unpaired t-test, n = 16, p = 0.0381)(Figure 8). And vanillin added can decrease cell survival of HMGB1 knock down cell (two-tailed unpaired t-test, n = 14, p = 0.0338)(Figure 9)

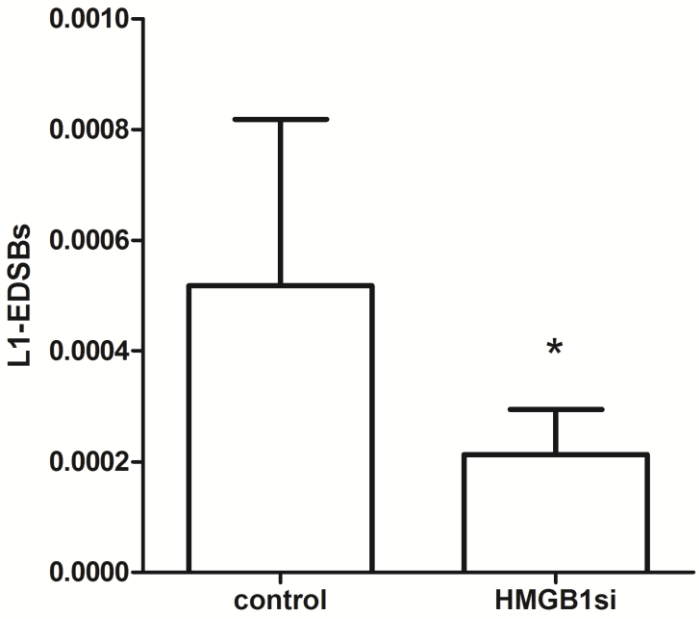


Figure 5 L1-EDSB-LMPCR quantities in HeLa cell line which were transient transfection by HMGB1 oligonucleotide (HMGB1si) compared with negative oligonucletide using as control.

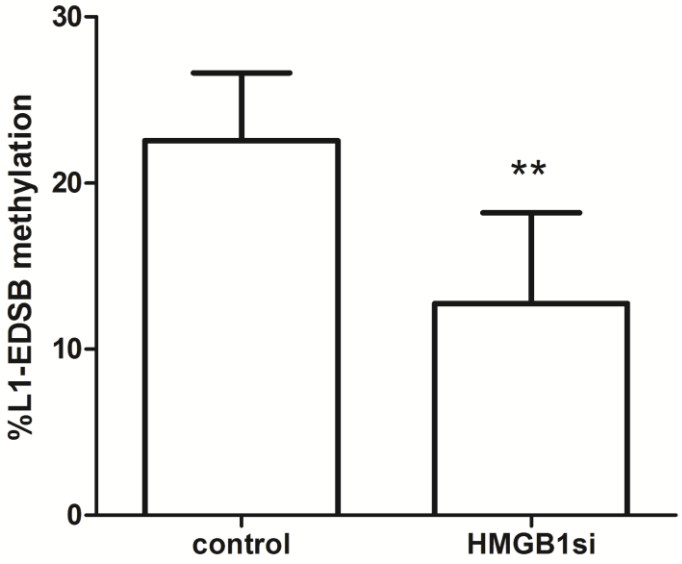


Figure 6 COBRA-L1-EDSBs show the methylation level of L1 located closely with RIND-EDSBs. *HMGB1* knock down cells by RNAi transient transfection had a L1-EDSB methylation level lower than negative control.

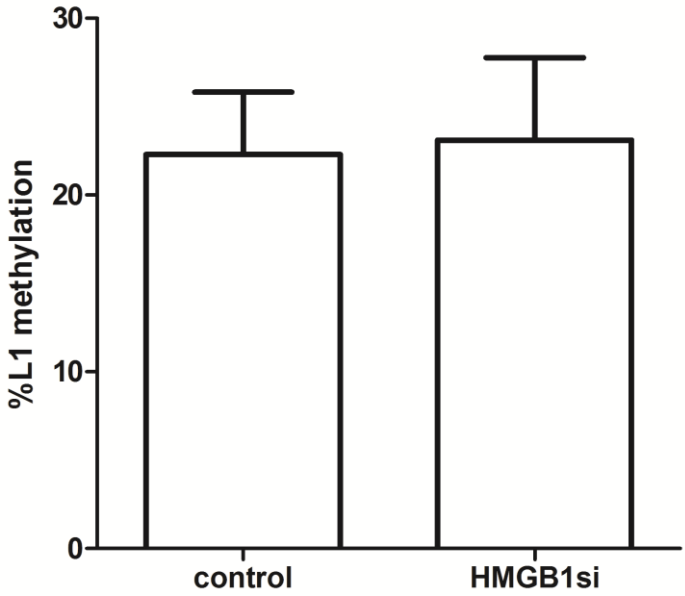


Figure 7 The percentage of methylation status in *HMGB1* transfected cells and negative control cells measured by COBRA-L1. There is no different between

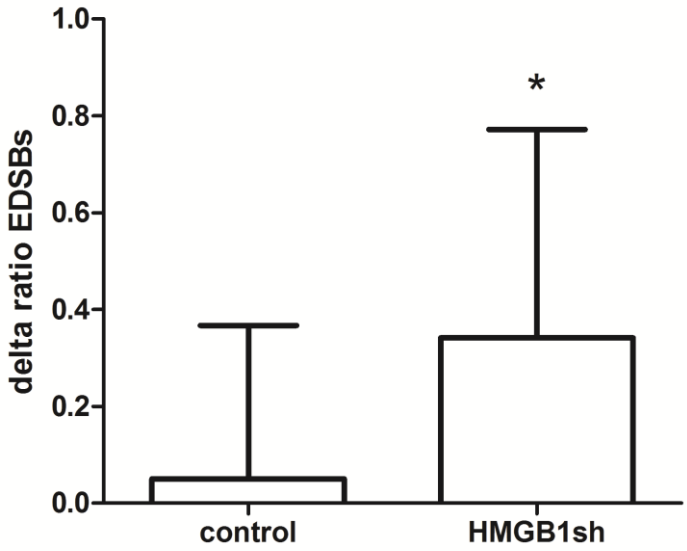


Figure 8 L1-EDSB- LMPCR quantities show the increasing of RIND-EDSBs in *HMGB1* knock down cells when were added by vanillin for 2 hrs compare with negative control.

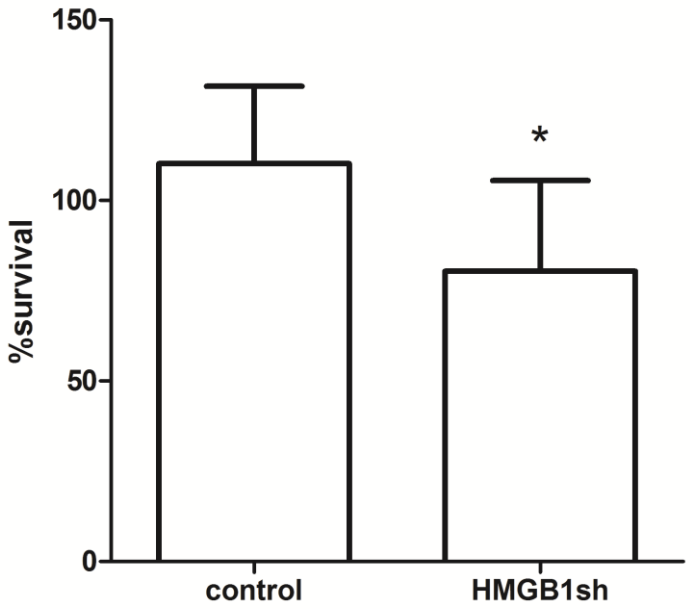


Figure 9 The percentage of cell survival in *HMGB1* knock down cells and negative control cells when non-homologous end joining repair, DNA-PKcs, was inhibited by vanillin for 2 hrs. *HMGB1* knock down cells have a percentage of cell survival lower than control when vanillin was added for 2 hrs.

The variation in RIND-EDSB levels and cell survival

Retained methylated RIND-EDSBs, unlike radiation induced DSBs, may not induce cell death. Retained RIND-EDSBs are present in all cells and do not correlate with decreased cell survival. As mentioned, retained RIND-EDSBs may be repaired pathways selectively mediated by methylation and consequently affect cell viability. Vanillin, a DNA-PKcs inhibitor, is not only induces apoptosis [37] but also prevents mutations. In experiment, we treated cells with vanillin for 24h and found that if prior to vanillin treatment cells possessed higher levels of retained RIND-EDSBs, more cells survived. We found significant results both in HeLa (spearman, $n = 18$, $r = 0.7833$, $p = 0.0172$)(Figure 10 upper) and HCT116 cell line (spearman, $n = 22$, $r = 0.7091$, $p = 0.0182$)(Figure 10 lower). The result may be caused by the production of euchromatic related RIND-EDSBs to cause cell death when DNA repair was inhibited. Moreover, retained RIND-EDSBs may prevent the production of euchromatic related RIND-EDSBs.

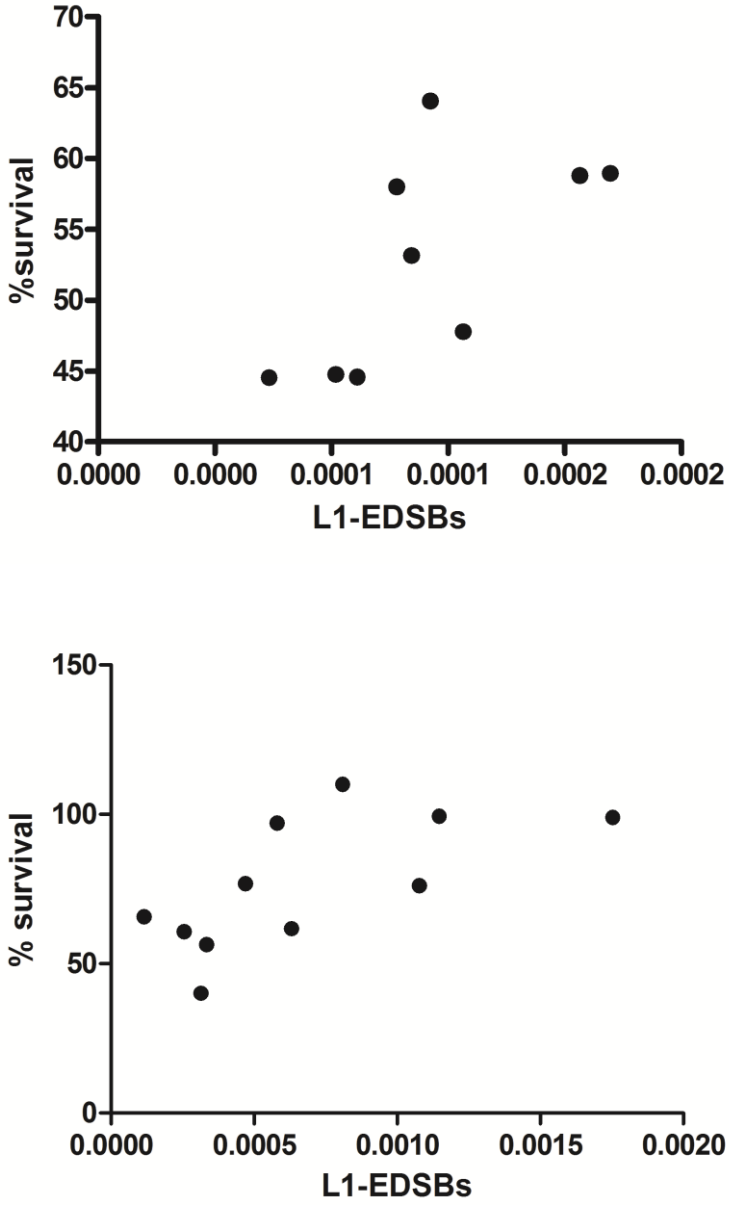


Figure 10 RIND-EDSBs and survival. The relation between RIND-EDSBs prior to vanillin treatments and cell survival, X-axis represented RIND-EDSB levels and Y-axis represents %survival. (upper) Comparison between % survival and L1-EDSB genomes per control genome of vanillin-treated HeLa. Fewer cells survived if they possessed fewer RIND-EDSBs. (lower) HCT116.

CHAPTER V

DISCUSSION

The inhibition of DNA-PKcs mediated non-homologous end joining (NHEJ) under drug induce histone acetylation reduced cell survival. However the mechanism is not yet known. Recently, we developed a new PCR technique to measure the amount and epigenetic modification of endogenous DNA double strand breaks (EDSBs). We found EDSBs in G_0 called these EDSBs as replication independent EDSBs (RIND-EDSBs). There are two classes of RIND-EDSBs. The first presents at a certain level in all cells underphysiologic condition. These RIND-EDSBs are hypermethylated, retained in facultative heterochromatin, devoid of γ-H2AX phosphorylation, and repaired by a slower but more precise ATM mediated NHEJ. The second class of RIND-EDSBs are hypomethylated and bound by γ-H2AX. These RIND-EDSBs are not usually detectable, and only are found when DSB repair is inhibited. Because the first class RIND-EDSBs are detectable in all cells, we proposed that this certain class of RIND-EDSBs may possess physiologic function. On the contrary, cell recognizes the second class similar to radiation induced DSBs. Therefore, the second class RIND-EDSBs may pathogenic. The numbers of RIND-EDSBs (NRIND-EDSBs) were reduced when chromatins were chemically induced by trichostatin A (TSA) to be hyperacetylated but increased when cells were treated with combination of TSA, vanillin (DNA-PKcs inhibitor) and caffeine (ATM inhibitor). RIND-EDSBs are hypermethylated because methylated and unmethylated EDSBs are processed unequally [(94)]. In this study, we show that histone hyperacetylation reduced NRIND-EDSBs and increased PRIND-EDSBs. Therefore, NRIND-EDSBs may be retained in heterochromatin and histone acetylation may facilitate their repair. We speculate that RIND-EDSBs may select a pathway to repair DNA via chromatin conformation. DNA methylation usually associates with heterochromatin [(46)], whose tightly

packed structure may brace the broken chromosome. Therefore, the consequent DNA repair processes between RIND-EDSBs associated with heterochromatin and those associated with euchromatin should be different. Recently, Cowell *et al*. found that H2AX foci form preferentially in euchromatin but not in heterochromatin after ionizing-radiation [95]. Moreover, heterochromatin radiation induces a slow repair of DSBs [38]. In the S phase, RIND-EDSBs are still hypermethylated, albeit with less significance than in the G_0 phase [94]. Because DNA replication does not occur simultaneously throughout the genome, heterochromatin may still capture the methylated RIND-EDSBs that locate away from replication forks.

Radiation-induced DSBs are hazardous to cells and can lead to faulty DNA recombination. Therefore, the discovery of RIND-EDSBs in all cells without environmental insults needs explanation. RIND-EDSBs can be produced without DNA breakage chemical agents or radiation. When vanillin was added, the level of RIND-EDSBs was sporadically increased. Therefore, RIND-EDSBs can be produced from both unmethylated and methylated DNA. The precise mechanism producing RIND-EDSBs is unknown. NRIND-EDSBs are different from PRIND-EDSBs [8] in that the levels of PRIND-EDSBs are less methylation dependent [94]. Moreover, we detected variable levels of PRIND-EDSBs [94].

Retained methylated RIND-EDSBs, unlike radiation-induced DSBs, may not induce cell death. RIND-EDSBs are present in all cells and do not correlate with decreased cell survival. In contrast, RIND-EDSBs may help the cell to survive. Vanillin not only induces apoptosis [96] but also prevents mutations [97, 98]. One of the mechanisms may be due to the fact that vanillin, a DNA-PKcs inhibitor, selectively kills genomically unstable cells, such as irradiated cells. In contrast, we demonstrated that vanillin may selectively do less harm to cells that possess more RIND-EDSBs. Therefore, this study reveals an unprecedented characteristic of RIND-EDSBs. Whereas radiation-induced DSBs are lethal to vanillin treated cells in a dose-dependent manner [99], RIND-EDSBs may help the cell survive vanillin toxicity, and a higher number of EDSB-NRs may be

relate to a more stable genome. These findings, while surprising, are reasonable. RIND-EDSBs that are retained in heterochromatin may possess a similar role as topoisomerase lesions that are important in maintaining DNA integrity by reducing the tension of DNA [21, 100].

Interestingly, in 2005, Yaneva *et al*. reported high cellular toxicity when NHEJ inhibitor and TSA were combined [51]. Higher L1-EDSB methylation levels suggest that there are more unrepaired RIND-EDSBs near methylated genomic regions. Since DNA methylation is usually associated with histone deacetylation, we determined whether RIND-EDSBs would be repaired if the chromatin became hyperacetylated. We treated HeLa cells with a histone deacetylase inhibitor, Trichostatin (TSA), to hyperacetylate histones and consequently decondense the chromatin. Histone acetylation was observed at the peake level at 4 hrs. TSA treatment of serum deprived HeLa cells significantly reduced the number of L1-EDSBs after 4 hrs. Interestingly, we demonstrated that TSA reduced RIND-EDSBs and cells with lower RIND-EDSBs survived less from vanillin treatment. Assuming that RIND-EDSBs were retained prior to TSA treatment and that histone hyperacetylation facilitated RIND-EDSB repair. This suggests that variable numbers of RIND-EDSBs maintained when chromatin is deacetylated. We concluded here that heterochromatin is a reservoir of RIND-EDSBs.

High mobility group box 1 (HMGB1) is an abundant chormatin protein involving in several functions. It is generally known as a DNA binding protein acting in maintenance of nucleosome structure. Transient interaction of HMGB1 with nucleosomal linker DNA enhances the ability bind nucleosomal DNA [101, 102] and can enhance DNA repair and chromatin modification after DNA damage. Its absence leads to increased mutagenesis, decreased cell survival, and altered chromatin reorganization after DNA damage [103]. RIND-EDSBs in HMGB1 knock down cell was decrease assuming that absent of HMBG1 affects to chromatin which might facilitate the DNA repair accessibility to RIND-EDSBs. However, HMGB1 knock down combined with NHEJ repair protein (DNA-PKcs) inhibitor, vanillin, causes the number of RIND-EDSBs rising up. This result

speculates that produced RIND-EDSBs should be PRIND-EDSBs in consideration of the lower cell survival.

Finally, this study may help speculate the connection how vanillin prevents mutation and how global hypomethylation leads to genomic instability. Genomic hypomethylation is a common molecular event in cancer [1, 104, 105], and it leads to chromosomal instability [31, 85, 106, 107]. The methylation levels of RIND-EDSBs are in direct correlation with the genome. The RIND-EDSBs methylation levels of hypermethylated genomes are higher than genomes that possess lower methylation levels [94]. Interestingly, vanillin not only inhibits NHEJ repair, increases PRIND-EDSBs, but also cells with lower RIND-EDSBs are more sensitive to vanillin treatment. It will be interesting to further explore whether retained NRIND-EDSBs contribute to genomic stability and whether lower levels of the RIND-EDSBs are directly associated with hypomethylation-related instability. RIND-EDSBs are maintained in normal cells to a certain levels by the functions of DNA methylation, histone acetylation and HMGB1. Reduction of these RIND-EDSBs produces PRIND-EDSBs and causing cell death when cells were treated with vanillin. We also postulated that when without repair inhibitors, these pathologic EDSBs may lead to DSB repair errors. Consequently, reduction of NRIND-EDSBs by global hypomethylation may cause genomic instability by this mechanism.

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APPENDICES

APPENDIX A

BUFFERS AND REAGENTS

1. Lysis Buffer 1

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

Sterilize the solution by autoclaving and store in a refrigerator (at 4^0C).

2. Lysis Buffer 2

5.0 M NaCl	15	ml
0.5 M EDTA (pH 8.0)	48	ml
Distilled water to	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	100	ml

Mix the solution and store at room temperature.

4. 20 mg/ml Proteinase K

Proteinase K	2	mg
Distilled water to	1	ml

Mix the solution and store in a refrigerator (at -20^{0}C).

5. 1.0 M Tris ─ HCl

Tris base	12.11	g

Dissolve in distilled water and adjusted pH to 7.5 with HCl

Distilled water to	100	ml

Sterilize the solution by autoclaving and store at room temperature.

6. 0.5 M EDTA (pH 8.0)

Disodium ethylenediamine tetraacetate.2H_2O	186.6	g

Dissolve in distilled water and adjusted pH to 8.0 with NaOH

Distilled water to	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

7. 1.0 M $MgCl_2$ solution

Magnesium chloride.$6H_2O$	20.33	g
Distilled water to	100	ml

Dispense the solution into aliquots and sterilize by autoclaving.

8. 5 M NaCl solution

Sodium chloride	29.25	g
Distilled water to	100	ml

Dispense the solution into aliquot and sterilize by autoclaving.

9. 10X Tris borate buffer (10X TBE buffer)

Tris ─ base	100	g
Boric acid	55	g
0.5 M EDTA (pH 8.0)	40	ml

Adjust volume to 1,000 ml with distilled water. The solution was mixed and store at room temperature.

10. 6X loading dye

Bromphenol blue	0.25	g
Xylene cyanol	0.25	g

Glycerol	50	ml
1M Tris (pH 8.0)	1	ml
Distilled water until	100	ml

Mixed and stored at 4^0C

11. 7.5 M Ammonium acetate (CH_3COONH_4)

Ammonium acetate	57.81	g
Distilled water	80	ml

Adjust volume to 100 ml with distilled water and sterilize by autoclaving.

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

Phenol	25	volume
Chloroform	24	volume
Isoamyl alcohol	1	volume

Mix the reagent and store in a sterile bottle kept in a refrigerator.

13. 8% Non-denature acrylamide gel (w/v)

40%acrylamide: Bis (19:1)	1	ml
10X TBE	0.5	ml

10% ammoniumpersulfate	100	µl
TEMED	5	µl
H_2O	3.5	ml

Dissolve by heating in microwave oven and occasional mix.

14. TE buffer

Tris base	1.21	g
5M EDTA	200	µl

Adjust pH to 7.5 with conc.HCL and adjust volume to 1.0 litre with H_2O.

APPENDIX B

Sequence of *HMGB1* and primer

RT-PCR

We created primers for tested *HMGB1* expression

HMGB1 sequence and primer

ATGGGCAAAGGAGATCCTAAGAAGCCGAGAGGCAAAATGTCATCATATGCATTTTTTG
TGCAAACTTGTCGGGAGGAGCATAAGAAGAAGCACCCAGATGCTTCAGTCAACTTCT
CAGAGTTTTCTAAGAAGTGCTCAGAGAGGTGGAAGACCATGTCTGCTAAAGAGAAAG
GAAAATTTGAAGATATGGCAAAAGCGGACAAGGCCCGTTATGAAAGAGAAATGAAAA
CCTATATCCCTCCCAAAGGGGAGACAAAAAAGAAGTTCAAGGATCCCAATGCACCCA
AGAGGCCTCCTTCGGCCTTCTTCCTCTTCTGCTCTGAGTATCGCCCAAAAATCAAAGG
AGAACATCCTGGCCTGTCCATTGGTGATGTTGCGAAGAAACTGGGAGAGATGTGGAA
TAACACTGCTGCAGATGACAAGCAGCCTTATGAAAAGAAGGCTGCGAAGCTGAAGGA
AAAATACGAAAAGGATATTGCTGCATATCGAGCTAAAGGAAAGCCTGATGCAGCAAAA
AAGGGAGTTGTCAAGGCTGAAAAAAGCAAGAAAAAGAAGGAAGAGGAGGAAGATGA
GGAAGATGAAGAGGATGAGGAGGAGGAGGAAGATGAAGAAGATGAAGATGAAGAAG
AAGATGATGATGATGAATAA

RT-PCR primer

Forward primer = ATATGGCAAAAGCGGACAAG

Reverse primer = GCAACATCACCAATGGACAG

PCR product = 193 bp

GAPDH primer

Forward primer = GTGGGCAAGGTATCCCTG

Reverse primer = GATTCAGTGTGGTGGGGGAC

PCR product = 460 bp

BIOGRAPHY

Miss Araya Thongnak was born in Phichit in 1986. In 2008, she graduated from faculty of Science, Chiang Mai University in Biology program and then attended to particulate in Medical Science program in Faculty of Medicine for her master degree. She plans to study in Ph.D. program.