บทบาทของ LINE-1 เมทิลเลชันต่อโปรโมเตอร์เมทิลเลชันของยีนในมะเร็งศีรษะและคอ

นายศุภกิจ โขวุฒิธรรม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

สาขาวิชาชีวเวชศาสตร์ (สหสาขาวิชา)

บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย

ปีการศึกษา 2553

The role of LINE-1 methylation

on gene promoter methylation in head and neck cancer.

Mr. Suphakit Khowutthitham

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biomedical Sciences
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2010

Thesis Title	The role of LINE-1 methylation on gene promoter methylation in head and neck cancer.
By	Mr. Suphakit Khowutthitham
Field of Study	Biomedical Sciences
Thesis Advisor	Professor Apiwat Mutirangura, M.D., Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

……...…… Dean of the Graduate School
(Associate Professor Pornpote Piumsomboon, Ph.D.)

THESIS COMMITTEE

……...…….. Chairman
(Associate Professor Prasit Pavasant, D.D.S., Ph.D.)

……..……... Thesis Advisor
(Professor Apiwat Mutirangura, M.D., Ph.D.)

……..……...…… Examiner
(Assistant Professor Virote Sriuranpong, M.D., Ph.D.)

….……..……..… Examiner
(Associate Professor Kanya Suphapeetiporn, M.D., Ph.D.)

…….……..…….. External Examiner
(Danai Tiwawech, Ph.D.)

ศุภกิจ โขวุฒิธรรม: บทบาทของ LINE-1 เมทิลเลชันต่อโปรโมเตอร์เมทิลเลชันของยีนในมะเร็งศีรษะและคอ (The role of LINE-1 methylation on gene promoter methylation in head and neck cancer) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.นพ.ดร. อภิวัฒน์ มุทิรางกูร, 75 หน้า

ภาวะการเกิดเมทิลเลชันระดับสูงที่โปรโมเตอร์ของยีนและภาวะการเกิดเมทิลเลชันระดับต่ำทั่วทั้งจีโนมเป็นลักษณะที่พบได้บ่อยในมะเร็ง โดยที่ภาวะการเกิดเมทิเลชันระดับต่ำทั่วทั้งจีโนมสามารถเหนี่ยวนำให้เกิดการเปลี่ยนแปลงการแสดงออกของยีนมะเร็ง การเกิดความไม่เสถียรของโครโมโซม รวมทั้ง การกระตุ้นให้ทรานโปซอน (Transposons) กลับมาทำงานอีกครั้งหนึ่ง LINE-1 เป็นรีโทรทรานโปซอนที่สามารถเคลื่อนที่ด้วยตัวเองและไม่มี LTR (Autonomous non LTR retrotransposon) ที่มีปริมาณมากที่สุดในจีโนมมนุษย์ตามจำนวนเบสและจะถูกยับยั้งการแสดงออกโดยกลไกที่เรียกว่า ดีเอ็นเอเมทิลเลชัน (DNA methylation) เราพบว่าระดับเมทิลเลชันของ LINE-1 ในเซลล์มะเร็งจะมีระดับที่ต่ำกว่าเซลล์ปกติในหลายเนื้อเยื่อ นอกจากนี้ ระดับเมทิลเลชันของ LINE-1 ยังมีความแตกต่างกันขึ้นอยู่กับเนื้อเยื่อแต่ละชนิด รวมทั้ง ตำแหน่งของ LINE-1 ด้วย อย่างไรก็ตาม เราสามารถพบภาวะการเกิดเมทิเลชันระดับสูงของ LINE-1 บางตำแหน่งในมะเร็งได้ เช่น LINE-1 ในยีน *CNTNAP5* ในการศึกษาครั้งนี้ เราสนใจที่จะศึกษาผลกระทบของระดับเมทิลเลชันของ LINE-1 ที่วางตัวอยู่ภายในยีน (Intragenic LINE-1 methylation) และ LINE-1 ที่กระจายตัวอยู่ทั่วทั้งจีโนม (Global LINE-1 methylation) ที่มีต่อโปรโมเตอร์ของยีนในมะเร็งศีรษะและคอ เราคัดเลือกยีนซึ่งมี LINE-1 อยู่ภายในยีนและไม่มี LINE-1 อยู่ภายในยีนเพื่อวัดระดับเมทิลเลชันของโปรโมเตอร์ของยีน, LINE-1 ที่วางตัวอยู่ภายในยีน และ LINE-1 ที่กระจายตัวอยู่ทั่วทั้งจีโนม เราพบว่า ระดับเมทิลเลชันของ LINE-1 ในเซลล์มะเร็งยังคงมีระดับที่ต่ำกว่าเซลล์ปกติ รวมทั้ง LINE-1 วางตัวที่อยู่ในยีนเดียวกัน โดยเฉพาะอย่างยิ่งใน intron เดียวกัน จะมีระดับเมทิลเลชันของ LINE-1 ที่มีความสัมพันธ์กันสูงที่สุด หลังจากนั้น เราได้ศึกษาความสัมพันธ์กันระหว่างระดับเมทิเลชันที่โปรโมเตอร์ของยีน, ระดับเมทิเลชันของ LINE-1 ที่วางตัวอยู่ภายในยีน และระดับเมทิลเลชันของ LINE-1 ที่กระจายตัวอยู่ทั่วทั้งจีโนม เราพบว่า ระดับเมทิลเลชันที่ต่ำของ LINE-1 ทั่วทั้งจีโนม (global LINE-1 hypomethylation) มีความสัมพันธ์กับระดับเมทิลเลชันของยีนทั้งยีนที่มี LINE-1 วางตัวอยู่ภายในยีน *(CNTNAP5)* และไม่มี LINE-1 วางตัวอยู่ภายในยีน (*CALCA*) ซึ่งโปรโมเตอร์ของทั้ง 2 ยีนมีลักษณะการเกิด demethylation จากข้อมูลนี้บ่งชี้ว่า การที่ระดับเมทิลเลชันที่ต่ำของ LINE-1 ทั่วทั้งจีโนม มีความสัมพันธ์กับระดับเมทิลเลชันของยีนนั้นไม่เกี่ยวข้องกับระดับเมทิลเลชันของ LINE-1 ที่วางตัวอยู่ภายในยีน รวมทั้ง กลไกในการเกิดระดับเมทิลเลชันที่ต่ำทั่วทั้งจีโนมนั้น น่าจะเป็นกลไลโดยทั่วไปที่ไม่มีความเฉพาะเจาะจงกับลำดับเบส

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2553 ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก .....

4689688220: MAJOR BIOMEDICAL SCIENCES
KEYWORDS: LINE-1 / GLOBAL LINE-1 METHYLATION/ GENE PROMOTER METHYLATION/ DNA METHYLATION / HEAD AND NECK CANCER

SUPHAKIT KHOWUTTHITHAM: THE ROLE OF LINE-1 METHYLATION ON GENE PROMOTER METHYLATION IN HEAD AND NECK CANCER.
ADVISOR: PROF. APIWAT MUTIRANGURA, MD, Ph.D., 75 pp.

In cancers, hypermethylation of CpG dinucleotides in promoter region and global hypomethylation are commonly featured. Global hypomethylation can lead to alterations in the expression of oncogenes, chromosomal instability and reactivation of transposons. LINE-1 elements are the most autonomous non LTR retrotransposon in human genome by mass and suppressed by DNA methylation process. The methylation levels of LINE-1 elements in cancerous cells were lower than wild-type cells in various tissues and different among tissue types and inserted locations. However, some intragenic LINE-1 elements hypermethylation can be found sporadically in cancer such as intragenic LINE-1 element that located on *CNTNAP5*. Here, we studied the influence of LINE-1 methylation, both intragenic LINE-1 and global LINE-1 elements, on gene promoter methylation in head and neck cancer cell lines. We selected candidate hypermethylated genes with and without LINE-1 insertion, and then investigated the methylation levels of their promoters, intragenic LINE-1 and global LINE-1 elements. The result showed that LINE-1 methylation in cancerous cells was also lower than normal cells. In addition, intragenic LINE-1 methylation in the same gene especially located on the same intron might have the highest correlation value. Then, we observed the correlation between gene promoter methylation and LINE-1 methylation; both intragenic and global LINE-1 methylation. We found that global LINE-1 hypomethylation was correlated to both promoter of LINE-1 inserted gene (*CNTNAP5*) and non-LINE-1 inserted gene (*CALCA*) which were demethylated promoters. These data indicated that the correlation between global hypomethylation and gene promoter methylation were associated in an intragenic LINE-1-independent manner, and that mechanism of global hypomethylation was general process which sequences-dependent manner.

Field of Study: Biomedical sciences Student's Signature: .....

Academic Year : 2010 Advisor's Signature: .....

ACKNOWLEDGEMENTS

This dissertation could not have been written without the support of the Royal Golden Jubilee Ph.D. program from Thailand Research Funds and the 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) from Chulalongkorn University.

I would like to express my appreciation to my advisor, Professor Apiwat Mutirangura who encouraged, suggested and supported me to complete this dissertation and Center of Excellence in Molecular Genetics of Cancer and Human Diseases, Faculty of Medicine, Chulalongkorn University for facilities to do research.

I would like to thank Dr. Pattamawadee Yanatatsaneejit, Dr. Keskanya Subbalekha, Dr. Nakarin Kitkumthorn, Ms. Khunta Panraksa, Dr. Narisorn Kongruttanachok, Dr. Wichai Pornthanakasem, Dr. Chotika Smarkchan and the rest in Center of Excellence in Molecular Genetics of Cancer and Human Diseases for the companionship and the emotional support including offering suggestion for my improvement.

My special thank to Dr. Chureerat Phokaew, Miss Wachiraporn Wanichnopparat and Mr. Prakasit Rattanatunyong for their assistance to my dissertation.

Finally, I would like to express my deepest gratitude to my mother and my elder brother for their love and understanding which have brought me today's success.

CONTENTS

PAGE

PAGE

PAGE

LIST OF TABLES

LIST OF FIGURES

FIGURES PAGE

LIST OF ABBREVIATIONS

5azaDC	5-aza-2'-deoxycytidine
BilIN	Biliary intraepithelial neoplasia
cDNA	Complementary DNA
CNTNAP5	Contactin associated protein-like 5
COBRA	Combined Bisulfite Restriction Analysis
COBRA-L1	COBRA of LINE-1
CU-L1	Cobra for unique sequence to LINE-1
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
dNTPs	Deoxynucleotide triphosphates
EDSBs	Endogenous DNA double strand breaks
EDTA	Ethylenediamine tetracetic acid
EHC	Extrahepatic cholangiocarcinoma
EN	Endonuclease
EOC	Epithelial ovarian cancer
FBS	Fetal bovine serum
FLC	Fibrolamellar carcinoma
GAPDH	Glyseraldehyde-3-phosphate dehydrogenase
GIST	Gastrointestinal stromal tumor
HDAC	Histone deacetylation
HERV	Human endogenous retroviral element
HNSCC	Head and neck squamous cell carcinoma
IRS	Interspersed repetitive sequences
IVS	Intervening Sequence
LINE-1	Long intersperse nuclear element type 1
LTR	Long terminal repeats
NOE	Normal oral epithelium
NSCLC	Non-small cell lung carcinoma
ORF	Open reading frame

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qRT-PCR	Quantitative Reverse Transcription-PCR
RNA	Ribonucleic acid
RNAPII	RNA polymerase II
RNP	Ribonucleoprotein particle
RPMI	Roswell park memorial institute (medium)
RT	Reverse transcriptase
SAM	S-adenosylmethionine
SDS	Sodium dodecyl sulfate
TPRT	Target-primed reverse transcription
UTR	Untranslated region
WSU-HN	Head and neck squamous cell carcinoma cell lines from Wayne State University

CHAPTER I
INTRODUCTION

Background and Rationale

Long intersperse nuclear element type 1 or LINE-1 elements are the most autonomous non LTR retrotransposon in human genome by mass. LINE-1 elements are widely distributed in the human genome both intragenic region and intergenic region [1-3]. LINE-1 elements can impact the genome structure that resulting to global instability, global rearrangement and global innovation [4, 5]. In normal cells, LINE-1 elements are transcriptional suppressed by DNA methylation however the methylation of LINE-1 elements is reduced in cancer [6, 7]. Global hypomethylation is one of the most common epigenetic changes during cancer development [6-8] as lead to alterations in the expression of oncogenes, chromosomal instability and reactivation of transposons. However, the cause of global hypomethylation mechanism is still unclear [9]. Chalitchagorn *et al*. studied the methylation level of genome-wide LINE-1 methylation from microdissected paraffin-embedded samples both adjacent wild-type cells and cancerous cells in several tissues. The result showed that the methylation levels of LINE-1 elements in cancerous cells are lower than wild-type cells in various tissues and the methylation levels of LINE-1 were significantly different among different tissue types [10]. This result confirmed to several studies that found the global hypomethylation in several cancers and the variation of LINE-1 methylation levels in normal tissues [11-19].

However, the consequences of global hypomethylation such as aberrant gene expression and chromosomal instability have been proposed on adjacent global hypomethylation region [20, 21]. Thus, LINE-1 elements in each locus should be different role in carcinogenesis. From this hypothesis, Phokaew *et al* evaluated the methylation of intragenic LINE-1 elements using COBRA for intragenic to LINE-1 sequence (CU-L1). 17 copies of intragenic LINE-1 elements in 16 genes are selected for methylated measurement in both normal cells and cancerous cells. The result showed that LINE-1 methylation levels both normal and cancer cells vary among locations and cell types. The methylation levels of several intragenic LINE-1 elements in cancerous cells are also lower than normal cells and not all LINE-1 elements are completely methylated in normal cells. However, some LINE-1 hypermethylation can

be found sporadically in cancer for example intragenic LINE-1 locus in *CNTNAP5* (L1-*CNTNAP5*) and intragenic LINE-1 locus in *MGC42174* (L1-*MGC42174*) [22].

Furthermore, Lomdee *et al* evaluated the methylation levels of five specific loci LINE-1 elements among common epithelial ovarian cancers (EOC) and normal ovarian specimens including observe the association between the methylation levels in each locus of LINE-1 and clinicopathological characteristics. Five copies of intragenic intragenic LINE-1 elements were selected to evaluate the methylation levels. These LINE-1 elements, L1-*CNTNAP5*, L1-*ANTXR2*, L1-*LRP2*, L1-*EPHA3*-IVS5 and L1-*EPHA3*-IVS15, were previous studied by Phokaew *et al*. The result showed that the methylation levels of intragenic L1-*CNTNAP5*-IVS11 and L1-*EPHA3*-IVS5 were correlated with disease free interval of ovarian cancer patients and L1-*CNTNAP5*-IVS11 had significant correlation with progression free survival.

Global LINE-1 hypomethylation and CpG islands hypermethylation were commonly featured in several cancers however the association between these two events was still investigated [23-26]. Although, the mechanism of global hypomethylation was still unclear, global hypomethylation frequently occurred at LINE-1 elements including Human endogenous retroviral (HERV) elements which were transposable elements [22, 27]. This features lead to our question that the mechanism of global hypomethylation was specific mechanism for transposable elements or general mechanism which did not depend on specific sequences.

Moreover, the loss of genome wide LINE-1 methylation related to tumor progression by induced global instability [5, 9, 28-30], we hypothesized that because of the mechanical DNA repair processes for methylated and unmethylated of endogenous DNA double strand breaks (EDSBs) should be different [9, 31, 32]. LINE-1 hypomethylation is directly associated with multistep carcinogenesis and a poor prognosis thus LINE-1 hypomethylation is a potential biomarker for the prediction of carcinogenesis [12, 13, 23, 33-39]. However, Aberrant LINE-1 methylation can also discover in other conditions for example tissue differentiation, particulate pollution exposure and dietary [40-46]. Therefore, we interested to study the role of LINE-1 methylation on cellular functions in cancer.

Research Questions

1. Does LINE-1 methylation, both intragenic LINE-1 methylation and global LINE-1 methylation, correlate to gene promoter methylation?
2. Does the correlation between global LINE-1 hypomethylation and gene promoter methylation depend on intragenic LINE-1 methylation?
3. Does the correlation between global LINE-1 hypomethylation and gene promoter methylation can observe in whole genome?

Objectives

1. To study the correlation between gene promoter methylation and LINE-1 methylation, both intragenic LINE-1 methylation and global LINE-1 methylation.
2. To study the correlation between global LINE-1 methylation and gene promoter methylation in with and without LINE-1 inserted genes.
3. To observe the highly methylated genes in global hypomethylated genome.

Hypothesis

1. LINE-1 methylation may associate to some gene promoter methylation which has highly methylation level at their promoters.
2. Global LINE-1 methylation may correlate to some gene promoter methylation both LINE-1 inserted gene and non-LINE-1 inserted gene.
3. Highly methylated genes can be found in global hypomethylated genome, and these highly methylated gene promoters may relate to global hypomethylation.

Key words

LINE-1, Global LINE-1 hypomethylation, Gene promoter methylation, DNA methylation, Head and neck cancer.

Expected result

This study will help us to understand about the correlation of the mechanism of LINE-1 methylation and gene promoter methylation in head and neck cancer.

Conception Framework

Research Question I: Does LINE-1 methylation, both intragenic LINE-1 methylation and global LINE-1 methylation, correlate to gene promoter methylation?

1. To select candidate genes.
    - CNTNAP5 was selected as our candidate gene. This gene was studied in previous studied by Phokaew *et al* which measured the methylation levels of intragenic LINE-1 elements in head and neck cancer cell lines.
2. To investigate the methylation levels of
    - Candidate genes promoter using COBRA.
    - Intragenic full-length LINE-1 elements located on *CNTNAP5* (L1-*CNTNAP5*) using CU-L1.
    - All LINE-1 elements which contain 5'UTR in whole genome, called global LINE-1 methylation, using COBRA-L1.
3. To observe the correlation between them.

Research Question II: Does the correlation between global LINE-1 hypomethylation and gene promoter methylation depend on intragenic LINE-1 methylation?

1. To select candidate genes.
    - Genes with intragenic LINE-1 insertion: *ME3*, *PDE3B* and *PDE4D* were chose as candidate genes.
    - Genes without intragenic LINE-1 insertion: DLEC1, CALCA and CHFR were chose as candidate genes.
2. To detect the methylation levels of candidate gene promoter methylation and intragenic LINE-1 methylation.
3. To observe the correlation between gene promoter methylation and global LINE-1 methylation including intragenic LINE-1 methylation among candidate genes.

Research Question III: Does the correlation between global LINE-1 hypomethylation and gene promoter methylation can observe in whole genome?

1. To investigate candidate hypermethylated genes in global hypomethylated genome by CU-DREAM.
2. To observe candidate hypermethylated genes with and without intragenic LINE-1 insertion by CU-DREAMX.

CHAPTER II

REVIEW of RELATED LITERATURE

Long Interspersed Nuclear Element

Transposable elements or transposons are mobile genetic elements that can transpose themselves to new genome locations. Approximately 45% of the human genome consists of transposable elements [47] . Mammalian transposable elements are separated into two board classes, known as DNA transposon and Retrotransposon (Figure 1).

1. **DNA transposons** are found in the genome of bacteria, many metazoa, insects, worms, including human [4]. They account for at least 3% of the human genome [5]. They generally transpose by excise themselves from the genome and then integrate themselves to a new position, called “cut and paste” mechanism. For example of this class are Tc1/mariner, PIF/Harbinger, hAT, Mutator, Merlin, Transib, and P [4, 48].

2. **Retrotransposons** contain approximately 42% of the human genome [49]. The transposition of retrotransposons is called “copy and paste” mechanism because they transcribe themselves into RNA intermediate and then reintegrate into the new genome sites. Furthermore, retrotransposons also are subdivided into two categories on the basis of the presence or absence long terminal repeats (LTR) that are repeated sequences about 300 – 1,000 base pairs at the ends of the elements.
   2.1 **LTR retrotransposon** contain about 8% in the human genome. LTR retrotransposon resemble to retrovirus, except that they has no an envelope glycoprotein gene that essential to exit from the cell [4, 5].
   2.2 **Non-LTR retrotransposon** accounts for approximately 34% in the human genome. This category composes of active elements and inactive elements. Moreover, non-LTR retrotransposable elements also can classify into autonomous and non-autonomous elements base on capability of the reverse transcriptase encoding that necessary for retrotransposition.

2.2.1 **Autonomous non-LTR retrotranspons**, such as Long intersperse nuclear elements or LINE elements, can encode essentially retrotransposable proteins that are required for their movement.

2.2.2 **Non-autonomous non-LTR retrotransposons** cannot encode essentially material for their retrotransposition thus the transposition of them depends on other transposons, the example of non-autonomous non-LTR retrotransposons is SINEs.

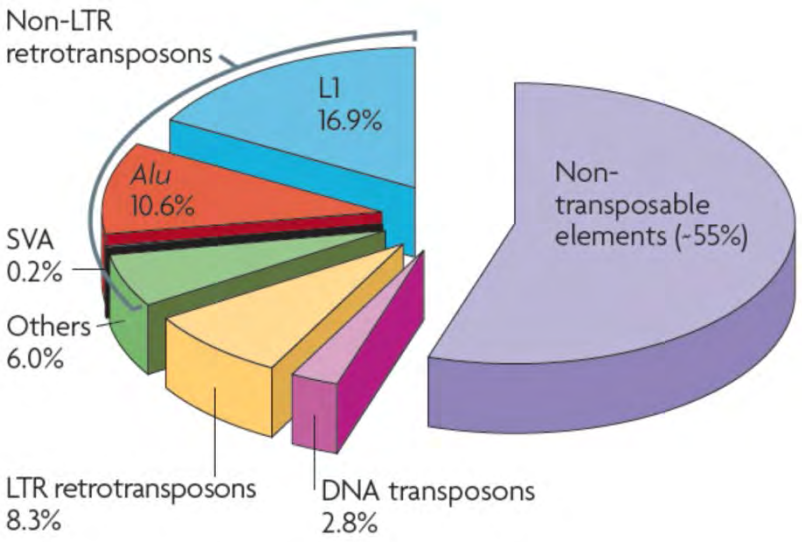

Figure 1. Human transposable elements. Approximately 45% of the human genome consists of transposable elements that can be divided into DNA transposons, LTR retrotransposons and Non-LTR retrotransposons. LINE-1 elements are the most transposon elements by mass that are about 17% in human genome. (Cordaux R *et al*, 2009) [5].

Long intersperse nuclear elements or LINE elements are the most active autonomous non-LTR retrotransposons. The majority of LINE elements in human genome are called LINE-1 elements. LINE-1 elements comprise approximately 500,000 copies or 17% which are the most transposable elements in the human genome by mass [47]. However, only about 3,000–5,000 copies represent full-length LINE-1 elements and only 100 copies are transcriptionally active [50]. The full-length form of LINE-1

elements is approximately 6 kilobase (kb) in length and the majority of these elements are nonfunctional in retrotransposition because mutation, truncation or rearrangement. The full length LINE-1 elements consist of a 910-bp 5' untranslated region (5'UTR) containing internal *RNA polymerase*II (RNAPII) promoter activity, two open reading frames (ORF1 and ORF2) that are separated by a 63-bp inter-ORF spacer region and a 205-bp 3' UTR containing an AATAAA polyadenylation signal, and a polyA tail [51] (Figure 2).

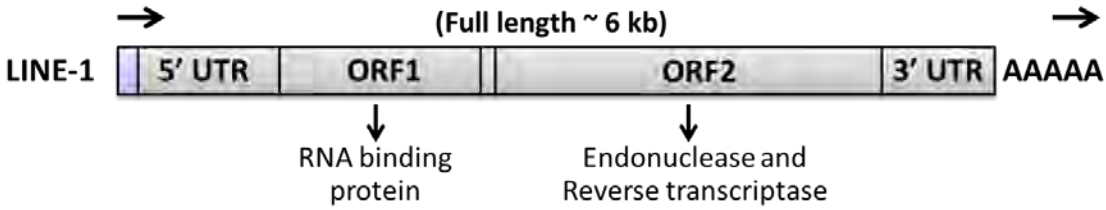

Figure 2. Structure of full length LINE-1 element. The full length LINE-1 element (Gray boxes) is approximately 6 kb consists of 5'UTR with internal promoter (Blue box) activity, ORF1 and ORF2 with endonuclease (EN) activity and reverse transcriptase (RT) activity that essential for retrotransposition and 3'UTR.

ORF1 is about 1 kb in length and encodes a 40 kDa protein (p40 or ORF1p) with RNA binding activity and ORF2 is about 4 kb in length encodes a 150 kDa protein (ORF2p) with endonuclease and reverse transcriptase activities. Both ORF1 and ORF2 are required for LINE-1 retrotransposition in the step referred to as target-primed reverse transcription (TPRT).

LINE-1 retrotransposition causes the increasing of copy number of LINE-1 elements in the human genome. LINE-1 retrotransposition initiates with transcription of them to LINE-1 RNA by RNA polymerase II. The LINE-1 RNA is exported to cytoplasm and translated to ORF1 and ORF2. ORF1 and ORF2 will interact with LINE-1 RNA to form ribonucleoprotein (RNP) particle, and then RNP particle will transport back into the nucleus. After that, the integration of the LINE-1 element into the genome is initiated called target-primed reverse transcription. ORF2 which have endonuclease

activity, will cleave between A and T of target DNA at 5′-TTTTAA-3′ consensus sites [52, 53]. The free 3′ hydroxyl group of target DNA exposes, and then used to prime reverse transcription of LINE-1 RNA by ORF2 which have also reverse transcriptase activity. The other stand of target DNA is cleaved and used to prime second-strand synthesis (Figure 3). This step was presented target-site duplication (TSD) which flanks the new inserted LINE-1 [3, 5, 54].

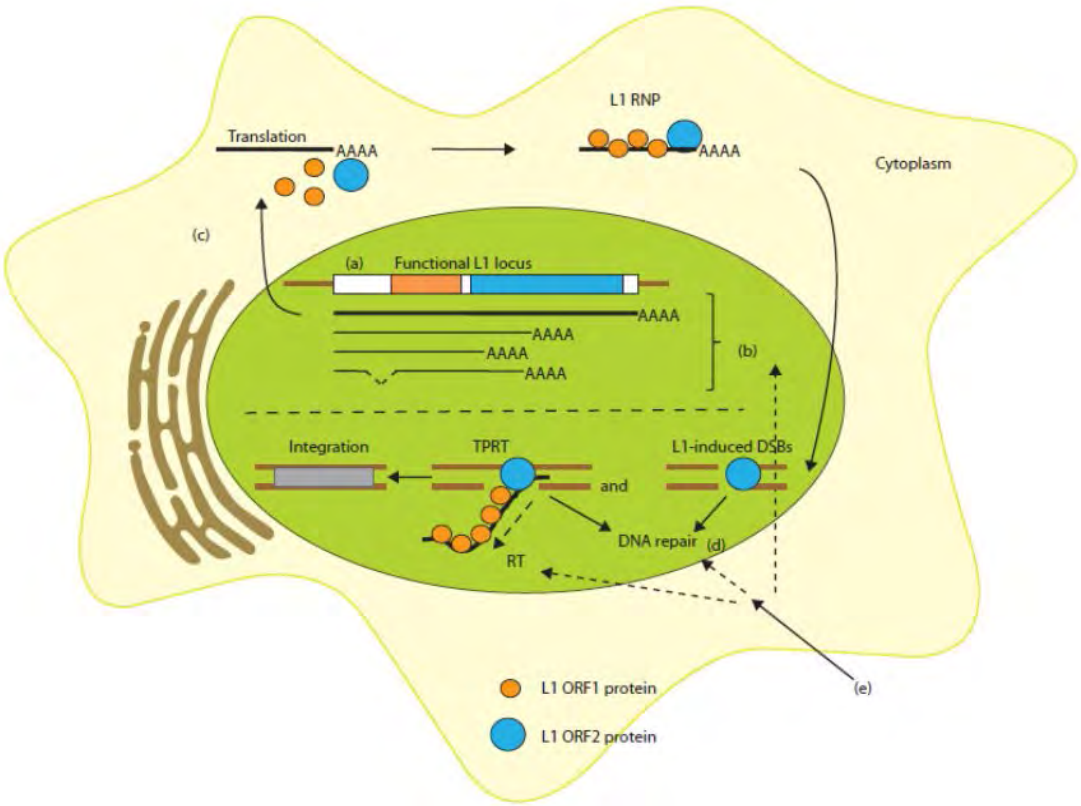

Figure 3. The mechanism of LINE-1 retrotransposition. a) and b) Full length LINE-1 elements can be transcript themselves to LINE-1 RNA. c) LINE-1 RNA exports to cytoplasm and translates ORF1 and ORF2 which interact with L1 RNA to form RNP particle. d) RNP particle transports back into the nucleus and integrates to new target DNA site. (Belancio et al, 2009) [54]

LINE-1 elements have been divided in sense and anti-sense LINE-1 by orientation. Moreover, LINE-1 elements can also be classified in 2 categories by location; inside and outside of genes, called intragenic LINE-1 elements and intergenic LINE-1 elements, respectively (Figure 4). Intergenic LINE-1 elements have approximately 9,355 copies whereas intragenic LINE-1 elements have about 2,546 copies and can be found in 1,454 genes [1]. The structural characteristics of intragenic LINE-1 elements and intergenic LINE-1 elements are different. Intragenic LINE-1 sequences have been conserved and contained C-G dinucleotide sites more than intergenic LINE-1 sequences. Beside, intergenic LINE-1 sequences are usually mutated and contain A and T nucleotides. These structural characteristics of intragenic LINE-1 elements may influence to their transcriptional activity and the methylation status of them [1].

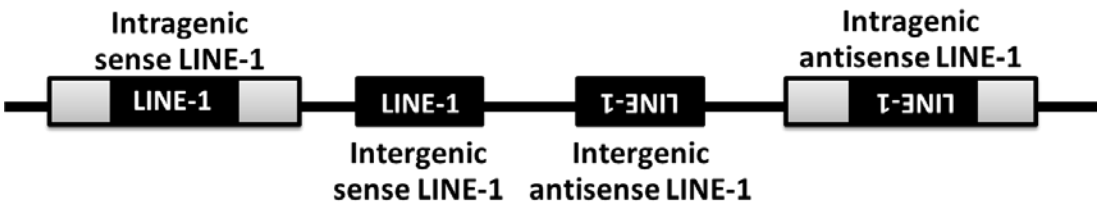

Figure 4. The characteristics of LINE-1 elements by orientation and location. LINE-1 elements can be classified in two group by orientation; sense and anti-sense LINE-1 elements. In addition, LINE-1 elements can be divided by location which located on inside and outside of genes, called intragenic LINE-1 elements and intergenic LINE-1 elements, respectively. The black boxes represent LINE-1 elements and the gray boxes are gene bodies. The black line is genome. The regular LINE-1 letters in the black boxes are sense LINE-1 elements whereas the inverted LINE-1 letters in the black boxes represent anti-sense LINE-1 elements.

LINE-1 and Human genome

LINE-1 elements can impact to the genome structure that resulting to global instability, global rearrangement and global innovation [5, 28]. The insertion of LINE-1 elements can affect to the host genes in several ways, If LINE-1 elements insert themselves into genic functional sequence such as promoters, exons and enhancers, these insertions are expected to severely compromise gene function or gene disruption (Figure 5A). The insertion of LINE-1 elements can cause human disease such as the LINE-1 element inserted to *c-myc* proto-oncogene resulting in breast carcinoma, *APC* gene cause to colon cancer predisposition [28, 55, 56]. In addition, LINE-1 retrotransposition can also be cause the rearrangement or deletion of neighboring sequences (Figure 5B and 5C) [57]. For example, Morisada *et al* reported the partial deletion of *EYA1* gene at least exons 5 to 7 including LINE-1 element insertion in Branchio-oto-renal syndrome [58]. Miné *et al* found the large deletion in the *PDHX* gene which directly connected to the insertion of a full-length LINE-1 element, in pyruvate dehydrogenase complex (PDHC) deficiency patient [59]. Due to LINE-1 elements have several copy numbers thus homologous recombination between non-allelic homologous LINE-1 elements may cause genome rearrangements [28] (Figure 5D). Moreover, LINE-1 elements can rearrangement the genome by co-transpose upstream or downstream flanking sequences with them to integrate themselves at the new genome locations that called 5' transduction and 3' transduction, respectively. 5' transduction and 3' transduction may represent a general mechanism for the evolution of new genes [60-62] (Figure 5E and 5F).

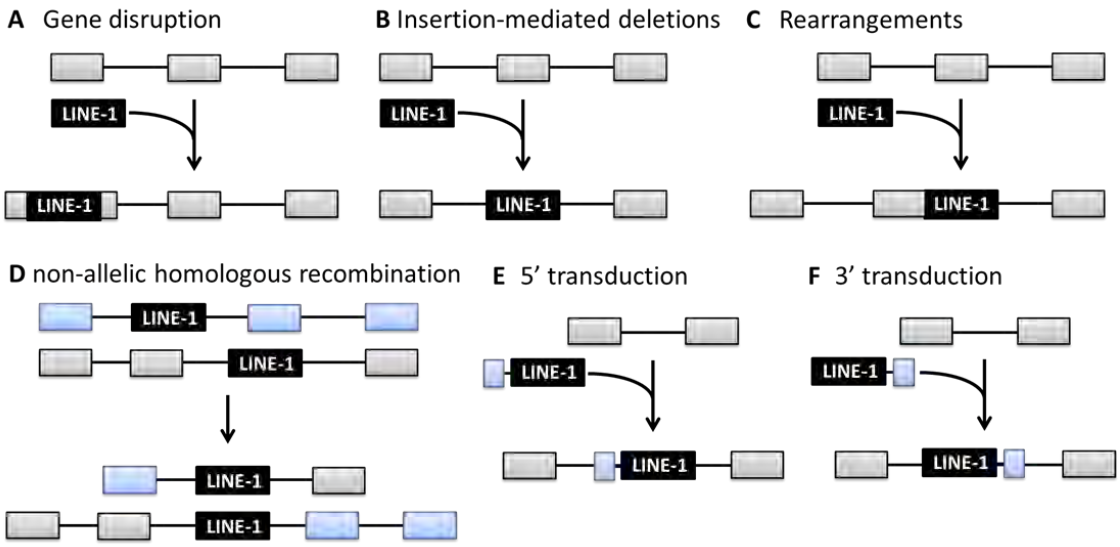

Figure 5. Impact of LINE-1 elements on the genome structure. A) Gene disruption. B) Insertion-mediated deletion. C) Rearrangements. D) non-allelic homologous recombination. E) 5'transduction and F) 3' transduction (Modified from Jeffrey S. *et al*) [63].

LINE-1 and gene expression

Besides DNA levels, LINE-1 elements also impact to transcriptional levels of host genes [1, 5, 64]. Because of LINE-1 elements contain multiple predicted the splice donor and the splice acceptor sites in both sense and antisense strands of their genome thus LINE-1 elements can provide new splice sites that may promote exonization and alternative splicing [60, 65, 66] (Figure 6A). In addition, LINE-1 elements can interfere to transcription elongation of the host genes by attenuation and premature polyadenylation. Intragenic LINE-1 elements can attenuate transcription of host genes because RNA polymerase II having a reduced capability to transcript through LINE-1 sequences and 3' UTR of LINE-1 containing polyadenylation signal, resulting in truncation of full-length host gene transcripts by premature polyadenylation [67, 68] (Figure 6B). Because of 5'UTR of LINE-1 elements contained internal polII promoter, promoter of LINE-1 elements can also initiate sense or antisense transcription through other genes. The antisense LINE-1 promoter can generate a chimeric mRNA which translated to chimeric protein in several cancers. Thus, the chimeric proteins may use as biomarker in cancers [69-72] (Figure 6C). Moreover, the methylation at 5' UTR of LINE-1 elements can also impact to host genes expression. The methylation is one type of epigenetic regulation in human genome. In general, LINE-1 elements are highly methylated however they are decrease methylated levels in cancers. LINE-1 hypomethylation can activate sense and antisense transcriptions using their promoter [73] (Figure 4D). Recently, Aporntewan *et al* reported intragenic LINE-1 hypomethylation can repress transcriptional of cancer genes. The intragenic LINE-1 hypomethylation could generate LINE-1 RNA, and then catch up with pre-mRNA of LINE-1 inserted genes as dsRNA. The dsRNA was targeted by RNA-Induced Silencing Complex (RISC) and was degraded through AGO2 [1] (Figure 6E).

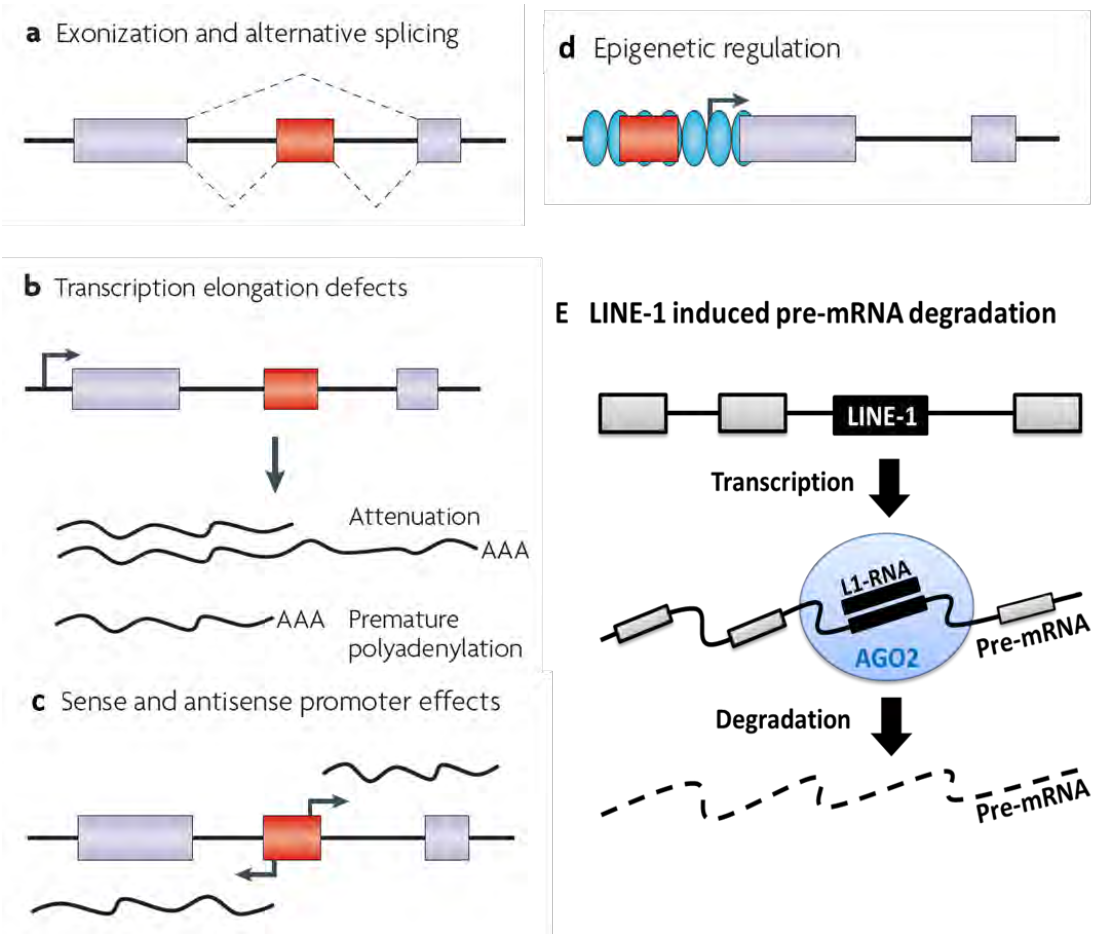

Figure 6. Impact of LINE-1 elements on gene expression. A) Exonization and alternative splicing. LINE-1 elements contain many splice donor sites and splice acceptor sites which that promote exonization and alternative splicing. B) Transcription elongation defects; attenuation and premature polyadenylation. 3' UTR of LINE-1 containing polyadenylation signal which can interfere the transcription of host genes by premature polyadenylation. C) Promoter effect; sense and antisense LINE-1 promoter can generate chimeric proteins which may use as biomarker in many cancers. D) Epigenetic regulation. LINE-1 elements are typically methylated which can alter the expression of neighboring genes and induce the formation of heterochromatin. E) LINE-1 induced pre-mRNA degradation. Pre-mRNA of host genes could bind to LINE-1 RNA, dsRNA form, and then was degraded through AGO2. The violet boxes and the gray boxes were the exon of host genes whereas the red boxes and the black represented LINE-1 elements. AGO2 was blue circle. (Modified from Cordaux *et al*, 2009) [1, 5].

Global LINE-1 hypomethylation and promoter methylation

Aberrant DNA methylation in global regions can be commonly found in several cancers [11, 23, 74]. The first region, global hypomethylation is related to the methylation reduction of intersperse repetitive elements and coding gene regions. The second is hypomethylation of oncogene promoters and the third region is hypermethylation of tumor suppressor genes. Although, global hypomethylation and aberrant regional methylation are important carcinogenic event in several cancers, the association between them remains unclear. There were many reports mention about the association between genes-specific promoter methylation and global LINE-1 hypomethylation.

- In 2004, Florl *et al* studied the methylation of cancer related-genes, *APC, GSTP1, RARB2, RASSF1A, CDH1, CDKN2A, SFRP1* and *ASC1*, including global LINE-1 methylation in prostate carcinoma (PCa). They found the correlation between gene specific hypermethylation and global LINE-1 hypomethylation. However, they proposed that the mechanisms causing DNA hypermethylation at specific sites and global LINE-1 hypomethylation were likely distinct because global LINE-1hypomethylation in PCa was a secondary event following DNA hypermethylation at specific sites during progression [26].
- In 2007, Cho *et al* reported the correlation between global LINE-1 hypomethylation and promoter CpG island hypermethylation in prostate adenocarcinoma. However, they proposed that the association between promoter CpG island hypermethylation and global LINE-1 hypomethylation was only found in the hypermethylated genes which showed cancer-related and associated with poor prognostic parameters. Thus, promoter CpG island hypermethylation and global LINE-1 hypomethylation were controlled by different mechanisms and these two processes were independent. [23].
- In 2008, Yamamoto *et al* studied the global LINE-1 methylation and intragenic genes methylation; *CDH1, CDH13*, and *PGP9.5* in enlarged-fold (EF+) gastritis which had an increased risk for gastric cancer. The result showed that LINE-1 methylation was significantly decreased and all gene promoters were significantly hypermethylated in patients with EF+ gastritis.

Furthermore, there was inverse correlated between the global LINE-1 hypomethylation and all genes methylation. They suggested that the methylation of the promoter regions of these genes was accompanied by global LINE-1 hypomethylation [74].

- In 2011, Daskalos *et al* studied about an extrinsic P1 promoter and an intrinsic P2 promoter of *p73* in the primary non-small cell lung carcinoma (NSCLC). They found that P2 promoter hypomethylation strongly correlated with global LINE-1 hypomethylation including increasing of ΔNp73 expression. They proposed that ΔNp73 overexpression might be a passive consequence of global DNA hypomethylation [24].
- In 2011, Woloszynska-Read *et al* studied about the correlation between cancer germline (CG) antigen genes and global hypomethylation in epithelial ovarian cancer (EOC). They found that the promoter hypomethylation of candidate genes were slightly correlated to some candidate genes expression and strongly correlated to global LINE-1 hypomethylation. In addition, the expression of some candidate genes was also correlated to global LINE-1 hypomethylation. Thus, they hypothesized that there was a shared mechanism promotes CG antigen promoter and global DNA hypomethylation in three possible mechanisms. First, *MTHFR* polymorphism (C667T) could reduce the intracellular pool of S-adenosylmethionine (SAM), methyl donor for DNA methylation, and then it induced for DNA hypomethylation. However, they could not found the association between *MTHFR* polymorphism and global LINE-1 hypomethylation in EOC. Second, enzymatical deficiency of DNMT3B isoforms could induce DNA hypomethylation but the result showed no difference in DNMT3B isoforms between high and low LINE-1 methylation. Third, they found the correlation between the *BORIS/CTCF* expression ratio and global LINE-1 methylation. In addition, they found the consensus CTCF binding sites in both LINE-1 elements and candidate genes which contained LINE-1 or Alu in proximity to their promoters. Thus, they proposed that BORIS and CTCF might regulate global DNA methylation status in EOC [75].

However, no correlation between global LINE-1 methylation and gene-specific methylation was also reported in many cancers.

- In 2002, Ehrlich *et al* was the first group which investigated the association between Global hypomethylation and CpG island hypermethylation in the same type of tumor. They reported that there was no association between CpG island hypermethylation and global DNA hypomethylation in Wilms tumors. In 2006, they studied the association between CpG island hypermethylation and global DNA hypomethylation in ovarian cancer and found no correlation between them. Thus, they proposed that tumor-associated DNA hypomethylation contributed to carcinogenesis separately from aberrant DNA hypermethylation [25, 76].
- In 2007, Iacopetta *et al* reported that, no associations were seen between global LINE-1 hypomethylation and CpG island hypermethylation in colorectal cancer. They proposed that global LINE-1 hypomethylation and CpG island hypermethylation were independent events and might contribute separately to tumorigenesis in colorectal cancer [77].
- In 2009, Kim *et al* found that CpG island hypermethylation occurred in the extrahepatic cholangiocarcinoma (EHC) and Biliary intraepithelial neoplasia (BilIN) which is the premalignant lesion of EHC. However, global LINE-1 hypomethylation could be detected in only EHC but not in BilIN. This result suggested that CpG island hypermethylation occurred earlier than global LINE-1 hypomethylation [78].
- In 2010, Trankenschuh *et al* studied global LINE-1 methylation and the methylation of gene-specific, *APC, CDH1, cyclinD2, GSTp1, hsa-mir-9-1, hsamir-9-2* and *RASSF1A* gene, in fibrolamellar carcinoma (FLC) and paired normal liver tissue specimens by quantitative high-resolution pyrosequencing. The result showed that FLC displayed gene-specific promoter hypermethylation without significant global hypomethylation, indicating that these two epigenetic aberrations were induced by different pathways [79].
- In 2010, Igarashi *et al* studied the methylation of tumor-related genes, *CDH1, MLH1, SFRP1, SFRP2, CHFR, APC, CDKN2A, RASSF1A and RASSF2*, and global LINE-1 methylation in gastrointestinal stromal tumors (GIST). The result showed no correlation between gene-specific promoter hypomethylation

and global LINE-1 hypomethylation. Because their result and recent studies reported that LINE-1 hypomethylation was inversely correlated with microsatellite instability and/or the CpG island methylator phenotype in colon cancer. Thus, they suggested that CpG island hypermethylation and global LINE-1 hypomethylation may reflect different tumor progression pathways [16].

Moreover, there were several reported about the global LINE-1 hypomethylation and aberrant promoter CpG island methylation in many human diseases such as neuroendocrine tumors [11]. In addition, global LINE-1 hypomethylation and aberrant promoter CpG island methylation was also investigated as the inheritance of DNA methylation patterns in maternal-infant pairs [80].

DNA methylation

Epigenetics can be defined as phenotypic alterations in the absence of DNA sequence changes. Epigenetics play a fundamental role in biological diversity such as such as embryonic development, cancer biology, and immune system response among many. Epigenetics can be classified in three groups; DNA methylation, histone modification and nucleosome positioning [81] however others complicated situation such as the RNA interferences phenomenon was reported. The most extensively studied epigenetic alterations are DNA methylation and histone modification.

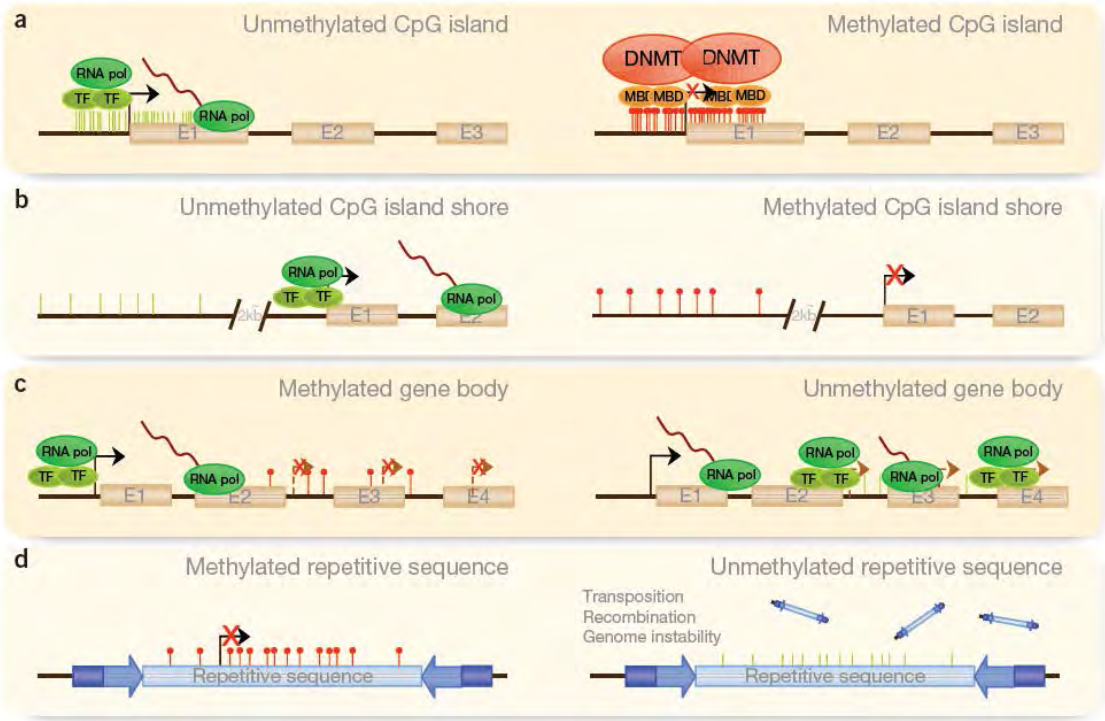

Figure 7. The DNA methylation patterns. **A)** DNA methylation occurs at CpG islands of gene promoters. **B)** The methylation status at CpG island shores, which are the low CpG density region, is essential for normal development and somatic cell reprogramming. **C)** The methylation of gene bodies also occur the methylation which relates to elongation efficiency and **D)** Interspersed repetitive elements are methylated in normal cells resulting in transcriptional repression. (Anna Portela *et al*, 2010) [81]

DNA methylation is important, as it is a well-known crucial epigenetically regulator. DNA methylation is associated with a several processes including differentiation of development, global imprinting, X-chromosome inactivation, suppression of interspersed repetitive elements and maintenance of chromatin structure. DNA methylation is the epigenetic modification of DNA by adding methyl group to 5'-position of cytosine base at CpG dinucleotides within the genome. CpG dinucleotides are concentrated in the regions called CpG islands that are sequences more than 200 bp in length with a GC content of at least 50% and an observed over expected ratio of CpG frequencies of 0.6 or greater [82, 83]. CpG islands reside approximately 60% of human gene promoter and are usually unmethylated in normal cells (Figure 7A). Beside promoters of genes, CpG islands are also observed at CpG island shores, gene bodies and interspersed repetitive elements. CpG island shores are lower CpG density region and locate up to 2 kb upstream of the traditional CpG islands. The methylation status of these regions is important to both normal development and somatic cell reprogramming [81, 84] (Figure 7B). DNA methylation can occur at the gene bodies that relate to elongation efficiency and prevention of false transcription initiations [85] (Figure 7C). Finally, DNA methylation is also found in region of interspersed repetitive sequences (IRS), which are normally hypermethylated to impact preventing to the genome structure for example global instability and global rearrangement. (Figure 7D)

DNA methylation is catalyzed by a family of DNA methyltransferase (DNMTs) that transfer the methyl group from S-adenosylmethionine (SAM) as the methyl donor to the cytosine residues. In mammals, DNMTs are reported in five members; DNMT1, DNMT2, DNMT3A, DNMTT3B and DNMT3L [86]. However, only 3 members of DNMTs, DNMT1, DNMT3A and DNMTT3B, are methyltransferase activity. DNMT1 is the maintenance DNMT, it prefers hemimethylated DNA. DNMT1 is the most abundant DNMT in the cells and methylates the newly synthesized DNA strand during the S phase of the cell cycle (Figure 8A). Whereas, DNMT3A and DNMT3B acts as *de novo* DNA methylation, they are highly expressed in embryonic stem cells including are downregulated in differentiated cells [87] (Figure 8B). Although DNMTs were originally classified as maintenance or *de novo* DNMTs, several evidence indicate that all three DNMTs not only cooperate, but also may possess both *de novo* and maintenance functions in *vivo* [87].

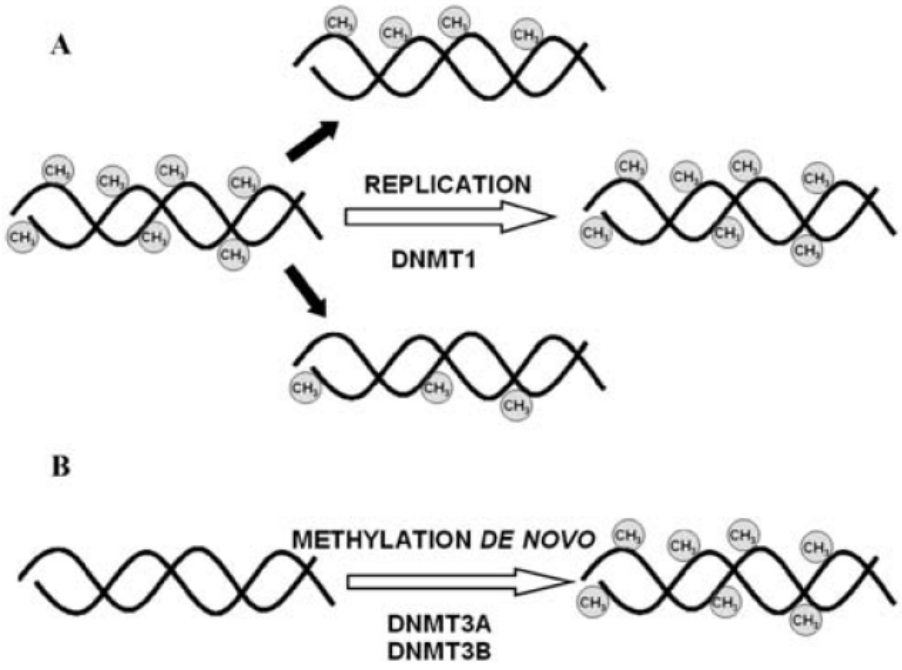

Figure 8. Maintenance and *de novo* DNA Methylation. A) Maintenance DNA methylation, DNMT1 add methyl groups to the hemimethylated DNA during replication, whereas, *de novo* DNA methylation, DNMT3A and DNMT3B can add methyl groups to CpG dinucleotides of unmethylated DNA (Luczak *et al*, 2006 [88]).

Dnmt2 was identified in 1998 but DNA methyltransferase activity cannot be detected in the first time. Dnmt2 is represented in several organisms such as human, fungi, plants, metazoa and protozoa [89]. Dnmt2 is the smallest enzyme among the eukaryotic DNMT and it comprises only the catalytic domain [90]. The biological function of Dnmt2 is still unclear however recent studies reported that DNMT2 methylates tRNAAsp in the cytoplasm [91, 92]. DNMT3L is the last member of DNA methyltransferase which involved in maternal genomic imprinting affects activity of DNMT3A and DNMT3B [93]. Moreover, DNMT3L can suppress transcription by interact directly to histone deacetylase (HDAC1) protein [94, 95].

DNA methylation and cancer

Global hypomethylation, genome-wide losses of DNA methylation, and aberrant promoter CpG islands methylation was commonly observed in several cancer types. There is proposed that more than 300 genes and gene products are epigenetically altered in various human cancers [96]. DNA hypermethylation is an early event in carcinogenesis which may play a role in tumor initiation and progression [97] however the mechanism of hypermethylation in cancers is not fully understood [98, 99]. Hypermethylation of tumor suppressor genes can be found in genes which involve in several cellular processes such as cell cycle, DNA repair, apoptosis, metas*TasI*s, carcinogen metabolism and hormone response etc [87, 98]. Global hypomethylation is one of normally epigenetic alteration during cancer development. There are many possible mechanisms which proposed for global hypomethylation potentially promotes carcinogenesis: activation of oncogenes, reactivation of transposable elements, chromosomal instability and loss of imprinting [99, 100].

Nowadays, aberrant DNA methylation, hyper- or hypo- methylation, of a particular gene or a set of selected genes is proposed as biomarker in several cancers [101-103]. Because DNA methylation has advantages more than other tumor makers such as it is easier to detect than the presence of mutations, their stability, their ability to be amplified at relatively low costs and their restriction to limited regions of DNA methylation [96, 97].

DNA methylation and LINE-1

DNA methylation is an important epigenetically regulator and associated with a several processes including suppression of interspersed repetitive elements. LINE-1 elements are the most non LTR retrotransposon in human genome. LINE-1 elements are widely distributed in the human genome. In normal cells, LINE-1 elements are usually methylated resulting in transcriptional repression of them. However, the methylation level of LINE-1 elements is reduced in several cancers called global LINE-1 hypomethylation. The reduction of wide LINE-1 methylation can induce several potential functional consequences for example LINE-1 retrotransposition and also various disturbances of the transcription by its promoters, the activity of LINE-1

encoded enzymes and the recombination between homologous LINE-1 elements. In 2004, Chalitchagorn *et al* [10] evaluated the methylation levels of global LINE-1 elements from several normal and cancerous tissues. They found that the methylation levels of LINE-1 elements in many normal tissues distributed within narrow range however some normal tissue types, esophagus and thyroid, had widen range. While, the methylation levels of LINE-1 elements in cancerous tissues revealed a lower percentage than normal tissues. Furthermore, Phokaew *et al* [22] investigated the specific intragenic LINE-1 methylation levels in both normal cells and cancerous cells. They selected 17 intragenic full-length LINE-1elements which located on 16 genes for methylation measurement. They found that the methylation levels distribution of specific intragenic LINE-1 elements were similar to the pattern of global LINE-1 methylation and no all specific intragenic LINE-1 elements completely methylated. Moreover, the methylation levels of specific intragenic LINE-1 elements in cancerous cells were still lower than normal cells. Nevertheless, some intragenic LINE-1 hypermethylation can be found sporadically in cancer for example intragenic LINE-1 element located on *CNTNAP5* and *MGC42174*. Additionally, the methylation levels of each intragenic LINE-1 loci in cancerous cells were correlated but no correlation in normal cells. Thus, the intragenic LINE-1 hypomethylation is also a commonly feature in several cancers. In addition, the methylation levels of intragenic LINE-1 elements are variable depend on tissue types and locations [10, 22].

The consequence of LINE-1 hypomethylation can impact to human genome and host genes transcription as previous review topic. For example, hypomethylation of a LINE-1 promoter which located on *MET* oncogene could induce an alternate transcript of the *MET* oncogene in bladder tumors [104]. Recent studied, Aporntewan *et al* reported the impact of hypomethylation of intragenic LINE-1 elements on altering gene expression. Intragenic LINE-1 hypomethylation could repress transcriptional of host gene in cancer cells through AGO2 [1]. Moreover, the hypomethylation of LINE-1 leaded to LINE-1 retrotransposition to the new genome sites such as Kazazian *et al* could detect truncated LINE-1 element on *Factor XIII* gene of hemophilia A patients [105]. Holmes *et al* reported the integration of LINE-1 elements in dystrophin gene that resulted in Duchenne muscular dystrophy [105, 106]. Morse *et al* compared *c-myc* proto-oncogene sequences from a ductal adenocarcinoma of the breast with normal breast tissue of the same patient. They found the insertion of

LINE-1 element into second intron of *c-myc* proto-oncogene in the ductal adenocarcinoma of the breast [72].

CNTNAP5

Contactin associated protein-like 5 (*CNTNAP5*), is also known as contactin associated protein 5 (caspr5) or FLJ31966, is located on chromosome 2, at 2q14.3. This gene is member of the contactin associated protein (Caspr) family that plays essential roles in the correct development and proper functioning of the peripheral and central nervous system. The *CNTNAP5* gene is conserved in mouse, rat, chimpanzee, dog, chicken, cow and zebrafish [107]. The global size (from start to stop codon) of this gene is 888,638 bp. The coding region of this gene is 24 exons and transcripts three splice variants in human (Table 1). Spiegel *et al*. identified Full-length cDNA of the human *CNTNAP5* in 2002 [108] and published in the genome databanks. The expression of this gene is low level however the adult brain tissues of mouse is highly expressed and different expression patterns are seen in other organs during fetal development and in the adult stage.

Table 1. Gene summary of *CNTNAP5* splice variants (Modified from Gene summary www.ensembl.org).

Name	**Length (bp)**	**Length (aa)**	**Biotype**
CNTNAP5-001	5284	1306	Protein coding
CNTNAP5-201	5284	1198	Protein coding
CNTNAP5-003	347	No protein product	Retained intron

- bp was base pair
- aa represented amino acid.

CNTNAP5 encodes contactin associated protein-like5 protein that belongs to the neurexin family, members of which function in the vertebrate nervous system as cell adhesion molecules and receptors. This protein, like other neurexin proteins, contains epidermal growth factor repeats and laminin G domains. In addition, it includes [1]an F5/8 type C domain, discoidin/neuropilin- and fibrinogen-like domains, and thrombospondin N-terminal-like domains. However, the function of *CNTNAP5* is still unclear.

CHAPTER III
MATERIALS AND METHODS

Materials

- MicroAmp PCR tube
- Cryotube
- Counting chambers
- Pipette tip : 10 µl, 100 µl,1,000 µl (Elkay, USA)
- Microcentrifuge tube : 0.2 ml, 0.5ml, 1.5ml (Bio-rad Elkay, USA)
- Beaker : 50 ml, 100 ml, 200 ml, 500 ml, 1,000 ml (Pyrex)
- Flask : 250ml, 500ml, 1,000ml (Pyrex)
- Reagent bottle : 100 ml, 250 ml, 500 ml, 1,000 ml (Duran, USA)
- Cylinder : 25 ml, 50 ml, 100 ml, 250 ml, 500 ml, 1,000 ml (Witeg, Germany)
- Glass Pipette : 5 ml, 10 ml (Witeg, Germany)
- Microcentrifuge tube rack (Scientific plastics, USA)
- Thermometer (Precision, Germany)
- Plastic wrap
- Stirring-magnetic bar

Equipments

- Light microscope
- Autoclave
- Microwave oven
- Hot air oven (Memmert, West Germany)
- Pipette boy (Tecnomara, Switzerland)
- Vortex (Scientific Industry, USA)
- pH meter (Eutech Cybernatics)
- Stirring hot plate (Bamstead/Thermolyne, USA)
- Balance (Precisa, Switzerland)
- Microcentrifuge (Fotodyne, USA)
- DNA Thermal cycler 480 (Perkin Elmer, Cetus USA)
- Thermal cycler (Touch Down, Hybraid USA) Power supply model 250 (Gibco BRL, Scothland)
- Power poc 3000 (Bio-Rad, USA)

- Heat block (Bockel)
- Incubator (Memmert, West Germany)
- Spectronic spectrophotometers (Genesys5, Milon Roy USA)
- UV Transilluminator (Fotodyne USA)
- UV-absorbing face shield (Spectronic, USA)
- Gel doc 1000 (Bio-RAD)
- Refrigerator 4 ^{0}C (Misubishi, Japan)
- Deep freeze -20 ^{0}C, -80 ^{0}C (Revco)
- Water purification equipment (Water pro Ps, Labconco USA)
- Water bath (Memmert, West Germany)
- Storm 840 and ImageQuaNT solfware (Molecular dynamics)
- Gel star nucleic acid gel stain (Cambrex Bio Science)
- Liquid nitrogen tank
- CO2 incubator
- Acrylamide gel electrophoresis

Reagents

General reagents

- Absolute ethanol (Merck)
- Agarose gel (FMC Bioproducts)
- Ammonium acetate (Merck)
- Bromphenol blue (Pharmacia)
- Chloroform (Merck)
- Disodium ethylenediamine tetracetic acid : EDTA (Merck)
- Dimethyl sulfoxide (DMSO) (Sigma)
- *Dnase*I (Gibco BRL)
- Diethylpyrocarbonate (DEPC) (Sigma)
- Fetal bovine serum (Sigma, St Louis, MO, USA)
- Glycogen (Sigma)
- Hydrochloric acid (Merck)
- Hydroquinone (Merck)
- Isoamyl alcohol (Merck)
- Isopropanol (Merck)
- Mineral oil (Sigma)

- Phenol (Sigma)
- Sodium acetate (Sigma, St Louis, MO, USA)
- Sodium bisulfite (Sigma, St Louis, MO, USA)
- Sodium chloride (Merck)
- Sodium hydroxide (Merck)
- Trizol reagent (Invitrogen)
- 25 base pair DNA ladder (Biolabs)
- 40%acrylamide/bis solution 19:1 (Bio-Rad)
- Wizard® DNA Clean-Up Kit (Promega)
- 2-Mercaptoethanol (2-ME) (Sigma)
- Dulbecco's modified Eagle's medium; DMEM (Sigma, St Louis, MO, USA)
- Roswell Park Memorial Institute (RPMI 1640) medium (Sigma, St Louis, MO, USA)
- 1% antibiotic/antimyotic (Gibco-Invitrogen)

PCR Reagents

- 10X PCR buffer (500 mM KCl, 200 mM Tris-HCl pH 8.4) (GibcoBRL, Perkin Elmer)
- Magnesium chloride (GibcoBRL, Perkin Elmer)
- Deoxynucleotide triphosphates (dNTPs) (Promega)
- Oligonucleotide primers (BSU, GENSET) in Table 1
- Hotstart*Taq* DNA polymerase (Qiagen)
- Bisulfite DNA samples or RNA samples

Bioinformatics and Statistical analysis programs

- ClustalX
- Align two (or more) sequences using BLAST (bl2seq) program, National Center for Biotechnology Information, USA
- Promoter 2.0 prediction program
- SPSS Statistics version 17.0 (IBM, NY, USA).

Methods

Cell culture

Head and neck squamous cell carcinoma (HNSCC) cell lines (WSU-HN), including WSU-HN 4, 6, 8, 12, 13, 17, 19, 22, 26, 30 and 31, were cultured in Dulbecco's modified Eagle's medium; DMEM (Sigma, St Louis, MO, USA) and ovarian carcinoma cell line (SKOV3) was grown in Roswell Park Memorial Institute (RPMI 1640) medium (Sigma, St Louis, MO, USA), both supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St Louis, MO, USA) and 1% antibiotic/antimyotic (Gibco-Invitrogen). Cells were incubated at 37 ^{o}C with 5% CO_2.

Samples preparation

Normal oral epithelium (NOE) samples were collected as previously described [22]. Briefly, subjects rinsed and gargled by 20 ml of sterile 0.9% NaCl solution for 20 seconds and spited out the solution into 50 ml sterile tube. The solution was centrifuged at 2,500 g for 15 minutes at 4°C, then discarded the supernatant and washed the pellets by sterile PBS.

Normal primary oral keratinocyte cells were collected from normal gingiva fresh biopsies in gingival hyperplasia cases. The trimmed gingiva skins were digested with collagenase type I (Gibco, cat. No.17100-017) 1 unit/mg of tissue overnight at 4°C in Phosphate buffered saline (PBS). After twice wash in PBS, cells were seed on Collagen type IV 0.67mg/cm2 coated dish following Sigma-Aldich standard protocol. Normal primary oral keratinocyte cells were supplemented in complete KBM-2 media (Bullet Kit, Cambrex, Walkersville, MD, USA) until 70% confluence, cells were collected for further Trizol RNA extraction by the ReagentPack™ Subculture Reagent Kit (CC-5002; Cambrex, Walkersville, MD, USA).

DNA Extraction

Total global DNA were extracted in 900 microliters of Lysis buffer (0.075 M NaCl, 0.024 M EDTA, pH 8.0), 50 ml of 10% SDS and 20 microliters of 20 mg/ml Proteinase K for overnight at 50^{o}C. Then, the samples were adding phenol/chloroform with ethanol precipitation. Global DNA was washed by 70% ethanol then spin down, following by air-dried and resuspended in dH_2O.

Combined Bisulfite Restriction Analysis (COBRA)

Global DNA was modified by sodium bisulfite as previously described protocol [22, 109]. Briefly, 2 µg of global DNA in 50 µl H_20 were denatured by 5.5 µl of 2M NaOH for 10 minutes at 37 ^oC. Then, 30 µl of fresh prepared 10 mM hydroquinone and 520 µl of 3M sodium bisulfite (Sigma, St Louis, MO, USA) were added for 16–20 hours at 50^oC. The samples were purified using the Wizard DNA Clean-Up System (Promega, Madison, WI). Subsequently, the global DNA were eluted by 50 µl of 95^oC H_20, and then incubated in 5.5 µl of 3M NaOH for 5 min at room temperature. The global DNA was precipitated by 100% ethanol with glycogen as a carrier, following by washed with 70% ethanol and air-dried. The bisulfited DNA was resuspended in 20 ml of dH_2O and stored at -20 ^oC. Then, The PCR was performed using bisulfite sample as template, and then the amplicons were digested in 10 µl reaction volumes by 2 units *TaqI* or 2 units *TaiI* in 1X R buffer (Fermentas, Maryland, USA). The reaction was incubated at 65 ^oC for overnight.

COBRA-L1 and CU-L1

For COBRA-L1, the PCR reaction was performed in total volume 20 µl contained with 1X PCR Buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.3 µM of LINE-1 inward and outward primers, 1 unit of HotStarTaq (QIAGEN) and 2 µl of bisulfited DNA as template. The PCR reaction was accomplished under the following conditions, 1 cycle of initial denaturation at 95 ^oC for 15 minutes, followed by 35 cycles of denaturing at 95 ^oC for 1 minute, annealing at 53 ^oC for 1 minute, extension at 72 ^oC for 1 minute, and 1 cycle of final extension at 72 ^oC for 7 minutes. The amplicons were double-digested in 10 µl reaction volumes by 8 µl of amplicons as template with 2 units of *TaqI* (Fermentas, Maryland, USA) and 2 units of *TasI* in 1X NEBuffer3 (NEW ENGLAND BioLabs®Inc, USA) with 0.1 µl BSA. The reaction was incubated at 65 ^oC for overnight. For COBRA for unique sequence to LINE-1 (CU-L1), the PCR reaction was performed similar to COBRA-L1 condition. However, the temperature of annealing step was depended on each primer and used 2 units of either *TaqI* or *TaiI* for the digestion step. The digested amplicons were separated on 8% non-denaturing polyacrylamide gel and stained by SYBR Gold stain. The intensities of DNA fragments were measured by PhosphoImager, using the ImageQuant software (Molecular Dynamics, GE Healthcare, Slough, UK).

5-aza-2'-deoxycytidine treatment

To inhibit DNMTs using 5-aza-2'-deoxycytidine (5azaDC) treatment, $1x10^6$ cells were seeded in 25 cm^2 tissue culture flask, then cells were treated with 10 μM 5-azaDC (Sigma-Aldrich) for 5 days. Medium were changed and added new drug every 24 hours. After that, cells were replaced by free 5azaDC media for 1 day and harvested cells in next day.

RNA extraction and Quantitative Reverse Transcription-PCR (qRT-PCR)

Total cellular RNA was extracted from cultured cells using the Trizol reagent (Invitrogen) following the manufacturer's protocol. The cDNA template was synthesized using both 5 micrograms of total RNA as template and 0.5 micrograms of oligo$(dT)_{18}$ primer, then added DEPC-treated water to 12 μl in volume. The reaction mixture was heated at 70^{o}C for 5 minutes and then quench on ice. Afterwards, the sample was added 5x reaction buffer (Fermentas), 20 units of Ribolock™ Ribonuclease inhibitor (Fermentas), 20 units of RevertAid™ M-MuLV Reverse Transcriptase (Fermentas) and 20 mM dNTPs. The reaction mixture was incubated at 42^{o}C for 60 minutes, 70 for 10 minutes and chilled on ice, respectively. cDNA was amplified as template using the exon primers of gene and Glyseraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control.

Real-time RT-PCRs were performed for 35 cycles with the annealing temperature set at 50 ^{o}C. Real-time RT-PCR was performed in a Light Cycler machine (Roche Molecular Biochemicals, Indianapolis, IN, USA) using QuantiTect SYBR Green I (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Bioinformatics

We used L1Base database for investigation of intragenic full length LINE-1 in candidate genes [110] and rechecked by NCBI/BLAST program. For NCBI/BLAST program, we inputted active LINE-1.2 sequence (accession number M80343) and candidate gene sequence to bl2seq program and directly compared their sequences for prediction of intragenic full length LINE-1 elements. After that, we selected candidate intragenic full length LINE-1 elements that their sequences were similar to intragenic L1-*CNTNAP5*-I11, and then the methylation measurment was performed.

For promoter prediction, we observed CpG islands at 5' upstream of candidate genes and blasted sequences to Promoter 2.0 prediction program for prediction of transcription start sites of vertebrate *PolII* promoters in DNA sequences [111].

Statistical analysis

The correlation between promoter methylation, intragenic LINE-1 methylation and global LINE-1 methylation was performed for statistical significance by Pearson correlation. Moreover, paired samples t-test was used to determine the differences between each intragenic LINE-1 methylation levels using SPSS statistics version 17.0 (IBM, NY, USA).

Table 2. List of Primers for experiments

Primers	**Sequences**		**Restriction Enzymes**
COBRA-L1	F	CGTAAGGGGTTAGGGAGTTTTT	*Taq I* and *Tas I*
	R	(A/G)TAAAACCCTCC(A/G)AACCAAATATAAA	
CNTNAP5 promoter	F	TTATTTGGGTTTGGAATTTAG	*Tai I*
	R	CTTAAATTAC(G/A)CATTTAATTTC	
ME3 promoter	F	GTTATTATATTGGATAGTATAAGGTAATAT	*Taq I*
	R	CTCCATCTTAACAAAACTAAATACAC	
PDE3B promoter	F	TTTAGTATTTTTATTAAGTATATTTTTTGGGG	*Taq I*
	R	CCACCGAAACTAAAAAAAAAAAACG	
PDE4D promoter	F	GTATATTAATTTTTTTTTTTTTAGATTTTATAATAAATG	*Taq I*
	R	CTTTACCTCTAAAAACAAAAAAAAAATC	
DLEC1 promoter	F	AGTTTATTTTTAAAAGGATAATG	*Taq I*
	R	ACTCCTAATCTCCATAACAACC	
CALCA promoter	F	TAGTTTTAGTTT(C/T)GGGTTTTGTGG	*Taq I*
	R	AAAATTAACCCCTAAATATACC	
CHFR promoter	F	TTTTTAGGAGTTATTTTTAGATTAG	*Taq I*
	R	AAACCAAAAACCTCTAC(G/A)CCCC	
L1-*CNTNAP5*-I1	F	AAGGAGATTTTGATTTGTAATTAT	*Taq I*
	R	CCCACTATCTAACACTCCCTAATA	
L1-*CNTNAP5*-I3A	F	GGATTATTTTGTTAATGATATTTA	*Taq I*
	R	CCCACTATCTAACACTCCCTAATA	
L1-*CNTNAP5*-I3B	F	GGGAGGGGATTGAAGATAAT	*Taq I*
	R	CCCACTATCTAACACTCCCTAATA	
L1-*CNTNAP5*-I11	F	GATTAAATTTTAATTGAATTAGAG	*Taq I*
	R	CCCACTATCTAACACTCCCTAATA	

Table 2. List of Primers for experiments (Continue)

Primers	**Sequences**		**Restriction Enzymes**
L1-*ME3*	F	GGTAATTGGTTGAAAGAGTTATTAGT	*Taq I*
	R	TTTATATTTGGTTCGGAGGGTTTTA	
L1-*PDE3B*	F	TTGGATTAAGAAAATGTGGTATATAG	*Taq I*
	R	TTTATATTTGGTTCGGAGGGTTTTA	
L1-*PDE4D*	F	AGGATTTAAAATTTATTAATTTTATG	*Taq I*
	R	TTTATATTTGGTTCGGAGGGTTTTA	
CNTNAP5 expression	F	CAAGTTACACATGGTGCCACCTG	
	R	CAGTGATATTGCAGTACACCTGGA	

CHAPTER IV

RESULTS

The methylation status of *CNTNAP5*.

Global LINE-1 hypomethylation is commonly featured in most cancers. Similar findings were found in intragenic LINE-1 elements which also had hypomethylation in many loci. However, hypermethylation of some intragenic LINE-1 elements could be found sporadically such as intragenic LINE-1 element located on intron11 at contactin associated protein-like 5 or *CNTNAP5* (L1-*CNTNAP5*-IVS11). Moreover, intragenic L1-*CNTNAP5*-IVS11 had significant correlation with progression free survival and disease free interval in ovarian cancers patients (unpublished data). Therefore, we interested to study the role of aberrant intragenic LINE-1 methylation, especially L1-*CNTNAP5*-IVS11, in cancer. The methylation levels of *CNTNAP5* promoter and LINE-1 elements, including both intragenic L1-*CNTNAP5*-IVS11 and LINE-1 elements in whole genome (global LINE-1 methylation), were investigated in normal oral epithelium (NOE) cells and head and neck squamous cancer (HNSCC) cell lines (WSU-HN). We detected the methylation levels of *CNTNAP5* promoter using COBRA, intragenic L1-*CNTNAP5*-IVS11 using CU-L1 and global LINE-1 methylation using COBRA-L1. The result showed that *CNTNAP5* promoter in cancers was higher methylated than normal epithelium tissues whereas the methylation of L1-*CNTNAP5*-IVS11 and global LINE-1 methylation in cancers were lower than normal epithelium tissues (Figure 9A). Then, the association between *CNTNAP5* promoter and LINE-1 methylation, both L1-*CNTNAP5*-IVS11 and LINE-1 elements in whole genome was investigated. Interestingly, we found that global LINE-1 hypomethylation, but not intragenic L1-*CNTNAP5*-IVS11 methylation, was significantly correlated to *CNTNAP5* promoter methylation (Figure 9B and 9C).

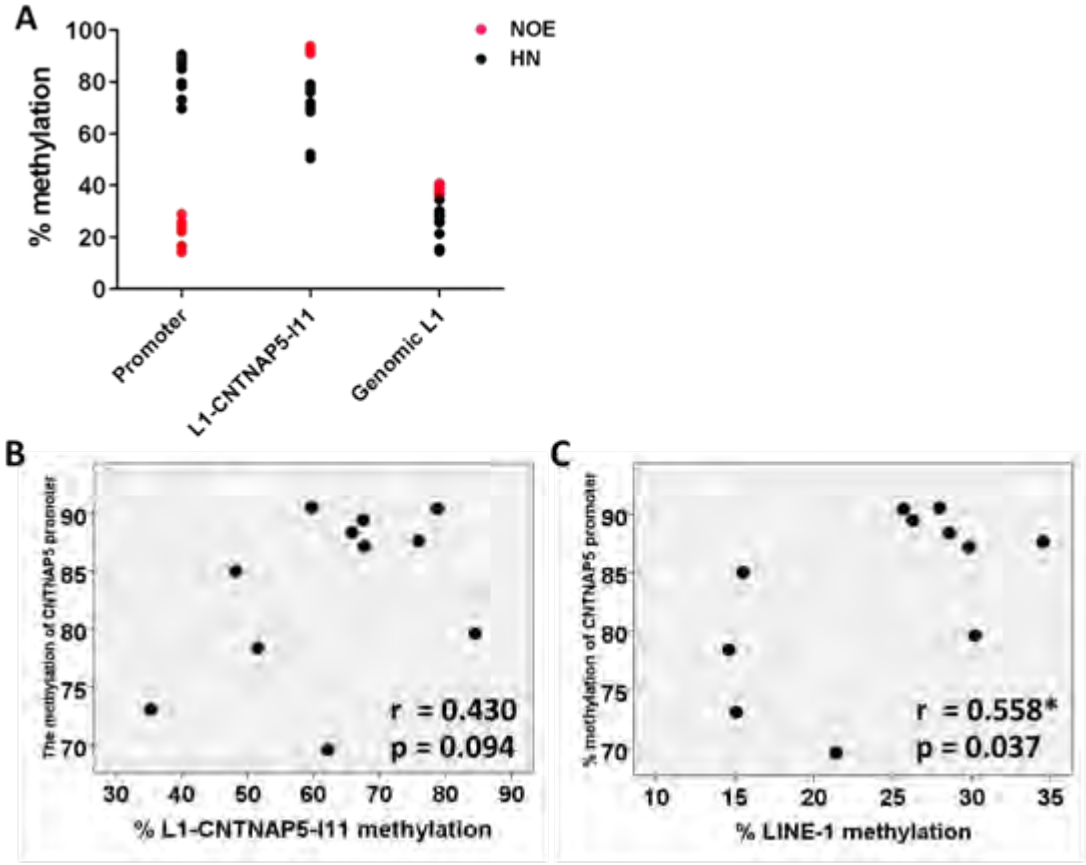

Figure 9. The methylation status of *CNTNAP5* promoter and intragenic L1-*CNTNAP5*-IVS11 including global LINE-1 methylation. **A)** The methylation levels of *CNTNAP5* promoter, intragenic L1-*CNTNAP5*-IVS11 and LINE-1 elements in whole genome. The black circles represented head and neck squamous cell cancer (HNSCC) cell lines (WSU-HN) and the red circles were normal oral epithelium (NOE) cells. **B)** The correlation between *CNTNAP5* promoter methylation and intragenic L1-*CNTNAP5*-IVS11 methylation. **C)** The correlation between *CNTNAP5* promoter methylation and global LINE-1 hypomethylation. r was Pearson correlation coefficient and p represented *P*-value.

Analysis of other intragenic LINE-1 methylation in *CNTNAP5*

Although, we could not find the significant correlation between intragenic L1-*CNTNAP5*-IVS11 methylation and *CNTNAP5* promoter methylation, there was trend to be correlated (Figure 9B). Thus, we interested to observe the correlation between *CNTNAP5* promoter methylation and the methylation levels of other intragenic LINE-1 elements which located on *CNTNAP5* (L1-*CNTNAP5*). To investigate other intragenic L1-*CNTNAP5* elements, sequence of this gene was analyzed using bl2seq program of Basic local alignment tool (BLAST) from NCBI website. We found that there were five full-length intragenic LINE-1 elements located on this gene. The first and second intragenic L1-*CNTNAP5* elements located on intron 1 (L1-*CNTNAP5*-IVS1A and L1-*CNTNAP5*-IVS1B), other two L1-*CNTNAP5* elements located on intron3 (L1-*CNTNAP5*-IVS3A and L1-*CNTNAP5*-IVS3B), and the other located on intron11 (L1-*CNTNAP5*-IVS11). The orientation of both intragenic L1-*CNTNAP5* elements located on intron1 and L1-*CNTNAP5*-IVS3B were anti-sense orientation whereas L1-*CNTNAP5*-IVS3A and L1-*CNTNAP5*-IVS11 were sense orientation. (Figure 10)

Figure 10. Mapping of intragenic LINE-1 elements located on *CNTNAP5*. Black boxes represented intragenic L1-*CNTNAP5* elements and white boxes were gene body of *CNTNAP5*. LINE-1 was LINE-1 element in sense orientation whereas LINE-1 represented LINE-1 element in anti-sense orientation. The number above mapping showed the distance between each intragenic L1-*CNTNAP5* elements.

All intragenic L1-*CNTNAP5* elements were investigated for the methylation levels using CU-L1. However, L1-*CNTNAP5*-IVS1A had more variable in its sequence, thus it cannot be detected the methylation levels. Therefore, only four intragenic L1-*CNTNAP5* elements were measured the methylation levels and we used L1-*CNTNAP5*-IVS1 to represent L1-*CNTNAP5*-IVS1B. For the methylation levels of each intragenic L1-*CNTNAP5* elements, we found that the percentage of all intragenic L1-*CNTNAP5* elements methylation levels distributed as ranged from 20% to 100% and the methylation levels of all intragenic L1-*CNTNAP5* elements were about 60 percentages by average (Figure 11). Then, the correlation among each intragenic L1-*CNTNAP5* elements was observed. We found that the methylation percentage was almost significantly correlated among each intragenic L1-*CNTNAP5* elements, except between L1-*CNTNAP5*-IVS3B and L1-*CNTNAP5*-IVS11. However, there was trend to be correlated between them (r=0.511, p=0.054) (Figure 11 and Table 5).

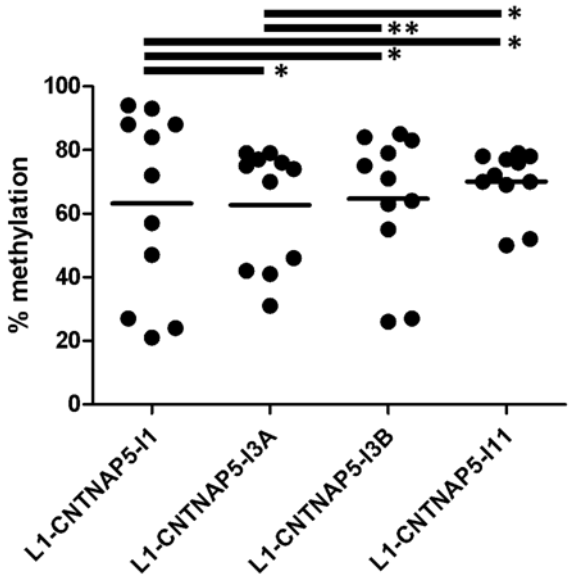

Figure 11. The methylation levels and the correlation of each intragenic L1-*CNTNAP5* elements. The lines in the graph represented the mean of the methylation levels of each intragenic L1-*CNTNAP5* elements and the black bold lines upper the graph showed the correlation between each intragenic L1-*CNTNAP5* elements. * was *P*-value at < 0.05 whereas ** was *P*-value at < 0.01.

Correlation between each intragenic L1-*CNTNAP5* methylation, global LINE-1 methylation and *CNTNAP5* promoter methylation.

The correlation between each intragenic L1-*CNTNAP5* methylation and *CNTNAP5* promoter methylation was observed. We found that only intragenic L1-*CNTNAP5* elements located on intron 3; L1-*CNTNAP5*-IVS3A and L1-*CNTNAP5*-IVS3B were significantly correlated to *CNTNAP5* promoter methylation (Figure 12A). Moreover, the association between each intragenic L1-*CNTNAP5* methylation and global LINE-1 hypomethylation was also observed. The result showed that all intragenic L1-*CNTNAP5* methylation was correlated to global LINE-1 hypomethylation (Figure 12B).

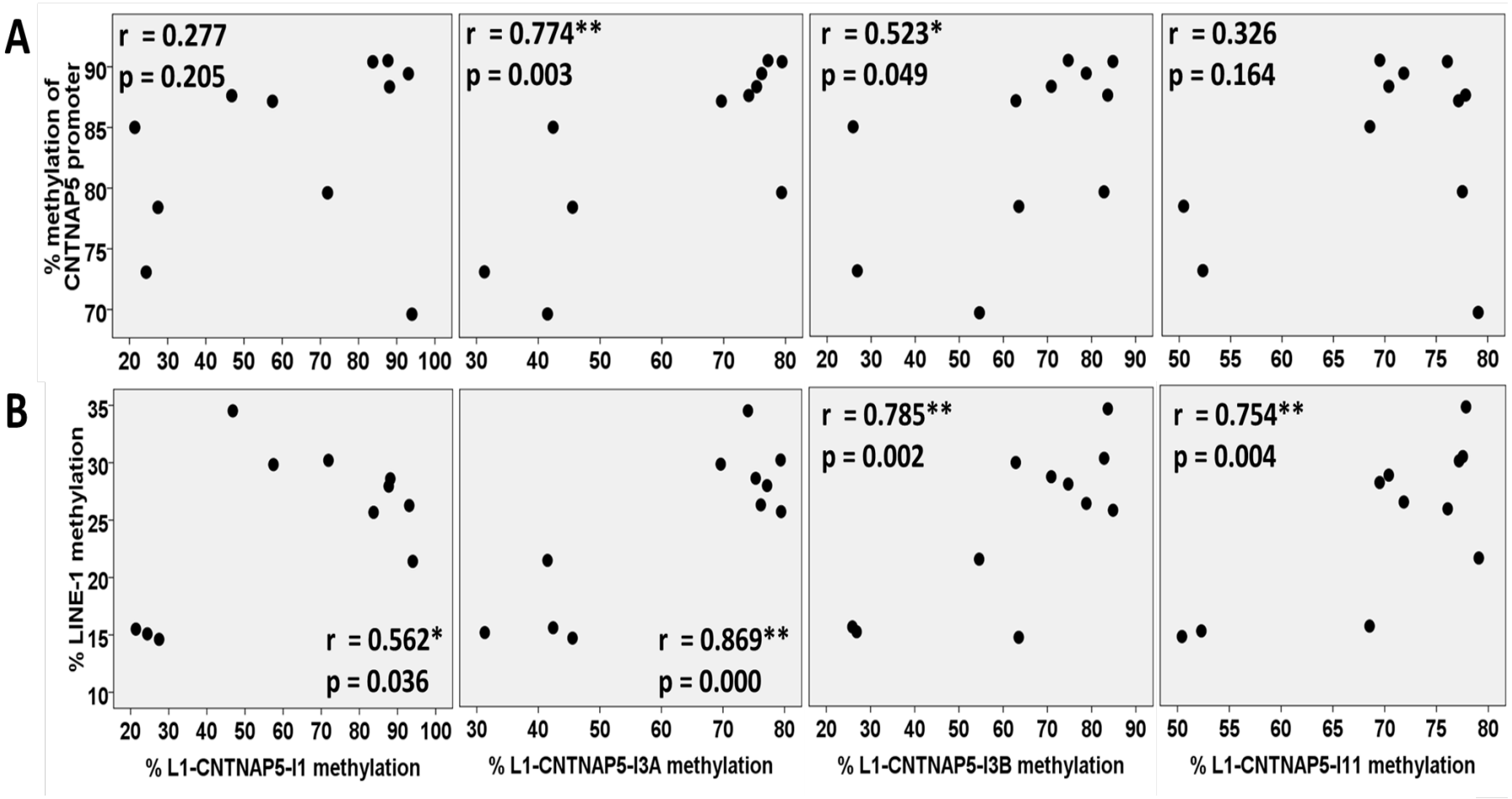

Figure 12. The correlation between each intragenic L1-*CNTNAP5* methylation and A) CNTNAP promoter methylation including B) global LINE-1 hypomethylation. r represented Pearson correlation coefficient and p was *P* value.

Interestingly, the trend of correlation between each intragenic L1-*CNTNAP5* methylation and *CNTNAP5* promoter methylation was similar to the correlation between each intragenic L1-*CNTNAP5* methylation and global LINE-1 methylation. It was mentioned that the Pearson correlation coefficient (r) and Probability value (p) of L1-*CNTNAP5*-IVS3A were the highest both r and p value (r = 0.774, p =0.003) following by L1-*CNTNAP5*-IVS3B (r = 0.523, p =0.049), L1-*CNTNAP5*-IVS11 (r = 0.326, p =0.164) and L1-*CNTNAP5*-IVS1 (r = 0.277, p =0.205), respectively (Table 6). Then, the graph using the Pearson correlation coefficient of both correlations was plotted and we found that the coefficient of determination (R^2) of the graph was 0.706 (Figure 13).

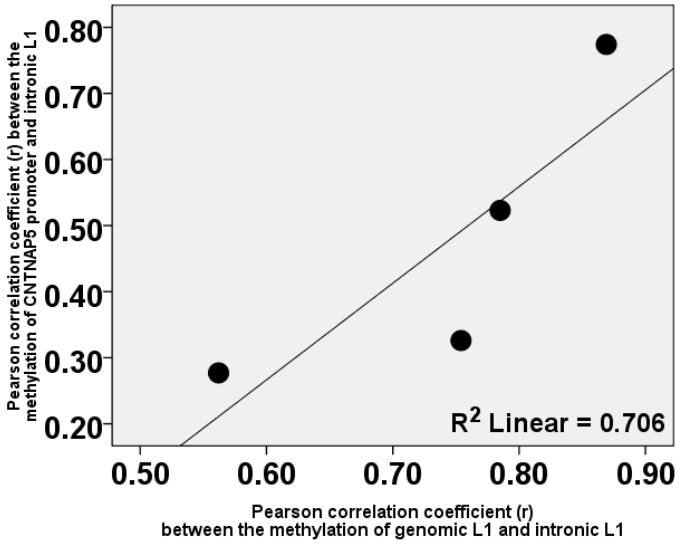

Figure 13. The relationship between two Pearson correlation coefficient (r). Y axis was the correlation between *CNTNAP5* promoter methylation and each intragenic L1-*CNTNAP5* methylation and X axis was the correlation between global LINE-1 hypomethylation and each intragenic L1-*CNTNAP5* methylation.

***CNTNAP5* promoter was controlled by DNA methylation.**

We found that *CNTNAP5* promoter was hypermethylation in head and neck squamous cancer cell lines (WSU-HN). We interested to prove that *CNTNAP5* promoter was controlled by DNA methylation. Thus, the expression of *CNTNAP5* in WSU-HN including SKOV3, ovarian cancer cell lines, was investigated. The result showed that *CNTNAP5* expressed in some cell lines (Figure 14 and 15B).

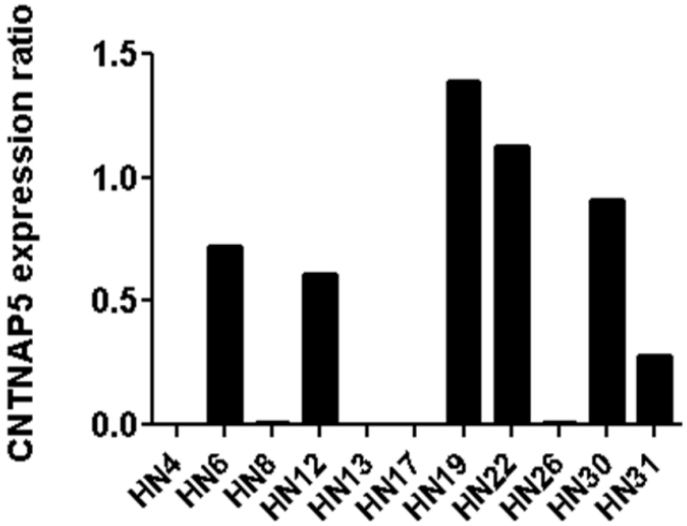

Figure 14. The expression ratio of *CNTNAP5* in head and neck squamous cancer cell lines.

After that, SKOV3 was treated with 5-Aza-2'-deoxycytidine (5azaDC) that acted as the demethylating agent, and then the expression of *CNTNAP5* and the methylation levels of LINE-1 elements, both L1-CNTNAP5-IVS11 and global LINE-1 elements were investigated. The result showed that the methylation levels of *CNTNAP5* promoter, L1-*CNTNAP5*-IVS11 and global LINE-1 elements were significantly decreased (Figure 15A). In addition, *CNTNAP5* was up-regulated after treated with 5azaDC (Figure 15B).

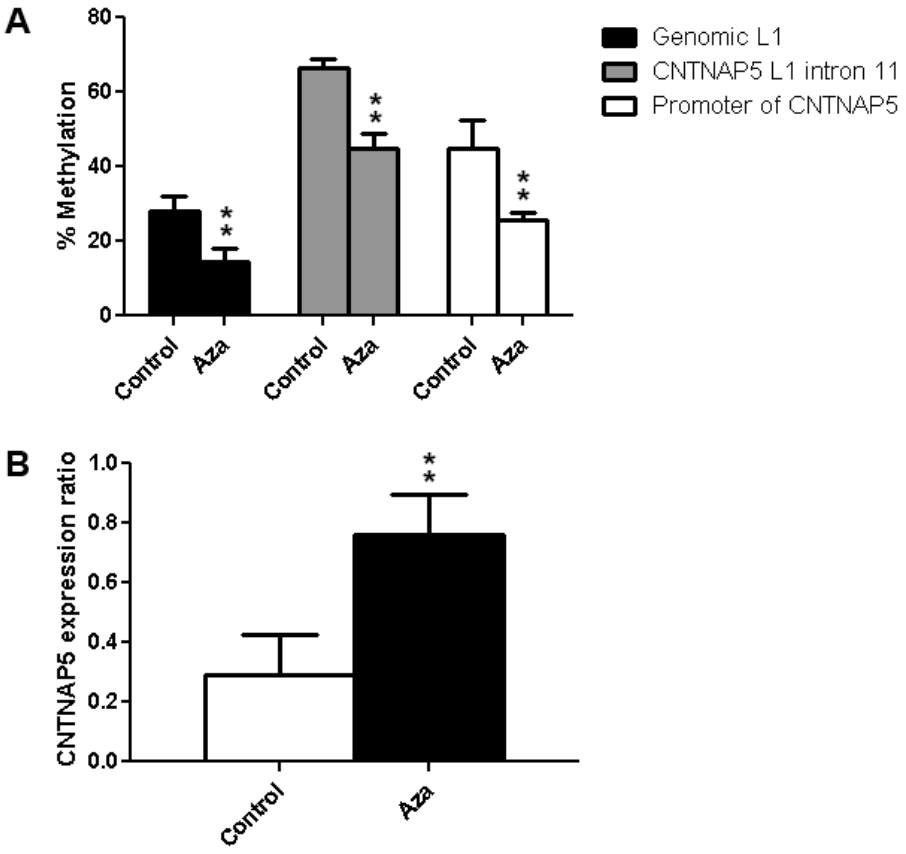

Figure 15. The methylation levels and *CNTNAP5* expression status in 5azaDC-treated SKOV3 cell lines. **A**) The methylation levels of global LINE-1 elements, intragenic L1-*CNTNAP5-IVS11* and *CNTNAP5* promoter. **B**) The expression status of *CNTNAP5*. Control was 5azaDC-untreated SKOV3 cell lines and Aza represented 5azaDC-treated SKOV3 cell lines. ** represented the *P*-value at < 0.01.

Correlation between the highly methylation of gene promoters and global LINE-1 hypomethylation in other full-length LINE-1 inserted genes.

To observe the correlation between promoter hypermethylation and global LINE-1 hypomethylation in other genes with full-length LINE-1 inserted on their sequences. Candidate hypermethylated genes were selected from microarray data which used 5azaDC-treated WSU-HN17 as test and WSU-HN17, head and neck squamous cell cancer (HNSCC) cell line, as control (unpublished data). 5'Aza-2-deoxycytidine (5azaDC) was DNMT inhibitor leading to loss of methylation in whole genome. Thus, genes which up-regulated after treated with 5azaDC were selected as candidate hypermethylated genes. From these criteria, 2,187 candidate hypermethylated genes were selected, and then we picked up only candidate genes that had many intragenic full-length LINE-1 elements located on theirs as our candidate genes. Thus, three candidate genes were selected; *ME3*, *PDE3B* and *PDE4D*.

The promoter of each candidate genes was observed the CpG islands on 5' upstream of candidate genes, and then predicted their promoter sites by Promoter 2.0 Prediction Server that was program for promoter predication in vertebrates. The methylation levels of candidate gene promoters were measured using COBRA. The result showed that the methylation status of promoter of *ME3* and *PDE3B* was highly methylation and the methylation levels of both genes were approximately 80 percentages by average. However, the methylation levels of *PDE4D* promoter were about 20 percentages by average that was lower than other candidate gene promoters (Figure 16).

Because there were many full-length intragenic LINE-1 elements located on each candidate genes. Intragenic LINE-1 elements in *ME3* had four copies; all of them were anti-sense orientation. Six intragenic LINE-1 copies in *PDE3B* separate to five sense orientation and one anti-sense orientation. The last candidate gene had 3 anti-sense LINE-1 elements for *PDE4D*. Therefore, intragenic LINE-1 elements that the sequences mostly resemble to active LINE-1 elements accession number M80343 (L1.2) and L1-*CNTNAP5-IVS11* were selected for the methylation measurement. From this criteria, intragenic LINE-1 elements located on intron 1 of *ME3* (L1-*ME3*), intron 1 of *PDE3B* (L1-*PDE3B*) and some LINE-1 element located on *PDE4D* (L1-*PDE4D*) were selected as our candidate intragenic LINE-1 elements. Then, the

methylation levels of intragenic LINE-1 elements in each candidate genes were investigated using CU-L1. We found that intragenic L1-*PDE3B* and L1-*PDE4D* methylation levels were scattered in wider range which between 40 to 85 percentage whereas the methylation levels of L1-*ME3* was slightly distributed at 80 percentage by average (Figure 16).

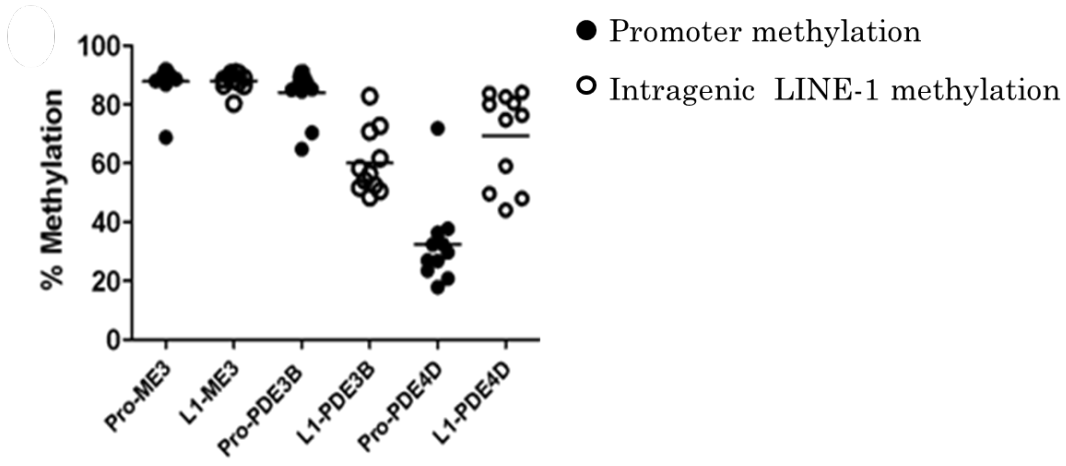

Figure 16. The methylation levels of *ME3*, *PDE3B* and *PDE4D* promoters and intragenic L1-*ME3*, L1-*PDE3B* and L1-*PDE4D*. Black circles represented promoter methylation and white circles were intragenic LINE-1 methylation.

The correlation between promoter methylation of each candidate genes and LINE-1 methylation, both intragenic LINE-1 methylation and global LINE-1 methylation, was observed. The result showed that there was no correlation between promoter methylation of these candidate genes and intragenic LINE-1 methylation (Figure 17A) including global LINE-1 methylation (Figure 17B). Thus, only *CNTNAP5* promoter methylation was correlated to global LINE-1 hypomethylation (Figure 9C).

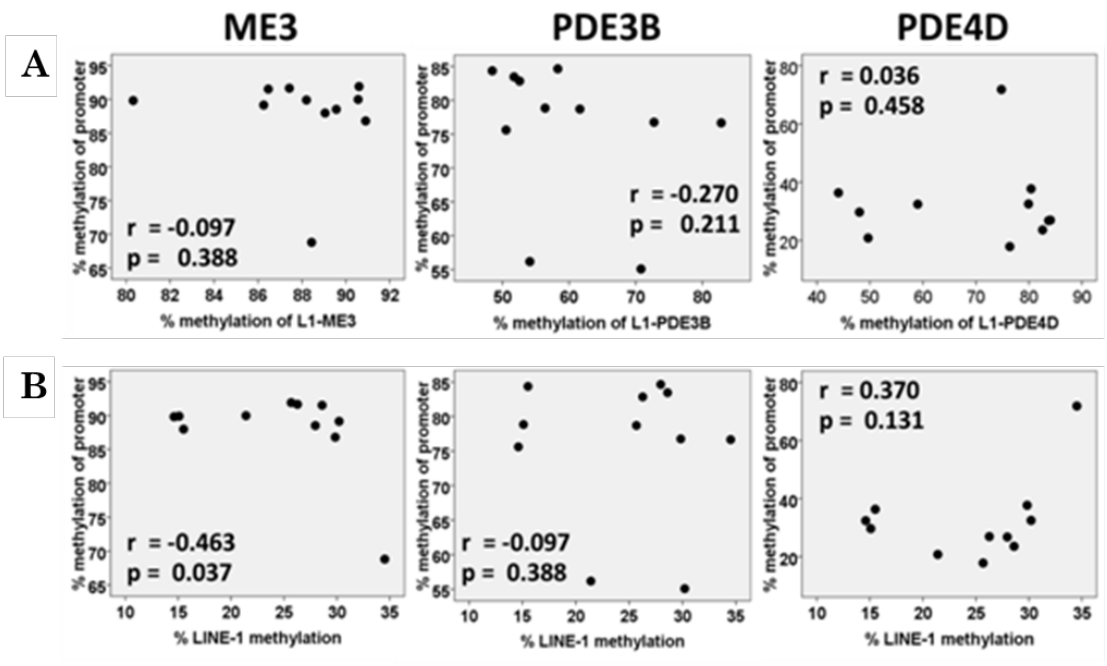

Figure 17. The correlation of promoter methylation of *ME3*, *PDE3B* and *PDE4D* and **A**) their intragenic LINE-1 methylation including **B**) global LINE-1 hypomethylation.

Correlation between the highly methylation of gene promoters and global LINE-1 hypomethylation in non-LINE-1 inserted genes.

To investigate the highly methylation of gene promoters that had no LINE-1 inserted into theirs, the candidate genes were chosen from Worsham M *et al* [112], and then selected only genes without intragenic LINE-1 insertion. From these criteria, *DLEC1*, *CALCA* and *CHFR* were picked up as our candidate genes. The promoter methylation of candidate genes was detected by COBRA. The result showed that the methylation levels of *DLEC1* promoter were highly methylation and approximately at 85 percentages by average. However, the methylation levels of *CALCA* promoter were distributed at 50 to 90 percentages whereas the methylation levels of *CHFR* promoter were separated in two groups that were highly methylation group and lowly methylation group (Figure 18A). Then, the correlation between the methylation levels of each candidate promoter methylation and global LINE-1 hypomethylation was observed. We found that *CALCA* promoter methylation, but not *DLEC1* and *CHFR*

promoter methylation, was significantly correlated to global LINE-1 hypomethylation (Figure 18B).

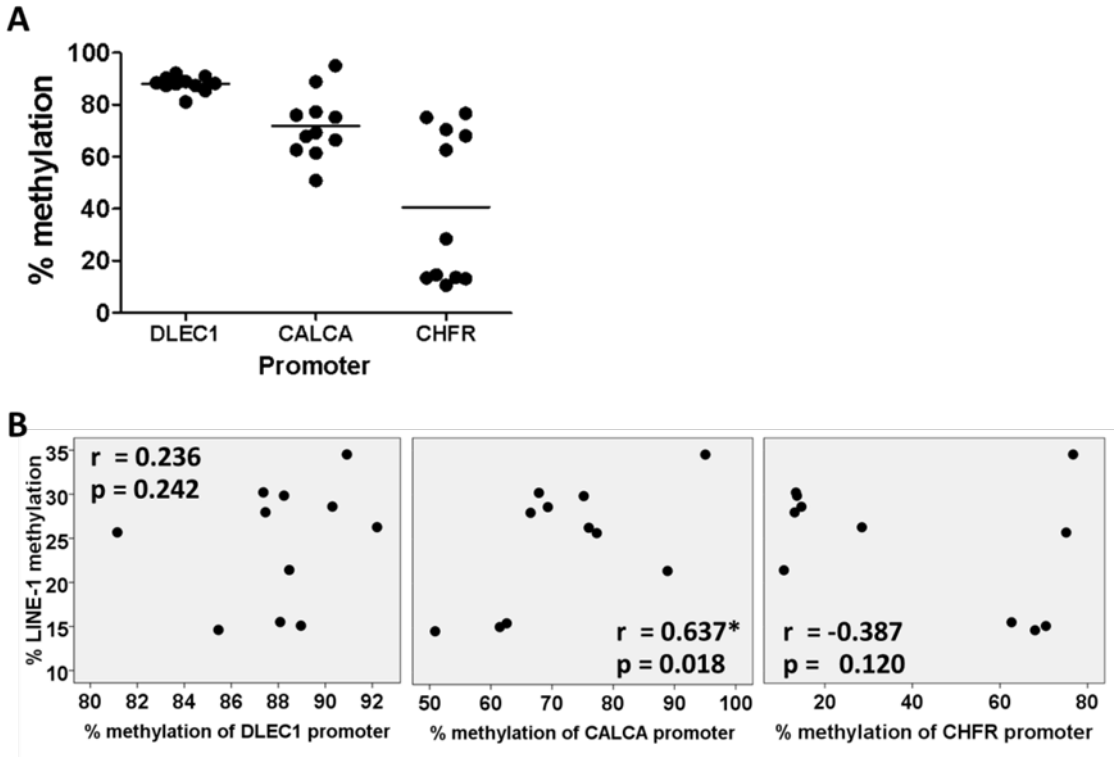

Figure 18. The methylation status in non-LINE-1 inserted genes. **A**) The methylation levels of *DLEC1*, *CALCA* and *CHFR* promoters. **B**) The correlation between global LINE-1 hypomethylation and promoter methylation of *DLEC1*, *CALCA* and *CHFR*, respectively.

The hypermethylated genes with and without LINE-1 insertion in global hypomethylated genome.

To investigate the correlation between global hypomethylation and hypermethylated genes in whole genome. CU-DREAM was performed using intersection between two independent microarray data. Because we interested to select the hypermethylated genes in global hypomethylated condition. Thus, the first microarray data used 5azaDC-treated WSU-HN17 which was global hypomethylated cell line by demethylated agent, 5azaDC, treatment as test and WSU-HN17 as control. Genes which up-regulated in 5azaDC-treated WSU-HN17 were selected as candidate hypermethylated genes. The second microarray data used WSU-HN31 which hypomethylated cell line when compared with WSU-HN17as test and WSU-HN17 as control. Then, both microarray data were intersected and genes which up-regulated in both 5azaDC-treated WSU-HN17 and WSU-HN31 when compared with WSU-HN17 were picked up as candidate hypermethylated genes in global hypomethylated cell lines.

The result showed that 1010 genes were significantly up-regulated in global hypomethylated cell lines compared with controls (Table 3). Then, these genes were separated into two groups, with and without LINE-1 insertion, by CU-DREAMX program. We found that both genes with and without LINE-1 insertion were significant up-regulated in global hypomethylated cell lines (Table 4).

Table 3. Hypermethylated genes in global hypomethylated genome.

		HN17 and 5azaDC treated HN17		
		Up (0.05)	**Not up**	**Sum**
HN17 and HN31	**Up (0.05)**	1010	3741	4751
	Not up	1177	12473	13650
	Sum	2187	16214	18401

P-value	7.2E-119
Odds ratio	2.861067

Table 4. Hypermethylated genes **A**) with and **B**) without LINE-1 insertion in global hypomethylated genome.

A) With LINE-1 insertion		**HN17 and 5azaDC treated HN17**		
		Up (0.05)	**Not up**	**Sum**
HN17 and HN31	**Up (0.05)**	50	262	312
	Not up	68	961	1029
	Sum	118	1223	1341

P-value	2.69E-07
Odds ratio	2.697014

B) Without LINE-1 insertion		**HN17 and 5azaDC treated HN17**		
		Up (0.05)	**Not up**	**Sum**
HN17 and HN31	**Up (0.05)**	960	3479	4439
	Not up	1109	11512	12621
	Sum	2069	14991	17060

P-value	1.7E-112
Odds ratio	2.864416

CHAPTER V

DISSCUSSION

LINE-1 elements are autonomous non LTR retrotransposon which widely distributed in the human genome, both intragenic region and intergenic region. LINE-1 elements can impact the genome structure that resulting in global instability, global rearrangement and global innovation [3, 5]. LINE-1 elements are transcriptional suppressed by DNA methylation however the methylation of LINE-1 elements is reduced in cancer. Global hypomethylation is one of the most common epigenetic changes during cancer development as lead to alterations in the expression of oncogenes, chromosomal instability and reactivation of transposons. However, the cause of global hypomethylation mechanism is still unclear [9]. The consequences of global hypomethylation such as aberrant gene expression and chromosomal instability have been proposed on adjacent global hypomethylation region. The methylation levels of several intragenic LINE-1 elements in cancerous cells are also lower than normal cells and not all LINE-1 elements are completely methylated in normal cells. However, some LINE-1 hypermethylation can be found sporadically in cancer for example intragenic L1-*CNTNAP5*-IVS11.

To understanding about the role of aberrant LINE-1 methylation effected to the host genes in cancers. We selected highly methylated LINE-1 elements located on *CNTNAP5* intron 11 (L1-*CNTNAP5*-IVS11) as candidate intragenic LINE-1 element to investigate their impact to host gene. First, we detected the methylation levels of host gene promoter and LINE-1 elements which both intragenic L1-*CNTNAP5*-IVS11 and global LINE-1 elements in head and neck squamous cell carcinoma cell lines. We found that both intragenic L1-*CNTNAP5*-IVS11 methylation and global LINE-1 methylation were reduced in cancerous cells compared with normal cells. This finding corroborated to previous studies that found the global LINE-1 elements and intragenic LINE-1 elements was hypomethylation in cancerous cells comparing to normal cells [11, 12, 18, 22-25, 38, 39, 113]. Whereas, the methylation levels of *CNTNAP5* promoter methylation in cancerous cells were higher than normal cells. This epigenetically feature indicated that *CNTNAP5* may be candidate tumor suppressor gene in head and neck squamous cell carcinoma. Then, we observed the association between *CNTNAP5* promoter methylation and LINE-1 elements, both LI-*CNTNAP5*-

IVS11 methylation and global LINE-1 hypomethylation. Interestingly, we found the correlation between global LINE-1 hypomethylation, but not L1-*CNTNAP5*-IVS11 hypermethylation, and *CNTNAP5* promoter hypermethylation.

Although, we could not observe the correlation between global LINE-1 hypomethylation and intragenic L1-CNTNAP5-IVS11 hypermethylation, there was trend to correlate between them. Therefore, *CNTNAP5* sequence was analyzed to investigate other intragenic LINE-1 elements which located on its sequence, and then we examined the methylation levels of them. We found five copies of LINE-1 elements located on *CNTNAP5*. However, only four copies, L1-*CNTNAP5*-IVS1, L1-*CNTNAP5*-IVS3A, L1-*CNTNAP5*-IVS3B and L1-*CNTNAP5*-IVS11, were measured the methylation levels because another had many variables on its sequence. The result showed that the methylation levels of all intragenic L1-*CNTNAP5* elements were reduced in cancerous cells more than normal cells. Moreover, the methylation levels of them had no significant different in the same cell lines and the methylation status of them were correlated especially the correlation between L1-*CNTNAP5*-IVS3A and L1-*CNTNAP5*-IVS3B which located on the same intron, had the highest correlation value ($P<0.00$, Pearson $r = 0.885$) (Table 5). Therefore, this finding suggested that the methylation levels of all intragenic LINE-1 elements in the same gene might be correlated especially intragenic LINE-1 elements that located on the same intron. This finding related to previous studied which reported that intragenic LINE-1 elements located on the same gene might have the highest correlation value [22].

The association between each intragenic L1-*CNTNAP5* methylation and global LINE-1 methylation including *CNTNAP5* promoter methylation was observed. We found the methylation levels of all intragenic L1-*CNTNAP5* elements were significantly correlated to global LINE-1 hypomethylation. This result confirmed to previous studied that found the correlation between intragenic LINE-1 methylation and global LINE-1 hypomethylation [22]. In addition, we found the significant correlation between *CNTNAP5* promoter methylation and some intragenic L1-*CNTNAP5* methylation, L1-*CNTNAP5*-IVS3A and L1-*CNTNAP5*-IVS3B. Interestingly, the trend of correlation between each intragenic L1-*CNTNAP5* methylation and *CNTNAP5* promoter methylation was similar to the trend of the correlation between each intragenic L1-*CNTNAP5* methylation and global LINE-1 hypomethylation (Table 6). It was mention that L-1*CNTNAP5*-IVS3A was the most

significantly correlated to *CNTNAP5* promoter methylation and global LINE-1 hypomethylation, following to L1-*CNTNAP5*-IVS3B, L1-*CNTNAP5*-IVS11 and L1-*CNTNAP5*-IVS1, respectively. Then, the graph using the Pearson correlation coefficient of both correlations was plotted and we found that the coefficient of determination (R^2) of the graph was 0.706. It seemed that global LINE-1 hypomethylation may influence *CNTNAP5* promoter methylation and each intragenic L1-*CNTNAP5* methylation. To prove this hypothesis, we observed the correlation between gene promoter methylation and global LINE-1 hypomethylation in other genes which contained intragenic LINE-1 insertion and without intragenic LINE-1 insertion on their sequences.

For intragenic LINE-1 inserted genes, we selected highly methylated genes, *ME3 PDE3B* and *PDE4D* as candidate genes, to study the correlation between their gene promoter methylation and LINE-1 methylation. However, we did not notice the correlation between genes promoter methylation and LINE-1 methylation, both intragenic LINE-1 methylation and global LINE-1 methylation. Thus, only *CNTNAP5* could be observed the correlation between gene promoter methylation and LINE-1 methylation.

After that, we selected non-LINE-1 inserted genes which were *DLEC1 CALCA* and *CHFR* as candidate gene, and then the methylation levels of each candidate gene promoters were investigated. Then, we observed the correlation between global LINE-1 methylation and promoter methylation of each candidate genes. We found the promoter methylation of *CALCA*, but not *DLEC1* and *CHFR*, was significantly correlated to global LINE-1 hypomethylation. This result suggested that promoter methylation was related to global LINE-1 hypomethylation even though no LINE-1 located on its sequence.

This finding corroborated to previous studies that reported the correlation between global LINE-1 hypomethylation and promoter methylation, in both hypermethylation and hypomethylation in several cancers. [11, 24, 25, 75-78]. However, a linked mechanism between them was still unclear. However, our results showed that only *CNTNAP5* and *CALCA* promoter methylation which were demethylated promoters correlated to global LINE-1 hypomethylation. We proposed that global LINE-1

hypomethylation correlated to only gene promoter methylation in which had trended to demethylation.

The correlation between hypermethylated genes and global LINE-1 hypomethylation was observed in global hypomethylated genome using CU-DREAM. The result showed that there were 1010 candidate hypermethylated genes significantly up-regulated in global hypomethylated genome and these candidate genes may relate to global LINE-1 hypomethylation. Moreover, we investigated the role of intragenic LINE-1 elements in the correlation between gene promoter methylation and global LINE-1 hypomethylation in global hypomethylated genome. We found that candidate genes, both LINE-1 inserted genes and non-LINE-1 inserted genes, significantly up-regulated in global hypomethylated genome. Thus, we proposed that the correlation between gene promoter methylation and global LINE-1 hypomethylation was not depended on intragenic LINE-1 insertion.

Although, the mechanism of global hypomethylation was still unclear, global hypomethylation frequently occurred at LINE-1 elements including Human endogenous retroviral (HERV) elements which were transposable elements [22, 27]. Moreover, our finding indicated that global hypomethylation could be observed in gene promoter too. Thus, we proposed that the mechanism of global hypomethylation was general mechanism which did not depend on specific sequences.

REFERENCES

[1.] Aporntewan, C., et al., Hypomethylation of Intragenic LINE-1 Represses Transcription in Cancer Cells through AGO2. *PLoS One*. 6(3): e17934.

[2.] Graham, T. and Boissinot, S. The genomic distribution of L1 elements: the role of insertion bias and natural selection. *J Biomed Biotechnol*, 2006. 2006(1): 75327.

[3.] Kazazian, H.H., Jr. and Moran, J.V. The impact of L1 retrotransposons on the human genome. *Nat Genet*, 1998. 19(1): 19-24.

[4.] Kazazian, H.H., Jr., Mobile elements: drivers of genome evolution. *Science*, 2004. 303(5664): 1626-32.

[5.] Cordaux, R. and Batzer, M.A. The impact of retrotransposons on human genome evolution. *Nat Rev Genet*, 2009. 10(10): 691-703.

[6.] Feinberg, A. and Vogelstein, B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. *Nature*, 1983. 301(5895): 89-92.

[7.] Feinberg, A. and Tycko, B. The history of cancer epigenetics. *Nat Rev Cancer*, 2004. 4(2): 143-53.

[8.] Goelz, S.E., et al., Hypomethylation of DNA from benign and malignant human colon neoplasms. *Science*, 1985. 228(4696): 187-90.

[9.] Hoffmann, M.J. and Schulz, W.A. Causes and consequences of DNA hypomethylation in human cancer. *Biochem Cell Biol*, 2005. 83(3): 296-321.

[10.] Chalitchagorn, K., et al., Distinctive pattern of LINE-1 methylation level in normal tissues and the association with carcinogenesis. *Oncogene*, 2004. 23(54): 8841-6.

[11.] Choi, I.S., et al., Hypomethylation of LINE-1 and Alu in well-differentiated neuroendocrine tumors (pancreatic endocrine tumors and carcinoid tumors). *Mod Pathol*, 2007. 20(7): 802-10.

[12.] Shuangshoti, S., et al., Line-1 hypomethylation in multistage carcinogenesis of the uterine cervix. *Asian Pac J Cancer Prev*, 2007. 8(2): 307-9.

[13.] Subbalekha, K., et al., Detection of LINE-1s hypomethylation in oral rinses of oral squamous cell carcinoma patients. *Oral Oncol*, 2009. 45(2): 184-91.

[14.] Choi, J.Y., et al., Association between global DNA hypomethylation in leukocytes and risk of breast cancer. *Carcinogenesis*, 2009. 30(11): 1889-97.

[15.] Saito, K., et al., Long interspersed nuclear element 1 hypomethylation is a marker of poor prognosis in stage IA non-small cell lung cancer. *Clin Cancer Res*. 16(8): 2418-26.

[16.] Igarashi, S., et al., A novel correlation between LINE-1 hypomethylation and the malignancy of gastrointestinal stromal tumors. *Clin Cancer Res*. 16(21): 5114-23.

[17.] Menendez, L., Benigno, B.B. and McDonald, J.F. L1 and HERV-W retrotransposons are hypomethylated in human ovarian carcinomas. *Mol Cancer*, 2004. 3: 12.

[18.] Suter, C.M., Martin, D.I. and Ward, R.L. Hypomethylation of L1 retrotransposons in colorectal cancer and adjacent normal tissue. *Int J Colorectal Dis*, 2004. 19(2): 95-101.

[19.] Schulz, W.A., L1 retrotransposons in human cancers. *J Biomed Biotechnol*, 2006. 2006(1): 83672.

[20.] Hellman, A. and Chess, A. Gene body-specific methylation on the active X chromosome. *Science*, 2007. 315(5815): p. 1141-3.

[21.] Qu, G.Z., et al., Frequent hypomethylation in Wilms tumors of pericentromeric DNA in chromosomes 1 and 16. *Cancer Genet Cytogenet*, 1999. 109(1): p. 34-9.

[22.] Phokaew, C., et al., LINE-1 methylation patterns of different loci in normal and cancerous cells. *Nucleic Acids Res*, 2008. 36(17): p. 5704-12.

[23.] Cho, N.Y., et al., Hypermethylation of CpG island loci and hypomethylation of LINE-1 and Alu repeats in prostate adenocarcinoma and their relationship to clinicopathological features. *J Pathol*, 2007. 211(3): 269-77.

[24.] Daskalos, A., et al., Global DNA hypomethylation-induced DeltaNp73 transcriptional activation in non-small cell lung cancer. *Cancer Lett*. 300(1): 79-86.

[25.] Ehrlich, M., et al., Hypomethylation and hypermethylation of DNA in Wilms tumors. *Oncogene*, 2002. 21(43): 6694-702.

[26.] Florl, A.R., et al., Coordinate hypermethylation at specific genes in prostate carcinoma precedes LINE-1 hypomethylation. *Br J Cancer*, 2004. 91(5): 985-94.

[27.] Iramaneerat, K., et al., HERV-K hypomethylation in ovarian clear cell carcinoma is associated with a poor prognosis and platinum resistance. *Int J Gynecol Cancer*. 21(1): 51-7.

[28.] Konkel, M.K. and Batzer, M.A. A mobile threat to genome stability: The impact of non-LTR retrotransposons upon the human genome. *Semin Cancer Biol*. 20(4): 211-21.

[29.] Daskalos, A., et al., Hypomethylation of retrotransposable elements correlates with genomic instability in non-small cell lung cancer. *Int J Cancer*, 2009. 124(1): 81-7.

[30.] Kazazian, H.H., Jr. and Goodier, J.L. LINE drive. retrotransposition and genome instability. *Cell*, 2002. 110(3): 277-80.

[31.] Kongruttanachok, N., et al., Replication independent DNA double-strand break retention may prevent genomic instability. *Mol Cancer*. 9: 70.

[32.] Pornthanakasem, W., et al., LINE-1 methylation status of endogenous DNA double-strand breaks. *Nucleic Acids Res*, 2008. 36(11): 3667-75.

[33.] Kawakami, K., et al., Long interspersed nuclear element-1 hypomethylation is a potential biomarker for the prediction of response to oral fluoropyrimidines in microsatellite stable and CpG island methylator phenotype-negative colorectal cancer. *Cancer Sci*. 102(1): 166-74.

[34.] Ogino, S., et al., A cohort study of tumoral LINE-1 hypomethylation and prognosis in colon cancer. *J Natl Cancer Inst*, 2008. 100(23): 1734-8.

[35.] Pattamadilok, J., et al., LINE-1 hypomethylation level as a potential prognostic factor for epithelial ovarian cancer. *Int J Gynecol Cancer*, 2008. 18(4): 711-7.

[36.] Tangkijvanich, P., et al., Serum LINE-1 hypomethylation as a potential prognostic marker for hepatocellular carcinoma. *Clin Chim Acta*, 2007. 379(1-2): 127-33.

[37.] Smith, I.M., et al., DNA global hypomethylation in squamous cell head and neck cancer associated with smoking, alcohol consumption and stage. *Int J Cancer*, 2007. 121(8): 1724-8.

[38.] Cho, N.Y., et al., Genomic hypomethylation and CpG island hypermethylation in prostatic intraepithelial neoplasm. *Virchows Arch*, 2009. 454(1): 17-23.

[39.] Moore, L.E., et al., Genomic DNA hypomethylation as a biomarker for bladder cancer susceptibility in the Spanish Bladder Cancer Study: a case-control study. *Lancet Oncol*, 2008. 9(4): 359-66.

[40.] Wright, R.O., et al., Biomarkers of lead exposure and DNA methylation within retrotransposons. *Environ Health Perspect*. 118(6): 790-5.

[41.] Schernhammer, E.S., et al., Dietary folate, alcohol and B vitamins in relation to LINE-1 hypomethylation in colon cancer. *Gut*. 59(6): 794-9.

[42.] Teneng, I., et al., Reactivation of L1 retrotransposon by benzo(a)pyrene involves complex genetic and epigenetic regulation. *Epigenetics*. 6(3): 355-67.

[43.] Bollati, V., et al., Changes in DNA methylation patterns in subjects exposed to low-dose benzene. *Cancer Res*, 2007. 67(3): 876-80.

[44.] Figueiredo, J.C., et al., Global DNA hypomethylation (LINE-1) in the normal colon and lifestyle characteristics and dietary and genetic factors. *Cancer Epidemiol Biomarkers Prev*, 2009. 18(4): 1041-9.

[45.] Perrin, D., et al., Specific hypermethylation of LINE-1 elements during abnormal overgrowth and differentiation of human placenta. *Oncogene*, 2007. 26(17): 2518-24.

[46.] Zhang, F.F., et al., Dietary Patterns Are Associated with Levels of Global Genomic DNA Methylation in a Cancer-Free Population. *J Nutr*, 2011.

[47.] Lander, E.S., et al., Initial sequencing and analysis of the human genome. *Nature*, 2001. 409(6822): 860-921.

[48.] Munoz-Lopez, M. and Garcia-Perez, J.L. DNA transposons: nature and applications in genomics. *Curr Genomics*. 11(2): 115-28.

[49.] Ostertag, E.M. and Kazazian, H.H. Jr., Biology of mammalian L1 retrotransposons. *Annu Rev Genet*, 2001. 35: 501-38.

[50.] Brouha, B., et al., Hot L1s account for the bulk of retrotransposition in the human population. *Proc Natl Acad Sci U S A*, 2003. 100(9): 5280-5.

[51.] Babushok, D.V. and Kazazian, H.H. Jr., Progress in understanding the biology of the human mutagen LINE-1. *Hum Mutat*, 2007. 28(6): 527-39.

[52.] Cost, G.J. and Boeke, J.D. Targeting of human retrotransposon integration is directed by the specificity of the L1 endonuclease for regions of unusual DNA structure. *Biochemistry*, 1998. 37(51): 18081-93.

[53.] Jurka, J., Sequence patterns indicate an enzymatic involvement in integration of mammalian retroposons. *Proc Natl Acad Sci U S A*, 1997. 94(5): 1872-7.

[54.] Belancio, V.P., Deininger, P.L. and Roy-Engel, A.M. LINE dancing in the human genome: transposable elements and disease. *Genome Med*, 2009. 1(10): 97.

[55.] Miki, Y., et al., Disruption of the APC gene by a retrotransposal insertion of L1 sequence in a colon cancer. *Cancer Res*, 1992. 52(3): 643-5.

[56.] Morse, B., et al., Insertional mutagenesis of the myc locus by a LINE-1 sequence in a human breast carcinoma. *Nature*, 1988. 333(6168): 87-90.

[57.] Chen, J.M., et al., Genomic rearrangements in inherited disease and cancer. *Semin Cancer Biol*. 20(4): 222-33.

[58.] Morisada, N., et al., Branchio-oto-renal syndrome caused by partial EYA1 deletion due to LINE-1 insertion. *Pediatr Nephrol*. 25(7): 1343-8.

[59.] Mine, M., et al., A large genomic deletion in the PDHX gene caused by the retrotranspositional insertion of a full-length LINE-1 element. *Hum Mutat*, 2007. 28(2): 137-42.

[60.] Moran, J.V., DeBerardinis, R.J. and Kazazian, H.H. Jr., Exon shuffling by L1 retrotransposition. *Science*, 1999. 283(5407): 1530-4.

[61.] Goodier, J.L., Ostertag, E.M. and Kazazian, H.H. Jr., Transduction of 3'-flanking sequences is common in L1 retrotransposition. *Hum Mol Genet*, 2000. 9(4): 653-7.

[62.] Pickeral, O.K., et al., Frequent human genomic DNA transduction driven by LINE-1 retrotransposition. *Genome Res*, 2000. 10(4): 411-5.

[63.] Han, J.S. and Boeke, J.D. LINE-1 retrotransposons: modulators of quantity and quality of mammalian gene expression?. *Bioessays*, 2005. 27(8): 775-84.

[64.] Ustyugova, S.V., Lebedev, Y.B. and Sverdlov, E.D. Long L1 insertions in human gene introns specifically reduce the content of corresponding primary transcripts. *Genetica*, 2006. 128(1-3): 261-72.

[65.] Belancio, V.P., Hedges, D.J. and Deininger, LINE-1 RNA splicing and influences on mammalian gene expression. *Nucleic Acids Res*, 2006. 34(5): 1512-21.

[66.] Belancio, V.P., Roy-Engel, A.M. and Deininger, The impact of multiple splice sites in human L1 elements. *Gene*, 2008. 411(1-2): 38-45.

[67.] Perepelitsa-Belancio, V. and Deininger, P. RNA truncation by premature polyadenylation attenuates human mobile element activity. *Nat Genet*, 2003. 35(4): 363-6.

[68.] Roy-Engel, A.M., et al., Human retroelements may introduce intragenic polyadenylation signals. Cytogenet *Genome Res*, 2005. 110(1-4): 365-71.

[69.] Cruickshanks, H.A. and Tufarelli, C. Isolation of cancer-specific chimeric transcripts induced by hypomethylation of the LINE-1 antisense promoter. *Genomics*, 2009. 94(6): 397-406.

[70.] Weber, B., et al., Demethylation of a LINE-1 antisense promoter in the cMet locus impairs Met signalling through induction of illegitimate transcription. *Oncogene*. 29(43): 5775-84.

[71.] Matlik, K., Redik, K. and Speek, M. L1 antisense promoter drives tissue-specific transcription of human genes. *J Biomed Biotechnol*, 2006. 2006(1): 71753.

[72.] Nigumann, P., et al., Many human genes are transcribed from the antisense promoter of L1 retrotransposon. *Genomics*, 2002. 79(5): 628-34.

[73.] Roman-Gomez, J., et al., Promoter hypomethylation of the LINE-1 retrotransposable elements activates sense/antisense transcription and marks the progression of chronic myeloid leukemia. *Oncogene*, 2005. 24(48): 7213-23.

[74.] Yamamoto, E., et al., LINE-1 hypomethylation is associated with increased CpG island methylation in Helicobacter pylori-related enlarged-fold gastritis. *Cancer Epidemiol Biomarkers Prev*, 2008. 17(10): 2555-64.

[75.] Woloszynska-Read, A., et al., Coordinated Cancer Germline Antigen Promoter and Global DNA Hypomethylation in Ovarian Cancer: Association with the BORIS/CTCF Expression Ratio and Advanced Stage. *Clin Cancer Res*.

[76.] Ehrlich, M., et al., Quantitative analysis of associations between DNA hypermethylation, hypomethylation, and DNMT RNA levels in ovarian tumors. *Oncogene*, 2006. 25(18): 2636-45.

[77.] Iacopetta, B., et al., Methylation levels of LINE-1 repeats and CpG island loci are inversely related in normal colonic mucosa. *Cancer Sci*, 2007. 98(9): 1454-60.

[78.] Kim, B.H., et al., CpG island hypermethylation and repetitive DNA hypomethylation in premalignant lesion of extrahepatic cholangiocarcinoma. *Virchows Arch*, 2009. 455(4): 343-51.

[79.] Trankenschuh, W., et al., Frequent and distinct aberrations of DNA methylation patterns in fibrolamellar carcinoma of the liver. *PLoS One*. 5(10): e13688.

[80.] Kile, M.L., et al., Correlation of global and gene-specific DNA methylation in maternal-infant pairs. *PLoS One*. 5(10): e13730.

[81.] Portela, A. and Esteller, M. Epigenetic modifications and human disease. *Nat Biotechnol*. 28(10): 1057-68.

[82.] Gardiner-Garden, M. and Frommer, M. CpG islands in vertebrate genomes. *J Mol Biol*, 1987. 196(2): 261-82.

[83.] Larsen, F., et al., CpG islands as gene markers in the human genome. *Genomics*, 1992. 13(4): 1095-107.

[84.] Doi, A., et al., Differential methylation of tissue- and cancer-specific CpG island shores distinguishes human induced pluripotent stem cells, embryonic stem cells and fibroblasts. *Nat Genet*, 2009. 41(12): 1350-3.

[85.] Zilberman, D., et al., Genome-wide analysis of Arabidopsis thaliana DNA methylation uncovers an interdependence between methylation and transcription. *Nat Genet*, 2007. 39(1): 61-9.

[86.] Klose, R.J. and Bird, A.P. Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci*, 2006. 31(2): 89-97.

[87.] Esteller, M., Epigenetic gene silencing in cancer: the DNA hypermethylome. *Hum Mol Genet*, 2007. 16 Spec No 1: R50-9.

[88.] Luczak, M.W. and Jagodzinski, P.P. The role of DNA methylation in cancer development. *Folia Histochem Cytobiol*, 2006. 44(3): 143-54.

[89.] Ponger, L. and Li, W.H. Evolutionary diversification of DNA methyltransferases in eukaryotic genomes. *Mol Biol Evol*, 2005. 22(4): 1119-28.

[90.] Jeltsch, A., Molecular enzymology of mammalian DNA methyltransferases. *Curr Top Microbiol Immunol*, 2006. 301: 203-25.

[91.] Goll, M.G., et al., Methylation of tRNAAsp by the DNA methyltransferase homolog Dnmt2. *Science*, 2006. 311(5759): 395-8.

[92.] Rai, K., et al., Dnmt2 functions in the cytoplasm to promote liver, brain, and retina development in zebrafish. *Genes Dev*, 2007. 21(3): 261-6.

[93.] Hermann, A., Gowher, H. and Jeltsch, A. *Biochemistry* and biology of mammalian DNA methyltransferases. *Cell Mol Life Sci*, 2004. 61(19-20): 2571-87.

[94.] Aapola, U., Liiv, I. and Peterson, P. Imprinting regulator DNMT3L is a transcriptional repressor associated with histone deacetylase activity. *Nucleic Acids Res*, 2002. 30(16): 3602-8.

[95.] Deplus, R., et al., Dnmt3L is a transcriptional repressor that recruits histone deacetylase. *Nucleic Acids Res*, 2002. 30(17): 3831-8.

[96.] Kanwal, R. and Gupta, S. Epigenetics and cancer. *J Appl Physiol*. 109(2): 598-605.

[97.] Taby, R. and Issa, J.P. Cancer epigenetics. CA Cancer J Clin. 60(6): 376-92.

[98.] Cheung, H.H., et al., DNA methylation of cancer genome. *Birth Defects Res C Embryo Today*, 2009. 87(4): 335-50.

[99.] Strathdee, G. and Brown, R. Aberrant DNA methylation in cancer: potential clinical interventions. *Expert Rev Mol Med*, 2002. 4(4): 1-17.

[100.] Esteller, M., Epigenetics in cancer. N Engl J Med, 2008. 358(11): 1148-59.

[101.] Zhang, Y., et al., Methylation of multiple genes as a candidate biomarker in non-small cell lung cancer. *Cancer Lett*. 303(1): 21-8.

[102.] Dong, W., et al., Hypermethylation-mediated reduction of LMX1A expression in gastric cancer. *Cancer Sci*. 102(2): 361-6.

[103.] Li, B., et al., Hypermethylation of multiple tumor-related genes associated with DMNT3b up-regulation served as a biomarker for early diagnosis of esophageal squamous cell carcinoma. *Epigenetics*. 6(3): 307-16.

[104.] Wolff, E.M., et al., Hypomethylation of a LINE-1 promoter activates an alternate transcript of the MET oncogene in bladders with cancer. *PLoS Genet*. 6(4): e1000917.

[105.] Miki, Y., Retrotransposal integration of mobile genetic elements in human diseases. *J Hum Genet*, 1998. 43(2): 77-84.

[106.] Holmes, S.E., et al., A new retrotransposable human L1 element from the LRE2 locus on chromosome 1q produces a chimaeric insertion. *Nat Genet*, 1994. 7(2): 143-8.

[107.] Traut, W., et al., New members of the neurexin superfamily: multiple rodent homologues of the human CASPR5 gene. *Mamm Genome*, 2006. 17(7): 723-31.

[108.] Spiegel, I., et al., Caspr3 and caspr4, two novel members of the caspr family are expressed in the nervous system and interact with PDZ domains. *Mol Cell Neurosci*, 2002. 20(2): 283-97.

[109.] Eads, C.A. and Laird, P.W. Combined bisulfite restriction analysis (COBRA). *Methods Mol Biol*, 2002. 200: 71-85.

[110.] Penzkofer, T., Dandekar, T. and Zemojtel, T. L1Base: from functional annotation to prediction of active LINE-1 elements. *Nucleic Acids Res*, 2005. 33(Database issue): D498-500.

[111.] Knudsen, S., Promoter2.0: for the recognition of PolII promoter sequences. *Bioinformatics*, 1999. 15(5): 356-61.

[112.] Worsham, M.J., et al., Novel approaches to global mining of aberrantly methylated promoter sites in squamous head and neck cancer. *Otolaryngol Head Neck Surg*. 143(1): 116-21, 121 e1-19.

[113.] Fabris, S., et al., Biological and clinical relevance of quantitative global methylation of repetitive DNA sequences in chronic lymphocytic leukemia. *Epigenetics*. 6(2): 188-94.

APPENDICES

APPENDIX A

SUPPLEMENTS

Table 5 The Pearson correlation coefficient (r) and the probability value (p) between each intragenic LINE-1 methylation in *CNTNAP5*, *ME3*, *PDE3B* and *PDE4D*.

	***CNTNAP5* Intron 3A**	***CNTNAP5* Intron 3B**	***CNTNAP5* Intron 11**	***ME3***	***PDE3B***	***PDE4D***	**Global LINE-1**
***CNTNAP5* Intron 1**	0.627* 0.020	0.628* 0.017	0.624* 0.020	0.343 0.151	-0.004 0.496	0.623* 0.020	0.562* 0.036
***CNTNAP5* Intron 3A**		0.885** 0.000	0.606* 0.024	0.153 0.327	0.474 0.071	0.951** 0.000	0.869** 0.000
***CNTNAP5* Intron 3B**	0.885** 0.000		0.511 0.054	-0.069 0.420	0.483 0.066	0.852** 0.000	0.785** 0.000
***CNTNAP5* Intron 11**	0.606* 0.024	0.511 0.054		0.645* 0.016	0.512 0.054	0.449 0.083	0.754** 0.000
ME3	0.153 0.327	-0.069 0.420	0.645* 0.016		0.278 0.204	0.055 0.436	0.329 0.162
PDE3B	0.474 0.071	0.483 0.066	0.512 0.054	0.278 0.204		0.422 0.098	0.740** 0.005
PDE4D	0.951** 0.000	0.852** 0.000	0.449 0.083	0.055 0.436	0.422 0.098		0.832** 0.001

- Upper value represented Pearson correlation coefficient (r) and lower value was the probability value (p).
- Dark gray boxes represented significant correlation value.
- * was *P* value at < 0.05 and ** was *P* value at < 0.01

Table 6 The Pearson correlation coefficient (r) and the probability value (p) of each intragenic L1-*CNTNAP5* methylation with *CNTNAP5* Promoter methylation, Global LINE-1 methylation and *CNTNAP5* expression.

Intragenic L1-CNTNAP5 elements	*CNTNAP5* Promoter methylation		Global LINE-1 methylation		*CNTNAP5* expression	
	r	p	r	p	r	p
L1-*CNTNAP5*-IVS3A	0.774	0.003**	0.869	0.000**	-0.489	0.063
L1-*CNTNAP5*-IVS3B	0.523	0.049*	0.785	0.002**	-0.425	0.097
L1-*CNTNAP5*-IVS11	0.326	0.164	0.754	0.004**	-0.151	0.328
L1-*CNTNAP5*-IVS1	0.277	0.205	0.562	0.036*	0.740	0.414

- Dark gray boxes represented significant correlation value.
- * was *P*-value at < 0.05 and ** was *P*-value at < 0.01

APPENDIX B

BUFFERS AND REAGENTS

1. Lysis Buffer 1

Sucrose	109.54	g
1.0 M Tris – HCl (pH 7.5)	10	ml
1.0 M MgCl2	5	ml
Triton X – 100 (pure)	10	ml
Distilled water to volume	1,000	ml

Sterilize the solution by autoclaving and store in a refrigerator at 40 ^{o}C.

2. Lysis Buffer 2

5.0 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. 20 mg/ml Proteinase K

Proteinase K	2	mg
Distilled water to volume	1	ml

Mix the solution and store in a refrigerator at -20 ^{o}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 volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

6. 0.5 M EDTA (pH 8.0)

Disodium ethylenediamine tetraacetate.2H2O	186.6	g
Dissolve in distilled water and adjusted pH to 8.0 with NaOH		
Distilled water to volume	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

7. 1.0 M $MgCl_2$ solution

Magnesium chloride.6H2O	20.33	g
Distilled water to volume	100	ml

Dispense the solution into aliquots and sterilize by autoclaving.

8. 5 M NaCl solution

Sodium chloride	29.25	g
Distilled water to volume	100	ml

Dispense the solution into aliquot and sterilize by autoclaving.

9. 20 mg/ml glycogen

Glycogen	200	mg
Distilled water to volume	10	ml

Sterilize the solution by filter through 0.2 μm membrane, aliquot and store in a refrigerator at -20 ^{o}C.

10. 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.

11. 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 to volume	100	ml

The solution was mixed and store at 4 ^{o}C

12. 10 M Ammonium acetate (NH_4OAc)

Ammonium acetate	77.08	g
Distilled water to volume	80	ml

Adjust volume to 100 ml with distilled water and sterilize by autoclaving. The solution was stored at room temperature.

13. 25:24:1 (v/v) Phenol-chloroform-isoamyl alcohol

Phenol	50	ml
Chloroform	48	ml
Isoamyl alcohol	1	ml

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

14. 8% Non-denature acrylamide gel (w/v)

40% Acrylamide: Bis (19:1)	1	ml
10X TBE	0.5	ml
10% ammonium persulfate	50	µl
TEMED	5	µl
H2O	3.5	ml

15. 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 liter with H_2O.

16. 3 M Sodium acetate (CH3COONH4)

Sodium acetate	40.82	g
dH_2O	80	ml

Adjust the pH to 5.3 by adding conc. HCl.

Adjust volume to 100 ml with dH_2O, and sterile by autoclaving

17. Sodium Bisulfite

Sodium Bisulfite	3.76	g
dH_2O	10	ml

Adjust the pH 5.0 by adding 10 M NaOH.

18. Hydroquinone

Hydroquinone	55.4	mg
dH_2O	50	ml

Protected from light by cover with foil

19. 10X PBS

NaCl	80	g
Na_2HPO_4	2	g
KCl	14.4	g
KH_2PO_4	2.4	g
Distilled water to volume	1,000	ml

Mix to dissolve and adjust pH to 7.4, and then sterilize the solution by autoclaving and store at room temperature

20. DMEM medium (Stock solution)

DMEM medium	1	bottle
$NaHCO_3$	3.7	g

Mix to dissolve and adjust volume to 1000 ml with distilled water. Adjust pH to 7.1-7.2 with conc.HCl and then sterilize the solution by filter and store at 4 ^{o}C.

DMEM medium (Working solution)

DMEM medium (Stock solution)	900	ml
Fetal bovine serum (FBS)	100	ml
Antibiotic	10	ml

The solution was mixed and store at 4 ^{o}C.

21. RPMI medium (Stock solution)

RPMI medium	1	bottle
$NaHCO_3$	2	g

Mix to dissolve and adjust volume to 1000 ml with distilled water. Adjust pH to 7.1-7.2 with conc.HCl and then sterilize the solution by filter and store at 4 ^{o}C.

RPMI medium (Working solution)

RPMI medium (Stock solution)	900	ml
Fetal bovine serum (FBS)	100	ml
Antibiotic	10	ml

The solution was mixed and store at 4 ^{o}C.

APPENDIX C

LINE-1 SEQUENCE

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

GGGGGGAGGAGCCAAGATGGCCGAATAGGAACAGCTCCGGTCTACAGCT
CCCAGCGTGAGCGACGCAGAAGACGGTGATTTCTGCATTTCCATCTGAGG
TACCGGGTTCATCTCACTAGGGAGTGCCAGACAGTGGGCGCAGGCCAGTG
TGTGTGCGCACCGTGCGCGAGCCGAAGCAGGGCGAGGCATTGCCTCACCT
GGGAAGCGCAAGGGGTCAGGGAGTTCCCTTTCTGAGTCAAAGAAAGGGG
TGACGGTCGCACCTGGAAAATCGGGTCACTCCCACCCGAATATTGCGCTT
TTCAGACCGGCTTAAGAAACGGCGCACCACGAGACTATATCCCACACCTG
GCTCGGAGGGTCCTACGCCCACGGAATCTCGCTGATTGCTAGCACAGCAG
TCTGAGATCAAACTGCAAGGCGGCAACGAGGCTGGGGGAGGGGCGCCCG
CCATTGCCCAGGCTTGCTTAGGTAAACAAAGCAGCCGGGAAGCTCGAACT
GGGTGGAGCCCACCACAGCTCAAGGAGGCCTGCCTGCCTCTGTAGGCTCC
ACCTCTGGGGGCAGGGCACAGACAAACAAAAAGACAGCAGTAACCTCTG
CAGACTTAAGTGTCCCTGTCTGACAGCTTTGAAGAGAGCAGTGGTTCTCCC
AGCACGCAGCTGGAGATCTGAGAACGGGCAGACAGACTGCCTCCTCAAGT
GGGTCCCTGACTCCTGACCCCCGAGCAGCCTAACTGGGAGGCACCCCCCA
GCAGGGGCACACTGACACCTCACACGGCAGGGTATTCCAACAGACCTGCA
GCTGAGGGTCCTGTCTGTTAGAAGGAAAACTAACAACCAGAAAGGACATC
TACACCGAAAACCCATCTGTACATCACCATCATCAAAGACCAAAAGTAGA
TAAAACCACAAAGATGGGGAAAAAACAGAACAGAAAAACTGGAAACTCT
AAAACGCAGAGCGCCTCTCCTCCTCCAAAGGAACGCAGTTCCTCACCAGC
AACAGAACAAAGCTGGATGGAGAATGATTTTGACGAGCTGAGAGAAGAA
GGCTTCAGACGATCAAATTACTCTGAGCTACGGGAGGACATTCAAACCAA
AGGCAAAGAAGTTGAAAACTTTGAAAAAAATTTAGAAGAATGTATAACTA
GAATAACCAATACAGAGAAGTGCTTAAAGGAGCTGATGGAGCTGAAAAC
CAAGGCTCGAGAACTACGTGAAGAATGCAGAAGCCTCAGGAGCCGATGC
GATCAACTGGAAGAAAGGGTATCAGCAATGGAAGATGAAATGAATGAAA
TGAAGCGAGAAGGGAAGTTTAGAGAAAAAAGAATAAAAAGAAATGAGCA
AAGCCTCCAAGAAATATGGGACTATGTGAAAAGACCAAATCTACGTCTGA
TTGGTGTACCTGAAAGTGATGTGGAGAATGGAACCAAGTTGGAAAACACT

CTGCAGGATATTATCCAGGAGAACTTCCCCAATCTAGCAAGGCAGGCCAA
CGTTCAGATTCAGGAAATACAGAGAACGCCACAAAGATACTCCTCGAGAA
GAGCAACTCCAAGACACATAATTGTCAGATTCACCAAAGTTGAAATGAAG
GAAAAAATGTTAAGGGCAGCCAGAGAGAAAGGTCGGGTTACCCTCAAAG
GAAAGCCCATCAGACTAACAGTGGATCTCTCGGCAGAAACCCTACAAGCC
AGAAGAGAGTGGGGGCCAATATTCAACATTCTTAAAGAAAAGAATTTTCA
ACCCAGAATTTCATATCCAGCCAAACTAAGCTTCATAAGTGAAGGAGAAA
TAAAATACTTTATAGACAAGCAAATGTTGAGAGATTTTGTCACCACCAGG
CCTGCCCTAAAAGAGCTCCTGAAGGAAGCACTAAACATGGAAAGGAACA
ACCGGTACCAGCCGCTGCAAAATCATGCCAAAATGTAAAGACCATCGAGA
CTAGGAAGAAACTGCATCAACTAATGAGCAAAATCACCAGCTAACATCAT
AATGACAGGATCAAATTCACACATAACAATATTAACTTTAAATATAAATG
GACTAAATTCTGCAATTAAAAGACACAGACTGGCAAGTTGGATAAAGAGT
CAAGACCCATCAGTGTGCTGTATTCAGGAAACCCATCTCACGTGCAGAGA
CACACATAGGCTCAAAATAAAAGGATGGAGGAAGATCTACCAAGCCAAT
GGAAAACAAAAAAAGGCAGGGGTTGCAATCCTAGTCTCTGATAAAACAG
ACTTTAAACCAACAAAGATCAAAAGAGACAAAGAAGGCCATTACATAAT
GGTAAAGGGATCAATTCAACAAGAGGAGCTAACTATCCTAAATATTTATG
CACCCAATACAGGAGCACCCAGATTCATAAAGCAAGTCCTCAGTGACCTA
CAAAGAGACTTAGACTCCCACACATTAATAATGGGAGACTTTAACACCCC
ACTGTCAACATTAGACAGATCAACGAGACAGAAAGTCAACAAGGATACC
CAGGAATTGAACTCAGCTCTGCACCAAGCAGACCTAATAGACATCTACAG
AACTCTCCACCCCAAATCAACAGAATATACCTTTTTTTCAGCACCACACCA
CACCTATTCCAAAATTGACCACATAGTTGGAAGTAAAGCTCTCCTCAGCA
AATGTAAAAGAACAGAAATTATAACAAACTATCTCTCAGACCACAGTGCA
ATCAAACTAGAACTCAGGATTAAGAATCTCACTCAAAGCCGCTCAACTAC
ATGGAAACTGAACAACCTGCTCCTGAATGACTACTGGGTACATAACGAAA
TGAAGGCAGAAATAAAGATGTTCTTTGAAACCAACGAGAACAAAGACAC
CACATACCAGAATCTCTGGGACGCATTCAAAGCAGTGTGTAGAGGGAAAT
TTATAGCACTAAATGCCTACAAGAGAAAGCAGGAAAGATCCAAAATTGA
CACCCTAACATCACAATTAAAAGAACTAGAAAAGCAAGAGCAAACACAT
TCAAAAGCTAGCAGAAGGCAAGAAATAACTAAAATCAGAGCAGAACTGA
AGGAAATAGAGACACAAAAAACCCTTCAAAAAATCAATGAATCCGGGAG
CTGGTTTTTTGAAAGGATCAACAAAATTGATAGACCGCTAGCAAGACTAA

TAAAGAAAAAAAGAGAGAAGAATCAAATAGACACAATAAAAAATGATAA
AGGGGATATCACCACCGATCCCACAGAAATACAAACTACCATCAGAGAAT
ACTACAAACACCTCTACGCAAATAAACTAGAAAATCTAGAAGAAATGGAT
ACATTCCTCGACACATACACTCTCCCAAGACTAAACCAGGAAGAAGTTGA
ATCTCTGAATAGACCAATAACAGGCTCTGAAATTGTGGCAATAATCAATA
GTTTACCAACCAAAAAGAGTCCAGGACCAGATGGATTCACAGCCGAATTC
TACCAGAGGTACATGGAGGAACTGGTACCATTCCTTCTGAAACTATTCCA
ATCAATAGAAAAAGAGGGAATCCTCCCTAACTCATTTTATGAGGCCAGCA
TCATTCTGATACCAAAGCCGGGCAGAGACACAACCAAAAAAGAGAATTTT
AGACCAATATCCTTGATGAACATTGATGCAAAAATCCTCAATAAAATACT
GGCAAACCGAATCCAGCAGCACATCAAAAAGCTTATCCACCATGATCAAG
TGGGCTTCATCCCTGGGATGCAAGGCTGGTTCAATATACGCAAATCAATA
AATGTAATCCAGCATATAAACAGAGCCAAAGACAAAAACCACATGATTAT
CTCAATAGATGCAGAAAAAGCCTTTGACAAAATTCAACAACCCTTCATGC
TAAAAACTCTCAATAAATTAGGTATTGATGGGACGTATTTCAAAATAATA
AGAGCTATCTATGACAAACCCACAGCCAATATCATACTGAATGGGCAAAA
ACTGGAAGCATTCCCTTTGAAAACCGGCACAAGACAGGGATGCCCTCTCT
CACCGCTCCTATTCAACATAGTGTTGGAAGTTCTGGCCAGGGCAATCAGG
CAGGAGAAGGAAATAAAGGGTATTCGATTAGGAAAAGAGGAAGTCAAAT
TGTCCCTGTTTGCAGACGACATGATTGTATATCTAGAAAACCCCATCGTCT
CAGCCCAAAATCTCCTTAAGCTGATAAGCAACTTCAGCAAAGTCTCAGGA
TACAAAATCAATGTACAAAAATCACAAGCATTCTTATACACCAACAACAG
ACAAACAGAGAGCCAAATCATGGGTGAACTCCCATTCGTAATTGCTTCAA
AGAGAATAAAATACCTAGGAATCCAACTTACAAGGGATGTGAAGGACCT
CTTCAAGGAGAACTACAAACCACTGCTCAAGGAAATAAAAGAGGACACA
AACAAATGGAAGAACATTCCATGCTCATGGGTAGGAAGAATCAATATCGT
GAAAATGGCCATACTGCCCAAGGTAATTTACAGATTCAATGCCATCCCCA
TCAAGCTACCAATGACTTTCTTCACAGAATTGGAAAAAACTACTTTAAAG
TTCATATGGAACCAAAAAAGAGCCCGCATTGCCAAGTCAATCCTAAGCCA
AAAGAACAAAGCTGGAGGCATCACACTACCTGACTTCAAACTATACTACA
AGGCTACAGTAACCAAAACAGCATGGTACTGGTACCAAAACAGAGATAT
AGATCAATGGAACAGAACAGAGCCCTCAGAAATAATGCCGCATATCTACA
ACTATCTGATCTTTGACAAACCTGAGAAAAACAAGCAATGGGGAAAGGAT
TCCCTATTTAATAAATGGTGCTGGGAAAACTGGCTAGCCATATGTAGAAA

GCTGAAACTGGATCCCTTCCTTACACCTTATACAAAAATCAATTCAAGATG
GATTAAAGATTTAAACGTTAAACCTAAAACCATAAAAACCCTAGAAGAAA
ACCTAGGCATTACCATTCAGGACATAGGCGTGGGCAAGGACTTCATGTCC
AAAACACCAAAAGCAATGGCAACAAAAGACAAAATTGACAAATGGGATC
TAATTAAACTAAAGAGCTTCTGCACAGCAAAAGAAACTACCATCAGAGTG
AACAGGCAACCTACAACATGGGAGAAAATTTTCGCAACCTACTCATCTGA
CAAAGGGCTAATATCCAGAATCTACAATGAACTCAAACAAATTTACAAGA
AAAAAACAAACAACCCCATCAAAAAGTGGGCGAAGGACATGAACAGACA
CTTCTCAAAAGAAGACATTTATGCAGCCAAAAAACACATGAAGAAATGCT
CATCATCACTGGCCATCAGAGAAATGCAAATCAAAACCACTATGAGATAT
CATCTCACACCAGTTAGAATGGCAATCATTAAAAAGTCAGGAAACAACAG
GTGCTGGAGAGGATGCGGAGAAATAGGAACACTTTTACACTGTTGGTGGG
ACTGTAAACTAGTTCAACCATTGTGGAAGTCAGTGTGGCGATTCCTCAGG
GATCTAGAACTAGAAATACCATTTGACCCAGCCATCCCATTACTGGGTAT
ATACCCAAATGAGTATAAATCATGCTGCTATAAAGACACATGCACACGTA
TGTTTATTGCGGCACTATTCACAATAGCAAAGACTTGGAACCAACCCAAA
TGTCCAACAATGATAGACTGGATTAAGAAAATGTGGCACATATACACCAT
GGAATACTATGCAGCCATAAAAAATGATGAGTTCATATCCTTTGTAGGGA
CATGGATGAAATTGGAAACCATCATTCTCAGTAAACTATCGCAAGAACAA
AAAACCAAACACCGCATATTCTCACTCATAGGTGGGAATTGAACAATGAG
ATCACATGGACACAGGAAGGGGAATATCACACTCTGGGGACTGTGGTGG
GGTCGGGGGAGGGGGGAGGGATAGCATTGGGAGATATACCTAATGCTAG
ATGACACATTAGTGGGTGCAGCGCACCAGCATGGCACATGTATACATATG
TAACTAACCTGCACAATGTGCACATGTACCCTAAAACTTAGAGTATAATA
AAAAAAAAAAAAAAAAAAAAAAAAA

BIOGRAPHY

Name Suphakit Khowutthitham

Birth 31st October 1980, Udon Thani, Thailand

Education

1999 – 2002	Bachelor of Science (Medical Technology) 1st class honor, Faculty of Allied Health Sciences, Chulalongkorn University
2003	Participated in Biomedical Sciences program, Graduate School, Chulalongkorn University

Scholarship

- Royal Golden Jubilee (RGJ) Ph.D. Scholarship from Thailand Research Fund (TRF)
- The 90th Anniversary of Chulalongkorn University Fund (Ratchadaphiseksomphot Endowment Fund) from Chulalongkorn University.
- Teaching assistant fellowship from Chulalongkorn University.