เมทิเลชั่นบนไลน์-1 ที่วางตัวในอินทรอนมีบทบาทควบคุมการแสดงออกของยีนในเซลล์มะเร็งศีรษะและคอ

นางสาว จุรีรัตน์ โพธิ์แก้ว

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INTRAGENIC LINE-1 METHYLATION CONTROLS GENE EXPRESSION IN HEAD AND NECK CANCER CELLS

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จุรีรัตน์ โพธิ์แก้ว : เมทิเลชั่นบนไลน์-1 ที่วางตัวในอินทรอนมีบทบาทควบคุมการแสดงออกของยีนในเซลล์มะเร็งศีรษะและคอ. (INTRAGENIC LINE-1 METHYLATION CONTROLS GENE EXPRESSION IN HEAD AND NECK CANCER CELLS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: ศ.นพ.ดร. อภิวัฒน์ มุทิรางกูร, 112 หน้า.

การสูญเสียหมู่เมทิลจากดีเอ็นเอในบริเวณของลำดับเบสซ้ำที่สามารถเคลื่อนที่ได้(transposable element, TE) ซึ่งรวมถึงไลน์-1 (long interspersed nuclear element-1, LINE-1)นั้นเป็นปรากฏการณ์สำคัญที่เกี่ยวข้องกับกระบวนการเกิดมะเร็ง ในธรรมชาติลำดับเบสซ้ำๆและไลน์-1ซึ่งมักจะแทรกตัวภายในยีน (intragenic) จะถูกกดการแสดงออกด้วยการเติมหมู่เมทิลเพื่อเป็นการควบคุมจุดเริ่มต้นการแสดงออก (Transcriptional start sites, TSSs) ที่ผิดปกติอันนำไปสู่การสูญเสียความควบคุมภายในจีโนม (genome instability) ทั้งนี้เพื่อเป็นการทำความเข้าใจเกี่ยวกับการสูญเสียหมู่เมทิลจากโปรโมเตอร์ของไลน์-1ซึ่งเป็นบริเวณที่ควบคุมการแสดงออกของไลน์-1ในเซลล์มะเร็งศีรษะและคอได้เลือกใช้เทคนิค CU-L1 เพื่อดูรูปแบบของหมู่เมทิลบนโปรโมเตอร์ของไลน์-1ตำแหน่งเดียวในจีโนมและ COBRALINE-1เพื่อศึกษารูปแบบของหมู่เมทิลบนโปรโมเตอร์ของไลน์-1ทั่วทั้งจีโนม จากการศึกษาพบว่ารูปแบบของหมู่เมทิลบนโปรโมเตอร์ของไลน์-1ตำแหน่งจำเพาะจะเปลี่ยนแปลงตามชนิดของเซลล์และหน้าที่ของยีนที่ไลน์-1วางตัวภายในยีนไลน์ จาก 16 ยีนที่ศึกษาด้วยเทคนิค CU-L1พบว่ามีแค่สองยีนคือ *EPHA3* และ *PPP2R2B* ที่น่าจะถูกกดการแสดงออกด้วยการเติมหมู่เมทิลบนพื้นที่ภายในยีนซึ่งหมายถึงบริเวณโปรโมเตอร์ของไลน์-1 และสามารถยืนยันด้วยการลดระดับของหมู่เมทิลภายในจีโนมด้วยสาร 5'-aza-2-deoxycytidine ในเซลล์ทดลองจะพบการลดการแสดงออกของยีน *EPHA3* นอกเหนือจากนี้การกดการแสดงออกของอาร์เอ็นเอของไลน์-1ด้วยหมู่เมทิลบนโปรโมเตอร์ของไลน์-1ทำให้เชื่อได้ว่าอาร์เอ็นเอของไลน์-1 ควรจะเป็นตัวแปรสำคัญต่อการคุมการแสดงออกของยีนที่มีไลน์-1แบบยีน *EPHA3* ซึ่งสามารถยืนยันได้จากการลดระดับอาร์เอ็นเอของไลน์-1 ส่งผลให้ยีน *EPHA3* แสดงออกได้มากขึ้น บทบาทของอาร์เอ็นเอของไลน์-1เท่าที่เป็นไปได้ไม่ว่าจะเป็นกลไกของ RNA interference หรือสภาวะเหนือพันธุกรรม (Epigenetics) ล้วนต้องการโปรตีนจากโครงสร้างที่ใช้อาร์เอ็นเอในการหยุดการแสดงออกของยีน (RISC) ซึ่งทำให้การลดการแสดงออกของโปรตีนจาก RISC น่าจะเป็นวิธียืนยันทิศทางที่อาร์เอ็นเอของไลน์-1เพื่อควบคุมการแสดงออกของยีนที่มีไลน์-1 โดยภายหลังจากการลดระดับของ *AGO2* ซึ่งเป็นโปรตีนจาก RISC ส่งผลให้ยีน *EPHA3* และอาร์เอ็นเอของไลน์-1แสดงออกได้ และเมื่อศึกษาการจับกันระหว่างอาร์เอ็นเอของไลน์-1และ *AGO2* ก็พบว่านอกเหนือจากที่ *AGO2* จะจับที่อาร์เอ็นเอของไลน์-1แล้ว *AGO2* ยังจับที่บริเวณโปรโมเตอร์ของไลน์-1 ซึ่งสามารถสรุปได้ว่าอาร์เอ็นเอของไลน์-1น่าจะควบคุมการแสดงออกของยีนโดยใช้กลไกของ epigenetics เป็นหลัก และเมื่อศึกษาระดับของเซลล์ที่ลดการแสดงออกของอาร์เอ็นเอของไลน์-1 และ *AGO2* ก็สามารถยืนยันว่าอาร์เอ็นเอของไลน์-1 และ *AGO2* ควบคุมการแสดงออกของยีนที่มีไลน์-1ด้วยบทบาทที่จำเพาะ (In cis) Epigenetics บริเวณภายในยีนที่มีไลน์-1 ทั้งนี้กลไกที่เซลล์เลือกใช้ในการควบคุมการแสดงออกของ retrotransposon นั้นผ่านทางกลไกของ endo-siRNA ซึ่งอาศัยโปรตีน *DICER1* ในการผลิต endo-siRNA จากโมเลกุลตั้งต้นที่รวมถึงสายอาร์เอ็นเอของ retrotransposon ทำให้เชื่อได้ว่าอาร์เอ็นเอของไลน์-1 อาจจะเกี่ยวข้องกับ endo-siRNA ในการควบคุม epigenetics ที่บริเวณภายในยีน และในการศึกษาส่วนสุดท้ายด้วยเทคนิค CU-DREAM เพื่อวิเคราะห์ความเกี่ยวข้องของอาร์เอ็นเอของไลน์-1, *DICER1*, การลดระดับของหมู่เมทิลภายในจีโนมด้วยสาร 5'-aza-2-deoxycytidineและการเกิดมะเร็งศีรษะและคอสามารถช่วยให้เข้าใจได้ว่ากลไกที่เกี่ยวข้องกับ *DICER1* จะกระตุ้นให้มีการเติมหมู่เมทิลภายในยีน แต่กลไกที่เกี่ยวข้องกับอาร์เอ็นเอของไลน์-1 มักจะทำให้มีการเติมหมู่เมทิลบนโปรโมเตอร์ของยีนที่ไม่มีไลน์-1 การพบว่ายีนที่ไม่มีไลน์-1 ถูกควบคุมด้วยอาร์เอ็นเอของไลน์-1ช่วยยืนยันบทบาทที่ไม่จำเพาะ (in tran) ของไลน์-1 โดยสรุปแล้ววิทยานิพนธ์ฉบับนี้สามารถอธิบายถึงการแสดงออกของไลน์-1 จากลดระดับของหมู่เมทิลจากโปรโมเตอร์ของไลน์-1 ในเซลล์มะเร็งศีรษะและคอที่มีผลต่อการควบคุมการแสดงออกของยีนผ่านโปรตีนจาก RISC โดยมีเป้าหมายต่อการเปลี่ยนแปลงภายใต้กลไก epigenetics ภายในเซลล์

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต.....

ปีการศึกษา 2553 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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KEYWORDS: LINE-1 PROMOTER HYPOMETHYLATION / HEAD AND NECK CANCER CELL / INTRAGENIC LINE-1 / GENE REGULATION / AGO2 / DICER1 / RISC / EPIGENETICS

CHUREERAT PHOKAEW : INTRAGENIC LINE-1 METHYLATION CONTROLS GENE EXPRESSION IN HEAD AND NECK CANCER CELLS. ADVISOR: Prof. Apiwat Mutirangura, MD., Ph.D., 112 pp.

One of most common events in carcinogenesis is loss of DNA methylation (hypomethylation) fromsequence of transposable element (TEs) including long interspersed nuclear element-1 (LINE-1). Naturally, LINE-1 and other TEs within gene body region (intragenic)will suppress by totallymethylated CpG sequences in order to keep prevent incorrect transcriptional start site (TSSs) that cause genome instability. To studycharacter of intragenic LINE-1 promoter hypomethylation or gene body methylation status, within Head and Neck squamous cell carcinoma (HNSCC) cell,LINE-1 promoter methylation status was detected with CU-L1 (unique intragenic LINE-1) and COBRALINE-1 (whole genome LINE-1) PCRs. Each intragenic LINE-1 promoter hypomethylation have unique pattern depending on cell types and impact ofLINE-1's host gene within cell. Next, from CU-L1 PCR 16 genes, *EPHA3* and *PPP2R2B* are only two genes that normally induce expression by hypermethylated in gene body region, intragenic LINE-1 promoter. LINE-1 promoter hypomethylation level induce global and specific LINE-1 expression and also repress LINE-1's host genes expression, which confirm by 5'-aza-2-deoxycytidine induced DNA hypomethylationin HNSCC cell that cause *EPHA3* become greater down-regulated.Then, sinceLINE-1 RNA can express via promoter hypomethylation, knockdown LINE-1 RNA can lead increasing of *EPHA3* in HNSCC cell. LINE-1 RNA was concern as key factor on LINE-1 host's gene regulation by consequence of gene body hypomethylation. RNA molecule can regulate gene by RNAi pathway and Epigenetics mechanism, within both machinery require RISC proteins. Next, knockdown RISC protein, *AGO2* protein or human *EIF2C2* can investigate possibility role that LINE-1 RNA regulate LINE-1 host gene. Knockdown *AGO2* within HNSCC cell, *EPHA3* become up-regulate that can conclude similar role between LINE-1 RNA and AGO2 on gene controlling. Immunoprecipitate by AGO2 antibody can reveal interaction of *AGO2* on both LINE-1 RNA and DNA from LINE-1 promoter sequence that indicate role of LINE-1 RNA and *AGO2* will mostly through Epigenetics pathway as confirm about AGO2 bind on LINE-1 promoter region. So that, by intragenic LINE-1 promoters methylation change in HNSCC cell that knockdown LINE-1 or *AGO2*, it may conclude that LINE-1 RNA and *AGO2* regulate LINE-1's host gene via in cis epigenetics mechanism within gene body region. Cell can control overexpress retrotransposon transcript by endo-siRNA pathway, which require *DICER1* for produce endo-siRNA from precursor molecule that include retrotransposon transcript. Combine endo-siRNA pathway to epigenetics mechanism within gene body region will be determine in last step. With CU-DREAM analyse microarray results by intersection datas for check the connection between factors on gene regulation including *DICER1*, LINE-1 RNA, DNA hypomethylation and HNSCC carcinogenesis model. Within HNSCC cell panel, *DICER1* involved pathway prefer to induce hypermethylation on gene body region while LINE-1 RNA involved pathway select to induce hypermethylation on gene without LINE-1 promoter, LINE-1 RNA have in tran function. In conclusion, intragenic LINE-1 promoter hypomethylation cause releases of LINE-1 RNA for regulate genes during HNSCC carcinogenesis via RISC protein.

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

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CONTENTS

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

Figure Page

Figure Page

Figure Page

LIST OF ABBREVIATIONS

TE	Transposable element
LINE-1	Long interspersed element -1
aza	5'-aza-2-deoxycytidine
COBRA	Combined bisulfite restriction analysis
COBRALINE-1	COBRA for genome wide LINE-1
CU-L1	COBRA for unique LINE-1 sequence
CU-L1-EPHA3IVS15	COBRA for unique LINE-1 sequence at intron 15 of gene EPHA3
L1-EPHA3IVS15	Intragenic LINE-1 at intron 15 of gene EPHA3
RNA-IP	RNA immunoprecipitation
HNSCC	Head and neck squamous cell carcinoma
WSU-HN17	Head and neck squamous cell cancer cell line number 17
DNMTs	DNA methyltransferase enzyme gene
AGO2	Argonaute2 , human *EIF2C2* gene
DICER1	Human *DICER1*, ribonuclease type III gene
CU-DREAM	Connection Up- or Down- Regulation Expression Analysis of Microarrays
TSSs	Transcriptional start site
RISC	RNA-Induced Silencing Complex
Endo-siRNA	endogenous small interference RNA
PTGS	Posttranscriptional gene silencing
TGS	Transcriptional gene silencing
RNAi	RNA interference
RNAa	RNA activation
RdDM	RNA-Directed DNA methylation

CHAPTER I

INTRODUCTION

Epigenetics mechanism is non-mutation machinery that occur to change gene expression pattern by DNA methylation[1, 2], Histone modification[3]and small RNA particles[4]. Among three major epigenetics mechanism, DNA methylation is the most considered event in many biology research topics. Many reports have confirmed the impact of DNA methylation change in serious pathogenesis such as in cancer, neurological diseases and autoimmune diseases[5]as shown in table 1. While gain of methylation correlate to tumor suppressor gene promoter, loss of methylation prefers to associate to oncogene promoter and repetitive sequence region in cancer genome as shown in figure 1[6]. Loss of DNA methylation or hypomethylation in repetitive elements region counting LINE-1, IAP and Sat2 was commonly found in many diseases including cancer as shown in table 1[7]. Hypomethylation status in repetitive sequence include LINE-1 can cause many serious consequence within genome that including activation of endoparasitic sequences, transposon transposition, genomic instability and incorrect transcriptional start site. In view of the fact that cell stress was one causein epigenetics deregulation within genome with the purpose of carcinogenesis [8]. Cell stress can induce repetitive element RNA expression, human Alu transcipts for rapid block cell stress response genes by binding binding RNA polymerase II (Pol II)[9]. Carcinogen such as benzo(a)pyrene (BaP)can induce LINE-1 RNA expression according to cell stress response[10] and also cause genomic repeats hypomethylation[11]. Loosen structure by CpGs hypomethylation will allow loading of transcription factor in gene promoter region and let gene and small RNA gene expression [12, 13]. LINE-1 related sequence can influence on gene expression and LINE-1 RNA expression[14]. LINE-1 promoter methylation is prefer occur with CpGs base and have equally among each strand [15]. Because loss of methylation in LINE-1 promoter cause

specific LINE-1 transcript in human cell thus CpGs methylation seem to be main factor in controlling each LINE-1 expression within cell[16]. DNA methylation is exist for control LINE-1 retrotransposon within normal and there is possibility of LINE-1 hypomethylation produce LINE-1 transcript in effect cell. As reviews here, the consequence of LINE-1 promoter methylation in this thesis willbe concerned as the significant factor on Head and Neck squamous cell carcinogenesis.

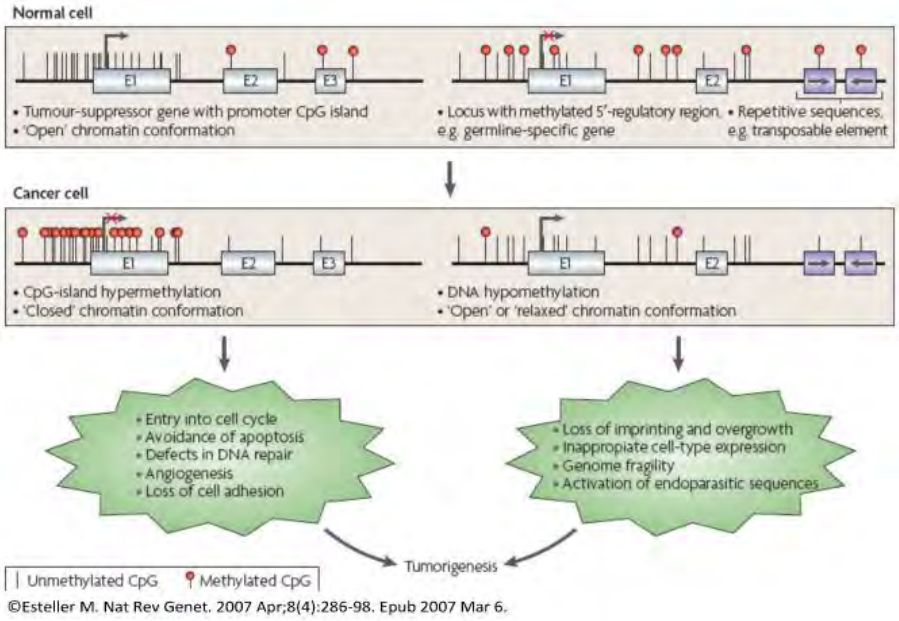


Figure 1: DNA methylation pattern normal cell compare to cancer cell [5].

LINE-1s (Long interspersed element 1) is the major non-LTR retrotransposons for the reason that 20% of human genome is LINE-1 sequence[16]. Retrotransposition is the initial mechanism that people concern about LINE-1 retrotransposon because of many works shown LINE-1 retrotransposition cause genomic instability [17, 18] and also mark LINE-1 as a mutagenesis factor because there is disease-linked LINE-1 insertion in many disease including cancer[19, 20]. LINE-1 retrotransposition for new location for human retrotransposon insertion polymorphisms (RIPs) was analyzed and confirm at very low rate

for this mechanism among each human genome[21]. If LINE-1 being just a junk particle it should found unrelated to any cellular mechanism butwith tissue specific methylated pattern and LINE-1 expression found in various normal adult human tissue such as esophagus, ovaries, placenta and prostate, LINE-1 become one interesting feature in cell differentiation[22, 23]. LINE-1 within normal genome will have dense methylated while methylated was decrease in adjacent cancerous tissue, which LINE-1 methylation may represent as tissue specific marker and also could indicate for carcinogensis character [7].Human LINE-1 retrotransposonin both sense and antisense orientation promoter confirm as factor that influence on gene in close proximity[24, 25]. Intragenic LINE-1 is conserve than intergenic LINE-1 along with human evolution so that intragenic LINE-1 epigenetics changing will reasonably have impact on human genome shown in figure 2[26]. Comparing potential of LINE-1 in genome, intragenic LINE-1 have more conserved characters than intergenic LINE-1 that may reflex significant impact of intragenic LINE-1 on LINE-1's host gene regulation during pathogenesis. Hypomethylation of LINE-1 antisense orientation promoter also cause cancer-specific chimeric transcript [27]. By epigenetics control, naturally LINE-1 antisense promoter driven transcription is common phenomenon in both cancer and normal cell which prove by evidenced with human expressed-sequence tag (EST) databases[28].

Recently, Aporntewan C et, al can prove that LINE-1 methylation require human *AGO2* or *EIF2C2* gene for repress gene in cancer cell line through the RNAi mechanism via potential complementary structure within LINE-1 sequence [26]. RNAi mechanism also influence on LINE-1 itself as LINE-1 5'UTR region contain both sense and antisense LINE-1 promoter which LINE-1 hypomethylation could release sense and antisense LINE-1 transcripts which may form double strand RNA structure between 400-600 nucleotide of LINE-1 promoter[25, 29]. *DICER1*, core cytoplasmic RISC protein, also damage LINE-1 RNA

molecule in order to prevent LINE-1 function within genome.Because RISC protein can control gene by various situations, RISC protein-dependent epigenetics change in order to prevent gene expression was recently report in many human genes. In Arabidopsis, noncoding RNA, including transposon transcript, found collaborate with RISC protein including Ago4 and Dicer-like protein for regulate gene via DNA hypermethylation in RNA-directed DNA methylation mechanism (RdDM) [30]. In Human *P21* gene, degradation of *P21* antisense transcript by *AGO2* protein, core nucleus RISC protein, cause *P21* gene up-regulate by loss of epigenetics silencing marker from *P21* gene promoter call as RNA activation mechanism (RNAa) [31]. Various factors that control LINE-1 promoter methylation pattern regarding some genes level change associate to LINE-1 hypomethylation status in Head and Neck related tissue. LINE-1 hypomethylation can induce by interleukin (IL)-6 inoral squamous cell carcinoma (OSCC)[32]. Loss of lymphoid-specific helicase (*LSH*) expression found associate to LINE-1 hypomethylation in somatic thymus tissue[33] and high level of *HELLS*(human lymphoid-specific helicase) was reported in HNSCC sample [34]. One interesting role of *TP53*,the important tumor suppressor gene, also found up-regulate LINE-1 expresssion by *TP53* binding site on LINE-1 promoter and *TP53*-dependent LINE-1 expression was report as genome protection mechanism[35]. According to current knowledge, LINE-1 existance was not relying only on retrotransposition process. Purpose of LINE-1s more than 500,000 copies especially some active full length LINE-1s existence withinhuman genome still enlist for determine in order to understand role of this endogenous repetitive element in many cellular systems.

Table 1 Epigenetic modifications in human diseases

Aberrant epigenetic mark	Alteration	Consequences	Examples of genes affected and/or resulting disease
Cancer			
DNA methylation	CpG island hypermethylation	Transcription repression	*MLH1* (colon, endometrium, stomach[11]), *BRCA1* (breast, ovary[11]), *MGMT* (several tumor types[11]), *p16*^{INK4a} (colon[11])
	CpG island hypomethylation	Transcription activation	*MASPIN* (pancreas[92]), *S100P* (pancreas[92]), *SNCG* (breast and ovary[92]), *MAGE* (melanomas[92])
	CpG island shore hypermethylation	Transcription repression	*HOXA2* (colon[20]),*GATA2* (colon[20])
	Repetitive sequences hypomethylation	Transposition, recombination genomic instability	*L1* (ref. 11), *IAP*[11], *Sat2* (ref. 107)
Histone modification	Loss of H3 and H4 acetylation	Transcription repression	*p21*^{WAF2} (also known as *CDKN1A*)[11]
	Loss of H3K4me3	Transcription repression	*HOX* genes
	Loss of H4K20me3	Loss of heterochromatic structure	*Sat2, D4Z4* (ref. 107)
	Gain of H3K9me and H3K27me3	Transcription repression	*CDKN2A, RASSF1* (refs. 115–116)
Nucleosome positioning	Silencing and/or mutation of remodeler subunits	Diverse, leading to oncogenic transformation	*BRG1, CHD5* (refs. 127–131)
	Aberrant recruitment of remodelers	Transcription repression	*PLM-RARa*[103] recruits NuRD
	Histone variants replacement	Diverse (promotion cell cycle/destabilization of chromosomal boundaries)	H2A.Z overexpression/loss
Neurological disorders			
DNA methylation	CpG island hypermethylation	Transcription repression	Alzheimer's disease (*NEP*)[135]
	CpG island hypomethylation	Transcription activation	Multiple sclerosis (*PADI2*)[135]
	Repetitive sequences aberrant methylation	Transposition, recombination genomic instability	ATRX syndrome (subtelomeric repeats)[135,143]
Histone modification	Aberrant acetylation	Diverse	Parkinson's and Huntington's diseases[135]
	Aberrant methylation	Diverse	Huntington's disease and Friedreich's ataxia[135]
	Aberrant phosphorylation	Diverse	Alzheimer's disease[135]
Nucleosome positioning	Misposition in trinucleotide repeats	Creation of a 'closed' chromatin domain	Congenital myotonic dystrophy[151]
Autoimmune diseases			
DNA methylation	CpG island hypermethylation	Transcription repression	Rheumatoid arthritis (*DR3*)[154,155]
	CpG island hypomethylation	Transcription activation	SLE (*PRF1, CD70, CD154, AIM2*)[6]
	Repetitive sequences aberrant methylation	Transposition, recombination genomic instability	ICF (*Sat2, Sat3*), rheumatoid arthritis (*L1*)[152,155]
Histone modification	Aberrant acetylation	Diverse	SLE (*CD154, IL10*, IFN-γ)[6]
	Aberrant methylation	Diverse	Diabetes type 1 (*CLTA4, IL6*)[159]
	Aberrant phosphorylation	Diverse	SLE (NF-κB targets)
Nucleosome positioning	SNPs in the 17q12-q21 region	Allele-specific differences in nucleosome distribution	Diabetes type 1 (*CLTA4, IL6*)
	Histone variants replacement	Interferes with proper remodeling	Rheumatoid arthritis (histone variant macroH2A at NF-κB targets)[157]

Table 1 : Epigenetics factor changing in many diseases[5].

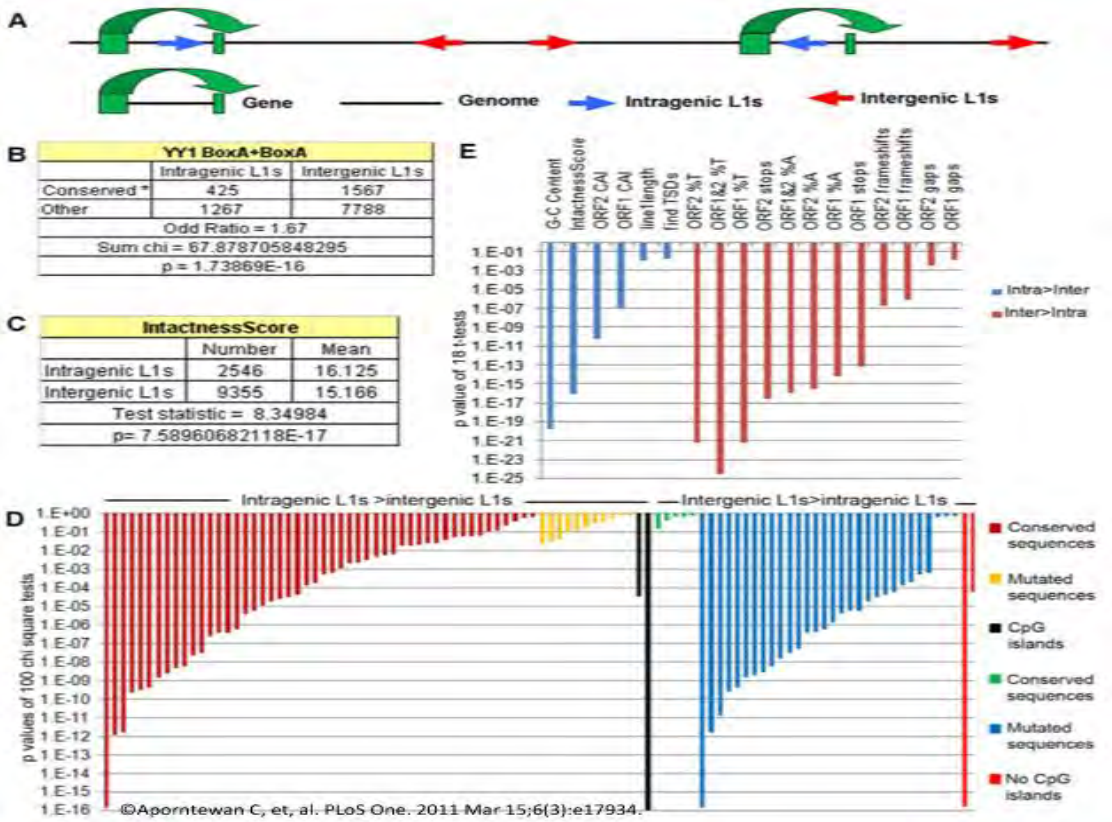


Figure 2: LINE-1 orientation within genome briefly divide into intragenic and intergenic LINE-1[26].

From previous study in Mutirangura's lab, reveal whole genome LINE-1 hypomethylation occur in many type of malignancy tissue when compare to counterparts normal tissue including breast, lung, colon, bladder, prostate, stomach liver and head and neck as shown in figure 3 [7]. Chalitchagorn K. et, al also found correlation of advance LINE-1 hypomethylation level in colon cancer progression which also found this similarity in uterine cervix cancer multistep process case by Shuangshoti S et, al [36]. LINE-1 hypomethylation level quantitate by COBRA LINE-1 method which develop by Chalitchagorn K et, al can be a prognostic marker in many cancer including epithelial ovarian cancer[37]and hepatocellular carcinoma[38]. By measure genomeLINE-1 hypomethylation status in 896 colon cancer cases, Baba Y. and colleagues found previously-unrecognized group among cancer patients which reflex meaning role of LINE-1 demethylated in epigenetics diversity cancer genome[39]. LINE-1 methylation in serum from of solid tumor case can be tumor marker for further specify cancer treatment by DNA methylation inhibitor drug such as decitabine[40]. Whole genomes LINE-1 hypomethylation level in cancers seems to be useful procedure in cancer categorization however with little understanding onhow LINE-1 promoter hypomethylation effects in cancer genome and complexity of LINE-1 sequence itself that cause people fail to notice impact of this phenomenon in carcinogenesis.

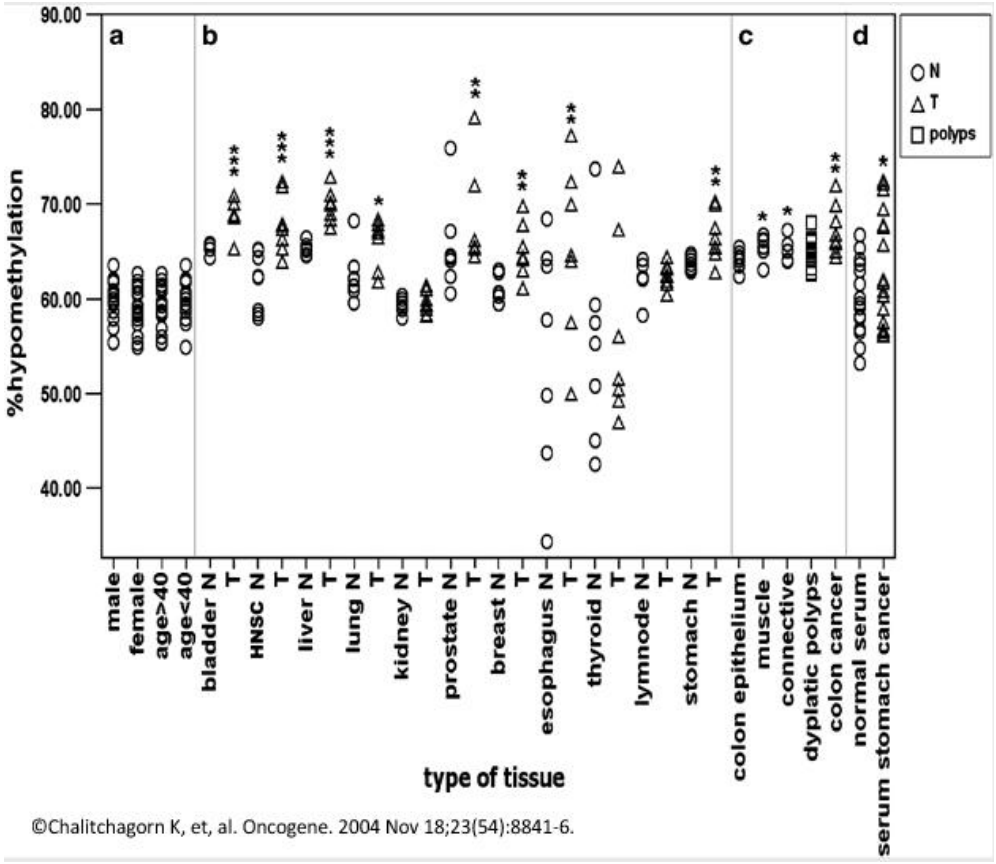


Figure 3: COBRALINE-1 study in various paraffin-embeded cancer tissues. Comparing tumor and normal counterpart cell from same pathologic dissection show LINE-1 hypomethylation occur in many cancer types. [7].

Recently, whole genomes studies with hybridization microarray technique [41] become popular according to less consume time, sample and budget for all genome condition datas reason. For example, in order to prove gene expression pattern within cell or disease, microarray technique can provide all genome datas from single experiment while general RT-PCR could consume more than 20,000 experiments in order to give same data set. Study impact of gene within cell or disease by knockdown specific gene follow with microarray for whole genome expression pattern is the most excellent and fastest protocol in current molecular biology science [42]. Whole genome datas will contains plenty

of numbers which require statistic method to make datas accurate and become really represent tcorrect whole genome result [43] by background cut out, sample intensity normalization and error model fixation. Recently, microarray data analysis base on altered gene expression pattern by chi-square test, odds Ratio test, and 95%CI test from 2x2 table, Connection Up- and Down-Regulation Expression Analysis of Microarrays (CU-DREAM) as show in figure 4, bea prefect procedure in searching connection between factors within gene regulation[44]. CU-DREAM, Connection Up- or Down- Regulation Expression Analysis of Microarrays, is a tool in analyse the correlation between two expression microarray datas from different experiment. In principle, first step, expression signal level from each microarrays was classified by student's t-test into up- and down- regulated and all remains will be not up- and not down- regulated. Second step, selected gene list will depend on experssion probe that only express in both microarray experiment. Third, selected gene list will classified into 4 group : 1) regulated gene in both experiment, 2) not regulated in only first experiment, 3) not regulated only in the second experiment and 4) not regulated in both experiment. Forth, numbers of gene list from each categories in third step was compare with chi-square test.The non-random distribution of gene numbers in forth step will indicate correlation between two factor from each microarray experiment that may assume role of this two factor in regulating gene in same biological process as result in changing of transcriptome of this biological process. Many standard datas from researchers in microarray technique including whole genome expression, whole genome DNA sequencing, whole genome RNA sequencing, whole genome epigenetics profiles, etc, was submit to Gene Expression Omnibus database, in order to share this important datas to scientists around the world for further concern into their currently project [45]. In this project, microarrays for whole genome expression were select to study impact of LINE-1 promoter hypomethylation and assumed partner like RISC protein in HNSCC cell lines.

	Up- or down-regulated genes of experiment A	Not up- or not down-regulated genes of experiment A
Up- or down-regulated genes of experiment B	Number of genes in the 1st group	Number of genes in the 2nd group
Not up- or not down-regulated genes of experiment B	Number of genes in the 3rd group	Number of genes in the 4th group

Figure 4: CU-DREAM principle. 2 X 2 table of chi-square test for study correlation among experiment A and B from test samples in both experiment [44].

Here in this thesis, Head and neck squamous cell cancer (HNSCC) cell lines (WSU-HN), will be used ascell panel for study the effect of LINE-1 promoter hypomethylation in cancer withinall steps.Since whole genome LINE-1 promoter hypomethylation relate to Head and Neck carcinogenesis [7], each specific LINE-1 promoter should also found hypomethylated-related to HNSCC carcinogenesis. First part of this thesis will study about specific LINE-1 promoter methylation pattern by using 17 COBRA Unique to LINE-1 (CU-L1) PCR. As intragenic LINE-1 conserved and all CU-L1 was designed base on intronic full length LINE-1s sequence, CU-L1s methylation status should reflex impact of each LINE-1's location. Since LINE-1 promoter methylation can reflex status of CpG rich within gene body region, many highly express genes found associate to gain of methylation on gene body sequence. For second step, I will focus about connection ofLINE-1 promoter methylation on LINE-1's host gene expression according to gene body methylation mechanism in order to show impact of intragenic LINE-1 on LINE-1's host genes in HNSCC cell lines. As DNA methylation in LINE-1 promoter repress LINE-1 expression in normal cell and LINE-1 RNA is the common target of RNA-induced silencing complex (RISC) protein in order to control genome stability. Third part of thesis will focus on consequence of LINE-1 RNA from LINE-1 promoter hypomethylation on LINE-1's host gene

regulation by knockdown LINE-1 with siRNA contructs in HNSCC cell line. And in forth part will demonstrate the interaction between LINE-1 RNA and RISC complex in order to confirm role of RISC protein in controlling LINE-1 RNA that should involve in role of LINE-1 RNA on LINE-1's host gene regulation within HNSCC cell line. At fifth step, works will focus on possibility of LINE-1 RNA and RISC protein in regulate LINE-1's host gene via gene body DNA methylation machinery in HNSCC cell line. And final part of thesis will study whole genome impact of LINE-1 RNA from LINE-1 promoter hypomethylation and RISC proteins in order to reveal important of LINE-1 hypomethylation process during HNSCC carcinogenesis. By intersection gene alteration datas from multiple microarray resultafter knockdowns each factor, LINE-1 RNA and RISC protein, role of LINE-1 promoter hypomethylation in HNSCC cell line will be clarify through RISC proteins involvedpathway. By hypothesizs that pathway of LINE-1 RNA from LINE-1 promoter hypomethylation requires RISC proteins for induce gene body methylation in order to control LINE-1's host gene expression should exist in HNSCC cell line.

Objective

1. To study unique intragenic LINE-1 promoter methylation pattern among specific LINE-1 loci in various HNSCC cells panel.
2. To evaluate connection of intragenic LINE-1 promoter methylation on LINE-1's hosts gene expression in HNSCC cell line.
3. To study affect of LINE-1 RNA from LINE-1 promoter hypomethylation on LINE-1's host gene expression in HNSCC cell line.

4. To illustrate possible mechanism that LINE-1 RNA control LINE-1's host gene expression by interaction with*AGO2*, nucleus RISC protein, within HNSCC cell line.

5. To show possible consequence of interaction between LINE-1 RNA and *AGO2* on gene body methylation in order to control LINE-1's host gene expression in HNSCC cell line.

6. To show whole genome consequences from LINE-1 promoter hypomethylation through RISC protein pathway in HNSCC cell.

Research Question

1. What is pattern of the specific intragenic LINE-1 promoter methylation in various HNSCC cell panel?

2. Whether there is the connection between intragenic LINE-1 promoter methylation on LINE-1's host gene expression in HNSCC cell lines?

3. Whether LINE-1 RNA from LINE-1 promoter hypomethylation have any effects on LINE-1's host gene expression in HNSCC cell line?

4. Whether LINE-1 RNA can regulate LINE-1's host gene expression by the machinerythat involve to *AGO2* protein within HNSCC cell line?

5. What is the consequence of on LINE-1's host gene from interaction between LINE-1 RNA and *AGO2* in HNSCC cell line?

6. What are the whole genome consequences from LINE-1 promoter hypomethylation all the way through RISC protein pathway in HNSCC cell line?

Hypothesis

1. Methylation level of each intrgenic LINE-1 promoter will varies depend on LINE-1's host gene impact on cell differentiation and in cancer cell, intragenic LINE-1 promoter should become hypomethylated similar to global LINE-1 hypomethylation in Head and Neck cell [7].

2. Some intragenic LINE-1 promoter methylation status should found correlate to LINE-1's host gene expression similar to gene body methylation mechanism in HNSCC cell line.

3. Knockdown LINE-1 RNA that release from LINE-1 promoter hypomethylation will demonstrate effect of LINE-1 RNA on LINE-1's host gene expression in HNSCC cell line.

4. LINE-1 RNA should found interact to *AGO2* and knockdown Ago2 should have effect to LINE-1's host gene expression, in order to show that LINE-1 RNA control LINE-1's host gene expression with *AGO2* proteinwithin HNSCC cell line.

5. Alteration of LINE-1's host gene expression from knockdown LINE-1 RNA or *AGO2* should link to gene body methylation status change by pathway that involve with LINE-1 RNA or *AGO2*.

6. By intersection datas from knockdown LINE-1 RNA, induced hypomethylation (that suppose to overexpress LINE-1 RNA) and knockdown *DICER1*

(cytoplasmic RISC protein), it sould be possible to reveal role of LINE-1 promoter hypomethylation in HNSCC cell line.

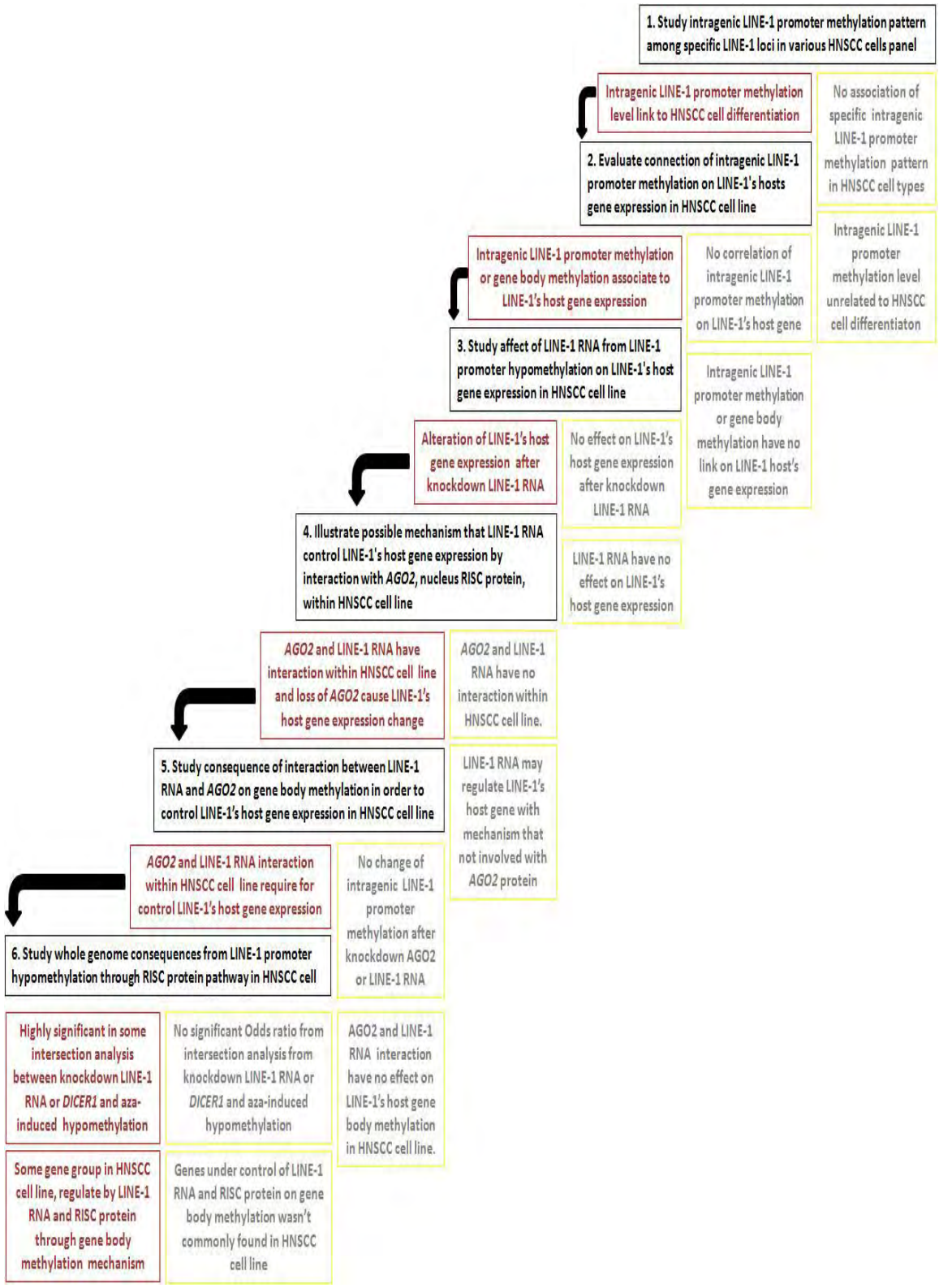
Key words

Long interspersed element 1 (LINE-1), Intragenic LINE-1 promoter methylation, Gene body DNA methylation, Head and neck squamous cell cancer, RISC protein, *AGO2*, *DICER1*, CU-DREAM, Gene expression microarray.

Expected Benefits and Application

Although, there are many association study of methylation status of LINE-1 in pathogenesis was report but none of it has clarify for reason of this phenomenon. Study cause and consequence LINE-1 methylation change in cancer genome will elucidate motive of active intragenic LINE-1 continued existence in human evolution. Understanding LINE-1 promoter hypomethylation effect on cancer genome will bring new noteworthy target for future cancer therapy approach as many active LINE-1 elements still exist in human genome.

Conceptual Framework



CHAPTER II

REVIEWS AND RELATED LITERATURES

DNA methylation mechanism

Epigenetics refer to changing of gene regulation by non-mutation mechanism which suppose to temporary modify of chromosome component structure for change gene expression pattern aimed at specific role in many pathways including gene and microRNA expression, nucleic acid-protein interaction, transposon silencing, embryogenesis, X-chromosome inactivation, genomic imprinting and cellular differentiation[5]. Important of epigenetics is shown in cell differentiation field according to development of pluripotent stem cell into various cell types is according to globally DNA methylation pattern altered [46]. Impact of epigenetics in individualize system is noticeable by evidence of epigenetics different between identical twins [47]. Recovery of how epigenetics mechanism effect on many human diseases including cancer make epigenenetics field become hot subject [5]. Mechanism of cytosine bases and histones modification, changing of nucleosomes position, recruitment of small RNA particles on target DNA is a common phenomenon in epigenetics concept [48]. DNA methylation seems to be the most well known phenomenon in epigenetics area because broadly studied of DNA methylation. DNA methylation can identify into two types of occurrences according to DNA methyltransferase (DNMT) that recruit methyl group onto DNA. *DNMT1* will maintain inheritance DNA methylation pattern and *DNMT3a*, *DNMT3b* will induce de novo DNA methylation for establish DNA methylation during embryonic development [5].

Finding many of abnormal epigenetics patterns on gene promoter, regulatory element, gene body sequence and repetitive sequence in several human diseases reveals critical role of epigenetics in maintaining normal genome homeostasis pattern [5, 22]. Disturbance of epigenetics mechanism with mutation, deletion or change expression of any epigenetics factor will cause aberrant transcriptome pattern which usually occur in

pathogenesis such as report of *DNMT3* mutation in acute myeloid leukemia (AML) case[49, 50]. According to the two-hit model for cancer initiation, silencing the only one active allele of tumor suppressor gene with epigenetics mechanism was proved case of DNA methylation on *MLH1* mismatch repair gene promoter in colorectal tumours[51]. Normally pattern of DNA methylation change in cancer cell refer to global hypomethylation and specific gene promoter hypermethylation[51]. DNA hypomethylation in cancer occur in many type of sequence including repetitive sequence, retrotransposon, intron region which cause genome instability [51]. There is high rate of chromosomal rearrangements on repeat sequence and retrotransposon relate to genomic translocation [52, 53]. For gene promoter in cancers, DNA hypomethylation can activate proto-oncogenes and lead loss of imprinting in insulin-like growth factor-2 (*IGF2*) gene for Wilm's tumor [54]. Disruption by hypermethylation on tumor suppressor gene promoter also found in many case including *MLH1* (mutL homolog-1), *BRCA1* (breast cancer–associated-1), *VHL* (von Hippel-Lindau tumor suppressor), *CDKN2A* (cyclin-dependent kinase inhibitor 2A) and microRNAs which control cell growth-inhibitory [55, 56]. Chimeric oncogene fusion also found for example: *SLC45A3-ELK4* in prostate cancer [57], *MLL-TET1* in AML and lymphocytic leukemias[58], *JAZF1-JJAZ1* in human endometrial stromal tumors[59]. Mutation and chromosomal deletion of *TET2* that cause genome hypomethylatios was report in various myeloid malignancies [60]. DNA methylation seems to be an important mechanism within genome in order to control genome stability.

Covalent modification on cytosine base in context of CpG dinucleotide is commonly symbolic of DNA methylation mechanism [61]. Cluster of CpG dinucleotide or CpG islands is refer to DNA region longer than 200 nucleotides which have GC content, percentage of base guanine and cytosine in DNA region, at least 50% and 0.6 ratio of CpG dinucleotide existence[62]. Nearly 60% of gene promoters in human genome associated to CpG islands and normally have no methylation in normal tissue, however 6% found methylated in tissue-specific process in tissue differentiation and early development stage[63]. Methylated DNA will recruit methyl-CpG-binding domain (*MBD*) proteins and this MBC protein species will induce binding of histone-modification and chromatin-modeling

complex to methylated sequence as result in gene repression[64]. While unmethylated sequence recruit Cfp1 protein for generating H3K4 trimethylation rich domain in euchromatin region [65]. Impact of DNA methylation on CpG island shores, 2 kb away from the CpG island but have lower CpG density, strong associate to disrupt transcription phenomenon which typically found in tissue-specific gene [66]. About 70% of methylation change by reprogramming process also associates to CpG island shores. Highly express genes within cell always have gene body methylation that may involve with elongation efficiency and prevent abnormal transcriptional initiation process [67, 68]. Hypermethylation always occur in repetitive element region within normal genome which is for silencing endogenous mobile elements sequence that likely to for produce genome instability, gene disruption, transcriptome deregulation and genomic rearrangement[6]. All currently describe DNA methylation concept within cell shown in figure 5.

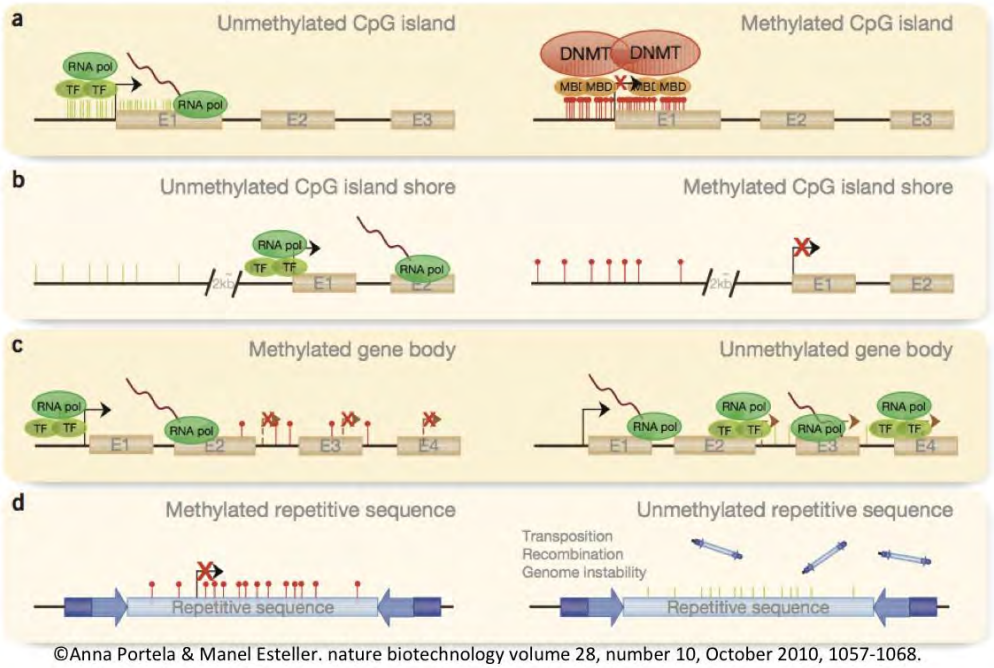


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Figure 5: DNA methylation pattern in genome [5].

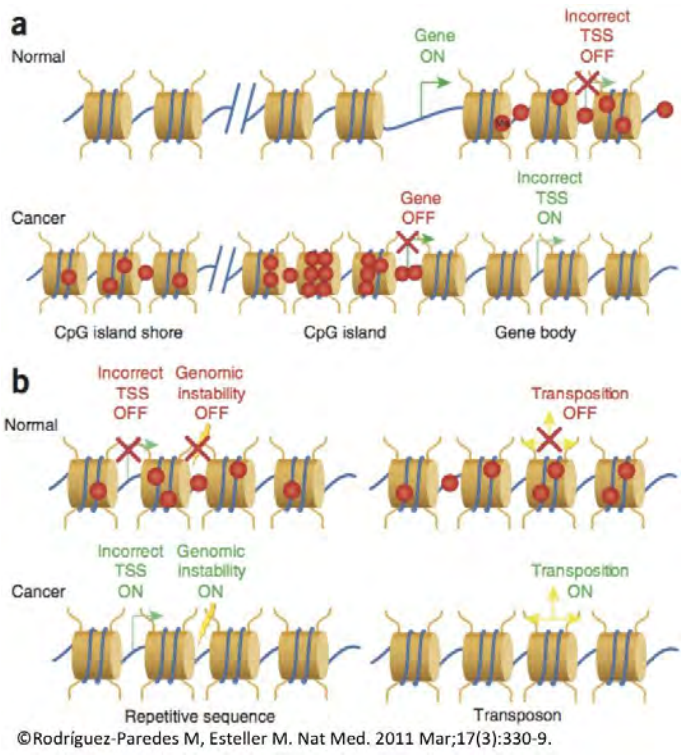

Figure 6 :Comparison of DNA methylation patterns among normal and cancer cells [69].

DNA methylation pattern in genome is shown in figure 6, normal pattern have high risk to change in cancerous condition[69]. According to scheme a, in normal cell CpG islands and CpG island shores always unmethylated which lead to gene expression and gene body always highly methylated in order to avoid spurious transcription initiations while in cancer cell this hypermethylation occur with CpG islands and CpG island shores but gene body have hypomethylation that can cause silencing of tumor suppressor gene including incorrect transcriptional start site (TSSs) from gene body region. In character b of figure 6, normal cell have hypermethylated on repetitive sequence and transposon for control this sequence but becom hypomethylated in cancer cell which cause genomic instability and aberrant transcription initiations and transposon trasnposition within cancer genome [69].

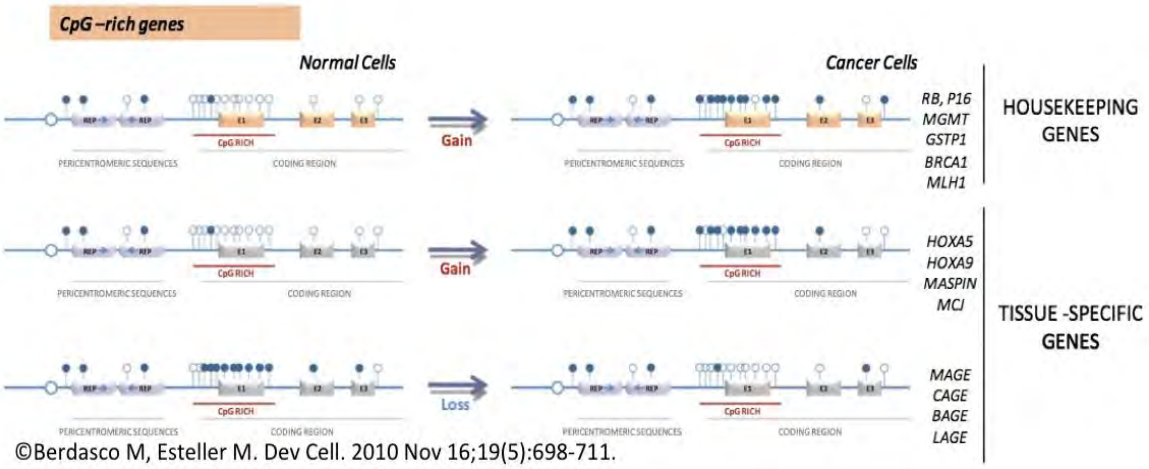

Figure 7 : Distribution of CpG Islands in Promoters of Housekeeping and Tissue-Specific Genes [70].

CpGs islands methylation promoter control half of tissue specific gene and most of house keeping gene expression and this epigenetics status change also described in carcinogenesis, tissue specific gene refer to tumor suppressor gene and oncogene while house keeping gene refer to genes that constitutively expressed in all tissues[71]. Normally, house keeping gene will have unmethylated CpG islands promoter while tissue specific gene can have methylated or unmethylated CpG islands according to genes function within cell [72]and seen in figure 7[70]. Some house keeping gene become hypermethylated in cancerous status while tissue specific gene may gain or loss of CpG hypermethylation depend on each gene function [70]. DNA methylation is unquestionably important mechanism within carcinogenesis phenomenon.

LINE-1 in human genome

After finish Human genome project, we found that 24 % of whole genome intronic region are occupy by transposable element at 60%[73]. Repetitive elements engage about 53% of whole human genome and half of them were long interspersed nuclear elements (LINEs, 21%). LINE-1 (L1) is the major group of LINEs because it is 17%

through human genome[74]. Human transposable elements divide in to 2 group; DNA transposon and retrotransposon, but only retrotransposon that still active. Although most of 520,000 LINE-1 retrotransposon are inactive because incomplete sequence (5' truncation, mutation, etc.) [75]. Full length LINE-1 is approximately at 12,000 copies, some of them still have transcriptionally activity [22]. Human LINE-1 structure is approximately 6 kb element as shown in figure 8; include 5' untranslated region (UTR) with internal promoter activity, 2 Open Reading Frame (ORF1 is p40 RNA binding protein and ORF2 encode Endonuclease and Reverse transcriptase) and 3' UTR that ends in an AATAAA polyadenylation signal, and a polyA tail[74]. LINE-1 5' UTR region contain regulation motif binding site of *YY1*[76], a putative runt-domain transcription factor (*RUNX*) site[77], the testis-determining factor gene SRY (the *SOX* family) site[78] that report as LINE-1 transcriptional regulation factor. LINE-1 involved with mammalian genome since before ~100 million years ago (the mammalian radiation) with ~100,000 LINE-1 have been inserted in human genome and exist in a single lineage of 16 distinct LINE-1 families (L1PA16-L1PA1)[79, 80]. The only most recently evolved human-specific L1 family, L1PA1 or cal las Ta [81] still active and have transcriptional, retrotranspositional abilitiy which can cause genome polymorphism[82] and disease[80, 83, 84].

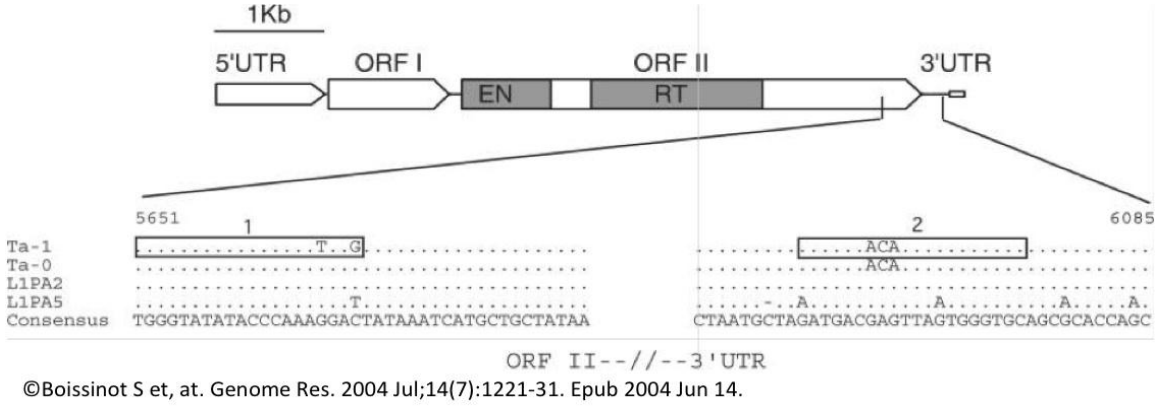

Figure 8: Structure of a typical full-length human L1 element [84].

LINE-1 Retrotransposition is life cycle of active LINE-1 that involve many disease including cancer[85]. Generally retrotransposition process including transcription, RNA processing, mRNA export, translation, posttranscriptional modifications and RNP formation, return to the nucleus, and reverse transcription and integration[74].LINE-1 retrotransposition, is the mechanism that base on target site-primed reverse transcription (TPRT) process which require both ORF proteins and LINE-1's RNA to form as cytoplasmic ribonucleoprotein particles (RNPs) to return back in nuclear region for novel AT-rich region cis insertion which may lead to pathogenic insertion incidence sometime. Mostly, LINE-1 within human genome will remains as 5'UTR missing form because incomplete trans efficiency of reverse transcriptase function of ORF2p. Moreover from LINE-1 retrotransposition, endogenous LINE-1 and Alu also have other impact on genome in various ways as shown in figure 9. Presence of LINE-1 can have impact on each location, according to figure 9, by LINE-1 character (1a) refer to sense LINE-1 and (1b) refer to antisense LINE-1 and those LINE-1 existence conseqence are including: low frequency of new insertion by retrotransposition (1C), Deletions occur at region of LINE-1 new insertion (2), 3' and 5' transduction by LINE-1 carry flanking sequence at it's end during LINE-1 retrotransposition (3,4), Mispairing and crossing over between LINE-1 lead to deletions or duplications (5), Premature termination of transcription by LINE-1 polyA signals (6), Antisense LINE-1 promoter cause new transcriptional start site for genes upstream LINE-1 that is on opposite strand (7), Splice site in LINE-1 sequence cause abnormal gene isoforms (8), LINE-1 change epigenetics status can cause altering gene expression (9), LINE-1 reverse transcriptase can mobilize Alu, SVA, mRNA and small non-coding RNA in order to genome expansion (10), LINE-1 reverse transcriptase U6 or Alu RNA in causing new chimeric insertion (11), RNA editing of Alus RNA cause gene suppression (12) and Alu cause microsatellites expansion can cause diseases[22].

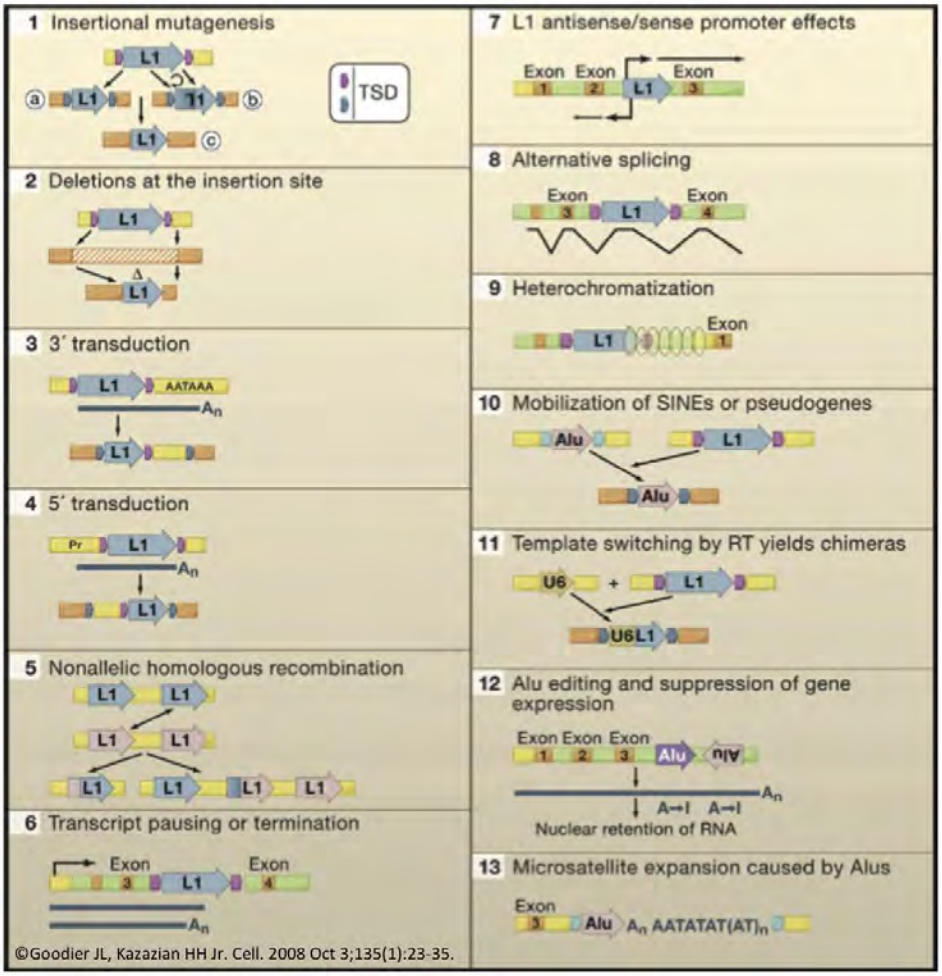

Figure 9: Effect of endogenous retrotransposon on genome [22].

Since LINE-1 is a massive element within human genome with potential in damage genome that cause cell have natural self defense mechanism for control LINE-1 and others retrotransposon as shown in figure 10 throughout most of LINE-1 sequence is in truncated, mutated and rearrangement inactive form (1), 5'UTR methylation cause LINE-1 silencing in normal cell (2), Premature termination or stop transcription can inhibit LINE-1 full length transcript (3), DNA/histone methylation cause LINE-1 and Alu suppression (4), Double strand RNA structure created bidirectional transcription from sense and antisense promoter, piRNA and endo-siRNA were cleavage targeted of RNAi mechanism (5), ORF1 protein and LINE-1 RNA can be sequestered in stess granules (6), Cytocine deamination by

APOBEC3s enzyme can stop LINE-1 retrotransposition (7), RNA editing occur with LINE-1 and Alus RNA (8), DNA nuclease, *TREX1*, prevent accumulation of LINE-1 single-stand DNA (ssDNA) within nucleus (9) and *MAEL* protein from chromatoid body in male germ cells can silence transposon in mice (10)[22]. DNA methylation is the first priority LINE-1 controlling factor because silencing LINE-1 transcription can prevent every serious consequence from LINE-1 expression such as genome instability[86].

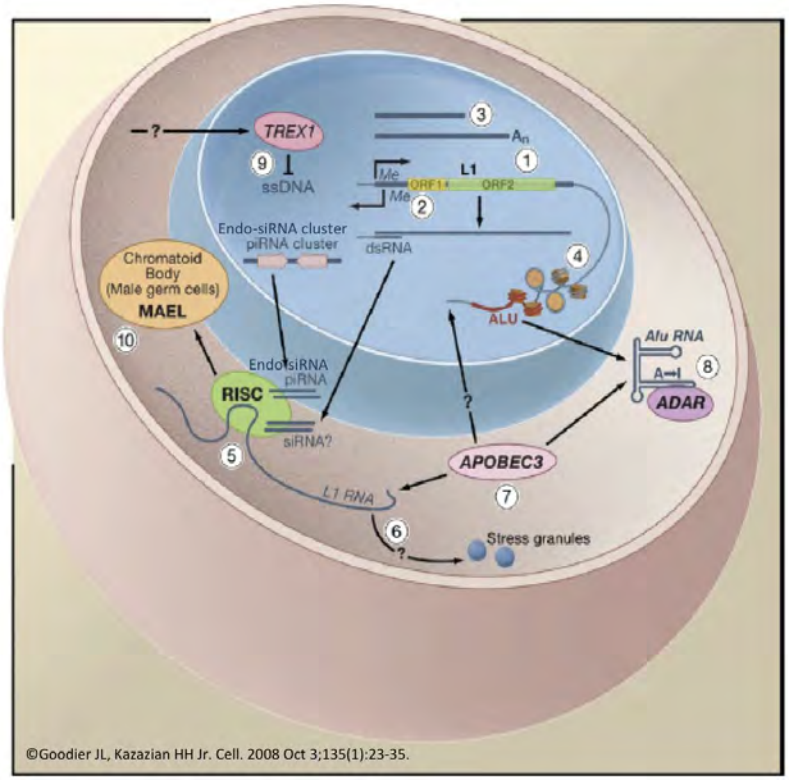

Figure 10: How Cell control endogenous retrotransposon[22] modified from Goodier JL and Kazazian HH Jr (2008).

RISC protein in regulating transposon within cell

RNAi mechanism is the high impact pathway in gene regulation which have RNA-Induced Silencing Complex (RISC) as operating particle by cooperate with microRNA

or siRNA for silencing gene target that can have complementary sequence with each guide small RNA [87]. In order to function in RNAi mechanism, siRNA will load on RISC-loading complex (RLC) consisting of *DICER1, TRBP* and Argonaute (*AGO2*) family[88, 89]. Duplex of siRNA will separated in to single strand RNA will assemble into Argonaute protein which become the core of RISC particle[90].Cellular mechanism of RNAi mechanism though RISC including (i) Translational silencing by pre-mRNA degradation with a perfect match siRNA to target sequence[91], (ii) Translational prevention by mismatch binding of miRNA sequence onto target pre-mRNA sequence[92], (iii) Threefold degradation of non-translated transcript when compare to protein transcript[93], (iv) Transcriptional silencing by RNA-induced transcriptional silencing (RITS) complex for induce and spread DNA/histone methylation with requirement of Argonaute and RdRP protein[94]and (v) Counteract with RNA editing for silencing endogenous genes and transgene[95]. In human, RISC loading and shuttle step between cytoplasm and nucleus in process details as shown in figure 11 are involving: (1) Loading of guide-strand small RNA on RLC in cytoplasm site, (2) Guide strand recognize target mRNA within cytoplasm, (3) Target mRNA was silenced by RNAi mechanism, (4) Dissociation of cytoplasmic RISC protein and only core of RISC, small RNA and *AGO2* or nucleus RISC, will import into nucleus, (5) Guide stand mediated target recognition in nucleus which cleavage by *AGO2* capacity and free nucleus RISC could import back to cytoplasm for unite in cytoplasm RISC again and (6) Remains structure of miRNA-like interaction in nucleus cause existence of nucleus RISC[96]. Circulation of nucleus RISC have guide stand small RNA from many type including endo-siRNA from transposon and repeat sequence which may involve with reason of huge part of human genome was repetitive sequence especially LINE-1.

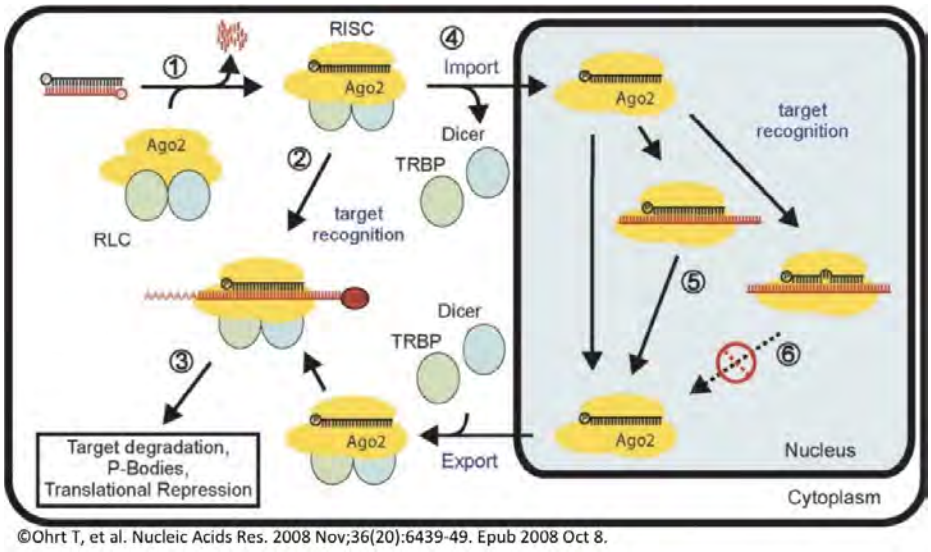

Figure 11: RISC complex within cell. *AGO2* was major protein in Nuclear RISC complex. Cytoplasmic RISC complex including *AGO2*, *DICER1, TRBP* protein[96].

Transposon can repress by epigenetics mechanism including DNA methylation and Histone modification [97, 98]. In post-transcriptional gene silencing (PTGS) of transposon transcript was occur by RNA-degradation complex, RNAi pathway [99]. Small non-coding RNA (sRNAs) will be released after dsRNA was cleaved by protein from *DICER1* classes that correlate to the sequence-specific silencing after transcription[100]. Small non-coding RNA will involve in DNA methylation of homologous DNA sequences in nucleus by RdDM and guide heterochromatin formation to silence transposon in transcriptional level [101, 102]. Function of small non-coding RNA related to 21nt long in case of post-transcriptional silencing while 24nt size for mediated silencing through the RdDM pathway and heterochromatin maintenance mechanism[103]. Transposon can mobilized in RNAi mutant C. elegan while RNAi mutant Arabidopsis have abnormal in DNA methylation and chromatin structure which cause releasing of transposon RNA[104-106].Beside, the silencing of transposon, small non-coding RNA also involved in several of biological phenomenon from developmental process to cell stress reponse[107, 108].

Name	Organism	Length (nt)	Proteins	Source of trigger	Function
miRNA	Plants, algae, animals, viruses, protists	20–25	Drosha (animals only) and Dicer	Pol II transcription (pri-miRNAs)	Regulation of mRNA stability, translation
casiRNA	Plants	24	DCL3	Transposons, repeats	Chromatin modification
tasiRNA	Plants	21	DCL4	miRNA-cleaved RNAs from the *TAS* loci	Post-transcriptional regulation
natsiRNA	Plants	22	DCL1	Bidirectional transcripts induced by stress	Regulation of stress-response genes
		24	DCL2		
		21	DCL1 and DCL2		
Exo-siRNA	Animals, fungi, protists	~21	Dicer	Transgenic, viral or other exogenous dsRNA	Post-transcriptional regulation, antiviral defense
	Plants	21 and 24			
Endo-siRNA	Plants, algae, animals, fungi, protists	~21	Dicer (except secondary siRNAs in *C. elegans*, which are products of RdRP transcription, and are therefore not technically siRNAs)	Structured loci, convergent and bidirectional transcription, mRNAs paired to antisense pseudogene transcripts	Post-transcriptional regulation of transcripts and transposons; transcriptional gene silencing
piRNA	Metazoans excluding *Trichoplax adhaerens*	24–30	Dicer-independent	Long, primary transcripts?	Transposon regulation, unknown functions
piRNA-like (soma)	*Drosophila melanogaster*	24–30	Dicer-independent	In *ago2* mutants in *Drosophila*	Unknown
21U-RNA piRNAs	*Caenorhabditis elegans*	21	Dicer-independent	Individual transcription of each piRNA?	Transposon regulation, unknown functions
26G RNA	*Caenorhabditis elegans*	26	RdRP?	Enriched in sperm	Unknown

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Table 2: Small RNA types and small RNA function within genome. ago2, Argonaute2; casiRNA, cis-acting siRNA; DCL, *DICER1*-like; endo-siRNA, endogenous small interfering RNA; exo-siRNA, exogenous small interfering RNA; miRNA, microRNA; natsiRNA, natural antisense transcript-derived siRNA; piRNA, Piwi-interacting RNA; Pol II, RNA polymerase II; pri-miRNA, primary microRNA; RdRP, RNA-dependant RNA polymerase; tasiRNA, trans-acting siRNA[109].

RNA interference (RNAi) mechanism was introduce into biological science as the evidence of small RNA involved in genome regulation [87]. Endogenous small RNA was believed to involved in silencing transposon by DNA methylation and degrade transposon RNA via RNA interference mechanism for stable normal genome [104, 110]. According to current knowledge, there are many types of endogenous small silencing RNAs as shown in table 2 including: microRNA (miRNA), natural antisense transcript-derived siRNA (natsiRNA), cis-acting siRNA (casiRNA), trans-acting siRNA (tasiRNA), piwi interacting RNAs (piRNA), long non-coding RNA (lncRNA) and endogenous siRNAs (endo-siRNA)

[109]. Most of repetitive elements related small RNA dedicate to endo-siRNA and piRNA through confirmed by suppressing retrotransposon complex sequence-based study [111-113]. Both endo-siRNA and piRNA will interact with Argonaute protein class during silencing of transposon mechanism happened[114]. With similar concept in transposon controlling, endo-siRNA will strictly control transposon in somatic cell while piRNA will control transposon in germ line cell[115].

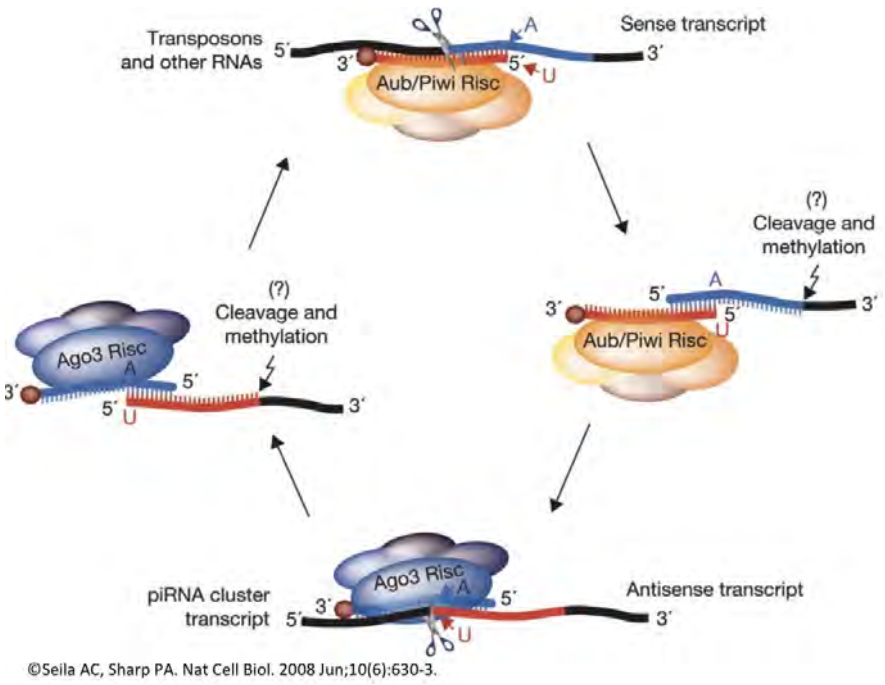

Figure 12: Model piRNA biogenesis in Drosophila [116].

Model of piRNA-mediated transposon silencing in germ line cell is completely proved in Drosophila and mice. There are two class of piRNAs partner with different Argonaute protein family. In Drosophila, piRNA from sense orientation of transposon transcript will assocate with Piwi and Ago3 (MILI in mice) while piRNA antisense transcripts will associate to Aubergine (MILI2 in mice)[117, 118]. According to piRNA biogenesis, “ping-pong” mechanism, sense strand piRNA will cleavage and produce antisense piRNA and vice versa[119]. Antisense piRNA transcript is originally produce from a small numbers

of long piRNA precursors from piRNA clusters within genome which some cluster reported as main regulator of transposon silencing[120, 121]. Through it sill unclear on how piRNA cycle start, the piRNA biogenesis pathway as shown in figure 12 always continue for cleavage transcript form master control loci with complementary sequence to transposons within genome[116]. DNMT3L is coordinator of de novo methylation in male germ line[122] by joining to piRNA regulator protein, MILI and MIWI2. DNMT3L-deficient will cause loss of LINE-1 methylation and increase of LINE-1 expression[118] that can further enter piRNA pathway. Mutation of endo-siRNA and piRNA Argonaute partners protein cause the up-regulate of transposon[123-125]. The overexpression of transposon induced abnormal germ cell development that emphasizes the impact of transposon silencing[126, 127]. In germ cells, the ping-pong amplification is believed in degrade transposon mRNA leading to post-transcriptional repression and also assumed to generate sequence-specific substrate to guide DNA methylation. As commonly known about loss of DNA methylation will reactivate transpable elementswithin genomes, DNA methylation was concern as high impact factor on long-term transposon silencing [128, 129].

Transposon controlling in somatic cell is correspond to function of endo-siRNA in parallel to piRNA role in germ line cell. Endo-siRNA production requires double stand RNA binding domain (dsRBD) protein Loquacious (Loqs) while exogenous siRNA, foreign small RNA, require R2D2, Loqs-homolog protein [130]. The difference isoform of Loqs protein according to prefer in Dcr-2 interaction is main factor for separate endo-siRNA precursor binding to Dcr-2 from pre-miRNA loading on Dcr-1 which result in distinguish endo-siRNA biogenesis pathway rather than mix in the known miRNA and siRNA pathways[131, 132]. Unlike piRNA, endo-siRNA can against an artificial sequence in high copy numbers which didn't integrate into genome[133]. Deep sequencing small RNA revealed that endo-siRNA originate from double strand RNA precursor of spliced mRNA parted to antisense transcript which cause no exon-exon junction in endo-siRNA[133]. Double stand RNA from 3'-UTRs overlapping among bidirectional transcript and trans form between two independent transcipts involved with endo-siRNA production [134]. In yeast, releasing of antisense transcript for endo-siRNA biogenesis in order to collaborate with

RNAi pathways protein have endogenous retrotransposon as main target within genome and according to dsRNA formation of antisense transcript, itmay become a transcript copy numbers scanner within cell and control transcripts number by endo-siRNA[135].

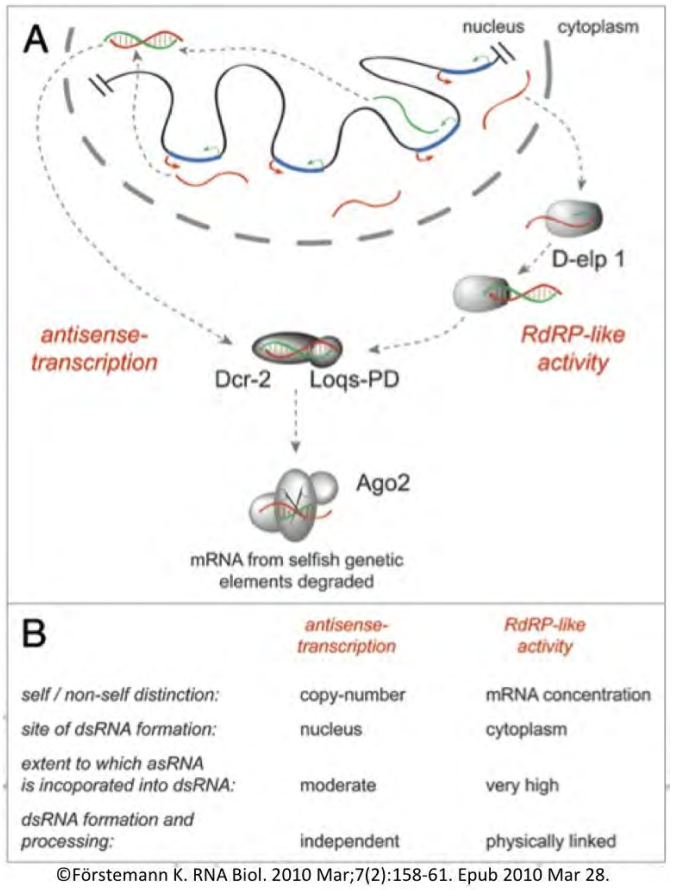

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Figure 13: The two possible of endo-siRNA precursor formation within cell [136]. According to this figure the red line refer to sense transcripts and green line represent the antisense transcripts/antisense RNA.

In current knowledge of endo-siRNA field, there is two proposed model in generation of a double-stands RNA precursor for endo-siRNA production[136] as shown in figure 13. First, in left side: the sporadic antisense transcript in nucleus involved in counting copy number of transposon element expression. Second, in right side: D-elp1 is the non-canonical RNA-dependent RNA polymerase that convert transposon mRNA into dsRNA. There is comparison feature among each two possibility model which still need more

experiment in order to understand on how endo-siRNA precursor formation within genome[136]. D-elp1 will synthesis dsRNA from various single strand RNA templates by primer dependent or independent initiation mechanism. D-elp1 depletion result in RNAi inhibition but have no effect on microRNA function. Interestingly, D-elp1 retarded cell will have higher transposon RNAs with decreasing of the corresponding transposon antisense transcripts and endo-siRNA. In Dcr-2 abnormal cell there is increased of transposon RNA and reduced the corresponding endo-siRNA with stable level of transposon antisense RNA level. While in D-elp1 occupy in unprimed pattern by resistance of ssRNA specific nuclease [137]. D-elp1 and Dcr-2 protein have some connection and can form into complex in some cell stage. Endo-siRNA production would responsible in genome defense and RNA silencing process with mainly target on transposon within genome.In somatic cell, rather than endo-siRNA, cell can also control transposon transcript by qiRNA according to cell stress by radiation, as LINE-1 expression by radiation will further induce genome instability [138]. Recently discover qiRNA in the filamentous fungus Neurospora by Yi Liu and colleagues, was one more evidence of transposon controlling through RISC protein involved pathway in eukaryotes [139].

RNA involved in epigenetics mechanism for gene regulation

Regulation in genome by DNA methylation mechanism always occurs within a specific region which may happen by interaction of DNA methytransferase with other epigenetics factor such as DNMT3a recruit by PRMT5 for mediate H4R3 methylation and DNA methylation in gene silencing[140], SET7-mediated lysine methylation and regulate DNMT1 stability[141]. Recently, small inhibitory (si) RNA-mediated, RNA-Directed DNA methylation have report in living including Arabidopsis[142], Saccharomyces cerevisiae, Drosophila, Caenorhabditis elegans[4], and human cell[143]. In Arabidopsis, RNA-Directed DNA methylation (RdDM) will initiate by double strand RNA particle recruit DNMTs for de novo DNA methylation in specific region including gene promoter and repetitive elements

sequence[144, 145]. Non-coding region in Arabidobsis genome can produce RNA transcripts which shown in recently experiment that siRNA and long-noncoding RNA can involved with de novo DNA methylation of Arabidopsis genome[146]. RNA-Directed DNA methylation is a conserved de novo DNA methylation which recognized as a general transcriptional silencing mechanism in plant as first found in transgenic potato containing viroid genes and most of RdDM mechanism study in plant model[142]. RdDM involve in many epigenetic phenomenon including transgene silencing, transposon suppression, gene imprinting and genome stability [147-149].

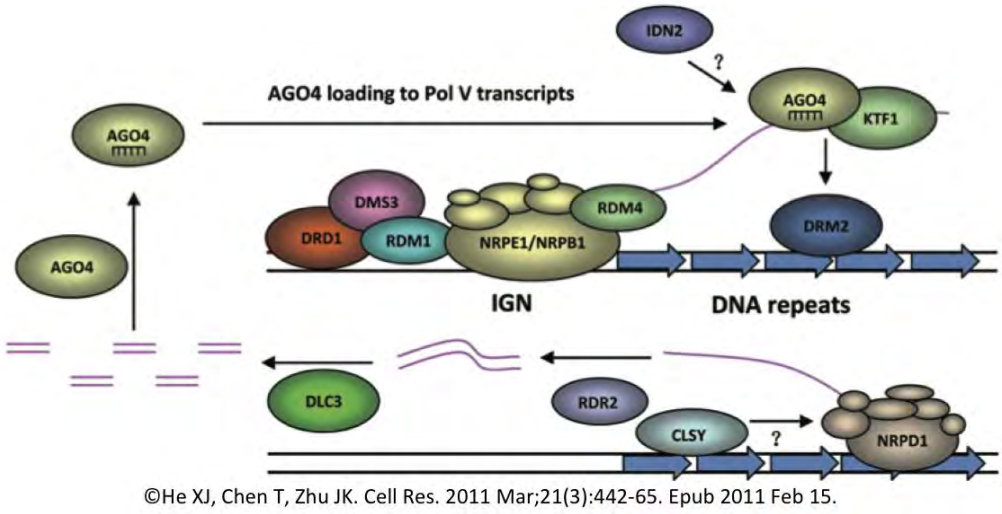

Figure 14: The RNA-Directed DNA methylation in plants [30].

The RNA-Directed DNA methylation mechanism in plant model[30] as shown in figure 14, will start by DNA-dependent RNA polymerase IV (Pol IV) will produce aberrant single strand RNA transcript from target of RdDM especially in transposon or DNA repeat region by enhance by chromatin remodeling protein CLSY. Next, RNA-dependent RNA polymerase (RDR2) will convert aberrant single RNA into double strand RNA which will cleaved to be 24nt siRNAs by DCL3, DICER1-like plant protein. The synthesis 24nt siRNA will load on Argonaute protein, AGO4, AGO6 or AGO9 for competent RdDM complex. While in intergenic non-coding (IGN) region, DNA-dependent RNA polymerase V (Pol V) will produce single stranded scaffold RNA transcripts, which require RDM4/DMS4, DRD1, DMS3 and RDM1 in RdDM process. RDM1 will bind single –strand methylated DNA for

recruit Pol V and Pol II in target region. DRD1, DMS3, and RDM1 will form DDR stable protein complex in this region. RNA binding protein KTF1 wills ties AGO4 onto Pol V or Pol II RNA transcripts to form RNA-Directed DNA methylation effector complex. IDN2 will stabilize base-pairing between the nescent scaffold transcript and 24nt siRNA. A perfect effector RdDM complex can guide the de novo DNA methytransferase DRM2 in specific the chromatin region for introduce new DNA methylation [30, 150].

RNA-based gene regulation have two major pathways : posttranscriptional gene silencing (PTGS) and transcriptional gene silencing (TGS)[151]. PTGS is first found in C. elegans and well known in messenger RNA (mRNA) regulation through Argonaute 2 (AGO2 or EIF2C2 in human) that normally call RNA interference pathways[152]. In RNA interference pathway, small interference RNAs (siRNAs) will load AGO2 on target mRNA and according to the complementary ability that lead AGO2 in cleavage mRNA or translational repression which finally result as gene repression. PTGS is transient gene regulation mechanism which can disappear by loss of siRNA in genome[152]. TGS is contrast to PTGS because TGS will target on DNA sequence and will induce long-term silencing phenomenon. TGS will related to non-coding RNA (ncRNA) creating epigenetics changes in gene promoter and repeat sequence region for reduce transcription from this target site. Both DNA methylation and histone modification occur in gene promoter or repetitive element sequences according to TGS mechanism have reported in plants [153, 154], Drosophila [155], yeast [156]and human[157].

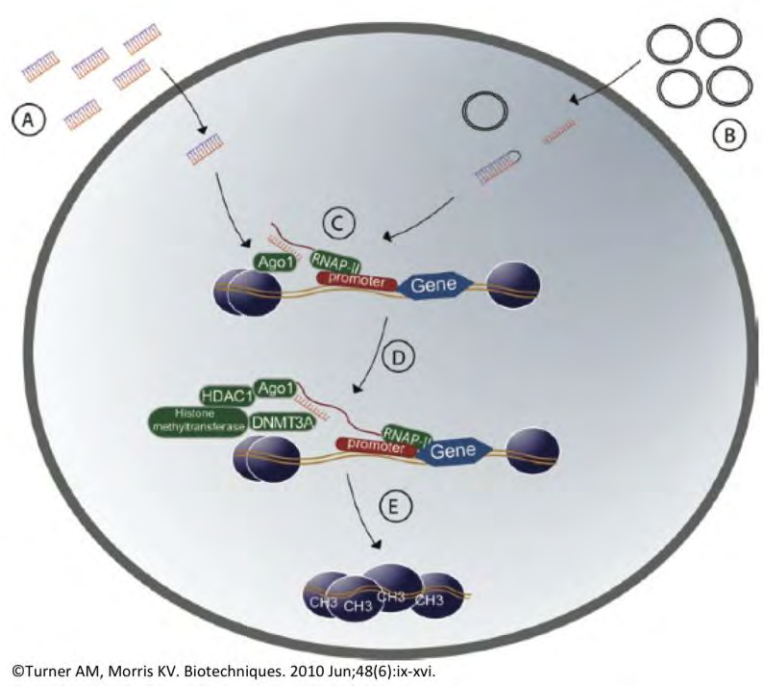

Figure 15: Small RNA in repress gene transcript in mammalian cells [151].

According to current research on TGS model as shown in figure 15, many protein involve in ncRNA for epigenetics silencing target gene regulation process. Introducing RNA polymerase III (RNA Pol III) driven shRNAs or antisense RNAs (B) or synthetic small RNA (A) into cell, *AGO1* with 24nt siRNA (C) will bind on targeted promoter and recruit a potential TGS complex that include *HDAC-1, DNMT3a* and unknown histone methyltransferase (D) for heterochromatin formation and suppress transcription process (E) as shown in figure 16 [151]. Others protein required in TGS pathway also refer to human *AGO2* [158], trans-activation response (TAR)-RNA binding protein[159] and histone methyltransferase *EZH2*[159]. However, non-coding RNA within genome can involved to epigenetics in gene regulationby RNA activation (RNAa) for activates gene transcription which is totally opposite with TGS mechanism in mammalian cell. In mammalian species including human, there is endogenous non-coding RNA that can induce gene expression. The naturally transcriptional level by bidirectional RNA mediated transcriptional mechanism[31] in human cell shown in figure 16 require balance between sense and antisense transcripts for keep genome homeostasis (A). Increasing of antisense transcript

(B) will result in recruitment of chromatin-modifying protiens such as histone methyltransferase *EZH2* and *SUV12* target on gene promoter (C) for induce TGS protein complex to silencing mechanism (D). Degradation antisense transcripts by *AGO2*-related in RNAi mechanism (E), gene become activate and increase gene expression (F) and if RNAi mechanism degrade sense/gene mRNA will result in up-regulate antisense transcript for switch into TGS pathway[31].

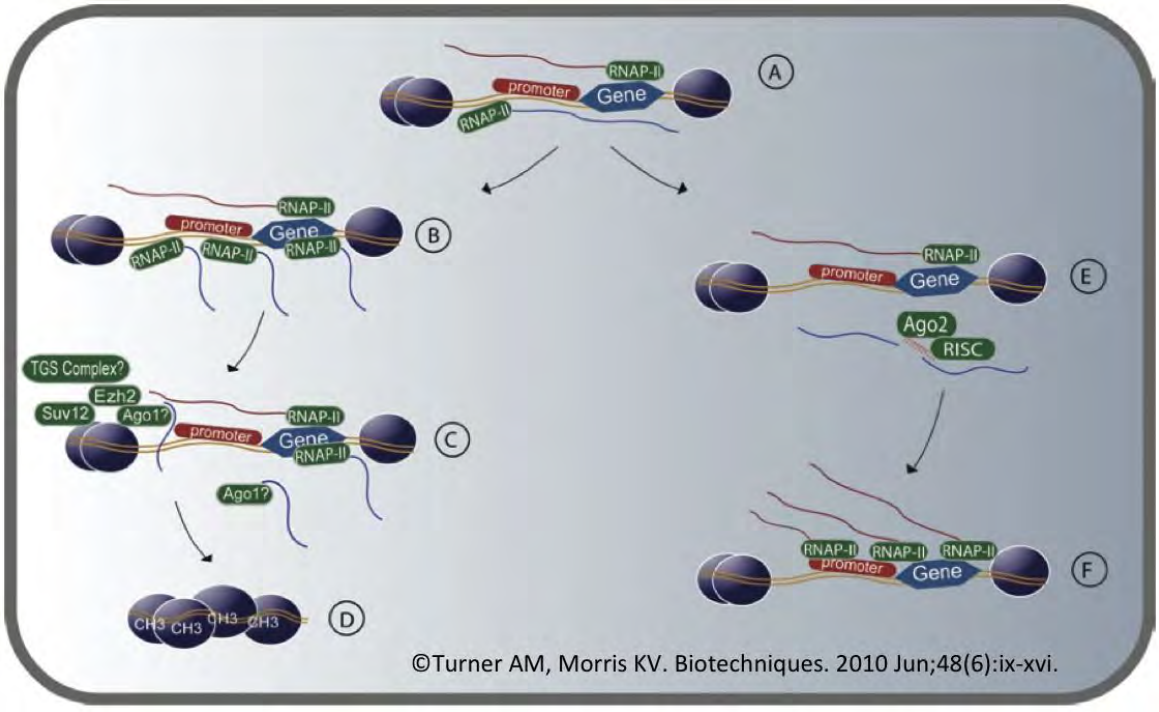

Figure 16: Model of endogenous long noncoding RNA in regulating gene expression in mammalian cells[151].

Gene activation in human cell was observed in E-cadherin, vascular endothelial growth factor (*VEGF*) and *P21* [160]. Requirement of *AGO2* and loss of H3K9Me for activate gene in RNA activation pathway is totally opposite to TGS concept. In RNA activation, siRNA would target AU-rich of gene promoter for change epigenetics profile to transcriptionally active chromatin by missing H3K4me marker [161]. As we know about miRNA is the gene suppressor in PTGS pathway but miRNA is also function through RNA activation in TGS level; miR-373 have reported as an activator of E-cadherin expression with

requirement of *DICER1* and RNA polymerase II[162]. Small RNA in RNA activation mechanism was observed direct binding to antisense transcript of progesterone receptor gene with the involvement of unidentify Argonaute protein [163]. In *P21* gene, the small RNA activator particle will not target on *P21* promoter but the *P21* activation through *AGO2*-dependent occur by complementary binding between siRNA and P21 antisense transcript, the degradation of *P21* antisense transcript will cause *P21* activation[31]. RNA activation mechanism also found in others mammalian cell line including nonhuman primate, mouse and rat[164]. Requirement of small non coding RNA in gene regulation still need to research more in the future which will let us understand concept of RNA-mediated transcriptional regulation[151].

MicroRNAs (miRNA) are the small non-coding RNA that not only control gene at post-transcriptional level but also regulate gene in epigenetics pathway that can lead abnormal level of DNA methylation [165, 166]. *DNMT3a* and *DNMT3b*was reported as target of miR-29 in 3'untranslated region of gene transcript. In lung cancer, lower expression of miR-29s (miR-29a, miR-29b and miR-29c) relate with up-regulated of *DNNT3a* and *DNMT3b* which also cause global hypermethylation poor prognosis of this type case. Reintroduction of miR-29s in lung cancer cell line can induce normal DNA methylation level and reexpess of DNA methylation-silenced tumor suppressor genes such as *FHIT* and *WWOX* with less tumorigenicity of experiment cell [167]. Function of miR-29b controlling *DNMT3a* and *DNMT3b* also confirm in acute myeloid leukemia because transfection of miR-29b in AML cell lines will cause DNA hypomethylation that will reexpress p15(INK4b) and *ESR1* for tumor suppressor pathway[168]. *DNMT3b* also can repress by miR-148 through coding sequence of *DNMT3b*[169].

CHAPTER III

MATERIALS AND METHODS

Cells and Tissue Preparation

Head and neck squamous cell cancer (HNSCC) cell lines (WSU-HN) [170], including WSU- HN 4, 6, 8, 12, 13, 17, 19, 22, 26, 30 and 31, were afforded by Dr. Silvio Gutkind, NIH, USA. HEK293 (Human embryonic kidney cell line) was purchased from ATCC (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Gibco BRL, Life Technologies, Pairly, UK) supplemented with 10% heat- inactivated fetal bovine serum (Sigma, St. Louis,MO, USA). Cells were incubated at 37^{o}C in 5% CO2. To inhibit DNMTs, HeLa cells were treated every 24 hours with 4 μM 5-aza-2- deoxycytidine for up to 16 days for genomic demethylation (Sigma-Aldrich). Normal oral epithelium (NOE) samples from 12 individuals were collected. 20 ml of sterile 0.9% NaCl solution were rinsed and gargled for 15 seconds then spitted into a sterile 50-ml closed container and kept at 4^{o}C until processed to collect DNA, within 24 hours. Cells from oral rinses were pelleted by centrifugation at 2500 g for 15 minutes at 4^{o}C. The supernatant was discarded and pellets were washed with sterile PBS. Several samples of white blood cells (WBCs) from normal healthy individuals were also collected.Primary normal human oral keratinocytes was isolate from normal healthy gingiva tissue by treat with collagenease type I (C0130, Sigma-Aldich.), and rinse normal oral keratinocyte cell in PBS 3 times, cell pellet in centrifuge at 150 g 5 minutes and culture on Collagen from human placenta, Bornstein and Traub Type IV coat plate (C5533,Sigma) in KGM^{TM}-2 Bullet Kit^{TM}media (©Lonza Walkersville, Inc.) according to the suggest protocol from company until cell number reach to target size.

Combined bisulfite restriction analysis (COBRA) PCR

According to principle of combined bisulfite restriction analysis (COBRA), here in this work will have whole genome LINE-1 methylation (COBRALINE-1) and COBRA for unique to L1 sequence (CU-L1) which performed as previously described[7, 171]. After extraction, all DNA samples were treated with sodium bisulfite as previously described[172]. Briefly, genomic DNA was denatured in 0.22 M NaOH at 37°C for 10 min. 30 μl of 10mM hydroquinone and 520 μl of 3M sodium bisulfite were added for 16-20 hrs at 50 °C. The DNA was purified and incubated in 0.33 M NaOH at 25 °C for 5 min, ethanol-precipitated, then washed with 70 % ethanol and re-suspended in 20 μl of TE buffer. 2μl of bisulfited DNA was subjected to 35 cycles of PCR with two primers as listed in Appendix at an annealing temperature of 53 °C. The amplicons were digested in 30 μl reaction volumes with 2U of TaqI or 8U of TasI in 1xTaqI buffer (MBI Fermentas) at 65 °C overnight and then electrophoresed in 8% non-denaturing polyacrylamide gels. The intensities of DNA fragments were measured by PhosphorImager, using ImageQuant software (Molecular Dynamics). The methylated amplicons, TaqI positive, yielded 80 bp DNA fragments; whereas the unmethylated amplicons, TasI positive, 97 bp fragments. The LINE-1 methylation level was calculated as a percentage (the intensity of methylated LINE-1 digested by TaqI (80bp), divided by the sum of the unmethylated LINE-1 digested by TasI (97bp)-and the TaqI-positive amplicons(80bp)). The same set of DNAs as use in previous work of Chalitchagorn K. et al. wasapplied as positive controls in each set of COBRA experiments.

Gene expresss quantitation

Total RNA was extracted from cell lines using the Trizol reagent (Life technologies, Inc.) according to the manufacturer's instructions. The RNA was prepared to DNA-free by Deoxyribonulease I (DNase I), RNase-free (Fermentas) and RiboLockTM

Ribonuclease Inhibitor (Fermentas) following the manufacturer's protocol. To synthesize cDNA, 5 μg DNA-free RNA was dissolved in 12 μl of DEPC-treated water containing 0.5 μg oligo(dT)18 primer (Fermentas). The RNA was incubated for 5 min at 70°C, chill on ice 5 min. To each sample, we added 200U of RevertAidTM M-MuLV Reverse Transcriptase (Fermentas), 20 U of RibolockTM Ribonuclease inhibitor (Fermentas), 20 mM dNTPs. The mixture was incubated for 1 hr at 42°C, 10 min at 70°C followed by chill on ice. cDNA was amplified using the exon primers as listed in Appendix. The RNA without reverse transcription was included as negative control and to evaluate the amount of LINE-1 DNA contamination. Real-time RT-PCRs were performed for 40 cycles with the annealing temperature set at 60°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.For quantitative RT-PCR, interest gene was amplify duplex with house keeping gene such as GAPDH or B-actin. PCR run in 6 % acrylamide gel (19:1,161-0144, Biorad.) in 1x TBE buffer 15 minute stain with GelStar™ Nucleic Acid Gel Stain (Cambrex Bio Science Rockland, Inc.) and scan by PhosphorImager, using ImageQuant software (Molecular Dynamics). Ratio expression analyse by compare intensity of target gene band per house keeping gene band.

Gene knockdown and siRNA transfection

Oligonucleotides specific target on LINE-1, *AGO2 (EIF2C2)* and *DICER1*were inserted into Psilencer 3.1 vector hygro H1 promoter. (Ambion, Austin, Texas, USA) and transfection was mediated by FuGENE ® HD (Roche), sequence shown in appendix. The GFP control insert was used as a control for transfection efficiency. Newly design, three LINE-1s siRNAs were used for transfection simultaneously. Stable knockdown *AGO2* and *DICER1* construct was design base previous experiment in HEK293T cell[173].

Oligosynthesis siRNA specific to *AGO2*, *DICER1* purchased from Santa Cruz biotechnology for transient tranfection, was dilute with RNASE free dH2O to reach 10μM. In a 12 well plate, seed 6 x 105 cells per well in 1 ml antibiotic-free normal growth medium supplemented with 10% FBS. In next day, perform transfection those siRNA at 100 nM with Lipofectamine 2000 reagent (Invitrogen Cat: 11668-027) according to the manufacturer's instructions. Cell was collect at 48h after transfection step by Trizol reagent (Life technologies, Inc.) in order to collect RNA, DNA and protein from each sample for further experiment. For stable cell culture, after 48 h of transfection with siRNA construct switch to culture with 200 ug/ml Hygromycin B (Cat. No. 10 843 555 001, Roche Applied Science) in Dulbecco's modified Eagle's medium (Gibco BRL, Life Technologies, Pairly, UK) supplemented with 10% heat- inactivated fetal bovine serum (Sigma, St. Louis, MO, USA). Growth curve plot by counting cell number at Day1, 3 and 5 after plate 10^4cell in 24 well plate (©Corning Incorporated,Lowell, MA USA) in normal cell culture condition of each cell.

RNA immunoprecipitation

RNA immunoprecipitation (RNA-IP) protocol was adapted method from previously propose[174]. Cells were grown in 75- cm2 flask at 80% confluence and washed with PBS and trypsinized. $1x10^8$ cells were added to a 15mlconical tube, pelleted, and resuspended in 10ml 1% formaldehyde in PBS. Crosslink reaction was performed for 30 minutes at room temperature and stop with Glycine in 125mM final concentration. The pellet was washed twice with ice-cold PBS containing 1x protease inhibitor cocktail. The cell pellet was resuspended in 200μl of Buffer A RNA-IP and placed on ice for 10 minutes. The crude nuclei fraction was pelleted by microcentrifugation for 5000 rpm for 5 minutes at 4^oC. The pellet was washed once in Buffer A without NP-40, then resuspended in 500μl of Buffer B and incubated on ice for 10 minutes. Lysates were sonicated three times on at 4^OC using a

Branson Sonifier at constant power, output at 70 %, and continuous sonication for 20 seconds. After sonication, insoluble elements were cleared by microcentrifugation at 14,000 rpm for 10 minutes at 4°C. The sonicated was diluted 10-fold into IP Buffer to a final volume of 1ml per immunoprecipitation reaction. A 1% aliquot was preserved as an input sample and frozen at -80°C until the reverse crosslinking step. 5 ug of antibodies or a normal IgG control were added to each tube. Immune complexes were allowed to form by slow mixing on a rotating platform at 4°C overnight. To collect immune complexes, 50μl of Protein A/G Agarose-PLUS (Santa Cruz) was added to each tube and slow mixing rotation continued for 2 hours. Immune complexes were "pulled down" by gentle centrifugation at 1000 rpm for 2 minutes at 4°C. Each immune complex was washed five times (1 ml wash, 5 minutes each). After each wash, Low salt wash, High salt wash, LiCl wash and 2 times of TE pH 8.0 respectively, complexes were pelleted by gentle centrifugation (1000 rpm, 1 minute) and the wash buffer aspirated using a clean pipet tip. Immune complexes were eluted by addition of 250μl Elution Buffer and collected by centrifugation (8000 rpm, 2 minutes). NaCl was added to a final concentration of 200mM (including the input samples) then placed at 65°C for at least 2 hours to reverse crosslinking. Samples were subjected to Trizol LS reagent extraction and resuspended in 20μl of DEPC-treated water. DNA from the samples was removed by the use of Deoxyribonuclease I (DNase I), RNase-free (Fermentas Inc.). RNA expression can be test by standard reverse-transcriptase-PCR protocol of SuperScript™ III RT kitfrom Invitrogen (Cat. No. 11752-050).Gene expression detect with quantitative PCR with 6% acrylamide gel electrophoresis stain after 15 minute stain withGelStar™ Nucleic Acid Gel Stain (Cambrex Bio Science Rockland, Inc.) and scan with PhosphorImager, using ImageQuant software (Molecular Dynamics). In order to quantitate specific LINE-1 noncoding RNA we choose EPHA3 intron 15 LINE-1 expression PCR at 58 OC, 1 min, 40 cycles.

Microarray and sample preparation

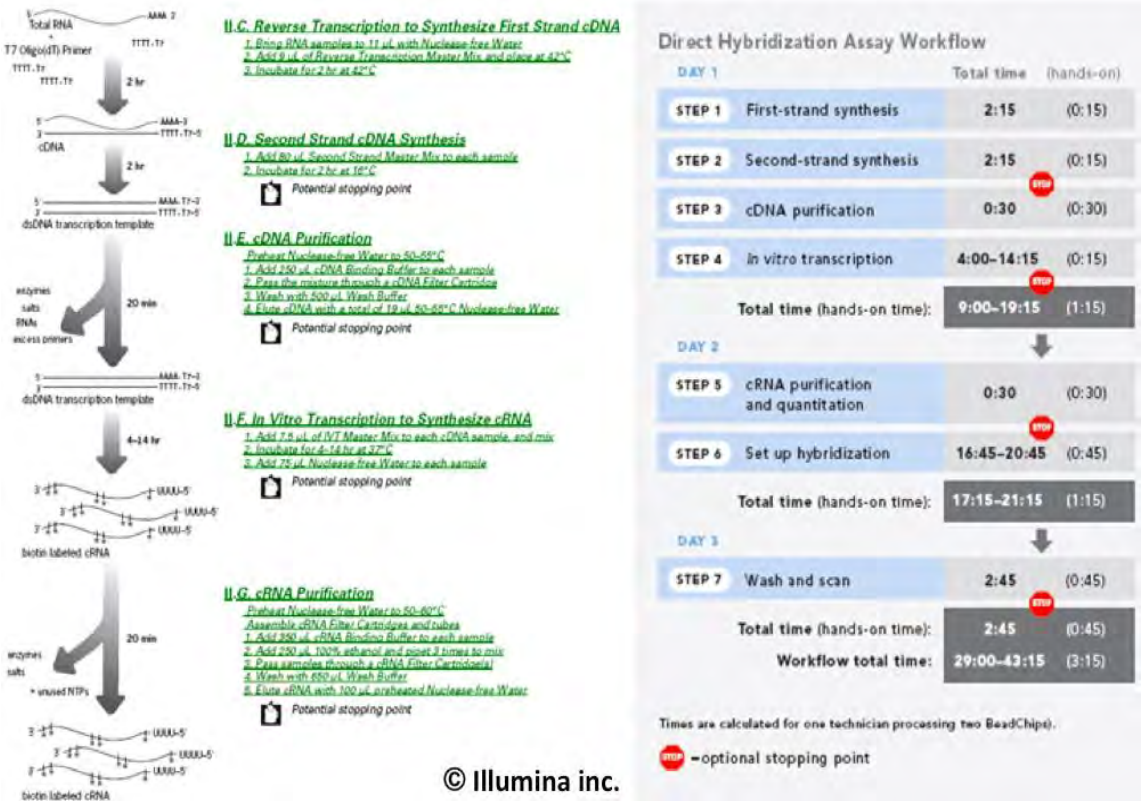

Figure 17: Protocol for prepare microarray chip for detect whole genome expression. Total preparation process for hybridize on illumine gene expression chip (Right) andIllumina TotalPrep RNA Amplification Procedure (Left).

RNA sample was keep in -80^{O}C until finish realtime PCR quatitation. RNA was check integrity by run 1ug RNA in 1% agarose gel for 1 hours at 90 volt constant. Ratio of 28s per 18s band should be around 2, with purity of RNA (260/280) at more than 2. 500 ng of RNA was synthesis cRNA by Illumina Total Prep RNA Amplification Kit (Ambion #1L1791) which briefly shown in figure 17. 250 ng cRNA of each sample were hybridize on Sentrix Human Ref-8 chip V3 according to manufacturer's standard protocol. After finish washing step, hybridized chip was scan with iScanTM System (Illumina inc.). Using Bead

studio software (Illumina inc.) for export intensity signal from each probe in the experiment after cut background and done normalization for further analysis.

Bioinformatic and statistic analysis

Scanning data is adjusted with Bead studio software (Illumina inc.) in order to identify gene expression group. Raw data of gene probe intensity of Cy5 dye was adjust by background subtraction follow with cubic spline normalization. Gene list and average intensity is export in to sample gene profile.txt file. Adjusted Data is open in excel and choose only average signal column for analysis. Independent t-test was use to confirm significant differential expression of experiment.Gene down and up expression group also separate in to each level of significant, 0.05, 0.01 and 0.001, respectively. We combine database of human whole genome LINE-1 from L1BASE and Homo sapiens gene list from Build 36.3 in order to identify whole genome gene with intragenic LINE-1 and gene without intragenic LINE-1 into each categories. Gene name from down and up regulate group was intersect with gene name from each gene character group. Chi-square was use for confirm correlation of LINE-1 insertion and gene regulation by methylation. Dr.Chatchawit Aporntewan, Department of Mathematics, Faculty of Science Chulalongkorn University,using CU-DREAM program [44] combine with LINE-1 database from L1xplorer[175] for analysis correlation between each hypothesized phenomenon in cell sample, transcriptome of WSU-HN17 si-DICER1 stable cell, WSU-HN31 si-3LINE-1 stable cell, WSU-HN17 dementhylated by 5'aza-2-deoxycytidine, primary normal oral keratinocyte. To intersect or find association between 2 dataset analysis two-by-two chi-square test, p, Odd Ratio, and 95%CI need to perfrom. Correlation studies consider only data that didn't contain 1 between upper and lower 95% CI.

CHAPTER IV

RESULT

1. LINE-1 promoter methylation study in HNSCC cell lines.

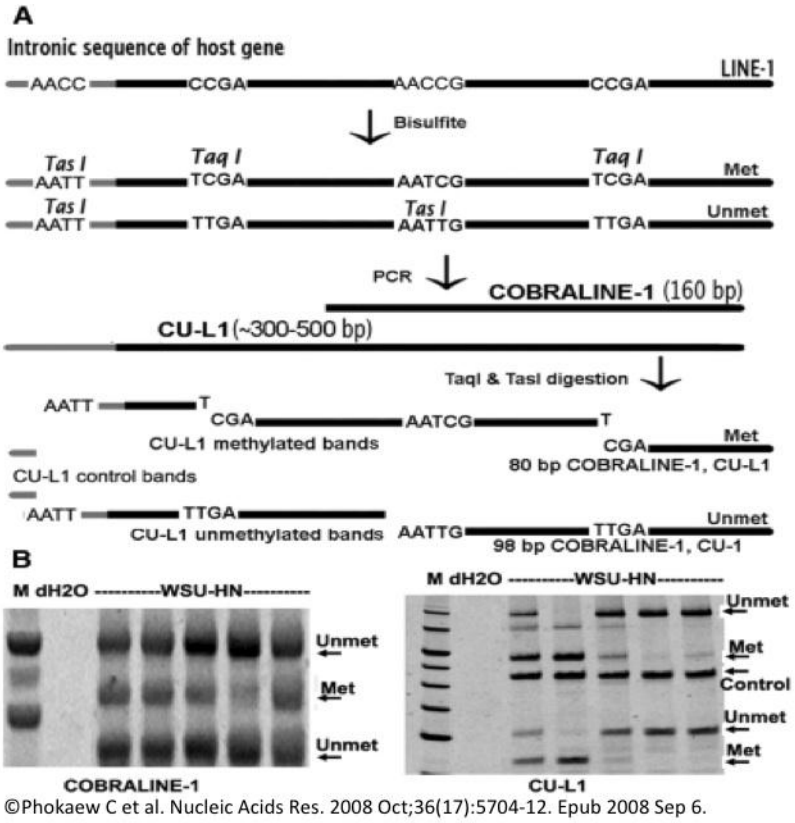

Figure 18: COBRALINE-1 vs COBRA unique to LINE-1 (CU-L1) in details [171].

COBRA for genome wide LINE-1 (COBRALINE-1) and COBRA for unique LINE-1 squence (CU-L1) in figure 18 was published in Chalitchagorn K. et al. [7] and Phokaew C. et al [171], respectively. As shown in figure 18, part A, both CU-L1 and COBRALINE-1 share same 3'primer sequence from 5'UTR of LINE-1 (M80343) while 5'primer of COBRALINE-1 still on 5'UTR of LINE-1, LINE-1 promoter region, 5'primer of CU-L1 extend into intronic upstream region of each specific 5'UTR of LINE-1 that cause COBRALINE-1's length at 160bp and CU-L1 will extend into 300-500bp size. Base on Bisulfite treatment,

PCR and RFLP principle, COBRALINE-1 and CU-L1 rely on AACCG and CCGA sequence which will become AATTG and TTGA in unmethylated DNA and TasI enzyme will cut at AATTG into 98bp band furthermore the methylated sequence will become AATCG and TCGA which cut by TagI enzyme at TCGA into 80bp band. The LINE-1 methylation level will measure GelStar™ Nucleic Acid Gel Stain (Cambrex Bio Science Rockland, Inc.)intensity and calculate proportion of 80bp(methylated DNA) band per summery between 98bp(unmethylated DNA) band and 80bp band into percentage.However because the length of CU-L1, there is a chance of additional AATTG and TCGA site on upstream 5'UTR of each LINE-1 which also report as specific CU-L1 additional band, CU-L1 unmethylated band and CU-L1 methylated band, respectively. Presence of both CU-L1 additional contol bands, can use for confirm the related methylation change character in each unique LINE-1 promoter location. In part B of figure 18 is the example of COBRALINE-1 PCR compare to CU-L1 PCR after detect by GelStar™ Nucleic Acid Gel Stain (Cambrex Bio Science Rockland, Inc.) intensity via the PhosphorImager machine (Molecular Dynamics). In both gels of COBRALINE-1 and CU-L1, as indicate by the arrow, there are 80bp methylated band, 98bp unmethylated band, additional methylated band (CU-L1 only) and additional unmethylated band (CU-L1 only). While COBRALINE-1 can represent whole genome LINE-1 promoter methylation change, CU-L1 can inform methylation status of each LINE-1 promoter and methylation character of 5'upstream region of each LINE-1 in CU-L1 PCR. Whole genome LINE-1 methylaton study by COBRALINE-1 method will represent average result of LINE-1 methylation from LINE-1 more than 500,000 copies. Along the evolution, there is only around 80-100 full length LINE-1 exist that still have retrotransposition activity [176]. CU-L1 is developedforstudy each putative active full length intragenic LINE-1 promoter methylation because all 17 CU-L1 PCR chosen from the most match region within gene coding sequence to LINE-1.2 (M80343) sequence. All 17 CU-L1 PCRs details were described in

the appendix part including PCR location, LINE-1 character, LINE-1's host gene and PCR condition. CU-L1 was alsoproving as tumor marker according to the Thailand patent request ID 0801002098. According to figure 18, sample lane 1 (WSU-HN4) and 2 (WSU-HN13) are both HNSCC cell line which seem to have equal global LINE-1 promoter methylation level by COBRALINE-1, 26.99 % and 27.06 %, respectively. In unique intragenic LINE-1 promoter status by CU-L1, WSU-HN4 (CU-L1 = 31.19 %) sample have more methylation on unique intragenic LINE-1 promoter than WSU-HN13 (CU-L1 = 5.7 %). The difference of methylation on LINE-1 promoter from COBRALINE-1 and CU-L1 PCR reveal fact about loss of methylation from each intragenic LINE-1 promoter will not occur in similar pattern among each HNSCC cell line that have same level of global LINE-1 promoter hypomethylation. CU-L1 is a new established PCR that can detect single intragenic LINE-1 promoter methylation status while COBRALINE-1 is a standard PCR for detect global LINE-1 promoter methylationlevel of sample.

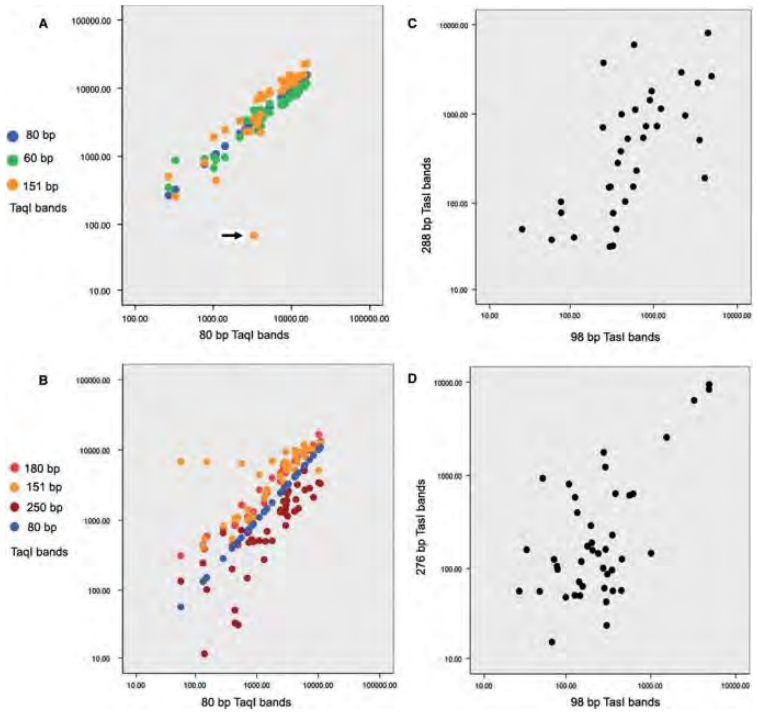

Figure 19: Correlation between the bands of CU-L1 [171].

The scatter plot in figure 19, each graph refer to direct correlation between intensity of CU-L1 unmethylated band (98 bp) or methylated band (80 bp) to the additional CU-L1 unmethylated and methylated band, respectively. The A to D graph, datas will represent the correlation analysis of methylated bands or unmethylated bands from CU-L1-*EPHA3*IVS5 PCR and CU-L1-*MGC4217* PCR. Graph A show direct correlation among three CU-L1 methylated bands (80, 60, 151 bp) from CU-L1-*EPHA3*IVS5 PCR in order to prove the intensity of band 80 bp from CU-L1-*EPHA3*IVS5 can reflex methylation status of LINE-1 promoter that settle intron 5 of *EPHA3* gene. Picture B also show the intensity of methylated band 80 bp that have direction correlation to the remains methylated bands from CU-L1-*MGC4217* PCR (180, 151, 250 bp) which confirm the methylation status by80 bp band in intragenic LINE-1 promoter of *MGC4217* gene. Picture C refer to direction correlation of additional unmethylated 288 bp band to 98 bp unmethylated band from CU-L1-*EPHA3*IVS5 PCR which used for show intragenic LINE-1 promoter methylation in intron 5 of *EPHA3*gene. Picture D show direction correlation between the additional unmethylated 276 bp band to 98 bp unmethylated band of CU-L1-*MGC4217* that reflex consistent of band 98 bp intensity in indicating intragenic LINE-1 promoter in *MGC4217* gene. All A to D association result will show the reliable of methylated band (80 bp) and unmethylated band (98 bp) that used for indicate intragenic LINE-1 promoter methylation status in all 17 CU-L1 PCRs as well as used for indicate global LINE-1 promoter methylation in COBRALINE-1 PCR.

In concerning about the false positive effectin CU-L1 PCR according to mutations or polymorphism in restriction site cut for example changing of CCGA into CTGA which will cause absence of 80 bp, the methylated band of CU-L1. The direct correlation in figure 19A and 19B between remains additional methylated bands and CU-L1 methylated band (80 bp) can comfirm CU-L1 result, however seeing that the one arrow pointed in 19A can be the example of possible mutation or polymorphism at a 151 bp additional

methylated band. According to the rare case of mutations or polymorphism in CU-L1 PCRs, it may conclude the requirement of conserved sequence in intragenic LINE-1 promoterregion that leading to further study on impact of intragenic LINE-1 on LINE-1's host gene regulation.

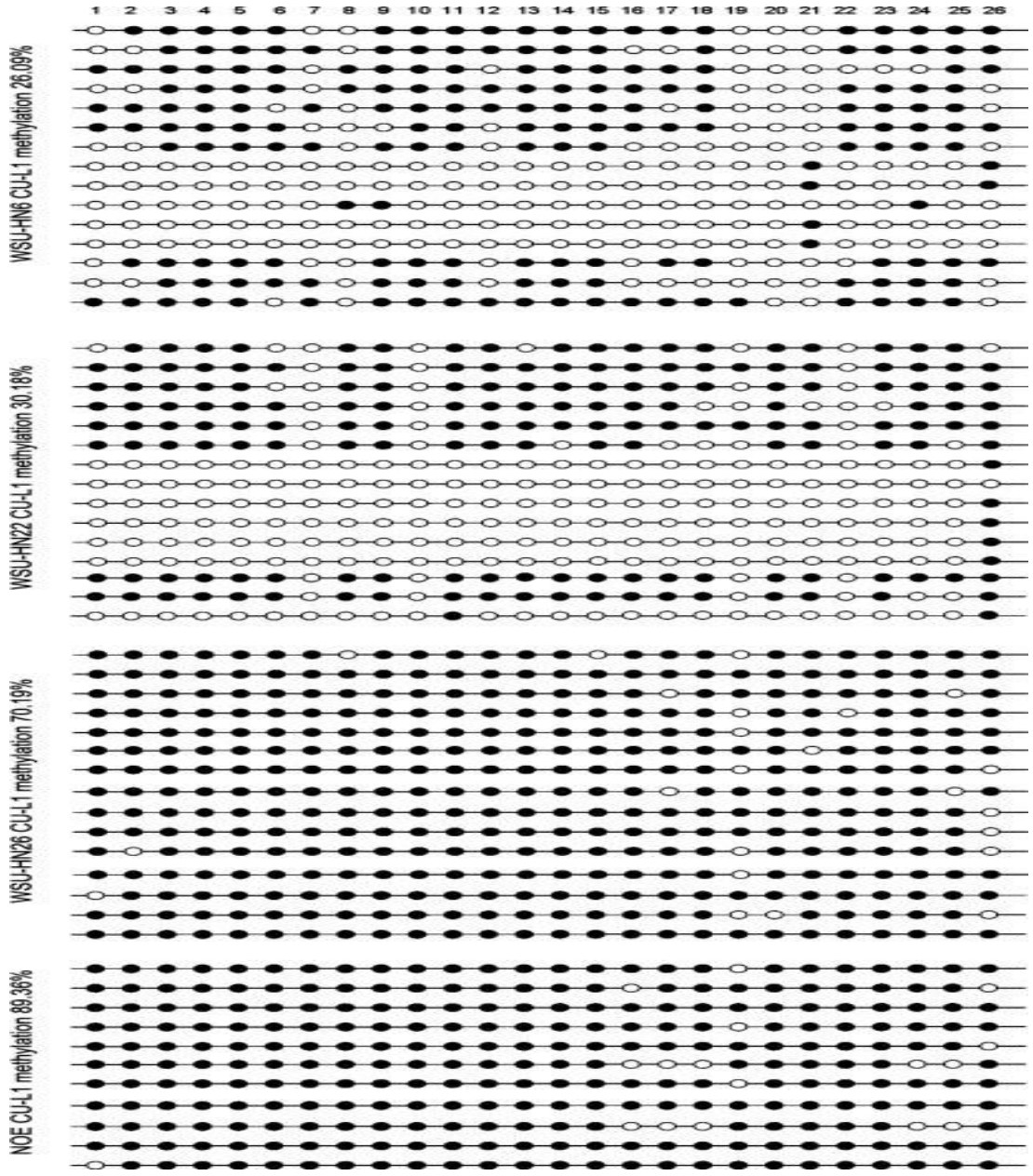

Figure 20: CU-L1s bisulfite sequences. The closed and opened circles refer to methylated CpGs dinucleotides and non-methylated CpGs dinucleotides respectively [171].

Data of all CpG dinucleotides on intragenic LINE-1 promoter of *LRP2* gene from CU-L1-*LRP2* PCRwas cloned and sequenced from WSU-HN6, WSU-HN22, WSU-HN26 and NOE in figure 20, with methylation level at 26.09%, 30.18%, 70.19% and 89.36%, respectively. The methylated band (80 bp) from TaqI restriction enzyme digestion and unmethylated band from TasI restriction enzyme digestion (98 bp) are the 19th and 20th CpGs, respectively. From figure 20, the direction correlation of intragenic LINE-1 promoter methylation status by CU-L1-*LRP2* PCR and bisulfite sequencing was revealed. Observing of more methylated CpG dinucleotides at 19th CpGs in samples that have higher CU-L1-*LRP2* level, in figure 20, WSU-HN26 and NOE have more closed circles than WSU-HN6 and WSU-HN22. As CU-L1 PCR was used for distinguish single intragenic LINE-1 promoter methylation in each genome, according to bisulfite sequencing result of CU-L1-*LRP2* PCR may indicate the preferred loss of methylation from 19th and 20th which is TaqI and TasI cut site that used for determine methylation level in CU-L1 PCRs. Bisulfite sequencing of CU-L1-*LRP2* PCR from WSU-HN6 and WSU-HN22 sample also show mix of two independent clonal cancer chromosome in same case, the patilally methylated and completely unmethylated pattern. The mixing of two type chromosome from case of CU-L1-*LRP2* PCR in WSU-HN6 and WSU-HN22 samples could reflex the unequally influenced of global hypomethylation process in HNSCC carcinogenesis.

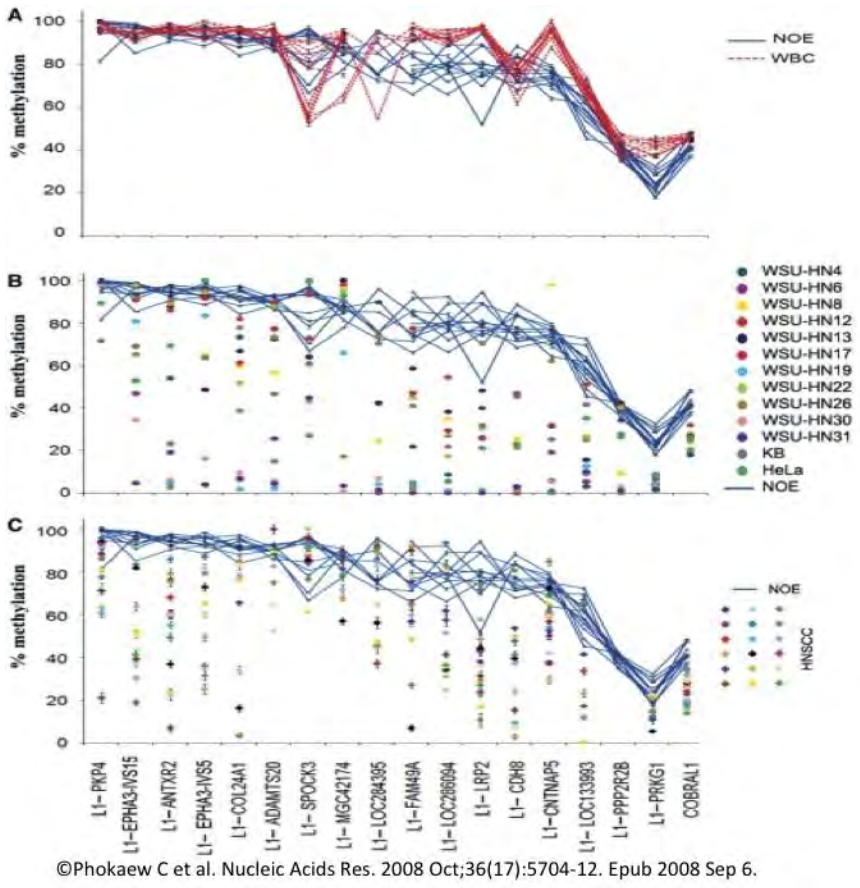

Figure 21: Detection of LINE-1 promoter methylation status by CU-L1 in HNSCC cell series [171].

As previously Chalitchagorn K. et al [7], study of whole genome LINE-1 promoter methylation by COBRALINE-1 PCR many tissues,the variation of methylation level in both normal or tumor among each case was dissimilarity depend on tissue type. With significant level of COBRALINE-1 in cancers, esophagous sample among cases has widest range at almost 35 % while smaller range of methylation among cases including bladder, head and neck squamous cell, liver, lung, prostate, breast and stomach. Here, in this thesis by using CU-L1 for study range of each intragenic LINE-1 promoter methylation among normal individual samples, NOE and WBC, there are varieties of methylation pattern by each CU-L1 PCR. Hypermethylated level in both NOE and WBC samples including CU-L1-

PKP4, CU-L1-*EPHA3*IVS5, CU-L1-*EPHA3*IVS15, CU-L1-*ANTXR2*, CU-L1-*ADAMTS20* and CU-L1-*COL24A1*. While the significant higher of methylation in WBC than NOE found in CU-L1-*CNTNAP5*, CU-L1-*LOC133993* and CU-L1-*PRKG1*. Mix of LINE-1 promoter methylation level about 40% rangeamong WBC and NOE was found in CU-L1-*SPOCK3*, CU-L1-*MGC42174* and CU-L1-*LOC284395*. In 30% methylation range, CU-L1-*CDH8* is only one locus that have trend of higher methylation in NOE while the remains loci including CU-L1-*FAM49A*, CU-L1-*LOC286094* and CU-L1-*LRP2* have methylation higher in WBC. However, only two CU-L1 loci have methylation nearly at 20%, CU-L1-*PPP2R2B* and CU-L1-*PRKG1*, while others CU-L1 have methylation level higher than 50% which can reflex hypermethylation of most LINE-1 promoter in normal tissue.

Global loss of LINE-1 promoter methylation was significantly prove in carcinogenesis of tissues including bladder, head and neck squamous cell, liver, lung, esophagous, prostate, breast and stomach [7]. Studying intragenic LINE-1 promoter methylation in carcinogenesis for this thesis, all 17 CU-L1 PCRs was used for compare NOE to 11 WSU-HN, Hela and KB cell line. According to graph B and C of figure 21, most of intragenic LINE-1 promoters from CU-L1 PCRs prefer to be hypomethylated than hypermethylated in cancerious tissues compare to NOE samples. Some location including CU-L1-*PKP4*, CU-L1-*SPOCK3* and CU-L1-*MGC42174*, intragenic LINE-1 promoters of 3-6 samples have hypomethylated status. Others group of intragenic LINE-1 promoters including CU-L1-*FAM49A*, CU-L1-*LOC286094*, CU-L1-*LRP2*, CU-L1-*CDH8* and CU-L1-*PRKG1*, almost cancer samples have hypomethylated status. Variation of methylation level among each LINE-1 promoters was obviously shown in graph B figure 21, WSU-HN19, CU-L1-*COL24A1* and CU-L1-*ADAMTS20* completely hypomethylated while CU-L1-*EPHA3IVS5* and CU-L1-*EPHA3IVS15* have hypermethylated status. Even cell from same persons but different tissue location, WSU-HN30 and WSU-HN31 according to graph B figure 21, CU-

L1-*EPHA3IVS15* have more than 10% methylation difference. Comparing between Hela and KB (Hela contaminate genome) will show CU-L1 difference among cell lines, for example in CU-L1-*ANTXR2* that KB have hypomethylated than Hela while CU-L1-*COL24A1*, Hela will have hypomethylated than KB cells. Moreover, since cancer cell have selective advantage potential, some LINE-1 promoter such as CU-L1-*CNTNAP5* of WSU-HN8 have hypermethylated status when compare back to NOE samples. As seen form CU-L1 results in HNSCC cell panel, each LINE-1 promoter methylation seem to reflex in cis impact of LINE-1 sequence in carcinogenesis. Effect of LINE-1 promoter methylation in HNSCC carcinogenesis can confirm be result in graph C figure 21, 17 HNSCC microdissected paraffin-embedded samples compare to NOE samples. Although some sample and some CU-L1 PCRs can't amplify in all HNSCC microdissected paraffin-embedded samples, according to sample process cause DNA break, fromachievable to CU-L1 PCR from this samples group have similar pattern as found in HNSCC cell line. Unique hypermethylation of CU-L1-*CNTNAP5* in WSU-HN8 also found in some case of HNSCC microdissected paraffin-embedded samples as shown in graph C figure 21.

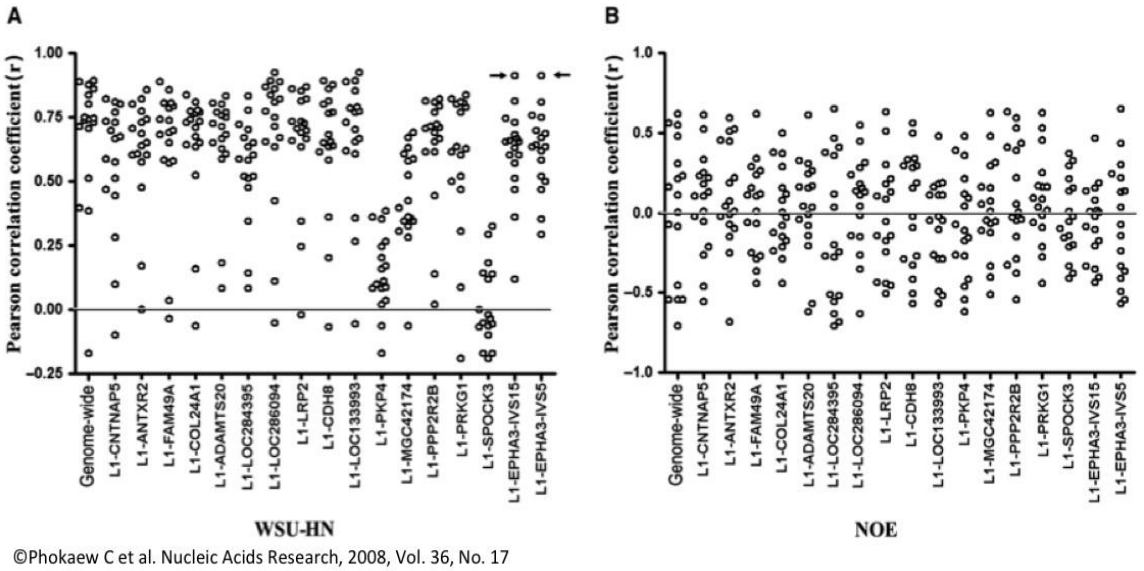

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Figure 22: COBRALINE-1 and CU-L1s correlation. Pearson correlation coefficient values between LINE-1 and genome-wide loci of (A) WSU-HN cells and (B) NOE cells. Each dot refer to pearson correlation of each specific CU-L1 to other specific CU-L1 within two cell types [171].

As CU-L1 hypomethylation become common character of HNSCC sample through carcinogenesis and there is a strong correlation between whole genome LINE-1 by COBRALINE-1 and average of 17 CU-L1 loci methylation level at $r = 0.8979$, $p = 0.003$. It can confirm the whole genome LINE-1 character though all 17 CU-L1 in DNA methylation analysis. In order to study the connection of methylation between whole genome LINE-1 (COBRALINE-1) to each CU-L1 of HNSCC sample and also among CU-L1s, the Pearson correlation coefficient (r) value analysis among each CU-L1 and COBRALINE-1 from HNSCC cell lines were plot in figure 22. The r value between -0.5 to 0.5 was not significant, r value lower than -0.5 shown reverse correlation among factor while r value higher than 0.5 reveal direct correlation among factor. In figure 22, r value of HNSCC samples in each CU-L1 of cancer group show more direct correlation than in normal samples, which have most of r value between -0.5 to 0.5. But some change of CU-L1 such as L1-*SPOCK3* and L1-*PKP4* have less connection than other CU-L1 and with result of this two loci in figure 22, both LINE-1s may not under influence of DNA methylation. LINE-1 promoter methylation in cancer cell has more connection between each LINE-1 of each sample than found in NOE samples. Intragenic LINE-1 methylation from same gene was study in *EPHA3* gene which have LINE-1 within intron 5 and 15, as indicate by arrows in graph A figure 22. From all 17 loci of CU-L1, L1-*EPHA3IVS5* and L1-*EPHA3IVS15* have strong Pearson correlation at 0.913 ($p<10^{-16}$). This can confirm synchronize LINE-1 methylation pattern within gene. It can conclude that even whole genome LINE-1 promoter hypomethylation generalized involve

with pathogenesis, the unique LINE-1 promoter methylation change will develop in carcinogenesis depend on impact of LINE-1's location and cell type specific mechanism.

In first part of thesis, because methylation statuses of most LINE-1 loci in HNSCC cells have positive correlation to each others in whole genome level, it can conclude the influence of a specific mechanism that cause global hypomethylation is commonly effect on every single loci in genome. Each specific locus is under controlling of epigenetics modification factor during carcinogenesis process such as hypermethylation of CU-L1-*CNTNAP5* and hypomethylation of CU-L1-*EPHA3IVS5* in WSU-HN8 cell line. In conclusion, global hypomethylation which generalized with repetitive sequence in carcinogenesis seem to occur with each specific locus depending on LINE-1's location and LINE-1's host gene function within genome. This lead us to investigate more on how each full length LINE-1 that we pick for CU-L1 study will involved with LINE-1's host gene, as all 17 CU-L1 is intragenic LINE-1 suppose to match to gene body methylation concept. Next study, it will focus more on the connection between LINE-1 promoter methylation level, as indicate for gene body region methylation, and LINE-1's host gene expression.

2. Correlation between intragenic LINE-1 promoter methylation on LINE-1's host gene expression in HNSCC cell lines.

DNA methylation in gene body region is the high impact factor mechanism that regulate normal gene expression pattern as prove in whole genome studies by George M. Church and his team [177]. In order to control incorrect transcriptional start site within gene body region from repetitive elements or CpG rich region, normal cell always found hypermethylated in repeat sequence region meanwhile pathogenic tissue always loss methylation within this kind of sequence [6]. Many report shown that LINE-1 existance will

bring some affect on gene nearby [24, 178]. As proved in the first step about loss of LINE-1 promoter methylation is occur under influence of global hypomethylation and LINE-1 methylation change in HNSCC cell line is depend on impact of each LINE-1 location in genome. All 17 CU-L1 PCRs was designed from intragenic full length LINE-1 in order to prove effect of each LINE-1 on LINE-1's host gene purpose. In this step, association study on each LINE-1 promoter methylation to LINE-1's host gene expression was studies by using qPCR normalized to *GAPDH* which is well known house keeping gene as shown in Figure 23. Ratio between genes expressions calculate by intensity of *EPHA3* gene per *GAPDH* was further compare back to CU-L1 methylation level in each LINE-1.

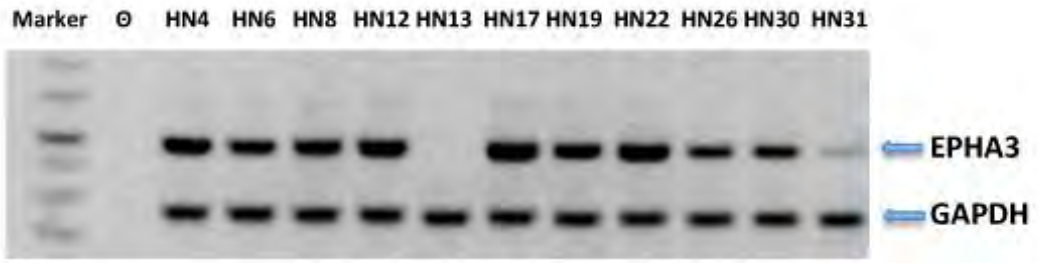

Figure 23: Acrylamide gel electrophoresis for analyse duplex PCR between EPHA3 and GAPDH gene from HNSCC cell lines sample.

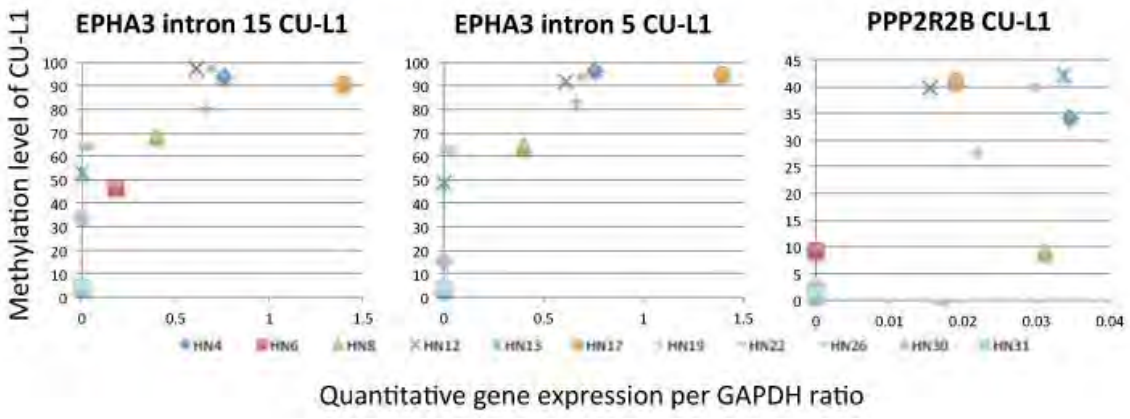

Figure 24: Association between intragenic LINE-1 promoter methylation and LINE-1's host gene expression in HNSCC cell lines. Pearson correlation scatter plot

between each intronic LINE-1 CU-L1 methylation level (X axis) vs LINE-1's host gene per *GAPDH* expression ratio (Y axis).

ByPearson correlation analysis, *EPHA3* and *PPP2R2B* are the only 2 from 16 genes that have correlation between gene expression and intragenic LINE-1s have hypermethylated status. These finding support common characters of normal highly expression gene always have hypermethylated gene body in order to prevent incorrect transcriptional start site (TSSs) [69], also found in HNSCC cell lines that we choose as carcinogenesis model for this thesis. L1-*EPHA3IVS5*, L1-*EPHA3IVS15* and L1-*PPP2R2B* have pearson correlation to host gene at r^2 = -0.7033 (p=0.0233), r^2 = -0.7237 (p=0.0118) and r^2 = -0.6295 (p=0.038) respectively in figure 24.

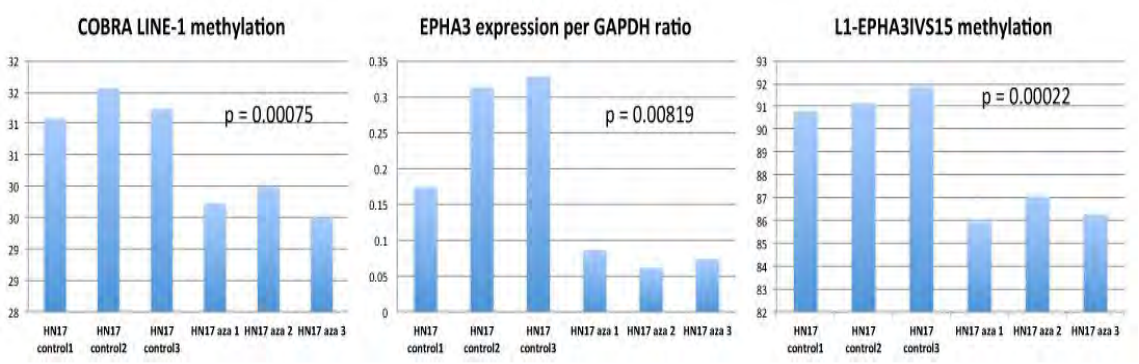

Figure 25: Hypomethylation induced in WSU-HN17 cell line by 5'-aza-2-deoxycytidine.

According the association between intragenic LINE-1 promoter methylation level with *EPHA3* and *PPP2R2B* expression level, it need to study effect of LINE-1 promoter methylationon LINE-1's host gene expression. In order to prove impact of DNA methylation, WSU-HN17 cell line treated with 5'-aza-2-deoxycytidine, demethylating agent, and check methylation change with COBRALINE-1 and CU-L1-*EPHA3* PCRs. After 5'aza-2-deoxycytidine treated, WSU-HN17 found loss of LINE-1 promoter methylation both whole

genome LINE-1 (COBRALINE-1) and specific LINE-1(L1-*EPHA3IVS15* CU-L1). According to result in figure 25, hypomethylation of L1-*EPHA3IVS15* and whole genome LINE-1 cause decease of *EPHA3* transcripts.As expected from gene body methylation machinery, hypomethylation in gene body sequence at LINE-1 promoter region will repress *EPHA3* expresssion in WSU-HN17 hypomethylated cell.It also interesting that all 3 LINE-1s that relate to *EPHA3* and *PPP2R2B* gene expression was intragenic antisense LINE-1.As many association studies confirmed about antisense LINE-1 orientation have impact on gene nearby especially in cancer cell lines [25, 27].

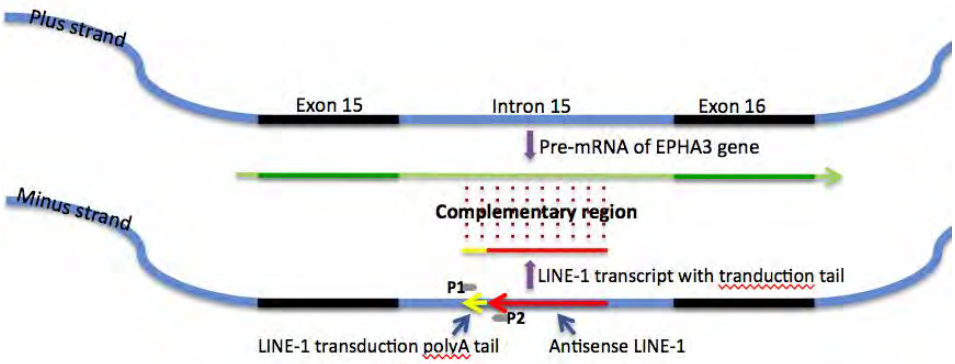

Figure 26: Details of LINE-1 transduction PCR at *EPHA3* intron 15 region (L1-*EPHA3IVS15*) for measure unique LINE-1 expression. L1-*EPHA3IVS15* PCR requires P1 and P2 primer as identify in this diagram.

According to alteration in LINE-1's host gene expression from hypomethylation event in figure 25, impact of LINE-1 promoter methylation on LINE-1 expression need to be verified in HNSCC cell lines in order to show consequence on LINE-1 sequence from LINE-1 promoter hypomethylation. Specific LINE-1 expression is hard to design because the full length LINE-1 that still active within genome is nearly 100 LINE-1s and each LINE-1 have same structure. But according to 3'transduction property of some LINE-1s [179], we may design a specificity detection method base on 3'transduction sequence for a unique LINE-1

expression study. Here in this step, in figure 26, specific L1-*EPHA3* RNA PCR was design in region between 3'UTR of L1-*EPHA3IVS15* to 3' transduction sequence of L1-*EPHA3IVS15*.

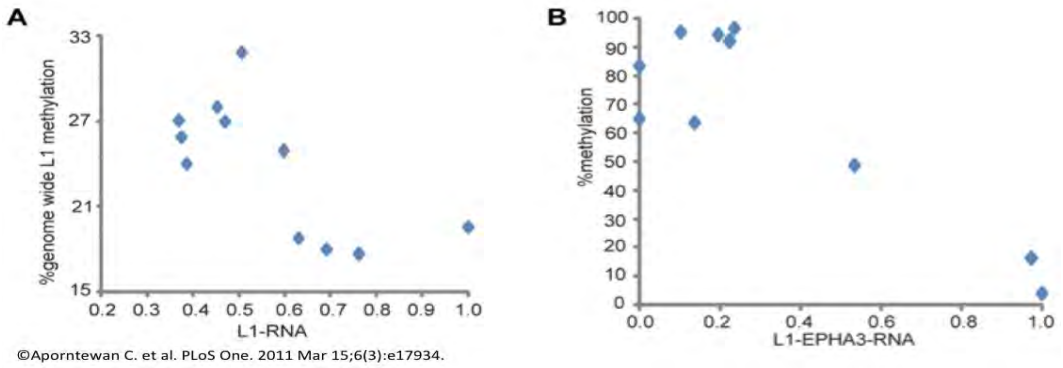

Figure 27: LINE-1 promoter hypomethylation can induce LINE-1 expression in HNSCC cell lines. Pearson correlation study for evidenced link between specific LINE-1 methylation on LINE-1's host gene (EPHA3) and specific LINE-1 itselfs expression [26].

As shown in graph B figure 27, we can detect specific LINE-1 expression, L1-*EPHA3IVS15*, and also found correlation between L1-*EPHA3IVS15* and CU-L1-*EPHA3IVS15* at r = 0.8686. Even there is no association between L1-*EPHA3IVS15* and *EPHA3* expression (r = -0.485, p = 0.065), but there is trend of reverse correlation between intragenic LINE-1 expression and LINE-1's host gene. Whole genome LINE-1 promoter methylation also have reverse correlation with LINE-1 expression at r = 0.6955. In HNSCC cell lines, there is a direct correlation between LINE-1 promoter hypomethylation and LINE-1 expression at both specifc LINE-1 and whole genome LINE-1 level. LINE-1 promoter methylation seems to be an important factor in controlling gene expression. As hypomethylated LINE-1 seem to release more LINE-1 transcript and LINE-1 hypomethylation is common feature in HNSCC carcinogenesis [7]. Role of LINE-1 RNA from LINE-1 promoter hypomethylation on LINE-1's host gene expression change, as shown in figure 25, need to further study in next step of this thesis.

3. Consequence LINE-1 RNA on LINE-1's host gene expressionin HNSCCcell line.

LINE-1 RNA can express from LINE-1 promoter hypomethylation and it may be the core factor on LINE-1's host gene regulation in HNSCC cell. Here in this part, it needs to clarify effect of LINE-1 RNA on LINE-1's host gene in HNSCC cell. As WSU-HN31, naturally hypomethylation HNSCC genome, have a very low level of *EPHA3* expression but have highest L1-*EPHA3IVS15* RNA expression among others HNSCC cell lines. WSU-HN31 should be the best candidate for knockdown full length LINE-1 to estimate impact of active LINE-1 RNA on genome by knockdown LINE-1 RNA base on RNAi pathway. As in figure 28,by using clustalX program [180], LINE-1 siRNA constructs was designed for transfect into WSU-HN31 cell since WSU-HN31 have high level of L1-EPHA3IVS15 expression among all WSU-HNSCC cell lines. Three selected sequence for RNAi mechanism target site were on conserve region of anreported active LINE-1s including LINE-1.2, LINE-1.3, LINE2.3, L19088, L19092, AF149422, AF148856 and all LINE-1 from 17 CU-L1 loci, as shown in figure 28. Combine tranfection all three si-LINE-1 constructs in the same WSU-HN31 cell, it assume to cleavage most of full length LINE-1 through RISC complex.

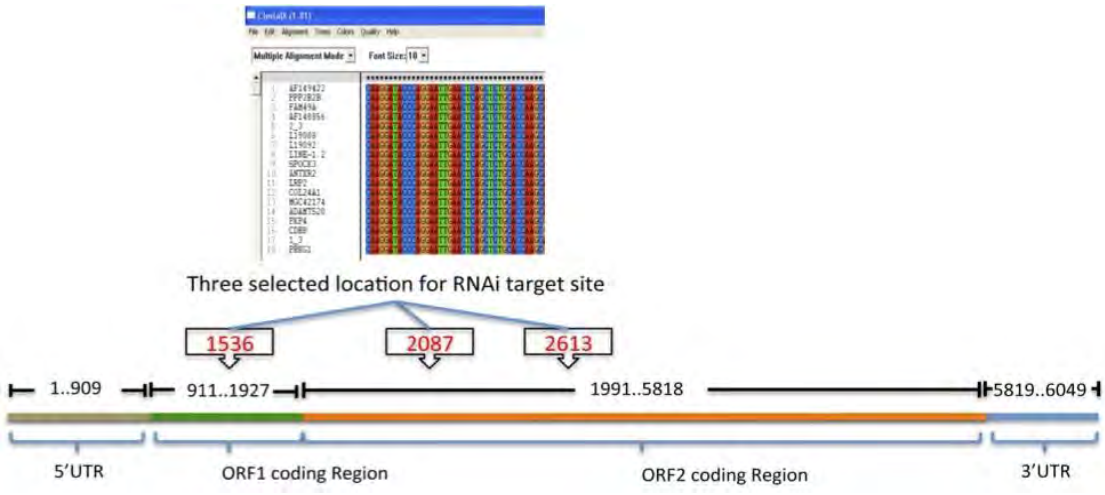

Figure 28: Details of full length active LINE-1 (L1.2: M80343) and target site of 3 contructs which target on mostly conserve region among active LINE-1.

After knockdown LINE-1 RNA in WSU-HN31 cell, both LINE-1 species, inactive LINE-1 GAG (refer L1PA2-L1PA5) and active LINE-1 ACA(refer to L1PA1 Ta element)[80], become down regulated by 3 si-LINE-1 construct transfection in figure 29. From both figure 29 and 30, *EPHA3* and *PPP2R2B*, which found regulate by gene body methylation (on LINE-1 promoter sequence), become up-regulated in stable knockdown si-3LINE-1 WSU-HN31 cell line. There is others gene, according to CU-L1 loci, that also have gene expression pattern change after LINE-1 RNA reduces within stable cell, result shown in figure 30. LINE-1 RNA seems to have significant impact on LINE-1's host gene expression.

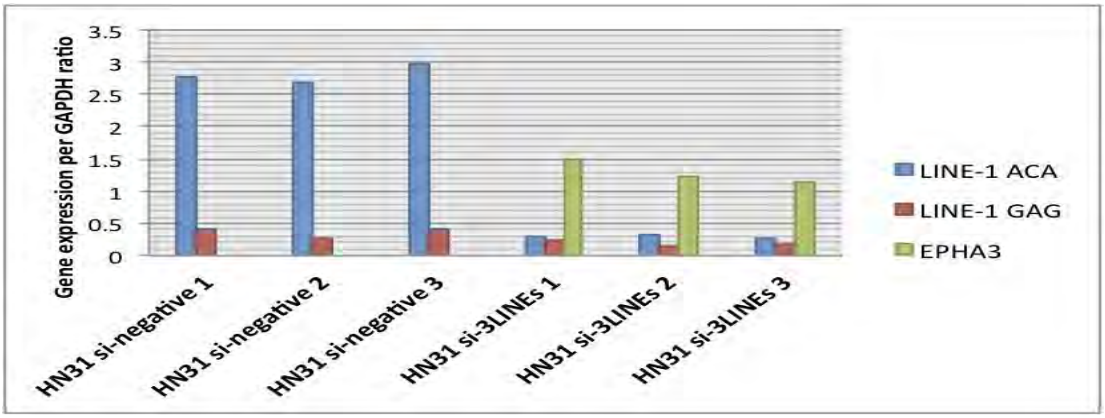

Figure 29: Stable LINE-1 knockdown in WSU-HN31 provoke *EPHA3* expression. By student t-test significant study, LINE-1 ACA p =0.00000376, LINE-1 GAG p = 0.01267 and *EPHA3* p = 0.000135.

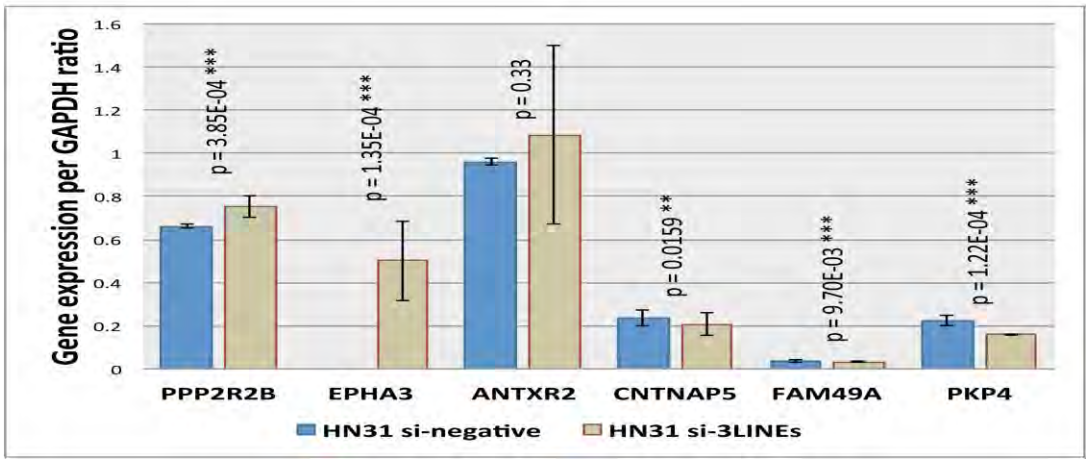

Figure 30: LINE-1's host gene expression change after LINE-1 stable knockdown in WSU-HN31 cell.

From result in figure 30, antisense intragenic LINE-1's host gene including *EPHA3* and *PPP2R2B* become up-regulated in LINE-1 RNA knockdown cell. However, down-regulated condition occur with sense intragenic LINE-1's host gene including *FAM49A* and *PKP4* and also antisense intragenic LINE-1's host gene such as *CNTNAP5*. *ANTXR2* is antisense intronic LINE-1's host gene that has unclear change after LINE-1 knockdown.*CNTNAP5* contain 5 LINE-1s with only 1 active LINE-1 (CU-L1-*CNTNAP5*), this may be the reason that *CNTNAP5* expression change differ from gene in the antisense categories under effect of LINE-1 RNA existence.

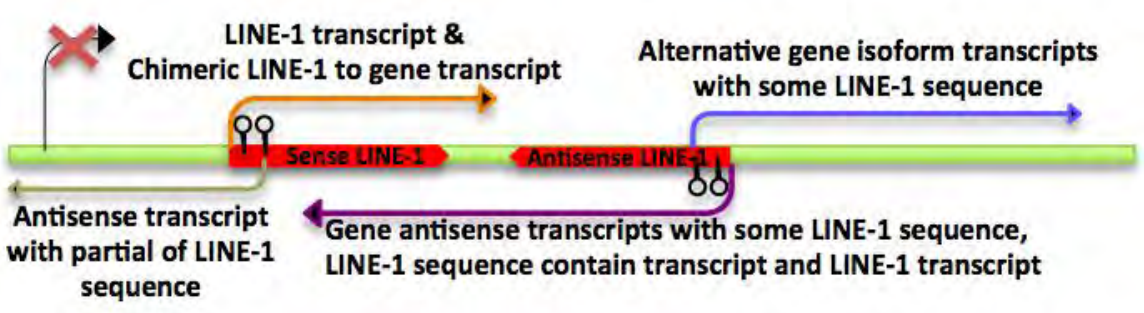

Figure 31: Various form of LINE-1 promoter hypomethylation releasing transcripts.

Loss of LINE-1 promoter methylation in both LINE-1 sense and antisense promoter directions can releasing others type of transcript moreover than LINE-1 RNA transcripts that includechimeric LINE-1 to gene transcripts, alternative gene isoform transcripts with some LINE-1 sequence, gene antisense transcripts with some LINE-1 sequence and LINE-1 sequence contain transcripts, as shown in figure 31. It should keep remind that, all possible various LINE-1 promoter hypomethylation related transcripts can interfere gene regulation as found in LINE-1's host gene. With all result until this step, it can

assume that LINE-1 RNA or LINE-1 promoter hypomethylation releasing transcript have some affect on LINE-1' host gene expression with unclear mechanism. Role of RNA in gene regulation involved report of many situation include small RNA (miRNA and siRNA) in RNAi mechanism and noncoding RNA for epigenetics in gene regulation. Both mechanisms have RISC proteins involved that cause the possibility of RISC protein in LINE-1 RNA influence on LINE-1's host gene expression. In next step, it should focus on how LINE-1 RNA regulates LINE-1's host gene expression via RISC protein.

4. Possibility of RISC protein and LINE-1 RNA on LINE-1's host gene regulation.

Loss of LINE-1 RNA or LINE-1 promoter hypomethylation releasing transcript has effect on LINE-1's host gene expression in HNSCC cell from previous steps.In this part, RISC protein as putative partner of LINE-1 RNA (LINE-1 promoter hypomethylation releasing transcript) will be clarified. In first step, for prove involvement of RISC protein like *AGO2* in LINE-1's host gene regulation, knockdown *AGO2* was perform in WSU-HN17. Although WSU-HN31 should be the good candidate cell of knockdown *AGO2* experiment because of highest level of LINE-1 RNA by LINE-1 promoter hypomethylation among all HNSCC cell line. In fact, WSU-HN31 si-*AGO2* stable cell line is hard to establish because cell with knockdown *AGO2* gene will have growth retard as shown in figure 32 and none of selected clone survive after hygromycin selection step. WSU-HN17, which has naturally higher growth level than WSU-HN31, was selected in this study. After hygromycin selection, WSU-HN17 si-*AGO2* stable cell line growth very slow when compare to WSU-HN17 si-negative stable cell line as shown in figure 32, doubling time at 71.7 h and 19.1 h respectively. On the other hand, WSU-HN31 si-LINE-1s stable cell line show higher growth rate than WSU-HN31 si-negative stable cell line as shown in figure 34, at 22.01 h and 29.35 h respectively.

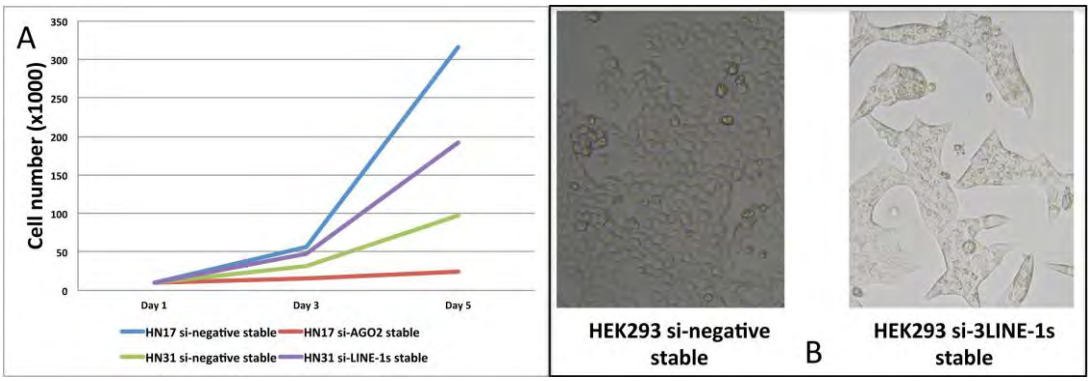

Figure 32: Knockdown LINE-1 RNA and *AGO2* can change cell proliferation and cell morphology. Growth curve of WSU-HN17 and WSU-HN31 stable knockdown cell line shown in picture B while cell morphology change in HEK293 si-3LINE-1s stable cell line.

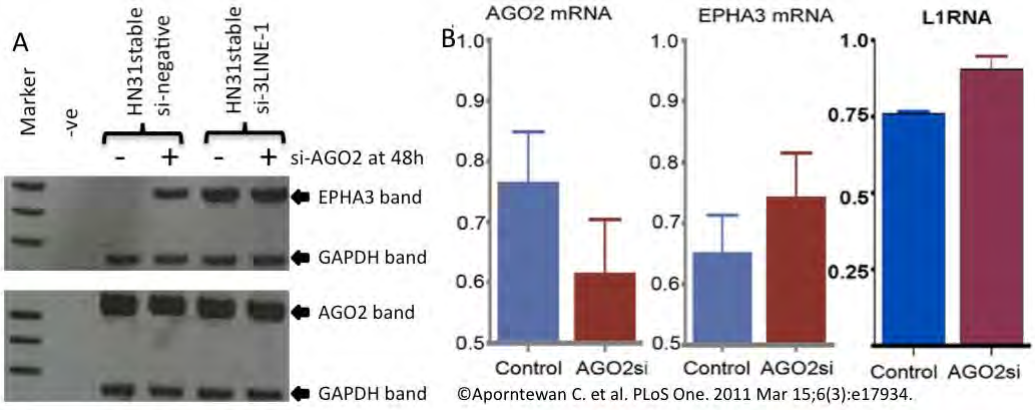

Figure 33: *EPHA3* expression study of transient knockdown human *AGO2* protein in HNSCC cell line. (A) is WSU-HN31 stable si-3LINE-1s cell line and (B) is WSU-HN17 cell line [26].

Both transient and stable knockdowns of *AGO2* can up-regulated *EPHA3* expression while LINE-1 expression can induce by *AGO2* transient knockdown in figure 33. Both, LINE-1 RNA (LINE-1 promoter hypomethylation releasing transcript) and *AGO2* protein (nucleus RISC protein) found regulate LINE-1's host gene expression. According to role of RNA in gene regulation through RISC proteins need interaction between both

molecules, here in this step, interaction among LINE-1 RNA and *AGO2* protein will be reveal by RNA immunoprecipitation method in figure 34.

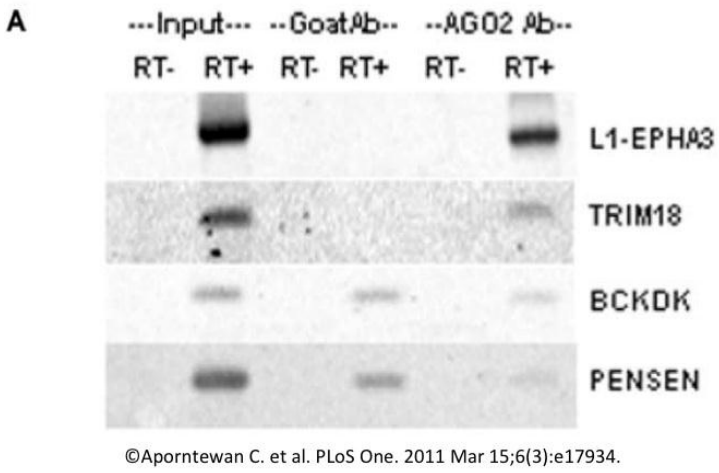

Figure 34: RNA-immunoprecipitation of *AGO2* in WSU-HN cell line [26].

The immunoprecipitate of *AGO2* (human *EIF2C2*) antibody system selected for study the presence of specific LINE-1 RNA on *AGO2* protein in order to confirm their interaction. The complex between *AGO2* and LINE-1 RNA found within nucleus pellet because this machinery should occur strictly within nucleus. In figure 34, specific LINE-1 RNA of L1-*EPHA3IVS15* was found bind on *AGO2* protein as well as *TRIM18* which is positive control gene with intronic full length LINE-1. *BCKDK* and *PENSEN* is negative control for *AGO2* immunoprecipitate experiment because this two gene didn't contain intronic full length active LINE-1. Interaction between AGO2 protein and LINE-1 RNA are obviously require for regulate LINE-1's host gene expression. However, it still need to evaluate effect of *AGO2* binding on LINE-1 RNA through two possible pathways: RNAi mechanism and Epigenetics mechanism. LINE-1 promoter methylation by CU-L1 PCR was selected to identify consequence of LINE-1 RNA and *AGO2* interaction.

5. Mechanism via RISC protein and LINE-1 RNA interaction on LINE-1's host gene regulation.

To prove LINE-1 RNA loading on *AGO2* protein underline purpose in association on LINE-1's host gene regulation. As double stands RNA structure was main target of RISC protein, a putative double stand structure location by active bidirectional LINE-1 promoter in 5'UTR LINE-1 sequence was amplify in figure 35. From Interestingly result in figure 35, it prove that *AGO2* protein bind with both DNA and RNA molecule of LINE-1 in 5'UTR region after compare to control IgG pull down sample. *AGO2* is a major protein of RISC complex in nucleus, which should involve with LINE-1 RNA on controlling intragenic LINE-1 promoter methylation (gene body region) within nucleus. From result in figure 35, it confirm the LINE-1 RNA and *AGO2* protein have interaction on DNA from intragenic LINE-1 promoter region that may support possible role of LINE-1 RNA and *AGO2* protein in epigenetics mechanism on LINE-1 promoter region. In order to prove this possibility role, pilot studies of CU-L1 PCRs were selected to reflex this epigenetics phenomenon.

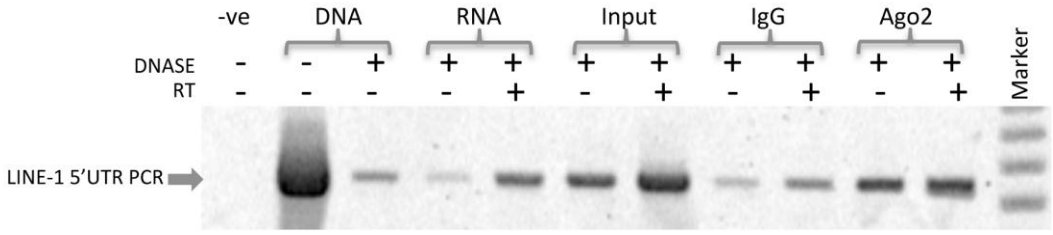

Figure 35: *AGO2* bind one LINE-1 5'UTR in both DNA and RNA form within nuclei pellet of WSU-HN31 cell line.

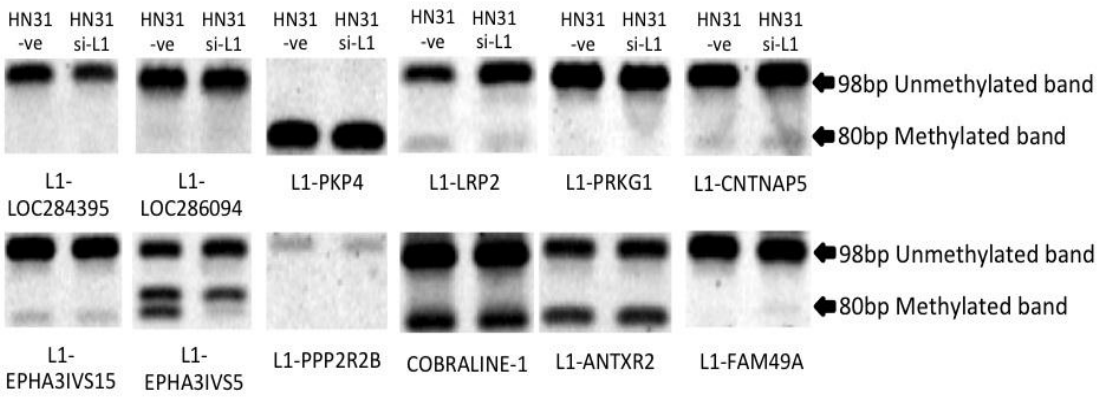

Figure 36: Pilot study of COBRALINE-1 and some CU-L1s PCRs instable knockdown LINE-1 WSU-HN31 genome.

Result of unique LINE-1 promoter methylation change in pilot study shown in figure 36. LINE-1 methylation alteration occur with CU-L1-*EPHA3IVS5*, CU-L1-*PKP4*, CU-L1-*LRP2*, CU-L1-*CNTNAP5*, CU-L1-*FAM49A*, CU-L1-*ANTXR2* and CU-L1-*EPHA3IVS15* may reflex role of LINE-1 RNA in epigenetics regulation in some LINE-1's host gene body region. In same gene but difference in gene body region, it interesting that L1-*EPHA3IVS15* becomes hypomethylated while L1-*EPHA3IVS5* becomes hypermethylated. This finding may reflex impact of species of LINE-1 sequence on LINE-1' host gene regulation, as L1-*EPHA3IVS15* is active LINE-1 (L1ACA) while L1-*EPHA3IVS5* is inactive LINE-1 (L1GAG). However knockdown LINE-1 RNA for proves effect of specific LINE-1 RNA on each specific LINE-1 methylation as found in the pilot study is currently impractical technique because homology sequence of active LINE-1 within genome. In this step we can only assume that some specific LINE-1 RNA from LINE-1 promoter hypomethylation may regulate each specific LINE-1's host gene by epigenetics mechanism on intragenic LINE-1 promoter or gene body region.

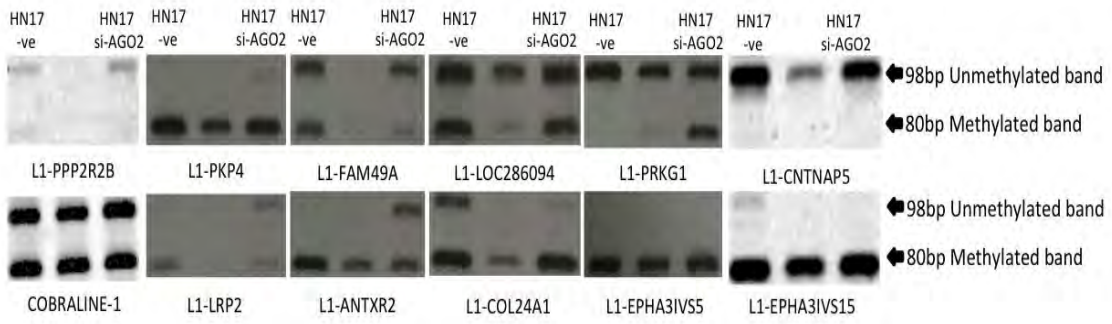

Figure 37: Specific LINE-1 methylation level of WSU-HN17 si-*AGO2* stable cell line compare to control cell in pilot study.

For confirm role of *AGO2* in regulate LINE-1's host gene by epigenetics machinery within gene body region as found in LINE-1 RNA effect from figure 36. Pilot COBRALINE-1 and CU-L1 PCRs studies in stable WSU-HN17 *AGO2* knockdown cell was performed and shown in figure 37. In stable knockdown *AGO2* protein cell, hypomethylatedon intragenic LINE-1 promoter include L1-*PPP2R2B*,L1-*PKP4*, L1-*LOC286094*, L1-*FAM49A*, L1-*LRP2* and L1-*ANTXR2*. And this cell, hypermethylated also occur with intragenic LINE-1 promoter such as L1-*PRKG1*, L1-*EPHA3IVS15* and L1-*COL24A1* while L1-*EPHA3IVS5* and L1-*CNTNAP5* seem to have stable level of DNA methylation in figure 36. However, it still need to repeat DNA methylation analysis of knockdown WSU-HNstable cells for confirm this possible role of LINE-1 RNA according to this pilot study. Until this step, it may conclude by this two pilot study, figure 35 and 36, for role of LINE-1 RNA and *AGO2* protein in regulate gene by epigenetics mechanism on LINE-1 promoter methylation or gene body methylation which depend on how LINE-1's host gene requirement in carcinogenesis. Is obvious that both LINE-1 RNA (LINE-1 promoter hypomethylation releasing transcript) and *AGO2* protein (nucleus RISC protein) found regulate LINE-1's host gene expression. It important to reveal more consequence of LINE-1

promoter hypomethylation via RISC protein since LINE-1 promoter hypomethylation associate to HNSCC carcinogenesis.

6. Whole genome effect of LINE-1 promoter hypomethylationand RISC protein in gene regulationwithin HNSCC cell line.

From work start, *EPHA3* was only one intragenic antisense LINE-1's host gene, that reflex role of LINE-1 RNA and RISC protein interaction. Term of antisense LINE-1 orientation seem to be main factor on identifytarget genes from LINE-1 RNA and RISC protein interaction. Many reports about LINE-1 promoter hypomethylation, focus on transcription level, can cause various events such as active LINE-1 RNA transcription, α-thalassemia via hemoglobin α-2 silence by antisense transcripts[181], cancer-specific chimeric transcript [27] and antisense transcription from antisense LINE-1 promoter [182]. Antisense transcripts function in many pathways including translational regulation, alternative splicing, RNA stablility, genomic imprinting and X-inactivation[183]. In human cell, natural antisense transcripts (NATs) are the inverse relation regulator of 15 % protein-encoding sense transcript in human transcriptome by epigenetics mechanism[184]. There is evidence of both long and short noncoding RNA in controlling DNA methylation status within genome such as *TXIS* and *P15* antisense respectively [185, 186]. In cancer cell, aberrant antisense transcript origin from reactivate transposable elements can cause abnormal DNA methylation pattern of oncogenes and tumor suppressor gene[181]. To suppress transposable element and control antisense transcription within eukaryote genome, cells have introduce non-coding RNA from repetitive region in term of endogenous small interference RNA (endo-siRNA) class which produce by Dicer1[112], Loqs-PD and D-elp1 and repress transposon by load on Ago2 protein, endo-siRNA partner molecule[130]. Endo-siRNA precursor molecule including long noncoding RNA molecule, longer than 200

nt size[130], transposon transcript and natural antisense transcripts[136]. By deep sequencing study for RNA loading on Ago2 protein, in S2 embryonic cell, endogenous siRNA originate form hypermethylated transposon and repeats region found at 26.6%, exonic origin at 26.6%, intronic origin at 17% [130], which may conclude that in somatic cell endo-siRNA and Ago2 molecule have major source from repetitive sequence and intragenic region. In many species including mammals, interaction between non-coding RNA [185] and RNAi mechanism was shown as controller of heterochromatin formation [187]. In mice decrease level of Dicer1 and Ago2 cell become decreased of endo-siRNA and increase of retrotransposon and protein-coding transcript that complementary to endo-siRNA [188]. In Arabidopsis, pseudogene-derived siRNA was reported in possibility in repress local transcription by RNA-Directed DNA methylation mechanism [189]. Both endo-siRNA and pseudogene-derived siRNA production involve with *DICER1*-like protein and RNA-dependent RNA polymerase (*RDRP*) [130, 190]. In relation to epigenetics pathway, gene can control by many mechanisms but DNA methylation on gene promoter and gene body region seem to be two main trails since many report support [69]. According to this briefly review, in final step, whole genome consequence of LINE-1 RNA from LINE-1 promoter hypomethylation and RISC protein will be determine on gene regulation through connect of RISC protein involved pathways and gene body mechanism with CU-DREAM protocol.

All 6 experiment sample group was design in order to show the essential of LINE-1 promoter hypomethylation and RISC core protein, *DICER1* is cytoplasmic part and *AGO2* is nucleus part, on gene expression controling within HNSCC cell line shown in table 3. According to table 3:(1) WSU-HN31 stable knockdown LINE-1 RNA for show impact of LINE-1 RNA from LINE-1 promoter hypomethylation (2) In vitro hypomethylation Hacat cell forstudy effect of DNA methylation on gene regulation in immortal human cell. (3) In vitro

hypomethylation WSU-HN17 will show effect from DNA methylation on regulate gene within HNSCC cell line. (4) Stable knockdown and (5) Transient knockdown of *DICER1* and *AGO2* in order to study role of RISC protein on gene regulation mechanism in HNSCC cell line. (6) Normal oral epithelial cell will represent normal gene expression pattern that comparing to WSU-HN cell line or Immortal human cell will refer to gene expression changing according to carcinogenesis or immortality process, respectively.

Sample_Plate	Sample_Group	Cell stage	description	Character in experiment
HN31 si-negative stable replicate 1	HN31 si-control	Tumor stage	replicate 1	Control in experiment 1
HN31 si-3LINE-1s stable replicate 1	HN31 si-3LINE-1	Tumor stage	replicate 1	Test in experiment 1
HN31 si-negative stable replicate 2	HN31 si-control	Tumor stage	replicate 2	Control in experiment 1
HN31 si-3LINE-1s stable replicate 2	HN31 si-3LINE-1	Tumor stage	replicate 2	Test in experiment 1
Hacat control replicate 3	Hacat control	Immortal stage	replicate 3	Control in experiment 2
Hacat aza treatment replicate 3	Hacat aza	Immortal stage	replicate 3	Test in experiment 2
Hacat control replicate 1	Hacat control	Immortal stage	replicate 1	Control in experiment 2
Hacat control replicate 2	Hacat control	Immortal stage	replicate 2	Control in experiment 2
Hacat aza treatment replicate 1	Hacat aza	Immortal stage	replicate 1	Test in experiment 2
Hacat aza treatment replicate 2	Hacat aza	Immortal stage	replicate 2	Test in experiment 2
HN17 control replicate 1	HN17 control	Tumor stage	replicate 1	Control in experiment 3
HN17 control replicate 2	HN17 control	Tumor stage	replicate 2	Control in experiment 3
HN17 aza treatment replicate 1	HN17 aza	Tumor stage	replicate 1	Test in experiment 3
HN17 aza treatment replicate 2	HN17 aza	Tumor stage	replicate 2	Test in experiment 3
HN17 control replicate 3	HN17 control	Tumor stage	replicate 3	Control in experiment 3
HN17 aza treatment replicate 3	HN17 aza	Tumor stage	replicate 3	Test in experiment 3
HN17 si-negative stable replicate 1	HN17 si-negative stable	Tumor stage	replicate 1	Control in experiment 4
HN17 si-negative stable replicate 2	HN17 si-negative stable	Tumor stage	replicate 2	Control in experiment 4
HN17 si-dicer1 stable replicate 1	HN17 si-dicer1 stable	Tumor stage	replicate 1	Test in experiment 4
HN17 si-dicer1 stable replicate 2	HN17 si-dicer1 stable	Tumor stage	replicate 2	Test in experiment 4
HN17 si-ago2 stable replicate 1	HN17 si-ago2 stable	Tumor stage	replicate 1	Test in experiment 4
HN17 si-ago2 stable replicate 2	HN17 si-ago2 stable	Tumor stage	replicate 2	Test in experiment 4
HN17 scramble 48h replicate 1	HN17 scramble 48h	Tumor stage	replicate 1	Control in experiment 5
NOE 4/10 scramble	Normal oral epithelial	Normal stage	replicate 1	Normal cell stage experiment
HN17 scramble 48h replicate 2	HN17 scramble 48h	Tumor stage	replicate 1	Control in experiment 5
HN17 si-dicer1 48h replicate 1	HN17 si-dicer1 48h	Tumor stage	replicate 2	Test in experiment 5
HN17 si-dicer1 48h replicate 2	HN17 si-dicer1 48h	Tumor stage	replicate 1	Test in experiment 5
HN17 si-ago2 48h replicate 1	HN17 si-ago248h	Tumor stage	replicate 2	Test in experiment 5
HN17 si-ago2 48h replicate 2	HN17 si-ago248h	Tumor stage	replicate 1	Test in experiment 5
Normal oral keratinocyte 19/7	Normal oral epithelial	Normal stage	replicate 2	Normal cell stage experiment
Normal oral keratinocyte 27/3	Normal oral epithelial	Normal stage	replicate 3	Normal cell stage experiment
HN17 control 1 at singapore	HN17 control	Tumor stage	replicate 4	Normalize microarray experiment

Table 3: Microarray design and sample purpose in each experiment.

All sample in whole genome expression microarray was check the hybridizaton quality control (QC) feature in order to estimate the microarray experiment quality and all requirement data in chip QC was shown in figure 38. As seen in figure 38, this microarray chip have good quality hybridization signal with control probe in chip that refer to

hybridization process and microarray chip preparation will correct protocol. Hybridization control probe low, med and high that each level refer to complementary level of each control probe, result show high > med > low as expect which confirm high complementary probe will bind on microarray chip better than med and low, respectively. Negative control probes both background and noice have low signal as noise (negative control stdev) is lower than background (negative control) which inform the background is at acceptable level because it higher than noise signal in signal detection by laser scanner. At low stringency probe control, PM (perfect match probe) have higher signal than MM2 (mismatch probe) which refer specificity of hybridization on microarray chip. Gene intensity panel show higher signal of housekeeping gene than all gene which refer to normal condition of gene expession pattern within cell that house keeping gene is critical gene that require in all cell than other type of genes. For biotin and high stringency control probe, higher level at biotin bar refer to correct process in washing for reduce perfect match non-specific sample in binding on probe in microarray chip. As shown result can confirm quality of microarray result in further analysis with low level of incorrect data. In order to analyse microarray data, after scanning process all raw data still need to be normalized and adjust by mathemetics method. By cut out noise signal at background level and normalized by accurate method all data will become comparable. Since each sample have difference background and comparison of microarray data with out normalization process will cause misinterpretation. Moreover, the correct normalization method will help in clustering similar sample together which will show correlation level among sample in the experiment. In figure 38, after background extaction and cubic spine normalization, all sample in the microarray experiment show correct clustering as confirm by high connection between sample in each experiment in table 1. According to result in figure 38 and 39, it confirm acceptable result by cubic spine normalization for further analysis.

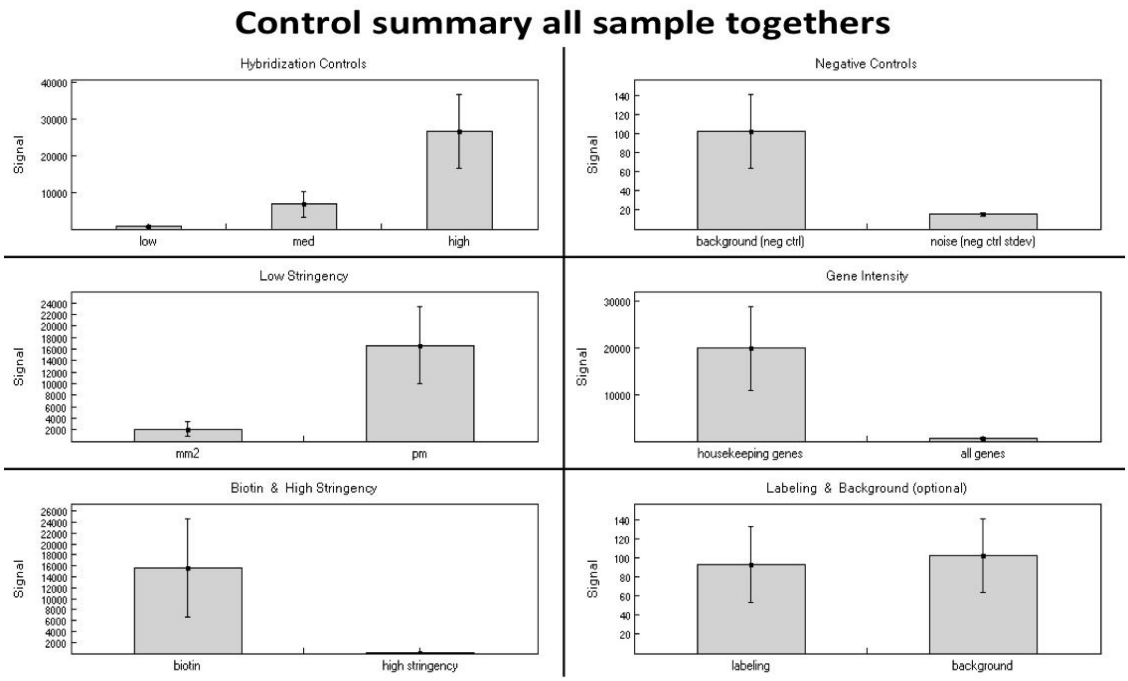

Figure 38 : Quality control of all samples in microarray experiment.

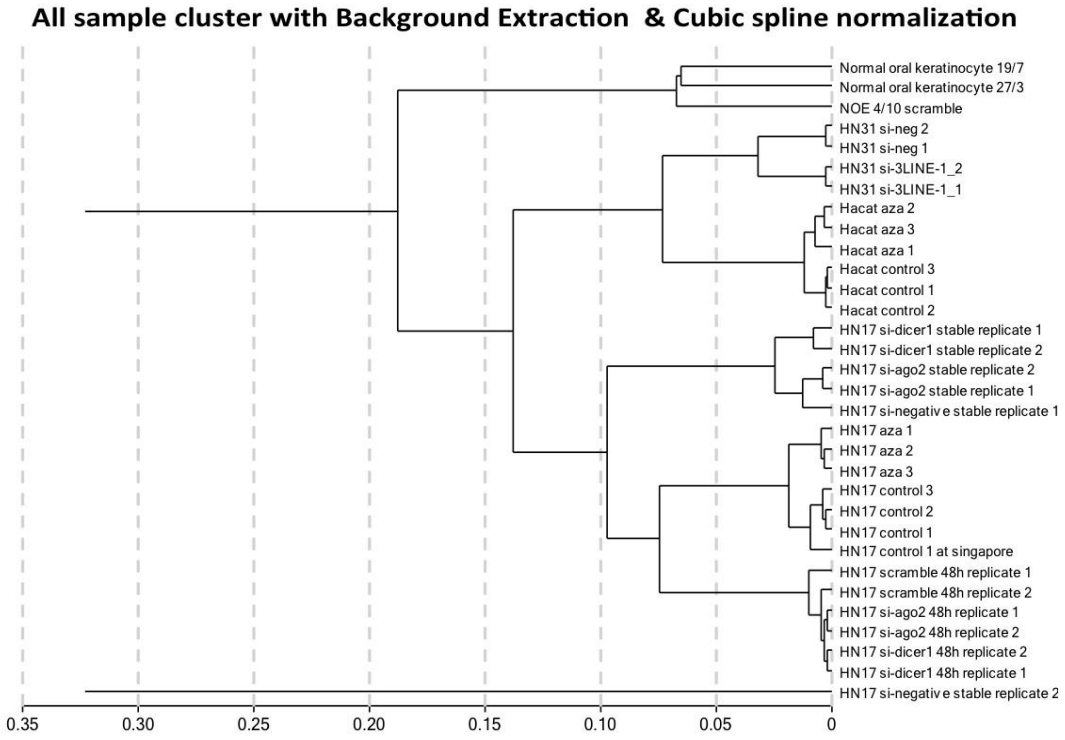

Figure 39 : Sample cluster analysis after data normalization from HNSCC sample expression array.

Because as this thesis start with LINE-1 methylation, analysis gene expression change should refer to existance of intrageneic LINE-1. Database of LINE-1 insertion within human genome name as “L1Base” [175] was used for identify gene with LINE-1 and gene without LINE-1. Bioinformatic tool in analysis based on the correlation between microarray experment in order to discover new interesting factor within each biological process name as “CU-DREAM”, which develop by Dr. Chatchawit Aporntewan and Prof. Apiwat Mutirangura [44]. Odd ratios and 95 % Confidence Interval (95% CI) was perform to study association factor in gene regulation according to gene number list in 2x2 table according to CU-DREAM concept.All CU-DREAM, Odd ratios, 95% CI and integrate intragenic LINE-1 database in CU-DREAM was calculated by Dr. Chatchawit Aporntewan, Department of Mathematics, Faculty of Science, Chulalongkorn University.

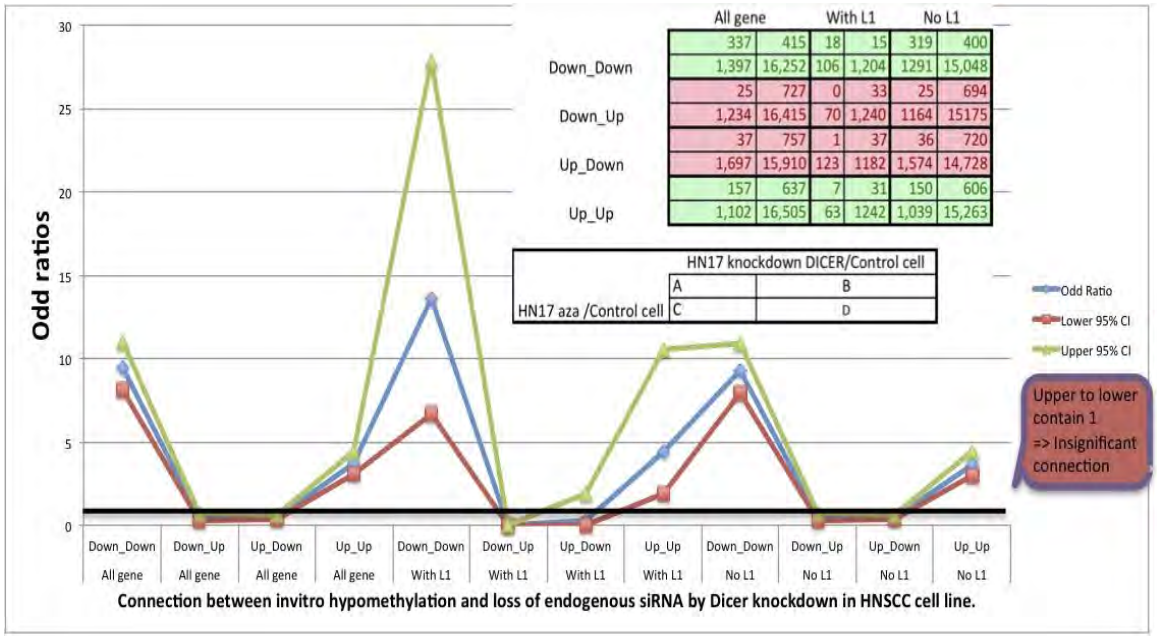

Figure 40: Intersection study of *DICER1* knockdown gene change to 5’-aza-2-deoxycytidine induced hypomethylation genes change in HNSCC cell line.

In figure 40, the selected correlation analysis between (A) HN17 knockdown *DICER1* per control cell and (B) HN17 aza per control cell line is choose for determine connection between *DICER1* involved pathway and DNA hypomethylation on gene regulation within HNSCC cell line. Down_Down category associate more than Up_Up that can refer to requirement of *DICER1* involved pathway will normally induce DNA hypermethylated status at gene body region (for induce gene expression) more than at gene promoter (for repress gene expression). All type of gene, with and without intragenic LINE-1, seem to control by role of this *DICER1* involved pathway.There is no significant connection in gene like *EPHA3* group that will up-regulated by RISC protein knockdown and down-regulated by 5'-aza-2-deoxycytidine induced hypomethylation. Pathway that regulate gene like *EPHA3* could require *AGO2* protein more than *DICER1* involved pathway.

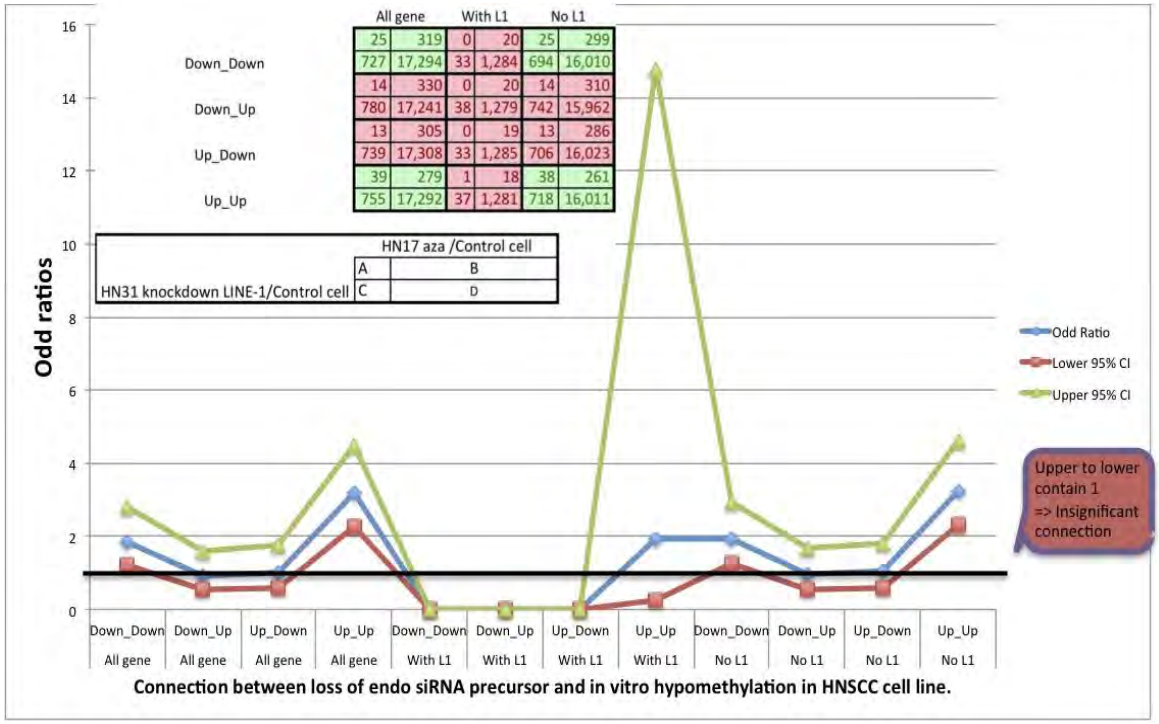

Figure 41 : Intersection study of knockdown LINE-1 RNA genes change to 5'-aza-2-deoxycytidine induced hypomethylation genes change in HNSCC cell line.

In figure 41, correlation analysis between (A) HN17 aza per control cell line and (B) HN31 knockdown LINE-1 per control cell is choose for determine connection LINE-1 RNA from LINE-1 promoter hypomethylation pathway and DNA hypomethylation on gene regulation within HNSCC cell line. Only in gene without intragenic LINE-1 groups, Up_Up category associate more than Down_Down category which indicate role of LINE-1 RNA from LINE-1 promoter hypomethylation will normally provoke DNA hypermethylation at gene promoter more than at gene body within HNSCC cell line, this strictly finding show that LINE-1 RNA have in trans machinery within genome. No significant of this intersection within gene with LINE-1 group can reflex effect of LINE-1 RNA from LINE-1 promoter hypomethylation on induce DNA methylation on gene promoter will strictly to gene without LINE-1 group. From this intersection, there is no gene like *EPHA3* group that will up-regulated by LINE-1 knockdown and down-regulated by 5'-aza-2-deoxycytidine induced hypomethylation, which show that gene that under control of LINE-1 RNA and LINE-1 promoter hypomethylation like *EPHA3* exist at limited number.

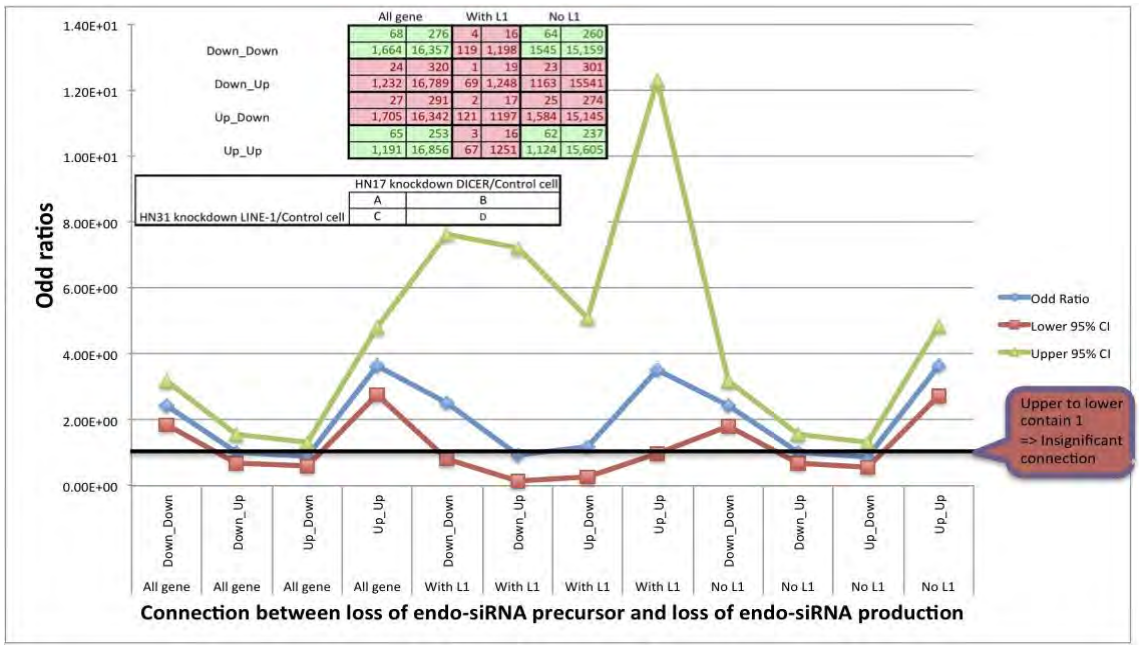

Figure 42: Intersection study of knockdown LINE-1 RNA genes change to *DICER1* knockdown genes change in HNSCC cell line.

In figure 42, correlation analysis between (A) HN17 knockdown DICER1 per control cell and (B) HN31 knockdown LINE-1 per control cell is choose for determine connection between *DICER1* and LINE-1 RNA involved pathway on gene regulation in HNSCC cell line. Only in gene without intragenic LINE-1,Up_Up category associate more than Down_Down category that indicate role of DICER1 and LINE-1 RNA involved pathway will normally repress gene expression than activate gene expression in HNSCC cell line. From this intersection, gene that will up-regulated by LINE-1 RNA and *DICER1* knockdown, which show that gene normally repress by LINE-1 RNA and *DICER1* like *EPHA3* exist but only obvious gene numbers in gene without LINE-1 group.

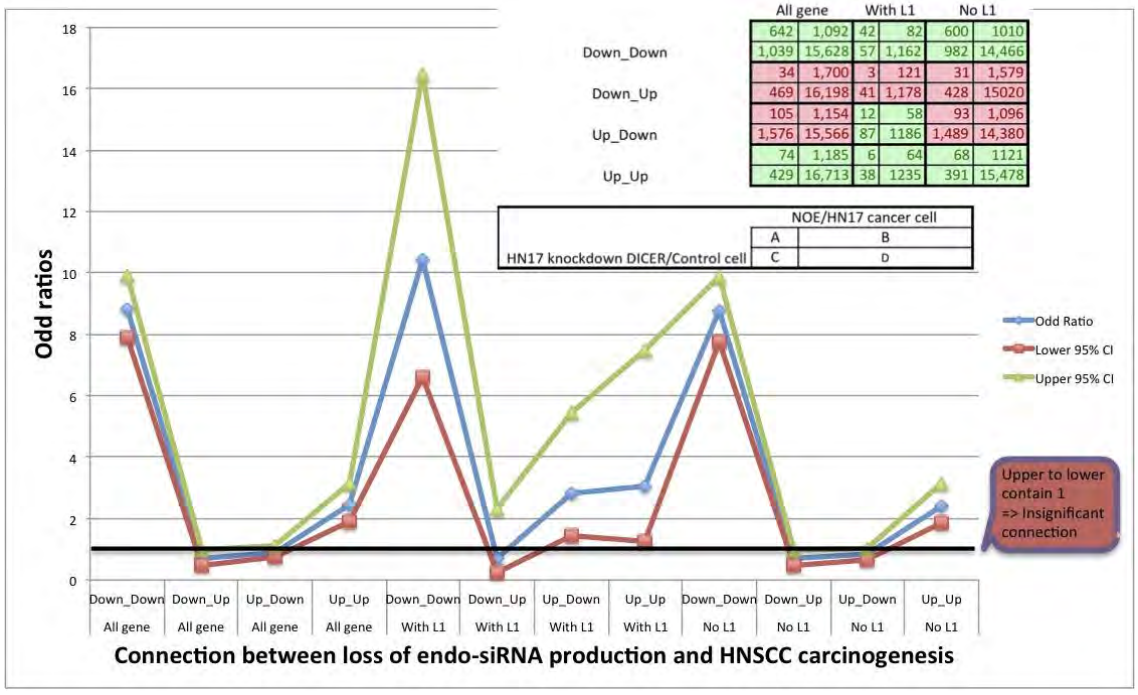

Figure 43: Intersection study of HNSCC carcinogenesis genes change to *DICER1* knockdown genes change in Head and Neck cell panel.

In figure 43, association study between (A) Normal oral epithelial per HN17 cell and (B) HN17 knockdown *DICER1* per control cell choose for determine connection between *DICER1* involved pathway and gene expression in HNSCC carcinogenesis. Down_Down category associate more than Up_Up category in all gene types, with and without intragenic LINE-1 indicate that role of *DICER1* involved pathway will prefer to activate gene more than repress gene for HNSCC carcinogenesis. If refer to global LINE-1 promoter hypomethylation status of WSU-HN17 when compare to NOE samples, *DICER1* involved pathway prefer to induce DNA hypermethylation on gene body region more than at gene promoter methylation during HNSCC carcinogenesis. However, this explaination is just possability that out of evidence in methylation level. Moreover in some genes with intragenic LINE-1, Up_Down panel also show significant correlation which reveal specific role of *DICER1* involved pathway can activate gene that normally repress by DNA hypermethylation at gene promoter during HNSCC carcinogenesis.

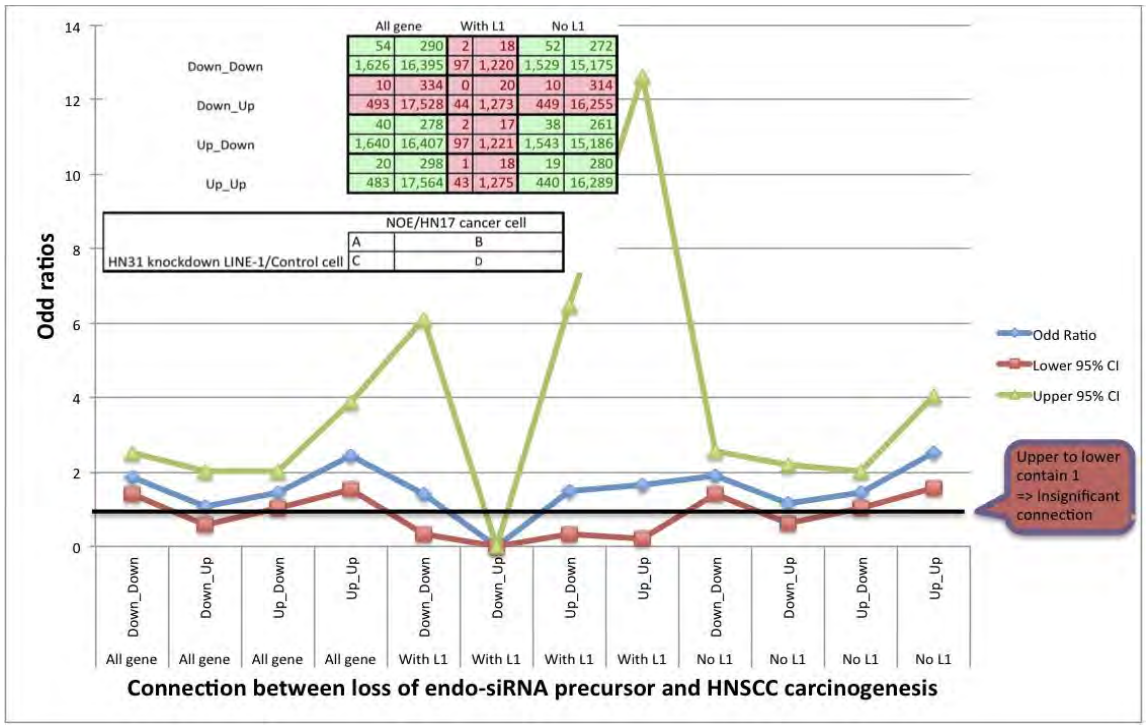

Figure 44: Intersection study of HNSCC carcinogenesis genes change to LINE-1 knockdown genes change in Head and Neck cell panel.

In figure 44, association study between (A) Normal oral epithelial per HN17 cell and (B) HN31 knockdown LINE-1 per control cell selected for analysis the association between LINE-1 RNA involved pathway on gene expression in HNSCC carcinogenesis.Only gene without intragenic LINE-1 group, Up_Up category associate more than Down_Down category that indicate role of LINE-1 RNA involved pathway will prefer to repress gene than activate gene in HNSCC carcinogenesis. If refer to global LINE-1 promoter hypomethylation status of WSU-HN17 when compare to NOE samples, LINE-1 RNA involved pathway prefer to induce DNA hypermethylation on gene promoter region more than at gene body methylation during HNSCC carcinogenesis. However, this explaination is just possability that out of evidence in methylation level. Moreover in gene without intragenic LINE-1, Up_Down panel also show significant correlation which reveal role of LINE-1 RNA involved pathway can activategene that normally repress by DNA hypermethylation at gene promoter duringHNSCC carcinogenesis, this strictly finding show that LINE-1 RNA have in trans machinery within genome as found in HNSCC cell line. Lack of gene that repress by LINE-1 involved pathway and up-regulated by DNA hypermethylation on gene body region during HNSCC carcinogenesis, especially in gene with intragenic LINE-1 group, can confirm limitation of genes like *EPHA3* within HNSCC genome.

LINE-1 promoter hypomethylation induce LINE-1 RNA expression that may become endo-siRNA precursor in endo-siRNA pathway. And *DICER1* require for produce endo-siRNA while *AGO2* is partner molecule of endo-siRNA as purpose in figure 45. Retrotransposon element within genome was report in strongly associate to gene promoter methylation which may be a critical factor in epigenetics regulation within in both normal

and abnormal cell stage [191]. This purpose mechanism will be interesting cooperation between endo-siRNA pathway and LINE-1 hypomethylation which should exist and important for gene regulation. DNA methylation can regulate gene in many region but main target site should be gene promoter and gene body [69]. By all statistic analysis, role ofLINE-1 promoter hypomethylation on gene regulation through RISC protein, *DICER1*, exist in HNSCC cell panel. *DICER1* involved pathway caninduce DNA hypermethylated status at gene body region for regulates all type of gene, with and without intragenic LINE-1, within HNSCC cell and HNSCC carcinogenesis. According to in trans purpose of LINE-1 RNA, some gene without intragenic LINE-1 could control by LINE-1 RNA from LINE-1 promoter hypomethylation.LINE-1 RNA involved pathway will normally induce DNA methylation on gene promoter within HNSCC cell and HNSCC carcinogenesis. *DICER1* involved pathway through LINE-1 RNA involved pathway will normally repress gene without LINE-1 in HNSCC cell line, one more evidence of in tran of LINE-1 RNA function.

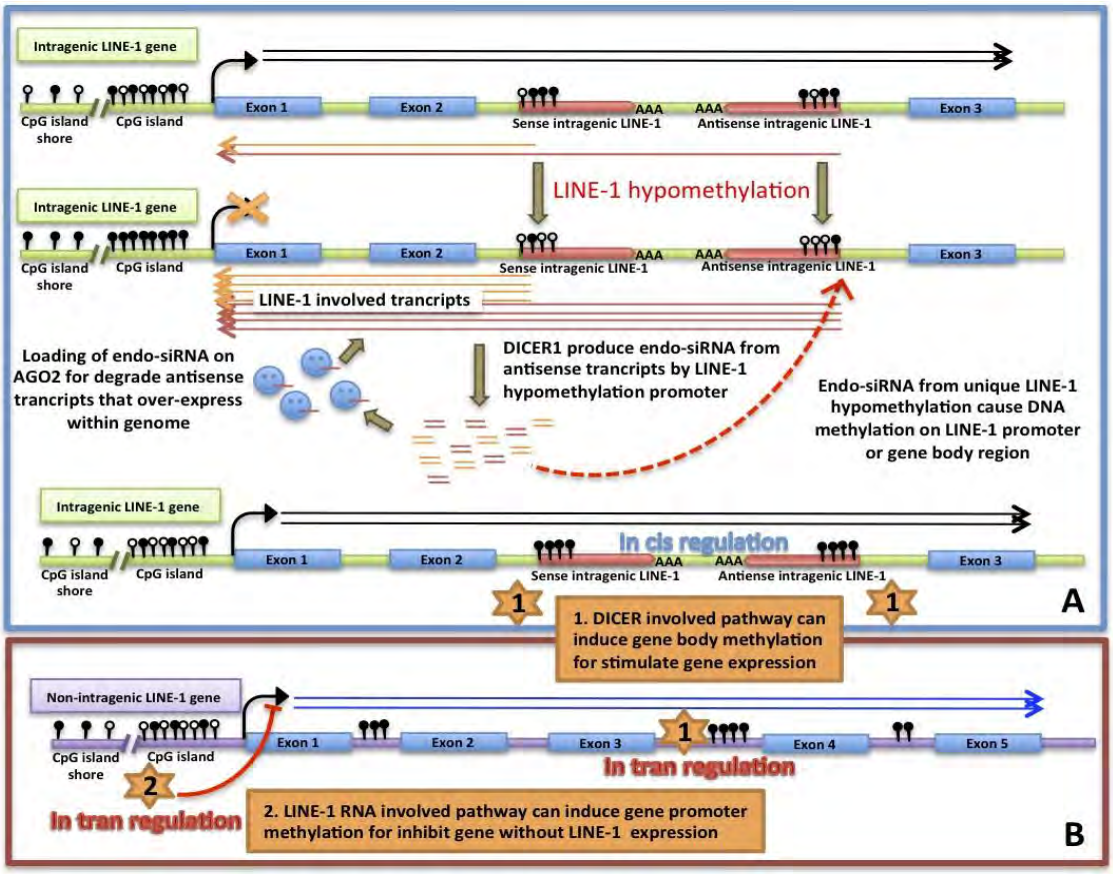

Figure 45: The possible mechanism that reflex impact of *DICER1* or LINE-1 RNA involved pathway both (A) in cis regulation and (B) in tran regulation. According to this illustrate, it may support finding from intersection study among whole genome expression study for impact of LINE-1 RNA, *DICER1, AGO2* and LINE-1 promoter hypomethylation in HNSCC cell panel.

CHAPTER V

DISCUSSION

Exploiting pattern of LINE-1 methylation status in Head and Neck squamous cell line in first part of this thesis with COBRALINE-1 and CU-L1 technique from Chalitchagorn K. et al. [7] and Phokaew C. et al [171]. Unique intragenic LINE-1 promoter methylation determine by each LINE-1's location within genome, according to location of LINE-1.Intragenic LINE-1 methylation status reflex the impact of each LINE-1's host gene within genome in carcinogenesis or tissue differentiation process while LINE-1 intergenic can cause genome instability [192-194]. In normal oral epithelial and white blood cell, there is specific level of each unique LINE-1 among 17 selected LINE-1 loci. For example, higher DNA methylation of L1-*CNTNAP5* in white blood cell sample than normal oral epithelial in graph A figure 21 may support by Pagnamenta AT et, al. work which found no exonic deletion of CNTNAP5 in normal samplebut also undetectable *CNTNAP5* RNA in blood [195].For discover epigenetics in whole genome phenomenon within cell, using CU-L1 method which base on bisulfite treatment DNA and restriction fragment length polymorphism (RFLP) could have efficiency as much as 1.4 million HpaII methyl-sensitive site cutting method in genomic scale [177]. From all result of CU-L1 and COBRALINE-1 result, LINE-1 methylationlevel altering in HNSCC cell compare to normal cell, most of LINE-1 becomes hypomethylated but some of LINE-1 becomes hypermethylated such as L1-*CNTNAP5* in WSU-HN8 cell line. Each intragenicLINE-1 promoter methylation associate to others LINE-1 promoter from remain 16 CU-L1 PCRs within HNSCC cell more than in normal cell. Most correlate CU-L1 associate is between CU-L1-*EPHA3IVS5* and CU-L1-*EPHA3IVS15*, different intron but locate in same gene, Pearson correlation at r = 0.913 show synchronize of gene body DNA methylation pattern. Intragenic LINE-1 promoter methylation

detect with CU-L1 method can show many interesting molecular data that may lead into individual genome change during carcinogenesis and it possibly that CU-L1 can be one of important marker for personal therapy in the future [196]. Support this concept by the new generation of sequencing, researcher can discover never reported LINE-1 specific between each individual [197] and there also have report about individual LINE-1 expression profile in human somatic cell [16] that make single LINE-1's impact on individual genome project in the future is more precise.

Difference of CU-L1 methylation level reflexes the essential of LINE-1 existence within each region. All 17 CU-L1 PCRs design from intragenic full length LINE-1 that sequence match to active LINE-1.2 (M80343), which will be an easy tool in studying impact of each LINE-1 on LINE-1's host gene. After measure all 16 LINE-1's host gene expression and analyze association to each intragenic CU-L1, only *EPHA3* and *PPP2R2B* that association to intragenic antisense LINE-1 promoter wihin gene body region methylation. Hypermethylated status of CU-L1-*EPHA3IVS5*, CU-L1-EPHA3IVS15 and CU-L1-*PPP2R2B* relate to higher expression of *EPHA3* and *PPP2R2B*, respectively.This finding match to the previous knowledge, the transcription process will prefer to start on loss of methylation promoter genes [198] with hypermethylated gene body condition [177]. DNA methylation be the important epigenetics factor in regulated gene, which prove in many studies of global demethylated gene promoter by 5-aza-2'deoxycytidine drug can cause expression of miRNA precursor [199], microRNAs [200], tumor suppressor gene[201], transcription factor [202] and transposon [203]. In this thesis, demethylating WSU-HN17 cell show possibility effect ofintragenic LINE-1 promoter hypomethylation (gene body region) on LINE-1's host gene expression, hypomethylation of CU-L1-*EPHA3IVS15* and COBRALINE-1 may cause down regulated of *EPHA3* gene at significant level. To be more specific on locally evidence, expression of intragenic LINE-1, L1-*EPHA3IVS15* expression PCR was established from

concept of 3' transduction sequence of some active LINE-1 [204]. Interestingly, reverse association between L1-*EPHA3IVS15* and CU-L1-*EPHA3* also found which will confirm concept of gene body hypermethylation in highly expressed gene[177]. Intragenic LINE-1 methylation, CU-L1, can reflex gene body methylation status and EPHA3 is example of gene that control by gene body hypermethylation in HNSCC cell line.

Impact of LINE-1 insertion on gene regulation is hazardous for normal genome such asin X-linked retinitis pigmentosa (XLRP) case that LINE-1 retrotransposition cause mutations in gene RP2[205], exon shuffling cause by LINE-1 retrotransposition[206], trans-splicing in ER-like variant contain LINE-1 sequence in human breast cancer[14]. Intact full length intragenic LINE-1 that still conserve along with human evolution should have reliable reason in involving with LINE-1's host gene. Since DNA methylation is the factor that control LINE-1 transcription within human cell [207] as also proved in this thesis in both whole genome and specific LINE-1. An active endogenous full length LINE-1 transcripts was detected in many kind of normal tissue and cancer cell lines[23] including Hela cell line which was hypomethylated genome by COBRALINE-1 method[7]. In demethylated MCF-7 cancer cell line by 5-aza-2'-deoxycytidine aslo show higher LINE-1 transcription begin from 5'UTR promoter [27]. Previously, LINE-1 transcription was mainly assumed to involve with retrotransposition[208]but scientists also know role of retrotransposon RNA in repress retrotransposon itself through endogenous siRNA form in RISC complex[112]. There is only 0.01 – 0.05 % for LINE-1 Retrotransposition event within genome[209], it prove that main purpose of LINE-1 RNA within genome isn't retrotransposition. As in this thesis, intragenic LINE-1 hypomethylation relate to host gene expression, there is possibility role of intragenic LINE-1 RNA on host gene regulation after LINE-1 demethylated. Influence of specific intragenic LINE-1 on its hosts gene expressionis hard perform because of homology among each active LINE-1 however with construct for RNA interference (RNAi) target on conserved

region in most report active LINE-1 sequences should covering on all active LINE-1 within genome. Remarkably, WSU-HN31 stable cellline which transfect with 3 RNAi construct that target on 3 conserve sequence of active LINE-1 have visibly EPHA3 up-regulation.Moreover, knockdown LINE-1 in WSU-HN31 also can alter expression level of *ANTXR2, PPP2R2B, FAM49A, CNTNAP5* and PKP4. In conclusion from LINE-1's host gene expression change, intragenic LINE-1 RNA that should release by LINE-1 promoter hypomethylation in HNSCC cell line have effect on LINE-1's host gene expression, as loss of LINE-1 expression associate with LINE-1's host gene expression change.

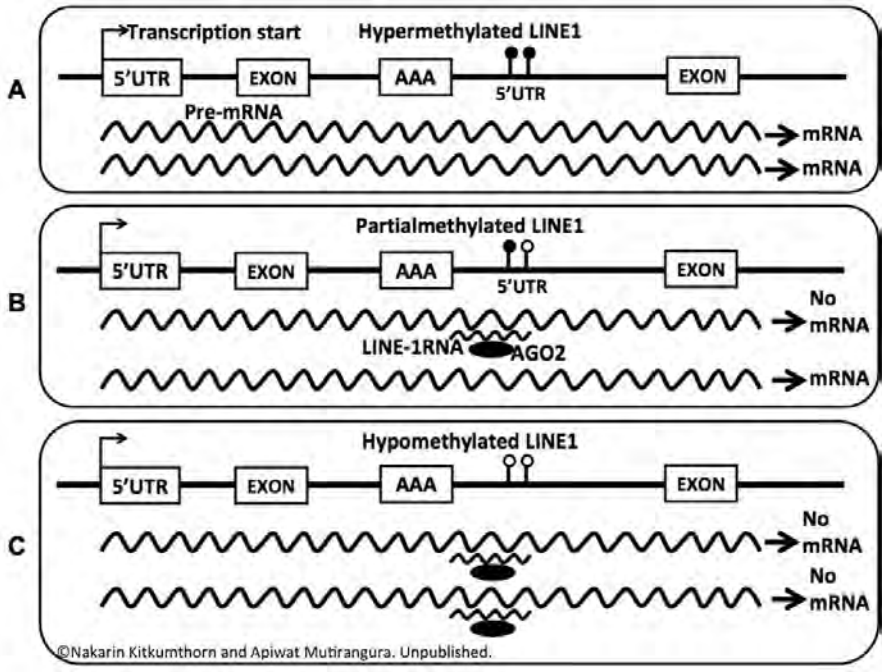

Figure 46: Intragenic hypomethylated LINE-1 represses host gene expression via *AGO2* [210].

From this impact of LINE-1 RNA finding, both RNA involved mechanism for gene regulation require RISC protein, through RNAi mechanism or Epigenetics mechanism. By knockdown *AGO2*, nucleus RISC protein, loss of *AGO2* experiment show up-regulated of *EPHA3* similar to knockdown LINE-1 within HNSCC cell line. Both *AGO2* protein and LINE-1

RNA have impact on LINE-1's host gene expression. Moreover, knockdown *AGO2* can induce LINE-1 expression which may relate to concept of RNAi mechanism, endogenous pathways that cell use for control LINE-1 transcipt, by target on double strand structure from both sense and antisense LINE-1 promoter.Interesting, role of intragenic LINE-1 hypomethylation in repressing LINE-1 host gene via *AGO2* protein in human cancer cell line by Prof. Apiwat Mutirangura [26] as shown in figure 46 [210], is an evidence for support impact of RNAi mechanism and LINE-1 RNA. In order to prove interaction between *AGO2* and LINE-1 RNA in RNAi mechanism, RNA-immunoprecipitate was performing by using *AGO2* antibody. Binding of RNA on *AGO2* confirm with reasonable controls, TRIM18 as positive control with intragenic LINE-1 and miRNA binding site, *PENSEN* and *BCKDK* is negative control because both lack of intragenic LINE-1 and miRNA binding site. More interesting from finding interaction between LINE-1 RNA and *AGO2* protein, *AGO2* protein also found have specific binding on DNA sequence from LINE-1 promoter within HNSCC cell line. This exciting result can confirm that LINE-1 RNA involved with *AGO2* in LINE-1's host gene regulation through mechanism that action on LINE-1 promoter sequence or gene body region. Epigenetics related mechanism was only remaining possible explanation in LINE-1 RNA and *AGO2* protein regulate LINE-1's host gene.Additionally, evidence of stable knockdown LINE-1 growth rate higher than control cell and knockdown *AGO2* growth very slow when compare to control cell in HNSCC cell line, can reveal impact of each factor on cell growth in previous study that cell proliferation enhance by *AGO2* [211] and LINE-1 RNA cause cell cycle abnormal [138]. The requirement of *AGO2* protein was report in Human umbilical vein endothelial cells (HUVECs) by si-*AGO2* HUVECs cell have growth retard and also repressed in angiogenesis events [212].

Many previous studies may reflex possible role of LINE-1 RNA on LINE-1's host gene through epigenetics mechanism. In 2008, Martienssen RA et, al can prove role of

transposon small RNA delete DNA methylation from transposon region in Arabidopsis[213]. Role of RNA molecule both short and long non-coding RNA can guide de novo DNA methylation factor, DNMT3a , into its binding region[214]. RNA-Directed DNA methylation phenomenon in human is *HBA2* gene which promoter DNA hypermethylaton occur by pathogenic expression of antisense *LUC7L-HBA2* chimeric thanscript from deletion of 16p13.3 that removes *HBA1* and *HBQ* in case of an alpha-thalassemia case [143].RNA-Directed DNA methylation or RNA activation found in many genes, with requirement of *AGO2* protein and gene promoter specific double stand RNA particle in activating gene expression via induce euchromatin status of gene promoter [160].Here in this thesis, specific LINE-1 RNA and *AGO2* protein may regulate LINE-1's host gene through epigenetics mechanism from LINE-1 promoter DNA and *AGO2* protein interaction was found. CU-L1s methylation pilot study of WSU-HN31 LINE-1 knockdown show changing pattern including CU-L1-*EPHA3IVS5* hypomethylated and hypermethylated of CU-L1-*ANTXR2*, CU-L1-*EPHA3IVS15*, CU-L1-*FAM49A* and CU-L1-*PKP4*. CU-L1s methylation pilot study of WSU-HN17 *AGO2* knockdown stable cell line also found LINE-1 methylation change including CU-L1-*COL24A1* and CU-L1-*EPHA3IVS15* hypermethylated and hypomethylated occur with L1-*PPP2R2B*,L1-*PKP4*, L1-*LOC286094,* L1-*FAM49A*, L1-*LRP2* and L1-*ANTXR2*. According to pilot study result, LINE-1 RNA or *AGO2* knockdown cause many LINE-1 promoter methylation change that show epigenetics mechanism within gene body region from LINE-1 RNA and *AGO2* protein interaction in HNSCC cell line. Role of *AGO2* and LINE-1 RNAas the epigenetic regulator on gene body region in HNSCC cell line, *AGO2* binding on 5'UTR of LINE-1 (LINE-1 promoter) also proved in this thesis.

Involvement of RISC protein and LINE-1 RNA alsonoticeable through the endo-siRNA pathway which cell create for control over express retrotransposon transcript within cell. As incorrect transcriptional start site associate to antisense promoter on 5'UTR of LINE-

1 [215] and 5'-aza-2-deoxycytidine induce hypomethylation on gene body region will stable than gene promoter region [216], LINE-1 antisene promoter hypomethylation within gene body region will cause long term effect on gene regulation. Incorrect transcriptional start site from LINE-1 promoter may become target of endo-siRNA pathway within genome.Thepurpose mechanism, as in figure 44, link between endo-siRNA pathway and gene body methylation should exist and important for genome balance mechanism according to essential of each factor in this mechanism. LINE-1 hypomethylation correlate to HNSCC carcinogenesis while *DICER1* cause cause hypomethylation in centromeric repeat sequence and result in activate those repetitive elements within genome [217]. According to final part of this project, role of LINE-1 promoter hypomethylation on gene regulation through *DICER-1* really exist within HNSCC cell line. *DICER1* involved pathways, may refer to endo-siRNA production, prefer to induce DNA hypermehtylation within gene body for induce correct gene expression within geneome. LINE-1 RNA, as endo-siRNA precursor, have in trans mechanism for control gene without intragenic LINE-1 by induce DNA methylation on gene promoter region in HNSCC cell panel. In trans function of LINE-1 RNA on gene without intragenic LINE-1 may explain bynatureof endo-siRNA pathwaythat some endo-siRNA precursor may be a double stand RNA tran formation by two independent transcripts [134]. During HNSCC carcinogenesis, somegenes which repressed by DNA hypermethylated promoter gene can induce for up-regulated by LINE-1 RNA in gene without intragenic LINE-1 and *DICER1* in gene with intragenic LINE-1.These purpose mechanisms that refer to role of DNA hypomethylation, HNSCC carcinogenesis, LINE-1 RNA and *DICER1* according to CU-DREAM study in figure 44 may be possible candidate pathway to clarify consequence of LINE-1 promoter hypomethylation in HNSCC cell.

Directions of the determination mechanism need to be clarify more by multiple intersection bioinformatics method and wet lab in order to confirm the existence and the essential of this mechanism within HNSCC genome. There should still have many factors that involve in endo-siRNA pathway from LINE-1 hypomethylation for gene regulation, which stillneed to illuminatemore as much as the uncertain endo-siRNA pathway in human cell. Endo-siRNA pathway in somatic cell prefers to regulate the intragenic regulation as much as transposon sequence which seem difference from piRNA pathway in germ line cell[130]. As endo-siRNA should presense in human global hypomethylated genome such as in pathogenesis step, endo-siRNA pathway from LINE-1 hypomethylation for gene regulation should study more in human pathogenic condition including cancer, neurological disorder and autoimmune disease[5]. Effect of endo-siRNA pathway from LINE-1 hypomethylation for gene regulation should study in the consequence event from LINE-1 hypomethylation including aberrant oncogenic expression, karyotypic instability[218] and LINE-1 reactivation induced transcriptional deregulation, genome disorganization, DNA break, various mutations, higher recombination frequency and chromosome instability[219]. In conclusion, LINE-1 promoter hypomethylation event may be center factor in HNSCC carcinogenesis mechanism through endo-siRNA pathway from LINE-1 hypomethylation for maintain and regulation genome.

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APPENDIX

APPENDIX: OLIGONUCLEOTIDES SEQUENCES AND PCR CONDITIONs

COBRALINE-1 primer sequence and PCR condition

Amplification with Qiagen's HotStarTaq DNA Polymerase start with first denaturation at 95°C 15 minutes, 35 cycles of 95°C 1 minute, 53°C 1 minute, 72°C 1 minute follow with final extension at 72°C 7 minutes and hold at room temperature. Primer sequence including F COBRALINE-1 = CCGTAAGGGGTTAGGGAGTTTTT and R COBRALINE-1 = RTAAAACCCTCCRAACCAAATATAAA.

Details of 17 unique LINE-1s for CU-L1 PCRs.

No	Gene	Map	locus	Intron sequence	LINE-1 site	LINE-1 start-end	LINE-1 orientation	LINE-1 Ta
1	COL24A1 collagen, type XXIV, alpha 1	1p22.3	intron 24	195146..195190,245006..245059	229,373-223,325	5-6,050	Antisense	ACAG
2	FAM49A family with sequence similarity 49,	2p24.3-2p24.2	intron 2	41813..41968,77703..77782	66,072-72,120	5-6,043	Sense	ACAG
3	CNTNAP5 contactin associated protein-like	2q14.3	intron 11	537931..538037,584515..584634	573,886-567,835	4-6,031	Antisense	GAGA
4	PKP4 plakophilin 4	2q24.1	intron 1	1..255,76217..76353	65,653-71,700	2-6,041	Sense	GAGA
5	LRP2 low density lipoprotein-related protein	2q31.1	intron 19	106268..106398,114989..115126	113,883-107,834	3-6,038	Antisense	ACAG
6	MGC42174 hypothetical protein MGC42174	2q37.1	intron 8	174789..175036,201776..201949	193,548-187,500	1-6,039	Antisense	ACAG
7	EPHA3 intron 5 EPH receptor A3	3p11.1	intron 5	234232..234567,288314..288438	244,122-238,079	5-6,045	Antisense	GAGA
8	EPHA3 intron 15 EPH receptor A3	3p11.1	intron 15	342654..342847,364941..365096	359,335-353,289	4-6,038	Antisense	ACAG
9	ANTXR2 anthrax toxin receptor 2	4q21.21	intron 16	95399..95479,165633..167529	135,388-129,338	6-6,050	Antisense	ACAG
10	SPOCK3 sparc/osteonectin, cwcv and kazal-	4q32.3	intron 7	448383..448502,485943..486164	484,799-478,750	27-6,050	Antisense	ACAG
11	LOC133993 hypothetical LOC133993	5q12.3	intron 3	1..29530	24,756-18,710	8-6,040	Antisense	GAGA
12	PPP2R2B protein phosphatase 2 (formerly 2	5q32	intron 8	443037..443201,480992..481161	471,974-465,923	8-6,050	Antisense	ACGG
13	LOC286094 hypothetical protein LOC28609	8q24.22	intron 1	1..65577	24,962-31,008	4-6,048	Sense	GAGA
14	PRKG1 protein kinase, cGMP-dependent, t	10q21.1	intron 9	1087357..1087431,1177038..1177134	1,158,767-1,164,816	33-6,045	Sense	AAGA
15	ADAMTS20 a disintegrin-like and metallopr	12q12	intron 7	61487..61527,83217..83322	80,951-74,900	3-6,036	Antisense	GAGA
16	CDH8 cadherin 8, type 2	16q21	intron 7	218401..218654,246651..246787	234,682-228,630	5-6,050	Antisense	AAGA
17	LOC284395 hypothetical protein LOC28439	19q12	intron 1	1..325,96939..97018	66,461-60,412	3-5,901	Antisense	AAAG

COBRA unique to LINE-1 (CU-L1) primer sequence and PCR condition

Gene	Primer of COBRA unique to LINE 1	CU L1 PCR product	Primer of RT PCR	RT PCR product	Location of RT PCR
COL24A1 ex	5'CUL1-COL24A1=GTTAAAGCGTTAAGAATGTGTGTAG	336 bp	5'RT-COL24A1=GCAGAAGTAGGAGATCAAGGAAA	94 bp	195146-195190,245906-245954
	3'CL1=GTAAAACCCTCCGAACCAAATATAAA		3'RT-COL24A1=CGCCAATGCTTCCAGTCAT		
FAM49A fa	5'CUL1-FAM49A=GTTTTAAAAAAAAATAAAGTTGG	385 bp	5'RT-FAM49A=GCCACCGTCCTGATTTGG	188 bp	41860-41968,77703-77781
	3'CL1=GTAAAACCCTCCGAACCAAATATAAA		3'RT-FAM49A=TTCAAAATCCAGCAAAAAGTGT		
CNTNAP5 c	5'CUL1 CNTNAP5=GATTAAATTTTAATTGAATTAGAG	403 bp	5'RT-CNTNAP5=CAAGTTACACATGGTGCCACCTG	150 bp	538008-538037,584515-584634
	3'CL1=GTAAAACCCTCCGAACCAAATATAAA		3'RT-CNTNAP5=CAGTGATATTGCAGTACACCTGGA		
PKP4 plako	5'CUL1-PKP4 =GGTATGATTTTAAAAAAAGAGAT	392 bp	5'RT-PKP4=CTGATCCCTGGAGCGACG	128 bp	215-255,76214-76303
	3'CL1=GTAAAACCCTCCGAACCAAATATAAA		3'RT-PKP4=TGCCTGGGCCAGTGGAG		
LRIF2 low de	5'CUL1-LRIF2=GGTATATAATTTTTATGGTGTTG	435 bp	5'RT-LRIF2=ATGACACATCCGTTTGGACTTG	109 bp	106365-106398,114989-115063
	3'CL1=GTAAAACCCTCCGAACCAAATATAAA		3'RT-LRIF2=CTGTCATTTCTCCACCATCTGC		
MGC42174	5'CUL1-MGC42=ATTGAGGTGTATTAAGAGATGGA	553 bp	5'RT-MGC42174=TCGACTGGAAGGAGGACTGC	115 bp	17503-175036,21776-201856
	3'CL1=GTAAAACCCTCCGAACCAAATATAAA		3'RT-MGC42174=AAATCCACGCCATACTCTGTTA		
EPHA3 intron	5'CUL1 EPHA3in5=TGTTATTGGAATATATGGAGATT	386 bp	5'RT-EPHA3=CTCCCTCGACAGTTTGGACTC	196 bp	234408-234567,288314-288349
	3'CL1=GTAAAACCCTCCGAACCAAATATAAA		3'RT-EPHA3=TCCGATCTTTCTTAATCGTCAG		
EPHA3 intron	5'CUL1 EPHA3in15=TAAGGATAAAAATTTTTGAAGTT	464 bp	5'RT-EPHA3=CTCCCTCGACAGTTTGGACTC	196 bp	234408-234567,288314-288349
	3'CL1=GTAAAACCCTCCGAACCAAATATAAA		3'RT-EPHA3=TCCGATCTTTCTTAATCGTCAG		
ANTXR2 an	5'CUL1-ANTXR=TATTGAGTATTAATTATGTATTTAGTAT	416 bp	5'RT-ANTXR2=TTGATGCTCTCTGGGCTTTG	127 bp	95406-95479,165633-165685
	3'CL1=GTAAAACCCTCCGAACCAAATATAAA		3'RT-ANTXR2=TTCCTGCTTCCCTTTACTGA		
SPOCK3 sp	5'CUL1-SPOCK3=GTGTAATTTTTTTAGATTTTGTAG	492 bp	5'RT-SPOCK3=CCTGGAGTTCAGGGAAGTGG	220 bp	442312-442421,479862-479971
	3'CL1=GTAAAACCCTCCGAACCAAATATAAA		3'RT-SPOCK3=AATGCTTCTGAGCTCTGACTGG		
LOC133993	5'CUL1-LOC133993=TTAGGATATTTTTTATTTTGGGA	446 bp	5'RT-LOC133993=TCAAAGATTAGACACTGGCCTTA	105 bp	5299-5356,27580-27627
	3'CL1=GTAAAACCCTCCGAACCAAATATAAA		3'RT-LOC133993=GATGCTGCATTTGAATTCAGG		
PPP2R2B pa	5'CUL1 PPP2R2B=GGGGAAAAATTGAAAGTT	590 bp	5'RT-PPP2R2B=GGACATTAAGCCAGCCAACA	267 bp	443044-443201,480992-481100
	3'CL1=GTAAAACCCTCCGAACCAAATATAAA		3'RT-PPP2R2B=TCCCTGGTCATGATATACCTCC		
LOC286094	5'CUL1 LOC286094=TATGTAAGTATGGAAATTTGAGG	429 bp	5'RT-LOC286094=CCTCAATGAATCCTGAGGACAG	337 bp	78-387,56992-57058
	3'CL1=GTAAAACCCTCCGAACCAAATATAAA		3'RT-LOC286094=CAAATGCAGAGACCTGGAGTTG		
PREG1 prot	5'CUL1 PREG1=AAAATTTTTAGTTGTTAAATGG	374 bp	5'RT-PREG1=TTTGATTGGAGGGCTGGAT	89 bp	1087369-1087431,1177038-1177063
	3'CL1=GTAAAACCCTCCGAACCAAATATAAA		3'RT-PREG1=CGAAGAAAGCCGCTTCAG		
ADAMTS20	5'CUL1-ADAMTS20=AAGTTGTGTGGTTTTTTGTAAAT	468 bp	5'RT-ADAMTS20=ATTTGTTCATCTAAAGAGAAATGTAA	107 bp	61494-61527,83217-83289
	3'CL1=GTAAAACCCTCCGAACCAAATATAAA		3'RT-ADAMTS20=GAAATGAGTCCTTTTTCTTCATTA		
CDH8 cadh	5'CUL1-CDH8=GGATTTGCGAGTTCGATAGTTAG	405 bp	5'RT-CDH8=GTCTTCTCTTCACCGACTTACCTA	170 bp	218545-218654,246651-246710
	3'CL1=GTAAAACCCTCCGAACCAAATATAAA		3'RT-CDH8=CCATCGTCTGCATTAATGTTGA		
LOC284395	5'CUL1-LOC284395=GAGAAATAGAATAGGTATGATTGATAA	473 bp	5'RT-LOC284395=CGAGAAGGGCGGAACG	227 bp	174-331,96945-97013
	3'CL1=GTAAAACCCTCCGAACCAAATATAAA		3'RT-LOC284395=CGCCTGAATTCCACTGTTAGA		

Quantitative RT-PCR and RNA-IP primers sequence

Primer name	Primer sequence	PCR test
eIF2C2_rtF_ex2-4_176bp	GGA-GAG-TTA-ACA-GGG-AAA-TCG-TGG-A	quantitative RT-PCR
eIF2C2_rtR_ex2-4_176bp	GCG-ATC-CTT-GCC-TTC-TCC-TGG	quantitative RT-PCR
DICER_rtF_ex13-15_190bp	AAA-TTG-GCG-AAC-TGG-ATG-AC	quantitative RT-PCR
DICER_rtR_ex13-15_190bp	GGC-TGA-TCA-GGT-CTG-GGA-TA	quantitative RT-PCR
GAPDH_rtF_ex1-2_138bp	TTC-GCT-CTC-TGC-TCC-TCC-TGT-TC	quantitative RT-PCR
GAPDH_rtR_ex1-2_138bp	CTG-GTG-ACC-AGG-CGC-CCA-A	quantitative RT-PCR
ACTB_rtF_ex2-3_137bp	GTC-GAC-AAC-GGC-TCC-GGC-AT	quantitative RT-PCR
ACTB_rtR_ex2-3_137bp	CCC-ACA-TAG-GAA-TCC-TTC-TGA-CCC-A	quantitative RT-PCR
LINE-1 RNA Forward	CAGGAAGGGGAATATCACACTC	quantitative RT-PCR
LINE-1 GAG RNA Reverse	TGCGCTGCACCCACTAACTC	quantitative RT-PCR
LINE-1 ACA RNA Reverse	TGCGCTGCACCCACTAATGT	quantitative RT-PCR
TRIM38_F	GCA-AAA-ACC-ACA-ATT-ACT-TTT-GCA-C	RNA-IP
TRIM38_R	AAG-AGA-GAA-AAT-TGG-TAA-TCA-GCT-TG	RNA-IP
L1.2DSloci5'UTR_F	GGC-CAG-TGT-GTG-TGC-GCA-CCG	RNA-IP
L1.2DSloci5'UTR_R	CCA-GGT-GTG-GGA-TAT-AGT-CTC-GTG-G	RNA-IP
BCKDK_F	CCC-ACC-ATG-ATG-CTC-TAC-GCT-GG	RNA-IP
BCKDK_R	CCT-TGA-TGC-GGT-GAG-CAA-TCC-TC	RNA-IP
PSENEN_F	GGC-ACC-CCA-GCC-GGA-GGA	RNA-IP
PSENEN_R	CGG-GTC-GTC-CCA-AGG-GTC-TG	RNA-IP
EPHA3L1polyAForward	CTAACCTGCACAATGTGCACATGTACCC	RNA-IP
EPHA3in15polyA245R3	ACAAATACCATATCCTTCAAGACAAATCG	RNA-IP

Oligonucleotide sequence of siRNA target gene in contruct

Target gene	Squence
1536_LINE-1 Upper	GATCCCGAGCAACTCCAAGACACATTTCAAGAGAATGTGTCTTGGAGTTGCTCTTTTTTGGAAA
1536_LINE-1 Lower	AGCTTTTCCAAAAAAGAGCAACTCCAAGACACATTCTCTTGAAATGTGTCTTGGAGTTGCTCGG
2087_LINE-1 Upper	GATCCCGACCCATCAGTGTGCTGTATTCAAGAGATACAGCACACTGATGGGTCTTTTTTGGAAA
2087_LINE-1 Lower	AGCTTTTCCAAAAAAGACCCATCAGTGTGCTGTATCTCTTGAATACAGCACA CTGATGGGTCGG
2613_LINE-1 Upper	GATCCCGTAAAGCTCTCCTCAGCAATTCAAGAGATTGCTGAGGAGAGCTTTACTTTTTTGGAAA
2613_LINE-1 Lower	AGCTTTTCCAAAAAAGTAAAGCTCTCCTCAGCAATCTCTTGAATTGCTGAGGAGAGCTTTACGG.

BIOGRAPHY

Miss Chureerat Phokaew was born in Nakornsawan in 1980. After finished high school education at Satri Nakhon Sawan School, Nakhon Sawan, in 1999 she starts her B.Sc. major in Genetics from Faculty of Science, Chulalongkorn university. She acquired her M.Sc.major on Molecular Biology and Genetics program in Depart in Faculty of Medicine, Chulalongkorn university, in 2006. From 2007 until now, she is candidate Ph.D. student in the interdisciplinary program in Biomedical Science, Graduate School, Chulalongkorn University. Her research interested includesthe motivation of LINE-1 existence within genome, role of uncontrolled RNA particle within genome and exploring new role of small RNA within genome.

During the Ph.D. studying, she receive two awards in Good research award in Ph.D. level from parts of her thesis work. Both awards are including, first from Chula Medical Conference “Leadership in Medicine” 2008 meeting and second from Joint Conference in MedicalSciences 2009. In first part of her Ph.D. thesis, CU-L1 technique that develop for unique LINE-1 methylation was approved in 25 April 2008 fro Thailand patent ID 0801002098. For data got from this thesis, can become 2 international publications by first is “LINE-1 methylation patterns of different loci in normal and cancerous cells”.Phokaew C, Kowudtitham S, Subbalekha K, Shuangshoti S, Mutirangura A. Nucleic Acids Res. 2008 Oct;36(17):5704-12. Epub 2008 Sep 6. The second is, “Hypomethylation of Intragenic LINE-1 Represses Transcription in Cancer Cells through AGO2”. Aporntewan C, Phokaew C, Piriyapongsa J, Ngamphiw C, Ittiwut C, Tongsima S, Mutirangura A. PLoS One. 2011 Mar 15; 6(3): e17934.