ความเป็นไปได้ของระบบพีไออาร์เอ็นเอในการควบคุมทรานส์โพซอนในเซลล์มะเร็ง

นางสาว ธัชวรรณ ธนาศุภวัฒน์

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THE POSSIBILITY OF piRNAs SYSTEM TO CONTROL TRANSPOSONS IN CANCER CELLS

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A Thesis Submitted in Partial Fulfillment of the Requirements
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ธัชวรรณ ธนาศุภวัฒน์ : ความเป็นไปได้ของระบบพีไออาร์เอ็นเอในการควบคุมทรานส์โพซอนในเซลล์มะเร็ง. (THE POSSIBILITY OF piRNAs SYSTEM TO CONTROL TRANSPOSONS IN CANCER CELLS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ศ. นพ. อภิวัฒน์ มุทิรางกูร, 60 หน้า.

Piwil2 (P-element induced wimpy testis like 2) มีความสำคัญในการพัฒนาของ stem cell และการพัฒนาของเซลล์สืบพันธุ์ ซึ่งจะแสดงออกเฉพาะระยะ spermatogenesis ของสัตว์เลี้ยงลูกด้วยนม การศึกษาหลังจากยับยั้งการแสดงออกของ *Piwil2* ในหนูทดลองพบการสูญเสียหมู่เมทิลของ LINE-1 นอกจากนั้นยังมีการศึกษาพบการแสดงออกของโปรตีน *Piwil2* ในเนื้อเยื่อมะเร็งหลายชนิด ซึ่งในเนื้อเยื่อมะเร็งจะพบการสูญเสียหมู่เมทิลของ LINE-1 ทั้งจีโนมและในตำแหน่งที่ LINE-1 แทรกอยู่ในยีนต่างๆ ดังนั้นเราจึงสนใจที่จะศึกษาถึงความสัมพันธ์ระหว่างการแสดงออกของ *Piwil2* ที่มีต่อระดับเมทิลเลชั่นของ LINE-1 ในเซลล์มะเร็ง โดยทำการศึกษาการแสดงออกของ *Piwil2* จาก semi-quantitative RT-PCR และศึกษาระดับเมทิลเลชั่นของ LINE-1 ในจีโนมโดยใช้เทคนิค COBRALINE-1 และใช้เทคนิค CU-L1 ในการศึกษาระดับเมทิลเลชั่นของ LINE-1 ในตำแหน่งที่แทรกอยู่ในยีน พบว่าใน 11 WSU-HN cancer cell lines การแสดงออกของ *Piwil2* ไม่มีความสัมพันธ์กับระดับเมทิลเลชั่นของ LINE-1 ในจีโนมแต่พบว่ามีความสัมพันธ์อย่างมีนัยสำคัญกับระดับเมทิลเลชั่นของ LINE-1 ที่แทรกอยู่ในยีน *EPHA3IVS5* และ *SPOCK3* ที่ Pearson $r = 0.7332$; $P \leq 0.01$ และ $r = 0.6124$; $P < 0.05$ นอกจากนั้นยังศึกษาความสัมพันธ์ระหว่างการยับยั้งการแสดงออกของ *Piwil2* แบบชั่วคราว เนื่องจากพบการตายของเซลล์เมื่อทำการยับยั้งแบบถาวร ใน HeLa cell กับระดับเมทิลเลชั่นของ LINE-1 พบการลดลงของการแสดงออกของ *Piwil2* ที่ 24 และ 48 ชม. แต่ไม่พบว่ามีความสัมพันธ์กับระดับเมทิลเลชั่น ของ LINE-1 ทั้งจีโนมและ LINE-1 ที่แทรกอยู่ในยีนต่างๆ ซึ่งอาจสรุปได้ว่าการแสดงออกของ *Piwil2* จะสัมพันธ์กับระดับเมทิลเลชั่นของ LINE-1 นั้นขึ้นกับตำแหน่งที่มีการแทรกตัวของ LINE-1 อยู่และชนิดของเซลล์มะเร็ง

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต ธัชวรรณ ธนาศุภวัฒน์

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THATCHAWAN THANASUPAWAT : THE POSSIBILITY OF piRNAs SYSTEM TO CONTROL TRANSPOSONS IN CANCER CELLS. ADVISOR : PROFESSOR APIWAT MUTIRANGURA, M.D., Ph.D., 60 pp.

Down-regulation of *Piwil2* (P-element induced wimpy testis like 2) expression induced hypomethylation, the loss of methylation levels, of long interspersed nuclear element-1 (LINE-1 or L1) sequences in the testes of mutant mice. Moreover, the expression of *Piwil2* can be found in various cancers. The levels of LINE-1 hypomethylation in cancers are not only generally varied, but also possess a locus-specific pattern. This study focused on the association between *Piwil2* and LINE-1 methylation. Eleven WSU-HN cancer cell lines were examined for the expression of *Piwil2* using semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Genome-wide and specific loci LINE-1 methylation levels were measured using Combined Bisulfite Restriction Analysis (COBRA) and COBRA for unique LINE-1 (CU-L1), respectively. Moreover, inhibit *Piwil2* expression used siRNAs. The levels of *Piwil2* expression and LINE-1 methylation were varied. Significant association between *Piwil2* RNA and LINE-1 methylation of two loci, L1-*EPHA3IVS5* and *L1-SPOCK3*, was observed (Pearson $r = 0.7332$; $P \leq 0.01$ and $r = 0.6124$; $P < 0.05$, respectively). There was no association with both genome wide LINE-1 methylation and the other 15 loci. We also reported transient inhibition of *Piwil2* expression. Unfortunately due to cell death, we can not establish stable cell line. We found no association between short term down-regulated *Piwil2* expression effect on methylation levels of LINE-1 in genome-wide and specific loci in HeLa. We suggested that *Piwil2* expression may be associated with LINE-1 methylation of selective loci in type of cancer cells.

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

Academic Year : 2008 Advisor's Signature : .....

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COBRA	=	Combined with bisulfite restriction analysis
COBRALINE-1	=	COBRA of LINE-1
CU-L1	=	COBRA to Unique LINE-1
LINE-1	=	Long interspersed nuclear element-1
PCR	=	Polymerase Chain Reaction
piRNAs	=	piwi-interacting RNAs
Piwil2	=	P-element induced wimpy testis like 2
siRNAs	=	short interfere RNAs

CHAPTER I

INTRODUCTION

BACKGROUND AND RATIONALE

The *Piwi* subfamily genes are essential for germline and stem cell development. These genes are highly conserved among organisms. In mammals, *Piwi* genes are expressed specifically during spermatogenesis in the testes (1, 2). Additionally, expression of *Piwil2* was also found in different tumors and cancer cell lines, whereas no expression was observed in normal tissues (3). However, *Piwi* interacts with piRNAs resulting in degradation of their target, mRNAs, which leads to gene silencing (4, 5). In fact, *Piwi*-piRNAs complexes are important for repressing expression of retrotransposons that are representative of repetitive elements such as long interspersed nuclear element-1 (LINE-1). Recently, it has been reported that *Piwil2*-null testes mice showed reduced methylation levels of LINE-1 (6). Therefore, *Piwil2* plays necessary roles in establishing DNA methylation of LINE-1 in germline development (7).

Genome wide hypomethylation is a common event in cancer and may be important in cancer development (8-11). This genome demethylation is associated with genomic instability (12-14) and may be associated with endogenous DNA double strand breaks (15, 16). The loss of methylation usually occurs at interspersed repetitive sequences (8). LINE-1 is a retrotransposon which contains 600,000 copies in a genome and 3,000 – 5,000 represent full-length elements (17). Our previous studies demonstrated varying degrees of reduced LINE-1 methylation levels in several carcinomas including bladder, head and neck, oral epithelium, liver, lung, prostate, breast, esophagus, stomach, ovary, cervix and colon (8, 18-22). Recently, we reported the methylation levels of LINE-1 from 17 loci, each located within an intron of a gene. In addition to a generalized hypomethylation pattern, locus-specific patterns of LINE-1 methylation were demonstrated (23).

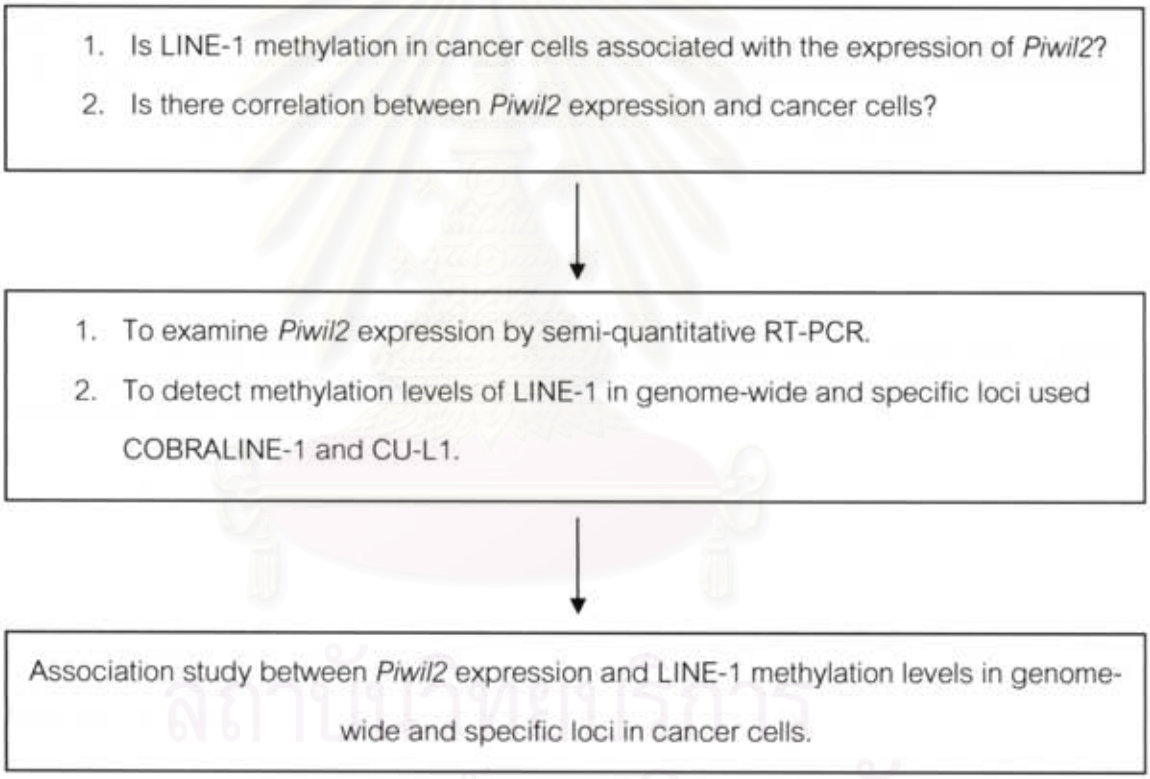
Because *Piwil2* is expressed in different tumors (20) and *Piwil2* is required for LINE-1 methylation in testis (7), it is interesting to evaluate the association between *Piwil2* expression and LINE-1 methylation levels in cancer cells. Furthermore, investigate

correlation between *Piwil2* expression and LINE-1 methylation in cells with the same genetic background.

Objectives

1. To evaluate the association between *Piwil2* expression and methylation levels of LINE-1 methylation in genome-wide and specific loci in cancer cells.
2. To examine correlation between *Piwil2* expression and LINE-1 methylation in cells with the same genetic background.

Concepture Framework



3. Is there correlation between *Piwil2* expression and LINE-1 methylation in cells with the same genetic background?

↓

1. To inhibit *Piwil2* expression by siRNAs.
2. To detect methylation levels of LINE-1 after inhibition of *Piwil2* expression in genome-wide and specific loci used COBRALINE-1 and CU-L1

↓

Association study *Piwil2* expression effect on LINE-1 methylation levels in genome-wide and specific loci in cells with the same genetic background.

Key Words

Piwil2; long interspersed nuclear element-1 (LINE-1); LINE-1 methylation; global hypomethylation; COBRA LINE-1; CU-L1

Expected Benefit

We can use the results of this study to understand and study mechanism of carcinogenesis.

Research Methodology

1. To observe *Piwil2* expression
    - 11 WSU-HN cancer cell lines and down-regulate *Piwil2* in HeLa cell lines
    - Extract RNA for RT-PCR
    - Agarose gel electrophoresis
    - Expression analysis

2. To evaluate methylation levels of LINE-1

    - Extract DNA for COBRALINE-1 PCR
    - Restriction Fragment Length Polymorphism (RFLP)
    - Agarose gel electrophoresis
    - Methylation analysis

CHAPTER II

REVIEW OF RELATED LITERATURES

Small ribonucleic acids (Small RNAs)

Small noncoding RNAs regulate essential processes for cell growth and differentiation. Moreover, small RNAs included mRNA degradation and translational repression. Characterization of small RNAs is classified 2 major classes (4), that is, short interfere RNAs (siRNAs) and micro RNAs (miRNAs). siRNAs are derived from 21 nucleotides double stranded RNA, whereas, miRNAs are endogenous small RNAs. Furthermore, both siRNAs and miRNAs interact with Agonaute (Ago) subfamily proteins and Dicer for induce mRNA cleavage and translational repression.

Recently, Aravin et al., 2006; Girard et al., 2006; Grivna et al., 2006 discovered a new class of small RNAs which interact with Piwi proteins have been called piwi-interacting RNAs (piRNAs). piRNAs are ~30 nucleotides single stranded RNAs, interact with *Piwi* subfamily protein and their activity don't require Dicer (1, 4, 5, 24). However, the importance of piRNAs are only detect in testes, express during spermatogenesis in mammalian. Therefore, they are essential for germline and stem cell development (Figure 1 and Table 1).

The characteristics of piRNAs are ~30 nucleotides single stranded RNAs contain 5' uracil end, 3' 2'-o-methylation end (25), consist of more than 50,000 species. They are found cluster disperse in genome including exon, intron, intergenic and mapped to repeat sequences. Mostly, retrotransposon, piRNAs are important for repetitive sequences repression, spermatogenesis inhibition also (1, 2, 4, 5, 24).

Table 1 Difference of small RNAs

	siRNAs	miRNAs	piRNAs
Size	~ 21 nucleotides	~ 21 nucleotides	~ 30 nucleotides
Origin	Exogenous Double strand RNA (dsRNA)	Endogenous Hairpin-loop Double strand RNA (dsRNA)	Endogenous Single strand RNA (ssRNA)
Ribonuclease	Dicer	Dicer	No
Argonaute family protein	Argonaute subfamily	Argonaute subfamily	*Piwi* subfamily

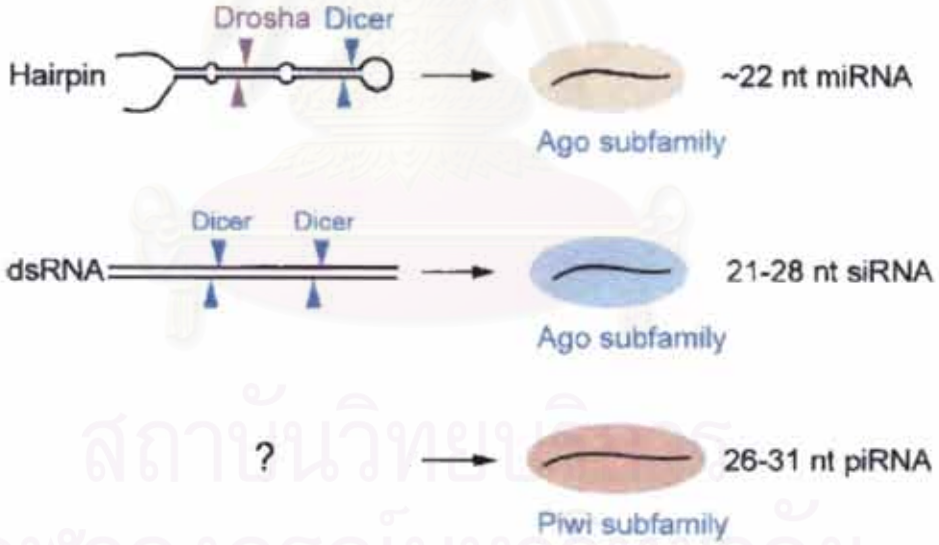


Figure 1 Difference of small RNAs. miRNAs and siRNAs are ~22 nucleotides double strands hairpin structure, whereas piRNAs is 31 nucleotides single strand. Furthermore, miRNAs and siRNAs use Dicer and Ago subfamily for their activity, in contrast, piRNAs use *Piwi* subfamily protein (Figure from Genes Dev. 2006 Aug 1;20(15):1993-7.) (2).

RNAi pathway, Small RNAs interact with Argonaute family proteins that highly conserved among eukaryotes. The small RNAs bind Argonaute proteins at PAZ domain for guide Argonaute proteins to their target. As a result, lead to gene silencing by Dicer. On the other hand, activity of *Piwi* domain is endonuclease. Besides, Argonaute family proteins are divided to 2 subfamilies based on their sequences similarities (26, 27).

1. Argonaute subfamily is associated with siRNAs and miRNAs, expressed in somatic cells.
2. *Piwi* subfamily is expressed in germ lines and stem cells. Recent, piRNAs are associated with *Piwi* subfamily proteins.

Piwi subfamily protein

Piwi subfamily is expressed specifically during spermatogenesis in testes. Two *Piwi* proteins, that is, MILI and MIWI, are found in different stage. MILI is expressed since spermatogonia in mitosis until pachytene spermatocyte stage (Figure 2). Nevertheless, MILI null effect to complete spermatogenesis arrest. On the contrary, MIWI is expressed after from mid-pachytene to early round spermatid. For this reason, MIWI null has not complete spermatogenesis process (28, 29).

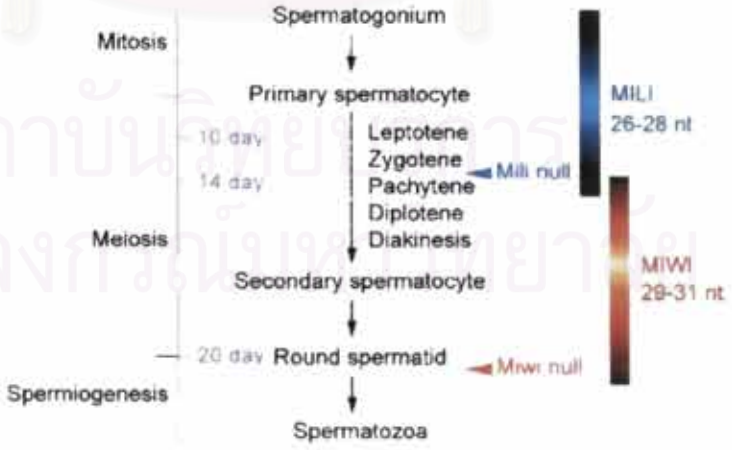


Figure 2 Schematic illustration of expression of *Piwi* proteins during spermatogenesis. MILI is expressed in early stage, whereas, MIWI is expressed in lately (Figure from Genes Dev. 2006 Aug 1;20(15):1993-7.) (2).

Ping-pong mechanism

Primary piRNAs is produced from transposon piRNAs cluster. First of all, primary antisense piRNAs are generated secondary sense piRNAs. Antisense piRNAs show uracil (U) at 5' end, bind Aubergine and *Piwi* proteins. Hence, *Piwi* proteins cleave their transposons targets between position of nucleotides 10 and 11 from 5' end of antisense piRNAs. Therefore, generate new 5' end of sense strand piRNAs-Ago3 complex that show adenine (A) at position 10. piRNAs bind Ago3 not only cleavage their targets but also generate antisense piRNAs. Moreover, piRNAs carry a 2'O-methylation t 3' end which is add by Hen-1 family RNA methyltransferase. However, an unidentified endonuclease cleave 3' end. Consequently, the combination of these steps can form a self amplification loop (Ping-Pong mechanism) (30, 31) (Figure 3).

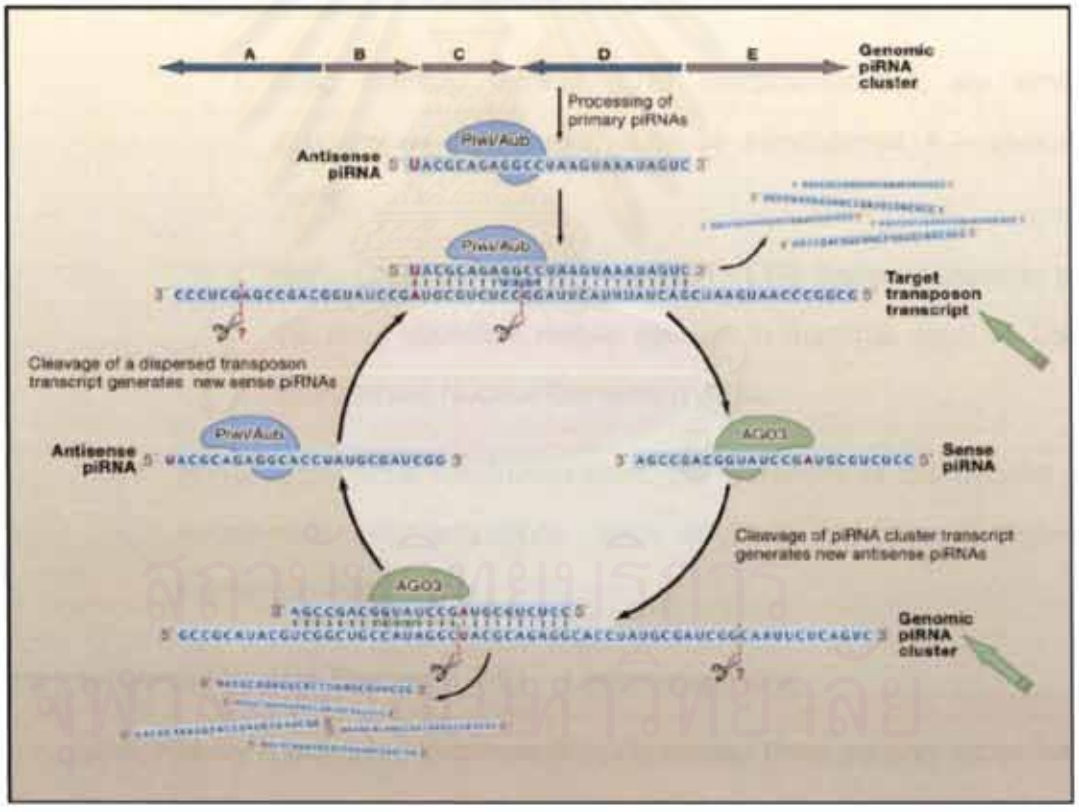


Figure 3 Schematic illustration of Ping-pong amplification loop mechanism model: biogenesis of piRNAs (Figure from Cell. 2007 Apr 6;129(1):37-44.) (32).

Transposable element (Transposons)

45% of the human genome consists of transposable elements; repetitive sequence DNA. Transposable elements compose of DNA transposons and Retrotransposon (17, 33).

1. DNA transposons; there are 3% in human genome. They move by "cut and paste" mechanism using transposase.
2. Retrotransposons; there are 42% in human genome. DNA retrotransposons encode reverse transcriptase (RT) and move by "copy and paste" mechanism. Moreover, Retrotransposable elements can be classified to Autonomous retrotransposons and Non - autonomous retrotransposons.
    - Autonomous retrotransposons can encode essential proteins which they require to move.
        i. Long Terminal Repeat (LTR) Retrotransposons; are similar structure as retroviruses, such as intracisternal A – particles (IAPs).
        ii. Non - Long Terminal Repeat (Non - LTR) Retrotransposons; are the most abundant mobile element in mammal, such as Long Interspersed Nuclear Elements (LINEs).
    - Non - autonomous retrotransposons; the movement of this depend on autonomous retrotransposons, such as Short Interspersed Nuclear Elements (SINEs).

Long Interspersed Nuclear Element (LINE) – 1 retrotransposons

LINE-1 family is estimated to contain 600,000 copies. There are only about 3,000 – 5,000 represent full-length elements. The most of LINE-1 elements have non-mobile elements because 5' truncatation, rearrangement or point mutation. On the other hand, full-length LINE-1 elements; ~6 kb, consists 5' untranslated region (5' UTR) with internal promoter. Furthermore, the consensus sequences contain 2 open reading frames (ORF1 and ORF2) that separated by intergenic. Besides, the 3' UTR end has AATAAA polyadenylation signal and poly A tail (17) (Figure 4).

Figure 4 Schematic illustration of structure of full-length LINE-1. There are 5' UTR, ORF1, ORF2, 3' UTR and poly A tail (Figure from Cell. 2002 Aug 9;110(3):277-80.) (34).
Abbreviation: TSD, target site duplication; 5' UTR, 5' untranslated region; ORF1, first open reading frame; ORF2, second open reading frame; EN, endonuclease domain; RT, reverse transcriptase domain; 3' UTR, 3' untranslated region; AATAAA, hexanucleotide poly(A) signal; and A_n, the poly(A) tract abuts the hexanucleotide signal in human.

ORF1 is ~1 kb, encoded 40 kDa protein which contains leucine zipper domain for RNA binding. Therefore, it is essential for move.

ORF2 is ~4 kb, encoded 150 kDa protein. Besides, there are 3 conserve domain, that is, endonuclease (EN) domain, reverse transcriptase (RT) domain and c – terminal cysteine - rich domain (17).

DNA methylation

DNA methylation is an epigenetic modification, reversible change in gene expression, whereas, DNA sequence alterations don't change. In addition, adding methyl group to carbon 5 position of cytosine; 5' - methyl cytosine, is found within cytosine-guanine dinucleotides (CpG) disperse whole genomic consists genes and repetitive sequences (Figure 5). Moreover, 5' - methyl cytosine is associated with condensation of chromatin, stabilization of chromosome, transcriptional silencing of X chromosome, genomic imprinting and tissue-specific silencing of gene expression (11, 35-37).

DNA methyltransferases (DNMTs) is the enzyme responsible for adding methyl groups to 5'-cytosine. Furthermore, it can be classified to maintenance and de novo methyltransferases (11, 38, 39).

DNMT1 is maintenance methyltransferases. During DNA replication, the new synthesis of DNA contains hemimethylated that recruits DNMT1 to transfer methyl groups to 5'-cytosine from its cofactor, S-adenosylmethionine (SAM).

DNMT3A and 3B are de novo methyltransferase, require for adding methyl groups to CpG dinucleotides of unmethylated DNA (Figure 6).

DNA hypermethylation is responsible for repression of tumor suppressor genes. In contrast, hypomethylation is a common epigenetics process in cancer induced protooncogenes overexpression. Moreover, hypomethylation of retrotransposons contribute active retrotransposition lead to activate oncogene by insertion.

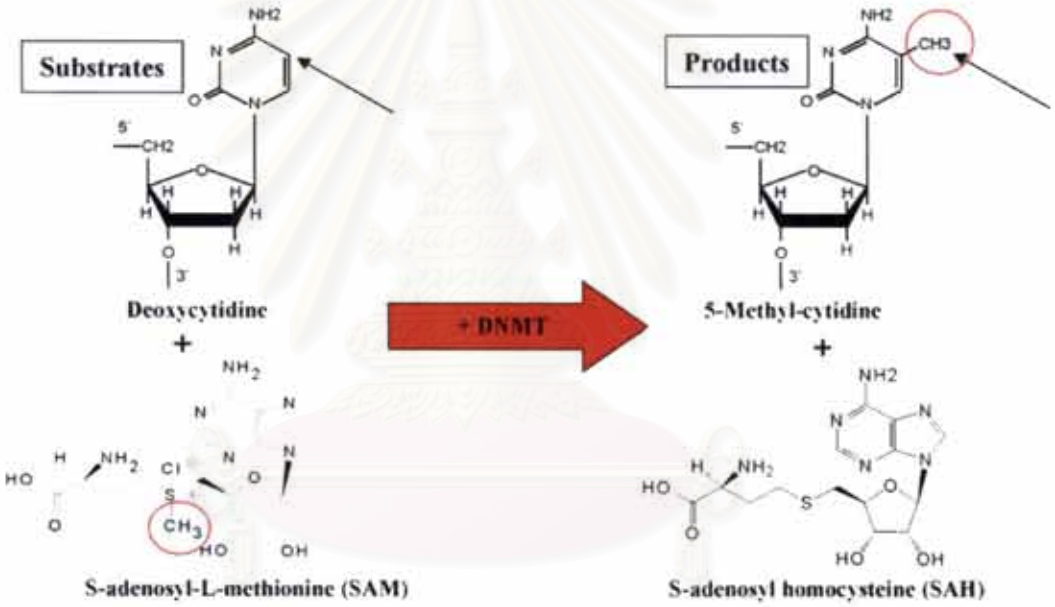


Figure 5 Methylation of cytosine. The DNA methyltransferase catalyze the transfer of the methyl group from S-adenosylmethionine to cytosine. It produces 5-methylcytosine and S-adenosylhomocysteine (Figure: http://www.med.ufl.edu/biochem/keithr/fig1pt.html).

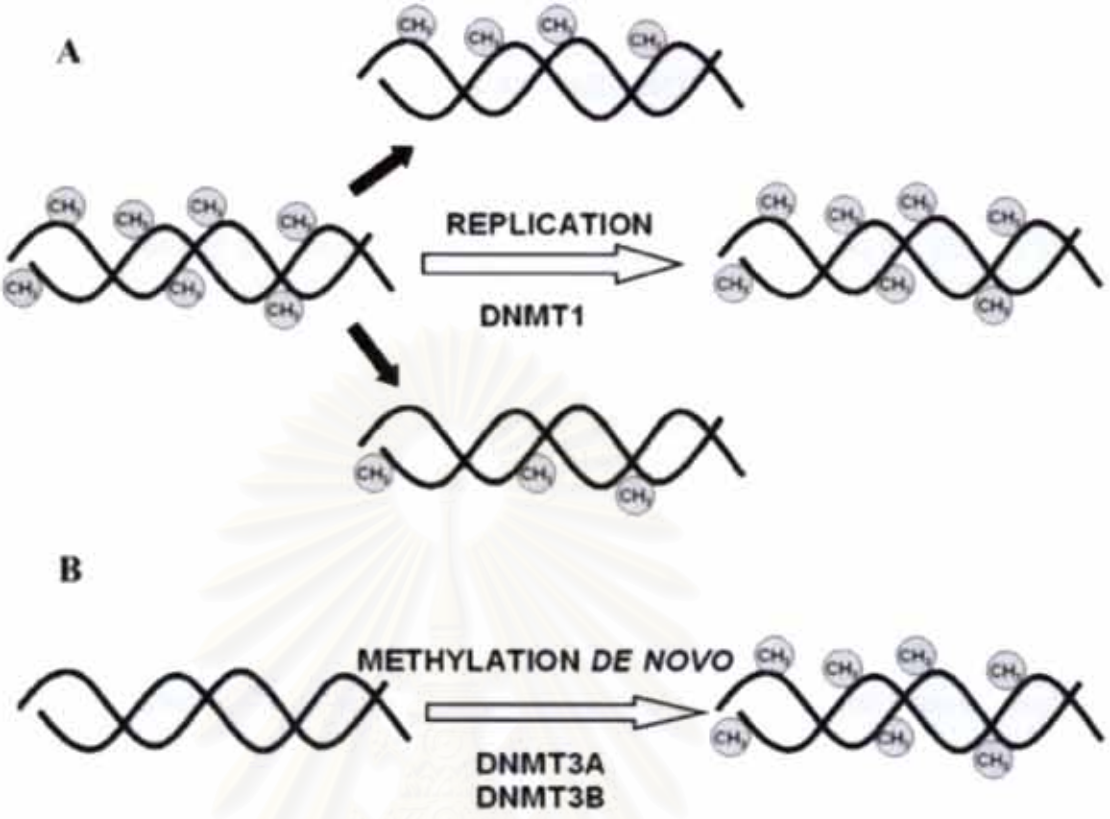


Figure 6 Classification of DNMT. (A) Maintenance DNA methylation, DNMT1 add methyl group to the hemimethylated DNA during replication. On the other hand, (B) de novo methlation, DNMT3A and DNMT3B add methyl group to CpG dinucleotides of unmethylated DNA (Figure from Folia Histochem Cytobiol. 2006;44(3):143-54.) (11).

CHAPTER III

MATERIALS AND METHODS

Cell Culture

WSU-HN cell lines, including WSU-HN 4, 6, 8, 12, 13, 17, 19, 22, 26, 30, 31, Fibroblast and HeLa cells were maintained in Dulbecco' modified Eagle's medium (DMEM) (Sigma-Aldrich, St Louis, MO, USA) containing 10% fetal bovine serum (FBS) and 100 unit/ml of antibiotic/antimycotic. All cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2.

siRNAs experiments

The *Piwil2* siRNAs sequences are following in Table 2. These sequences were ligation with p*Silencer*™ 3.1 hygro vectors (Ambion, Austin, TX). Ligated plasmids were transformed to E. coli DH5 alpha. Then, plate the transformed cells on 100 mg/ml ampicillin LB agar plate, incubated at 37 °C overnight. Afterward, the bacteria colonies were selected then cultured in LB broth containing 100 mg/ml ampicillin overnight and extracted plasmids by Qiagen midiprep (Qaigen, Valencia, CA). Plasmids were performed DNA sequencing to confirm the siRNAs sequence without any mutation. Applied Biosystems DNA sequencer using M13 primer was used.

HeLa cells were used for siRNAs transfection. Cells were plated at 5.0×10^5 cells per 25 cm^2 flask. Following 24 hr in culture, cells were transfected with 2 µg *Piwil2* siRNAs and negative siRNAs from p*Silencer*™ kit was used as control. FuGENE® HD Transfection Reagent (Roche Applied Sci-ence, 2006a) was used to transfection reagent. After transfection 24 and 48 hr, HeLa cells with siRNAs expression were harvested for RNA and DNA.

Harvested cells

WSU-HN cell lines, Fibroblast and HeLa cells were washed with PBS and trypsinized by trypsin. Stop the reaction with DMEM containing 10% fetal bovine serum (FBS) and 100 unit/ml of antibiotic/antimycotic. The pellet of cells was separated by

centrifuged at 150 g for 5 minutes. Moreover, the pellet was washed twice with PBS before extracted RNA and DNA.

RNA preparation

The RNA was extracted from fibroblast and HeLa cells using 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA, USA). Separation of RNA with chloroform then precipitated with 100% Isopropanol, washed the pellet with 70% ethanol and resuspened with DEPC dH2O. The RNA was stored at – 80 °C before was changed to cDNA.

Semi quantitative reverse transcriptase PCR (RT-PCR)

To synthesis cDNA, 5 μg of RNA was dissolved in 12 μl of DEPC dH_2O containing 0.5 μg of oligo(dT)18 primer (RevertAid™ First Strand cDNA Synthesis Kits) (MBI Fermentas). The RNA was denatured by incubated for 5 minutes at 70 °C, chilled on ice. Then, the samples were added 1X reaction buffer, 1 mM of dNTP mix, 20 units of RiboLock RNase Inhibitor and 200 units of RevertAid M-MulV Reverse Transcriptase, incubated for 60 minutes at 42 °C for cDNA synthesis and terminated the reaction by heating at 70 °C for 5 minutes.

For RT-PCR, 10 μl PCR was carried out in 1X PCR buffer containing 1.5 mM MgCl2, 0.2 mM dNTPs mix, 0.25 units of HotStarTaq (Qaigen, Valencia, CA), 0.1 μM of primer and 2 μl of cDNA. There are PCR conditions as following below.

Initial denaturation	95 °C for 15 minutes	
Denaturation	95 °C for 1 minute	35 cycles
Annealing	X °C for 1 minute	
Extension	72 °C for 1 minute	
Final extension	72 °C for 7 minutes	

The annealing temperature of *Piwil2* and G3PDH were 58 °C and 60 °C, respectively. The amplicons were electrophoresed in 2% agarose gel. As a result, *Piwil2* and G3PDH expression were found at 265 and 151 bp, respectively. The density of bands was measured with PhosphorImager using Image Quant software (Molecular

Dynamic). The *Piwil2* expression was calculated as the percentage of *Piwil2* densities divided by G3PDH amplicon (13).

DNA extraction

The pellet of cells was resuspended in 950 µl of Lysis Buffer II, 10% SDS and 400 mg/ml of proteinase K. Then, incubate at 50°C overnight. The DNA was purified by phenol/chloroform extraction, 100% ethanol and 10 M of NH_4OAC precipitation. The pellet of DNA was washed with 70% ethanol and was resuspended with dH_2O. The DNA was stored at – 20 °C.

Bisulfite Treatment

Dilute 1 µg of DNA into 50 µl with dH_2O, 5.5 µl of 2M NaOH were added, incubated for 10 minutes at 37 °C to create single-stranded DNA. Then, 30 µl of 10 mM hydroquinone and freshly prepared 520 µl of sodium bisulfite at pH 5.0 were added and mixed. The sample was incubated at 50 °C for 16 – 18 hours. Furthermore, the bisulfite–treated DNA was isolated using Wizard® DNA Clean-Up System (Promega, Madison, WI). The DNA was eluted by 50 µl of dH_2O at 95 °C and 5.5 µl of 3 M NaOH were added and incubated at room temperature for 5 minutes. The DNA was precipitated by adding 17 µl of 10 M Na_4OAc, 220 µl of 100% ethanol and 1 µl of 20 mg/ml glycogen as a carrier then incubated at -20 °C for 2 hours. After incubation, DNA was centrifuged at 14,000 rpm for 10 minutes. The pellet of DNA was washed with 70% ethanol then centrifuged at 14,000 rpm for 5 minutes and was resuspended with 20 µl of dH_2O. Bisulfite-treated DNA was stored at -20 °C until ready for used.

COBRA LINE-1

For COBRA LINE-1, a 20 µl PCR was carried out in 1X PCR buffer contained with 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix, 1 unit of HotStarTaq, 0.3 µM of B-L1-inward, 0.3 µM of B-L1-outward and 2 µl of bisulfite-modified DNA. There are PCR conditions following below.

Initial denaturation	95 °C for 15 minutes	
Denaturation	95 °C for 1 minute	35 cycles
Annealing	50 °C for 1 minute	
Extension	72 °C for 1 minute	
Final extension	72 °C for 7 minutes	

The amplicons were double-digested in 10 μl reaction volumn with 2 units of *Taq*I and 8 units of *Tas*I in 1X *Taq*I buffer (MBI Fermentus) then incubated at 65 °C overnight. Moreover, digested products were electrophoresed in 8% non-denaturing polyacrylamide gel. As a result, detection of methylated and unmethylated sequences was found at 80 bp (methylated), 63 and 98 bp (unmethylated). The intensity of DNA fragment was measured with PhosphorImager using Image Quant software (Molecular Dynamic). The LINE-1 methylation level was calculated as the percentage of *Tag*I intensity divided by the sum of *Taq*I and *Tas*I positive amplicons.

COBRA for unique to L1 sequence (CU-L1)

For CU-L1, the technique detected LINE-1 methylation levels in several genes with intronic insertion of full-length LINE-1 (23). Two μl of bisulfited DNA was subjected to 35 cycles of PCR with two primers as listed in Table 4 at an annealing temperature of 53 °C. Then, the amplicons were double-digested. The detection of methylated and unmethylated sequences was found many fragments. We calculated LINE-1 methylation levels by methylated sequences at 80 bp and unmethylated at 98 bp. Because, there was linear correlation between methylated fragment from the CUL-1 locations. Also, there were direct correlations between unmethylated fragments.

Statistical Analysis

Statistical significance was determined according to Pearson's correlation coefficient.

Table 2 Sequences of siRNAs of *Piwil2*

Primer Name	Sequence 5'-3'
siPiwil2_1	CGTCACTGCGTTTGATGGA
siPiwil2_2	AGGATAGCTTCACGATGTC
siPiwil2_3	TGTTCGAACCATTGGTCAG

Table 3 List of primers in experiments

Primer Name	Sequence 5'-3'
B-L1-inward	CGTAAGGGGTTAGGGAGTTTTT
B-L1-outward	RTAAAACCCTCCRAACCAAATATAAA
RT_*Piwil2*_F	CGAGGCTTGTCTGCTAATCTG
RT_*Piwil2*_R	GAGCTGGTGGTGATGACAGC
RT_G3PDH_F	CCATGGCACCGTCAAGGCTGA
RT_G3PDH_R	CTCCATGGTGGTGAAGACGC

Table 4 List of CU-L1 primer (23)

Gene	COBRA unique sequence oligonucleotides (5'-3')	Size (bp)	Methylated bands (bp)	Unmethylated bands (bp)
COL24A1	GTTAAAGGGTTAAGAATGTGTGTAG RTAAAACCCTCCRAACCAAATATAAA	336	47, 151, 60, 54, 80	294, 98
FAM49A	GTTTTAAAAAAAAATAAAGTTGG RTAAAACCCTCCRAACCAAATATAAA	385	41, 151, 113, 80	287, 98
CNTNAP5	GATTAAATTTTAATTGAATTAGAG RTAAAACCCTCCRAACCAAATATAAA	403	43, 151, 60, 53, 80	5, 6, 5, 289, 98
PKP4	GGTATGATTTTAAAAAAAGAGAT RTAAAACCCTCCRAACCAAATATAAA	392	48, 211, 53, 80	294, 98
LRP2	GGTATATAATTTTTATGGTGTTG RTAAAACCCTCCRAACCAAATATAAA	435	44, 150, 60, 53, 80	7, 27, 14, 289, 98

Gene	COBRA unique sequence oligonucleotides (5'-3')	Size (bp)	Methylated bands (bp)	Unmethylated bands (bp)
MGC42174	ATTGAGGTGTATTAAGAGATGGA RTAAAACCCTCCRAACCAAATATAAA	553	181, 60, 53, 80	25, 154, 276, 98
EPHA3-IVS5	TGTTATTGGAATATATGGAGATT RTAAAACCCTCCRAACCAAATATAAA	386	42, 151, 60, 53, 80	288, 98
EPHA3-IVS15	TAAGGATAAAAATTTTTGAAGTT RTAAAACCCTCCRAACCAAATATAAA	464	60, 150, 60, 53, 80	10, 33, 18, 305, 98
ANTXR2	TATTGAGTATTAATTATGTATTTAGTAT RTAAAACCCTCCRAACCAAATATAAA	416	28, 150, 60, 53, 80	11, 34, 273, 98
SPOCK3	GTGTAATTTTTTTAGATTTTGTAG RTAAAACCCTCCRAACCAAATATAAA	492	300, 60, 36, 17, 80	6, 22, 23, 37, 46, 262, 98
LOC133993	TTAGGATATTTTTTATTTTGGGA RTAAAACCCTCCRAACCAAATATAAA	446	101, 264, 80	374, 98
PPP2R2B	GGGGAAAAAATTGAAAGTT RTAAAACCCTCCRAACCAAATATAAA	590	8, 24, 151, 60, 53, 80	94, 28, 20, 42, 21, 9, 270, 98
LOC286094	TATGTAAGTATGGAAATTTGAGG RTAAAACCCTCCRAACCAAATATAAA	429	43, 151, 60, 53, 80	16, 20, 290, 98
PRKG1	AAAATTTTTAGTTGTTAAATGG RTAAAACCCTCCRAACCAAATATAAA	374	152, 60, 53, 80	2, 27, 247, 98
ADAMTS20	AAGTTGTGTGGTTTTTTGTAAAT RTAAAACCCTCCRAACCAAATATAAA	468	81, 151, 60, 36, 17, 80	22, 328, 98
CDH8	GGATTTGGGAGTTGGATAGTTAG RTAAAACCCTCCRAACCAAATATAAA	405	21, 211, 53, 38	30, 10, 276, 56, 42
LOC284395	GAGAAATAGAATAGGTATGATTGATAA RTAAAACCCTCCRAACCAAATATAAA	473	23, 151, 60, 53, 80	27, 5, 33, 36, 270, 98

CHAPTER IV

RESULTS

Expression of *Piwil* 2 in different cell lines

Recently, it has been reported that *Piwil2* was expressed specifically during spermatogenesis and different cancer cell line. Therefore, we examine expression of *Piwil2* in fibroblast, HeLa, and 11 WSU-HN, RNAs were subjected to RT-PCR analysis (Figure 7). *Piwil2* was observed in all cancer cell lines. No expression was detected in fibroblast which represents normal cell (Figure 7A).

Nevertheless, expression levels in each 11 WSU-HN cell lines were different (Figure 7B). We found that WSU-HN 6 possessed the highest level of *Piwil2* expression, whereas WSU-HN13 possessed the lowest level. *Piwil2* expressions levels of 7 WSU-HN cell lines were higher than mean including HN 4, 6, 8, 17, 19, 22 and 26. On the other hand, low expressions levels of *Piwil2* were found in the others WSU-HN cell lines.

LINE-1 methylation level and *Piwil2* expression in WSU-HN

COBRALINE-1 and CU-L1 data were the same as previously reported (23). Technique of COBRALINE-1 and CU-L1 are provided (Figure 8-9). We reported that LINE-1 methylation levels of head and neck cancer cell lines were generally lower than normal oral epithelium. Moreover, in cancer, LINE-1 methylation levels of each locus are generally directly correlated. Nevertheless, some loci possessed differential methylation levels depending on the repeat sequence locations.

Hence, we evaluated the correlations between *Piwil2* expression and LINE-1 methylation levels of genome-wide and specific loci are reported as Pearson's correlation coefficients (r) (Figure 10-12). There was no statistically significant association of *Piwil2* expression with genome-wide methylation of LINE-1 (Pearson r = 0.027; P = 0.938) (Figure 10). Interestingly, methylation levels of L1-*EPHA3-IVS5* and L1-*SPOCK3* were directly correlated with *Piwil2* expression (Pearson r = 0.7332; $P \leq 0.01$ and Pearson r = 0.6124; $P < 0.05$, respectively) (Figure 11). In contrast, no association

was found in other loci (Figure 12). These data provided preliminary evidence that, in cancer, LINE-1 methylation levels of some loci may be *Piwil2* dependent.

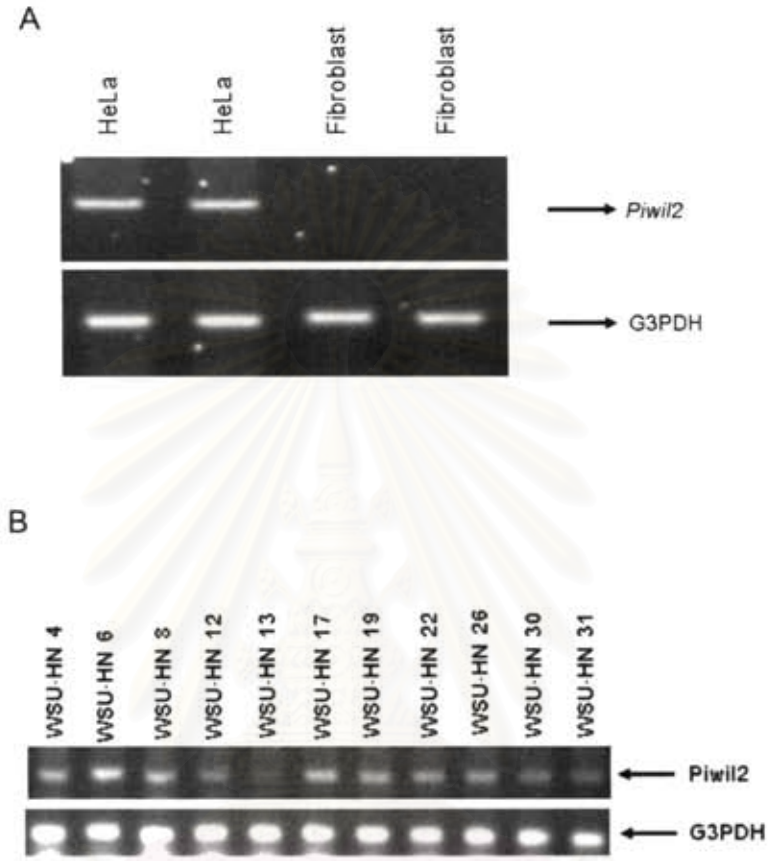


Figure 7 Expression analysis of *Piwil2* in (A) fibroblast and HeLa, (B) WSU-HN. Expression analysis was performed by semi-quantitative RT-PCR. Expression of *Piwil2* was observed in all cancer cell lines, whereas, fibroblast was no expressed. Nevertheless, expression levels in each cell lines are different. Furthermore, *Piwil2* expression was calculated as the percentage of *Piwil2* densities divided by G3PDH amplicon.

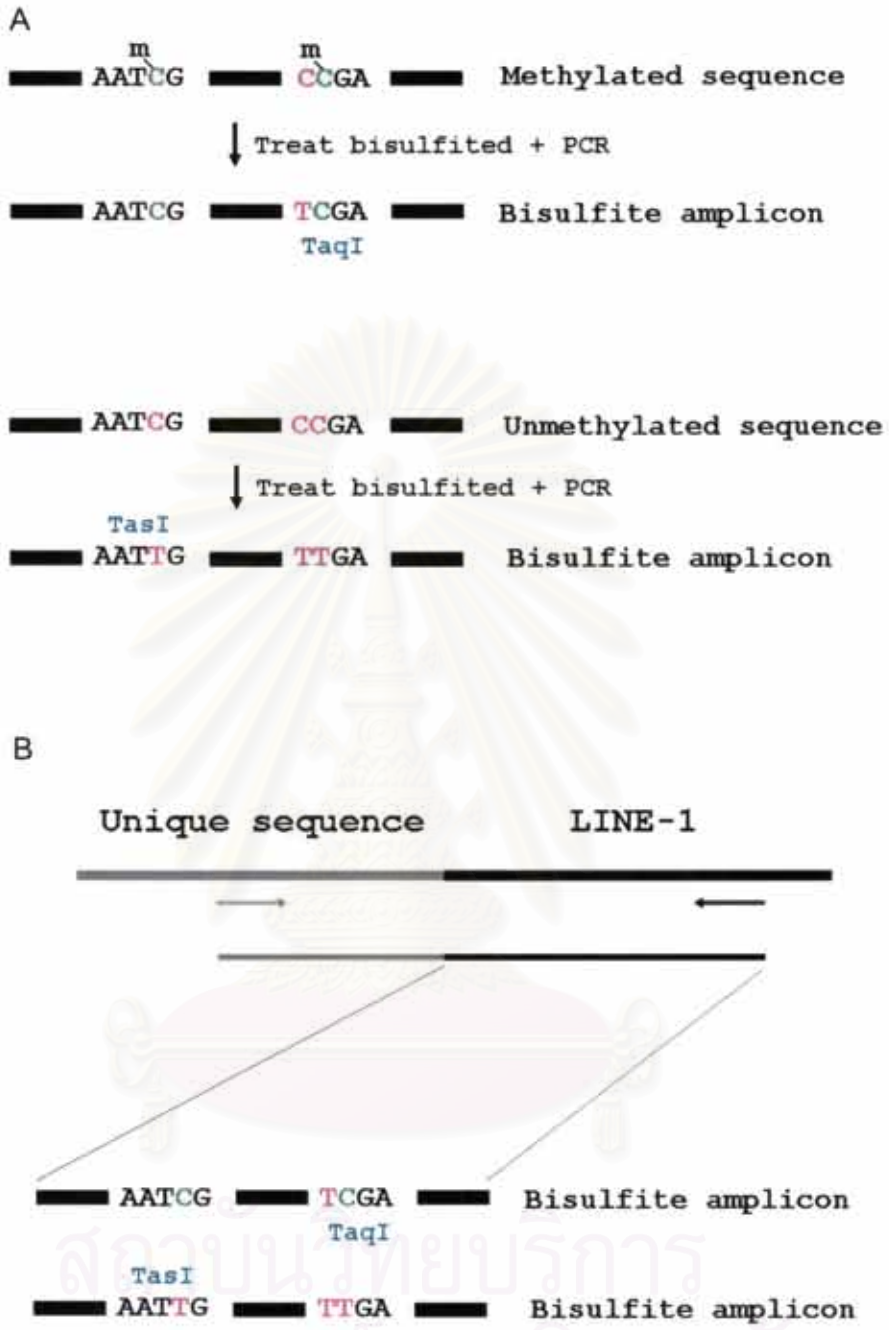


Figure 8 Schematics illustration of (A) COBRALINE-1 and (B) CUL-1. Arrows are PCR primers. When treated DNA with bisulfite and PCR, unmethylated sequences; AATCG, converted to AATTG (*Tas*I site), whereas, methylated sequences; CCGA, converted to TCGA (*Taq*I site)

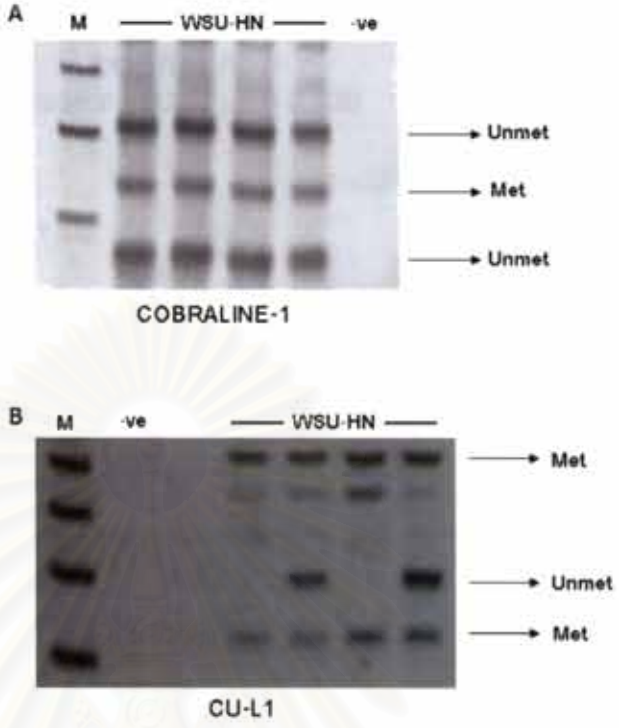


Figure 9 The example of result from COBRALINE-1 (A) and L1-*PPP2R2B* (B) in WSU-HN cell lines. Met and unmet are methylated and unmethylated sequences. M is a 25 bp size marker and –ve is dH_2O.

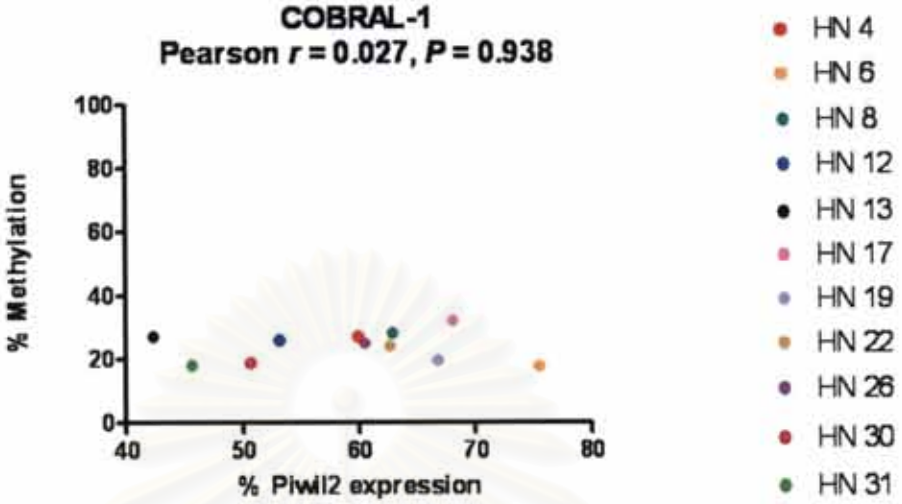


Figure 10 Correlation between *Piwil2* expression and genome-wide LINE-1 methylation levels. Pearson's correlation coefficient was determined.

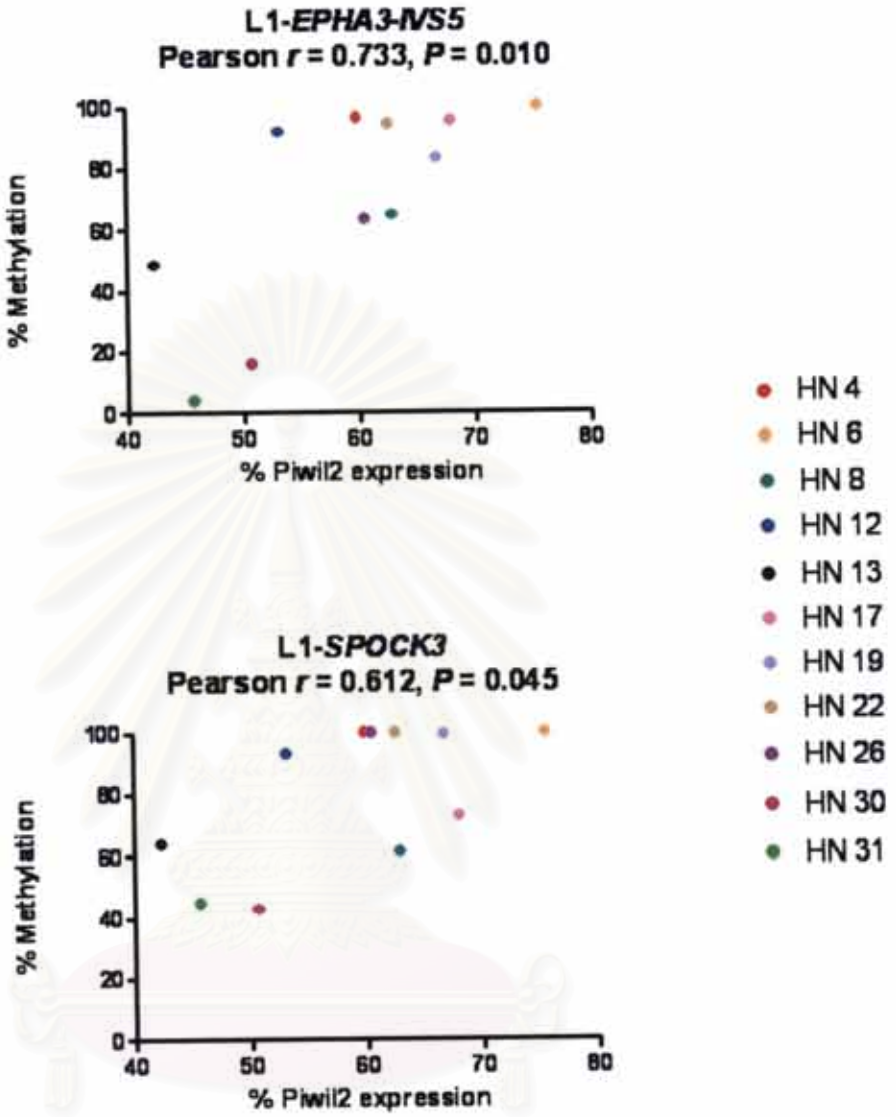


Figure 11 Correlation between *Piwil2* expression and LINE-1 methylation levels of L1-*EPHA3IVS5* and L1-*SPOCK3*. Pearson's correlation coefficient was determined.

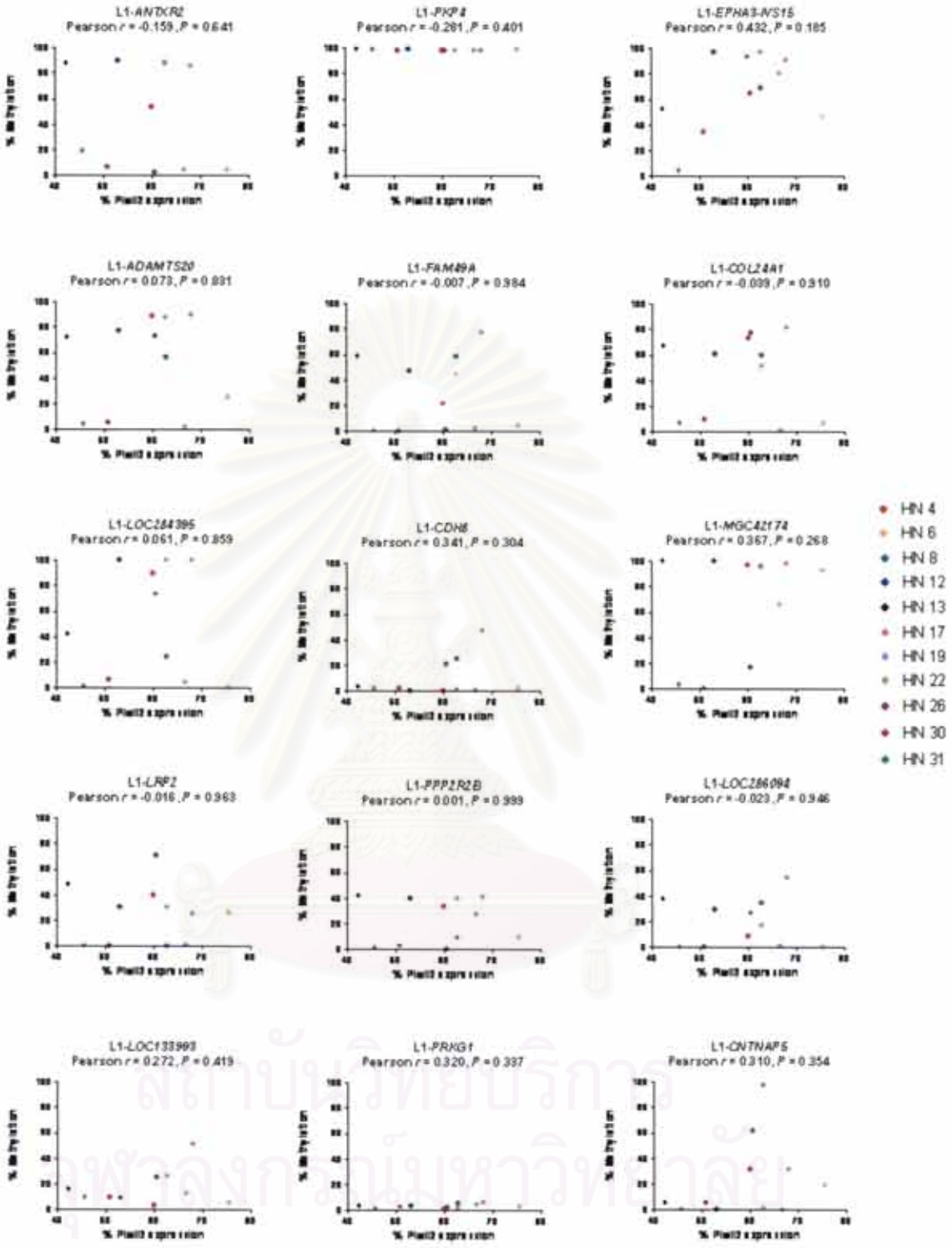


Figure 12 Correlation between *Piwil2* expression and LINE-1 methylation levels of 15 specific loci. Pearson's correlation coefficient was determined.

Correlation between *Piwil2* down-regulation and LINE-1 methylation

As previous studies reported that down-regulate of *Piwil2* in mice testes, showed loss of LINE-1 methylation levels (6). Consequently, we examined LINE-1 methylation levels in genome-wide and specific loci after down-regulate of *Piwil2* in cancer cell lines.

We inhibited expression of *Piwil2* in HeLa used 3 siRNAs target sequences, that is, siPiwil2_1, siPiwil2_2 and siPiwil2_3. First of all, we establish *Piwil2* siRNAs stable cell line in HeLa. We found apoptosis in HeLa cell after transfection *Piwil2* siRNAs. As a result, we change to transient transfection. After transfection 24 and 48 hr, we observed expression of *Piwil2* by semi-quantitative RT-PCR. We found that expression levels of *Piwil2* after 24 hr decrease than after transfection 48 hr. (Figure 13).

After down-regulate *Piwil2* expression by using siRNAs, we examined LINE-1 methylation in genome-wide and specific loci by using COBRALINE-1 and CUL-1 (Figure 14). We found there was not difference of LINE-1 methylation levels in genome-wide after inhibition Piwil2 expression after 24 and 48 hr. Therefore, we suggested that there was no statically significant association of decreasing of *Piwil2* expression with genome-wide methylation of LINE-1 after 24 and 48 hr (Pearson r = 0.8632, P = 0.1368 and Pearson r = 0.-0.1630, P = 0.8370, respectively) (Figure 15). Moreover, we found no association between decreasing of *Piwil2* expression and LINE-1 methylation in each specific locus (Figure 16).

For L1-*LOC284395*, PCR product was not detected in all samples.

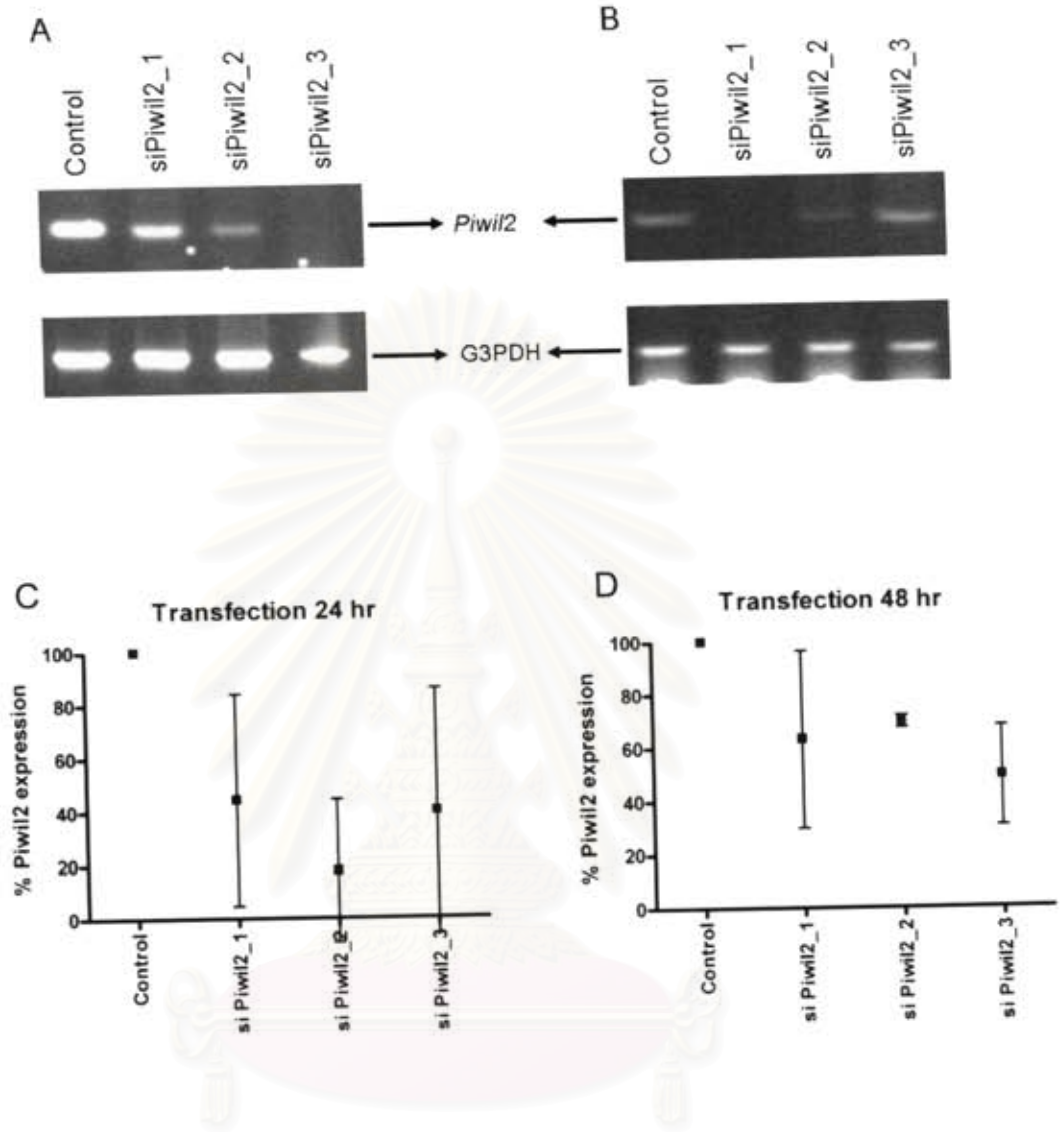


Figure 13 Down-regulate of *Piwil2* expression was performed by using siRNAs in HeLa cell. After siRNAs transfection for (A, C) 24 and (B, D) 48 hr. mRNA expression was determined by semi-quantitative RT-PCR. *Piwil2* expression was calculated as the percentage of *Piwil2* densities divided by G3PDH amplicon.

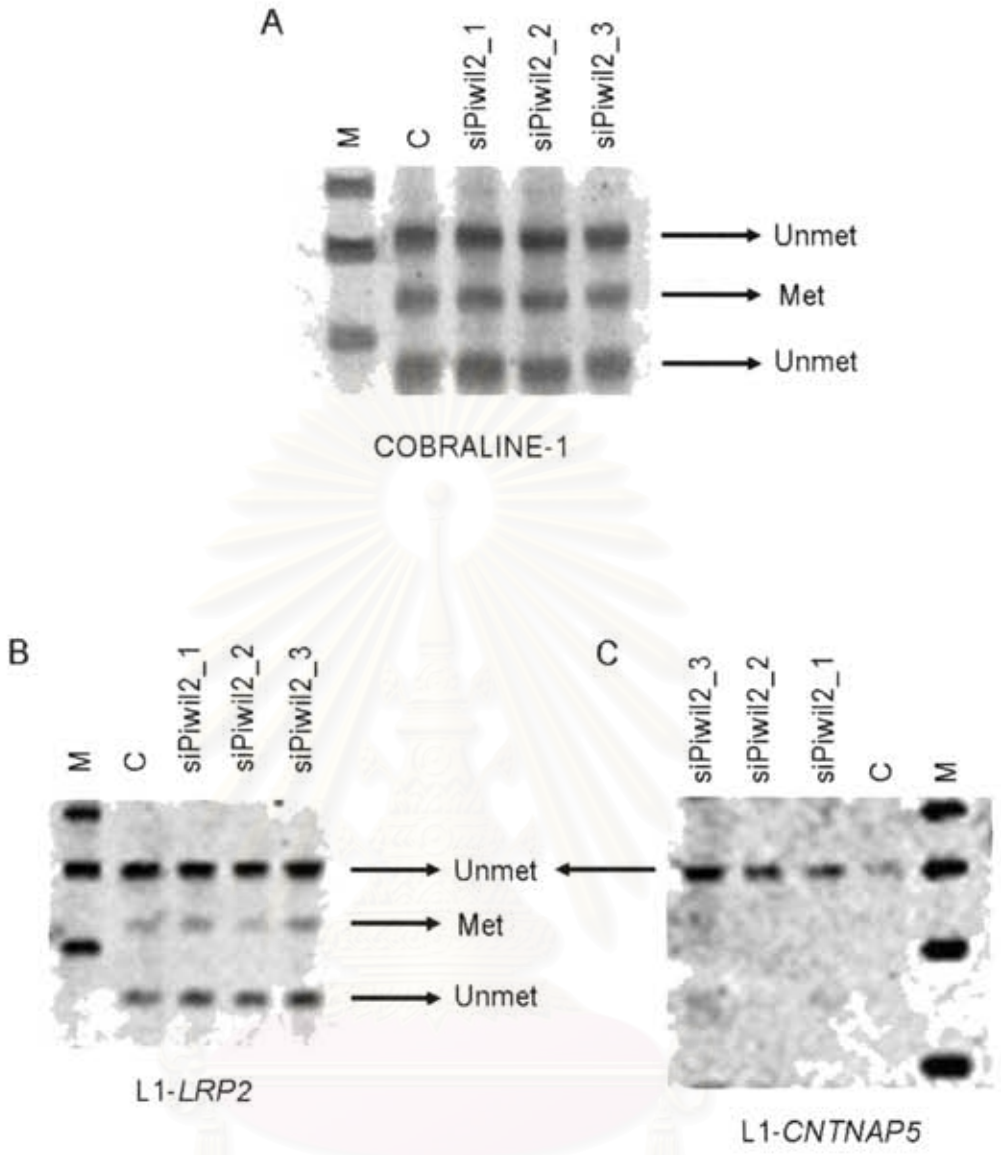


Figure 14 The example of (A) COBRALINE-1 and CU-L1; (B) L1-*LRP2* and (C) L1-*CNTNAP5* after inhibit *Piwil2* expression in HeLa. M is a 25 bp size marker, C is a control siRNAs. The Unmet and met are Unmethylated and methylated sequences.

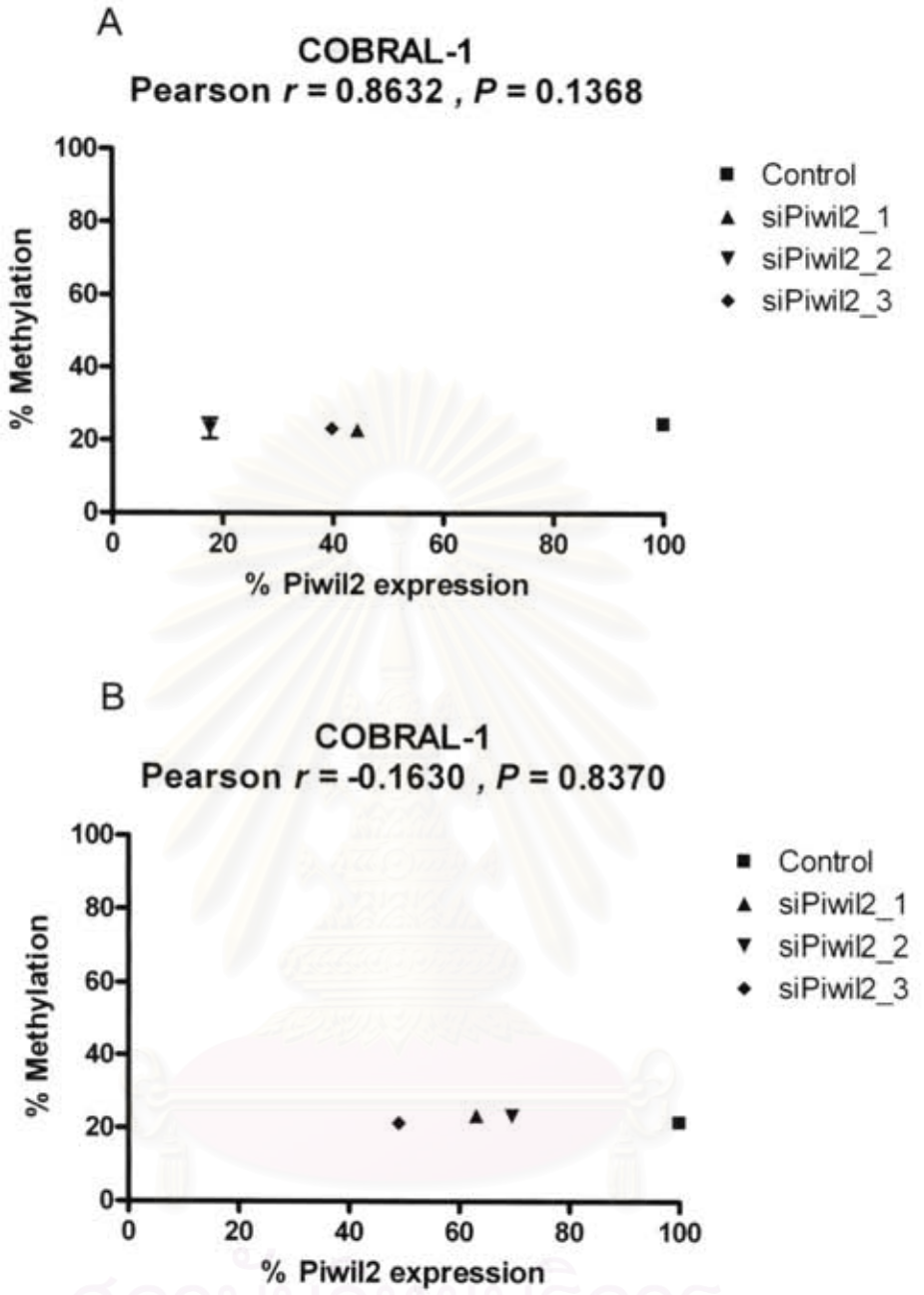
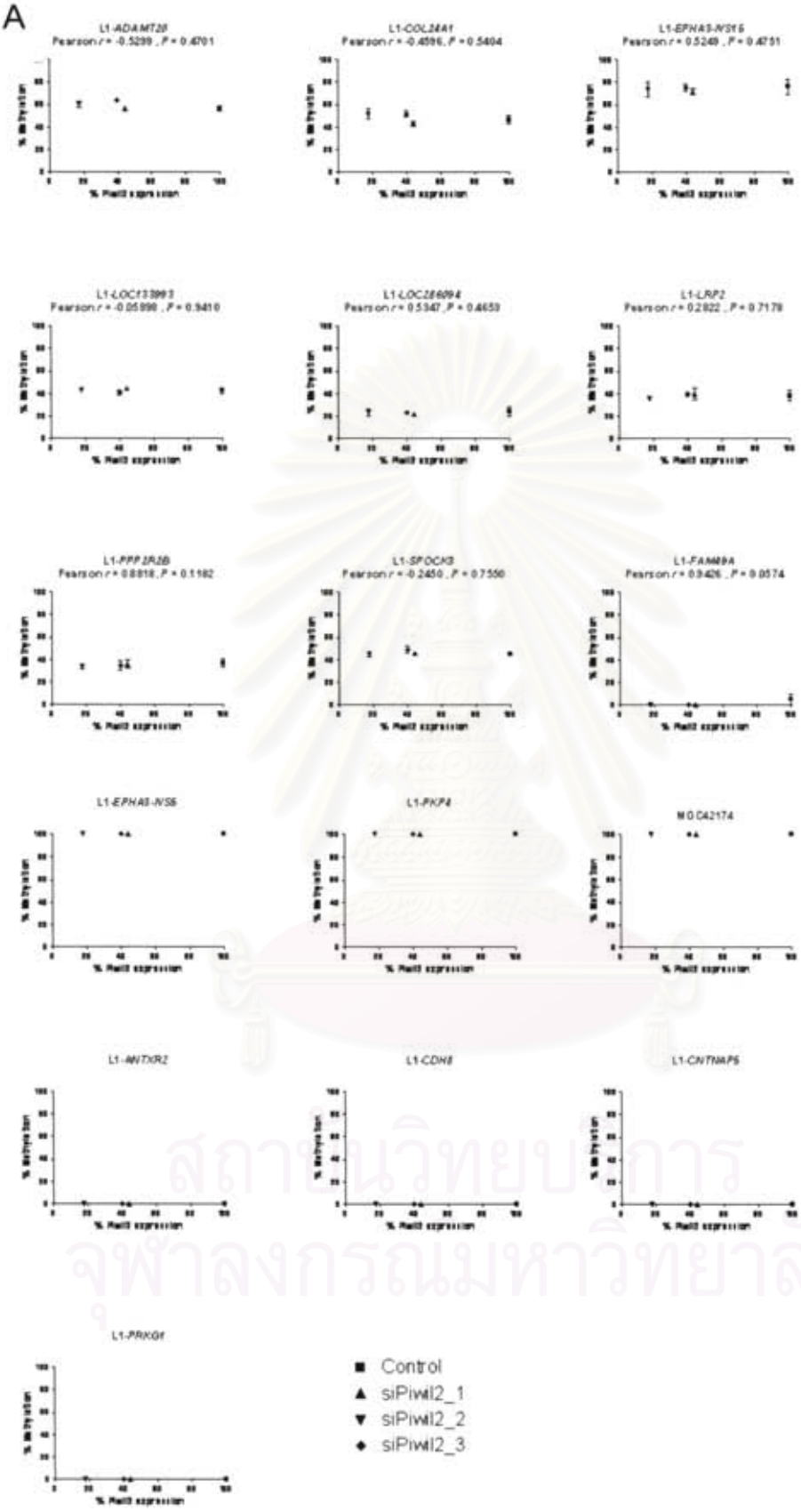


Figure 15 Correlation between down-regulate *Piwil2* expression and genome-wide LINE-1 methylation levels after transfection (A) 24 and (B) 48 hr. Pearson's correlation coefficient was determined.

A



B

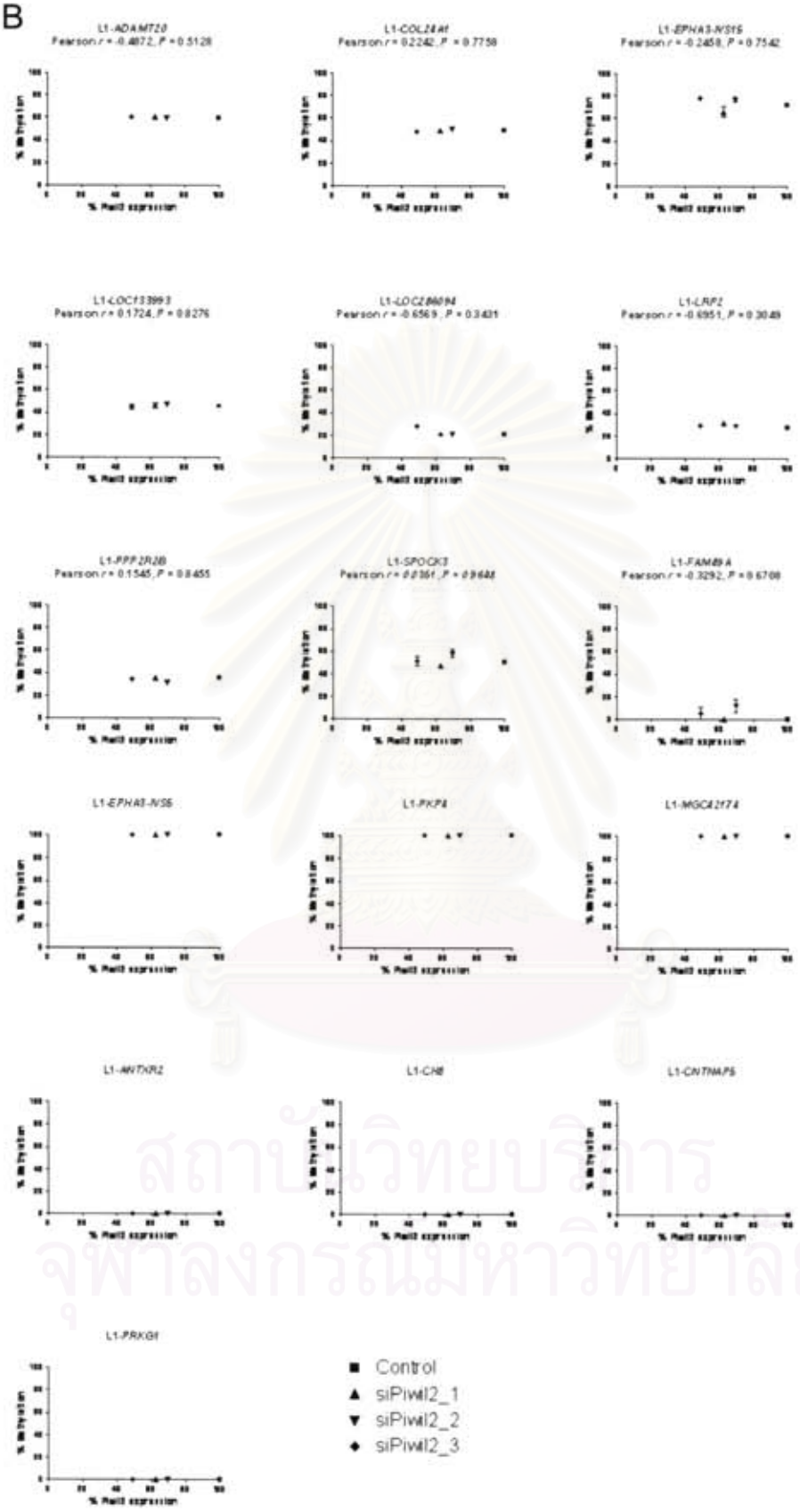


Figure 16 Correlation between down-regulate *Piwil2* expression and LINE-1 methylation levels of 15 specific loci, after transfection (A) 24 and (B) 48 hr. Pearson's correlation coefficient was determined.

CHAPTER V

DISCUSSION AND CONCLUSION

This study aimed to investigate if there is an association between *Piwil2* expression and LINE-1 methylation. We found that in WSU-HN cancer cell lines, LINE-1 methylation of some loci was directly correlated with the *Piwil2* expression level. Under normal physiological conditions, *Piwil2* is expressed and LINE-1s are methylated in mouse testis. Moreover, when *Piwil2* was mutated, thus preventing expression, LINE-1s in the germ cells were hypomethylated (6). In humans, *Piwil2* is not expressed in somatic cells but is up-regulated in cancer (40). Moreover, LINE-1s in cancer cells are generally hypomethylated (8). Therefore, *Piwil2* should directly influence LINE-1 methylation in cancers less than in the testis. Moreover, because the association between *Piwil2* expression and LINE-1 methylation was direct, the up-regulation of *Piwil2* in cancer may help compensate for global hypomethylation in cancer.

We require evaluating methylation of LINE-1 after inhibit *Piwil2* expression in long term. Because DNMT1 is the enzyme responsible for maintenance methylated DNA during replication and DNMT 3A and 3B require for de novo methylation. These mechanisms have longer period to effect on methylation. On the other hand, a previous study reported that knock-down *Piwil2* expression leaded to inhibition of apoptotic pathway via Stat3/ Bcl-X_L (40). As a consequence, we attempt to establish *Piwil2* siRNAs stable cell lines but this experiment failed. Therefore, we used transient transfection instead. After transient transfection, we found no association between *Piwil2* expression and LINE-1 methylation in specific loci, that is, LI-*EPHA3IVS15* and L1-*SPOCK3*. Since, these experiments have short period so we can not found methlation of LINE-1 change.

Recently, we reported that even though cancerous genomes generally have reduced LINE-1 methylation levels, LINE-1 methylation can be influenced differentially depending on where the particular sequences are located (23). This also suggests a specific role of LINE-1 methylation in cis. For example, we reported a striking correlation between LINE-1 methylation levels within two introns of the same gene (23). The mechanism and consequence of locus specific patterns of LINE-1 methylation are

unknown. It will be interesting to explore how *Piwil2* selects LINE-1 loci for methylation and to determine the effects of altered methylation levels.

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APPENDICES

APPENDIX A

BUFFER AND REAGENT

1. Lysis Buffer II

5 M NaOH	15	ml
0.5 M EDTA	48	ml
Distilled water to volume	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

2. 10% SDS Solution

Sodium dodecyl sulfate	10	g
Distilled water to volume	100	ml

Mix the solution and store at room temperature.

3. Proteinase K

Proteinase K	20	mg
Distilled water to volume	1	ml

Mix the solution and store at – 20°C

4. 0.5 M EDTA (pH 8.0)

Disodium ethyleneidamine tetraacetate	18.66	g

Dissolve in distilled water and adjust pH to 8.0 with NaOH

Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

5. 10X TBE Buffer

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

Mix the solution and store at room temperature.

6. 6X loading dye

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

Mix well and store at room temperature.

7. 10 M NH4OAc

NH4OAc	77.08	g
Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

8. 25:24:1 (v/v) phenol : chloroform : isoamyl alcohol

Saturated phenol	50	ml
Chloroform	48	ml
Isoamyl alcohol	2	ml

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

9. TE buffer (pH 8.0)

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

Sterilize the solution by autoclaving and store at room temperature.

10. 20 mg/ml glycogen

Glycogen	200	mg
Distilled water to volume	10	ml

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

APPENDIX B

SEQUENCE OF PIWIL2

>gi|24431984|ref|NM_018068.2| Homo sapiens piwi-like 2 (Drosophila) (PIWIL2), mRNA

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SEQUENCE OF LINE-1

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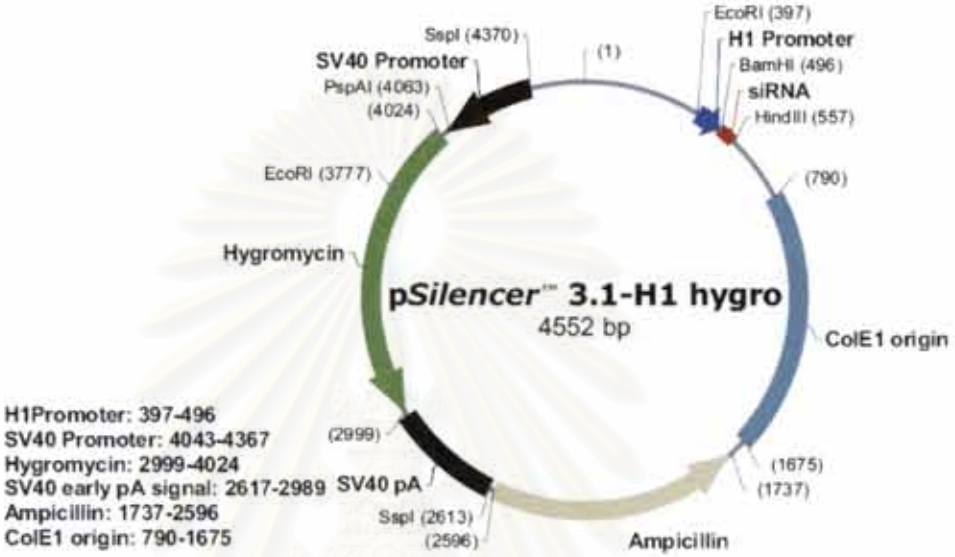
APPENDIX C

PLASMID

p*Silencer*™ 3.1-H1 hygro



BIOGRAPHY

Miss Thatchawan Thanasupawat was born on January 24, 1984 in Bangkok, Thailand. She received her Bachelor degree of Science (Medical Technology), (Second Class Honors) in 2006 from Faculty of Allied Health Sciences, Chulalongkorn University. She enrolled Chulalongkorn University in graduate program for Master degree of Medical Science since 2006. Her publication was published in Pornthanakasem W, Kongruttanachok N, Phuangphairoj C, Suyarnsestakorn C, Sanghangthum T, Thanasupawat T, et al. LINE-1 methylation status of endogenous DNA double-strand breaks. Nucleic Acids Res. 2008 Jun; 36(11):3667-75 and Thanasupawat T, Phokeaw C, and Mutirangura. The association between Piwil2 expression and LINE-1 methylation in cancer cells. Asian Biomedicine (in press).