ความสัมพันธ์ระหว่างการเกิดเมทิลเลชั่นของไลน์วันกับการใช้บุหรี่และเมทแอมเฟตามีน

นายกรกต ไกรจักร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
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Association between LINE-1 methylation with nicotine and methamphetamine use

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หัวข้อวิทยานิพนธ์	ความสัมพันธ์ระหว่างการเกิดเมทิลเลชั่นของไลน์วันกับการใช้บุหรี่และเมทแอมเฟตามีน
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คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย อนุมัติให้นับวิทยานิพนธ์ฉบับนี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญามหาบัณฑิต

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.....กรรมการภายนอกมหาวิทยาลัย

(แพทย์หญิง ปองขวัญ ยิ้มสอาด)

กรกต ไกรจักร์ : ความสัมพันธ์ระหว่างการเกิดเมทิลเลชั่นของไลน์วันกับการใช้บุหรี่และเมทแอมเฟตามีน (Association between LINE-1 methylation with nicotine and methamphetamine use) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. พญ. รัศมน กัลยาศิริ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. นพ. ดร. อภิวัฒน์ มุทิรางกูร, 53 หน้า.

นิโคตินจากบุหรี่และสารเมทแอมเฟตามีนเป็นสารเสพติดที่ออกฤทธิ์กระตุ้นจิตประสาทซึ่งสามารถทำให้เกิดความผิดปกติของการแสดงออกของยีนการศึกษาก่อนหน้าพบว่าการใช้สารเสพติดชักนำให้เกิดการเปลี่ยนแปลงการควบคุมสภาวะเหนือพันธุกรรมซึ่งอาจก่อให้เกิดการเปลี่ยนแปลงทั้งโครงสร้างเซลล์ประสาทการสร้างสารสื่อประสาทและนำไปสู่การเปลี่ยนแปลงทางพฤติกรรมโดยการเปลี่ยนแปลงการควบคุมเหนือพันธุกรรมนั้นสามารถเกิดได้ในระดับโครโมโซมหรือการเติมหมู่เมทิลเพื่อปรับเปลี่ยนสายพันธุกรรมที่เรียกว่าการเกิดดีเอ็นเอเมทิลเลชั่น มีรายงานการศึกษาถึงบทบาทของไลน์วัน ซึ่งใช้เป็นตัวแทนของการเกิดเมทิลเลชั่นทั่วทั้งจีโนมกับการใช้บุหรี่และการใช้เมทแอมเฟตามีนโดยเราสามารถแบ่งระดับการเกิดเมทิลเลชั่นของไลน์วันได้ทั้งสิ้น 4 ประเภท ดังนี้ $^{m}C^{m}C$, $^{m}C^{u}C$, $^{u}C^{m}C$, และ $^{u}C^{u}C$ โดย ^{m}C และ ^{u}C แสดงถึงการถูกเติมหมู่เมทิลและไม่ถูกเติมหมู่เมทิลตามลำดับ การเกิดภาวะไฮโปเมทิลเลชั่น ($^{u}C^{u}C$) ของไลน์นั้นสามารถกระตุ้นให้เกิดความไม่เสถียรของจีโนมได้ จุดมุ่งหมายของการศึกษาในครั้งนี้เป็นการศึกษารูปแบบของการเกิดเมทิลเลชั่นของไลน์วันที่เปลี่ยนแปลงไปเมื่อได้รับนิโคตินจากการสูบบุหรี่และการใช้สารเมทเอมเฟตามีนซึ่งในการศึกษาครั้งนี้พบว่าผู้ที่ได้รับสารนิโคตินจากบุหรี่ในช่วงชีวิตจำนวนมากกว่า 100 มวน และการใช้เมทแอมเฟตามีนในช่วงชีวิตจำนวนมากกว่า 1,000 ครั้งมีระดับของเมทิลเลชั่นทั่วทั้งจีโนม (^{m}C) และ ไฮเปอร์เมทิลเลชั่น ($^{m}C^{m}C$) ลดลง อย่างไรก็ตามกลับมีผลในการเพิ่มขึ้นของไฮโปเมทิลเลชั่น ($^{u}C^{u}C$) และในการศึกษาครั้งนี้ไม่พบการเปลี่ยนแปลงของ $^{m}C^{u}C$ และ $^{u}C^{m}C$ นอกจากนี้ยังทำการศึกษาผลของจำนวนการใช้บุหรี่ต่อหนึ่งวันหรือจำนวนวันที่สูบในหนึ่งสัปดาห์ต่อการเกิดเมทิลเลชั่นของไลน์วันในกลุ่มผู้ใช้บุหรี่มากกว่า 100 มวน พบว่าปริมาณการใช้ต่อวันที่มากน้อยต่างกันหรือจำนวนวันที่สูบไม่เท่ากันในหนึ่งสัปดาห์ ไม่ได้ส่งผลให้เกิดความแตกต่างของไลน์วันเมทิลเลชั่นในการศึกษาในครั้งนี้น่าจะสรุปได้ว่า การใช้บุหรี่นั้นมีผลต่อการเปลี่ยนแปลงของไลน์วันเมทิลเลชั่นโดยไม่ว่าใช้บุหรี่ปริมาณที่ต่างกันหรือจำนวนวันที่สูบที่ไม่เท่ากันไม่มีผลให้เกิดเปลี่ยนแปลงของไลน์วันเมทิลเลชั่นที่มากขึ้นหรือน้อยลงการศึกษาต่อมาคือทำการศึกษาผลของ ระยะเวลาการใช้เมทแอมเฟตามีนในสองกลุ่ม คือ กลุ่มที่ใช้เมทแอมเฟตามีนอย่างหนักและกลุ่มที่ไม่ได้ใช้เมทแอมเฟตามีนอย่างหนักร่วมกับการศึกษาเปรียบเทียบประวัติการใช้เมทแอมเฟตามีนในปีที่แล้วอย่างหนักกับกลุ่มที่ไม่มีประวัติการใช้ในปีที่แล้วอย่างหนักซึ่งพบว่าการใช้เมทแอมเฟตามีนของกลุ่มที่ใช้เมทแอมเฟตามีนอย่างหนักในระยะเวลาที่นานมีความสัมพันธ์กับระดับของ ^{m}C ที่ลดลงร่วมกับการเพิ่มขึ้นของ $^{u}C^{u}C$ ในขณะที่ไม่พบความสัมพันธ์ดังกล่าวในกลุ่มที่ไม่ได้ใช้เมทแอมเฟตามีนอย่างหนักอย่างไรก็ตามเมื่อเปรียบเทียบระดับการเกิดเมทิลเลชั่นของไลน์วัน ในกลุ่มที่มีและไม่มีประวัติการใช้เมทแอมเฟตามีนในปีที่แล้วอย่างหนักพบว่าไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติซึ่งสรุปได้ว่าการเปลี่ยนแปลงของการเกิดไลน์วันเมทิลเลชั่นหลังจากได้รับเมทแอมเฟตามีนยังคงอยู่แม้จะใช้ในปริมาณน้อยกว่า 150 ครั้ง ในช่วงที่ผ่านมาในการศึกษาครั้งนี้สรุปได้ว่าการได้รับนิโคตินจากบุหรี่ในช่วงชีวิตมากกว่า 100 มวน และการใช้สารเมทแอมเฟตามีนในช่วงชีวิตจำนวนมากกว่า 1,000 ครั้ง ส่งผลให้เกิดการเปลี่ยนแปลงระดับของการเกิดเมทิลเลชั่นและรูปแบบเมทิลเลชั่น ของไลน์วันเมื่อเทียบกับผู้ที่ใช้สารดังกล่าวจำนวนน้อยกว่าซึ่งการเปลี่ยนแปลงดังกล่าวอาจส่งผลให้เกิดความไม่เสถียรของจีโนมได้

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2558 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5574102430 : MAJOR MEDICAL SCIENCE

KEYWORDS: NICOTINE , METHAMPHETAMINE , LINE-1S , GLOBAL METHYLATION , COBRA LINE-1S

KORAKOT KRAIJAK: Association between LINE-1 methylation with nicotine and methamphetamine use. ADVISOR: ASST. PROF. RASMON KALAYASIRI, M.D., CO-ADVISOR: PROF. APIWAT MUTIRANGURA, M.D., 53 pp.

Psychostimulants such as nicotine and methamphetamine (MA) cause abnormal changes in gene expression. Previous studies suggest that substance may induce epigenetic alteration that contributing to the changes in the neuronal structure and neurotransmitters and resulting in behavioral change. The epigenetic alteration happens at the chromosomal level, by histone modification, and at the DNA level, by DNA methylation where the base cytosine is methylated. In DNA methylation, Long interspersed nuclear element 1s (LINE-1s) has been used as surrogate of overall global DNA methylation level. LINE-1s methylation pattern is classified into 4 patterns including, $^{m}C^{m}C$, $^{m}C^{u}C$, $^{u}C^{m}C$, and $^{u}C^{u}C$ when ^{m}C and ^{u}C stand for methylated and unmethylated cytosine respectively. In addition, the hypomethylation ($^{u}C^{u}C$) pattern of LINE-1s causes genomic instability of the genome. The aim of this study was to measure the methylation pattern of LINE-1s in peripheral blood that affected by nicotine and MA use in human. Our result revealed that participants with nicotine experienced (≥100 instances use in lifetime) and MA heavy use (≥ 1,000 episodes in lifetime) use had significant lower % overall methylation (^{m}C) and hypermethylation ($^{m}C^{m}C$) but higher % hypomethylation pattern ($^{u}C^{u}C$) than the control group. However, those with nicotine and MA use did not differ regarding % partial methylation ($^{m}C^{u}C$ and $^{u}C^{m}C$). Moreover, we evaluated the correlation with number of cigarette use per day/ frequency of cigarette smoking per week (2-3 days per week or 7 days per week). We found no correlation between the number of cigarette use and LINE-1s methylation. We also did not find any significant differences in smoking 2-3 days per week and smoking 7 days per week. From these results demonstrates that the effect of nicotine experienced on LINE-1s methylation will occur, no matter how frequency or amount of cigarette use. Next, we examined the association among MA duration and LINE-1s methylation pattern, we found that long time use of heavy MA may promote increased in hypomethylation ($^{u}C^{u}C$) of LINE-1s, while we did not find any correlation between non-heavy MA use and LINE-1s methylation. Then we compared percentage of LINE-1s methylation among past year MA use (≥ 150 episodes) and non MA use past year (< 150 episodes). We did not find any differences of all pattern of LINE-1s in two groups. This result suggest that even though using MA fewer than 150 episodes in the past year did not turn LINE-1s methylation to normal level. In conclusion, cigarette smoking and MA use may change the methylation pattern of LINE-1s that might cause the instability of the genome.

Field of Study: Medical Science

Academic Year: 2015

Student's Signature .....

Advisor's Signature .....

Co-Advisor's Signature .....

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สารบัญ

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CHAPTER I

INTRODUCTION

1.1 Background and rationale

Addiction is a neuropsychiatric disorder that characterized by compulsive substance seeking and taking even though the occurrence of harmful effects [(1)]. Administration of substance including psychostimulants such as methamphetamine (MA; as known as ya ba or ya ice in Thai) and nicotine cause changes in the neuronal plasticity that may lead to substance addiction [(2),(3)].The ventral tegmental area (VTA) is a crucial brain area that plays the major role in regulating the reward and motivation. VTA is composed of dopaminergic neurons that releases dopamine in many brain areas, for example nucleus accumbens (NAc), prefrontal cortex, amygdala, and hippocampus [(4)]. Nicotine is the major chemical that presents in tobacco that activates nicotinic acetylcholine receptors (nAChRs) in the dopaminergic neurons in the VTA resulting in the increase of the dopamine release [(5)]. MA is a psychoactive substance, also known to transiently facilitate dopamine transmission from nerve terminals and also block dopamine reuptake[(6)]. Nevertheless, long lasting of substance intake may cause epigenetic alterations mediated by substance-induced gene expression. Currently, increasing reports suggest that changes in neuronal plasticity or gene expression after substance administration alters the neurotransmitter transmission such as dopamine, the alteration of neurotransmitter by substance-induced gene expression may be a key mechanism capable of affecting reward processes [(7), (8)]. Epigenetics is the process that changes in gene expression but do not change the DNA sequence. Some environmental factors promote alterations in the chromatin structure by affecting the enzymatic activity including histone acethyltransferases (HATs), histone deacetylases (HDACs) or modifications of DNA by DNA methylation [(8)].

DNA methylation is a part of epigenetics that occurs at 5' position of cytosine bases mostly found in the gene promoter that converted to 5-methylcytosine (5mc) by using DNA methyltransferase (DNMTs) [9]. Long interspersed element-1s (LINE-1s) are retrotransposon (repetitive sequence that some are capable to copy themselves and insert into another region) located in the human genome for more than 500,000 copies. LINE-1s pattern is classified into 4 groups depending on the methylation status, including 2 methylated CpGs or hypermethylation ($^{m}C^{m}C$), 2 unmethylated CpGs or hypomethylation ($^{u}C^{u}C$), 5' methylated with 3' unmethylated CpGs ($^{m}C^{u}C$) and 5' unmethylated with 3' methylated CpGs ($^{u}C^{m}C$) or partial methylation[10]. Hypomethylation of LINE-1s promoter induced the LINE-1s expression and might correlate with many human diseases such as cancer [11], [12] and could be a potential mechanism of substance addiction [13]. Recently, the roles of nicotine and MA on the global methylation are largely unknown. Previous preclinical studies found that chronic MA exposure led to alteration of the methylation levels at CpG regions of many genes that expressed in frontal cortex and hippocampus [14]. Similarly, nicotine exposure also reduces the DNA methylation levels at many loci from a Genome-Wide Association Study (GWAS) [15]. In addition, changes in LINE-1s methylation of the oral mucosa of nicotine smokers were found from Dr. Apiwat Mutirangura laboratory. Specifically, oral mucosa of the nicotine smokers had the increase of % hypermethylation ($^{m}C^{m}C$) and % hypomethylation ($^{u}C^{u}C$) and decrease of the % 5' methylated with 3' unmethylated ($^{m}C^{u}C$) without change in 5' unmethylated with 3' methylated ($^{u}C^{m}C$) and overall methylation (^{m}C). The study found that a reduction of $^{m}C^{u}C$ may associate with increases $^{m}C^{m}C$ and $^{u}C^{u}C$ while decreases of $^{u}C^{m}C$ might correlate with increases only $^{u}C^{u}C$ but no correlate with $^{m}C^{m}C$ [10]. Nevertheless, no study has been investigated LINE-1s methylation in blood samples or post mortem brain tissue of nicotine smokers or other psychostimulant users. Since nicotine may have an effect on DNA methylation and nicotine has been known to be a gateway drug (a soft drug such as nicotine or alcohol whose use is thought to lead to the use of hard drug such as MA or cocaine). For example nicotine enhances the long term synaptic potentiation that induced by cocaine [16]. Hence we speculate that DNA methylation of LINE-1s of nicotine smokers may influence other substance of abuse such as MA as well. In this study we measured

the global methylation level of LINE-1s by using technique combined bisulphite restriction analysis (COBRA) to investigate the methylation pattern and association between the methylation pattern with nicotine and MA use. We hypothesize that not only the methylation pattern are differed between persons with and without substance use, the relationship between each methylation pattern of the nicotine smokers and MA users may be clarified. In addition, related factors such as nicotine and MA use variables may be related to the methylation pattern.

1.2 Research questions

1. What are the patterns of LINE-1s methylation in nicotine, MA, nicotine&MA, and healthy control groups.
2. Are there any differences of LINE-1s methylation pattern between nicotine, MA, nicotine&MA, and healthy control groups.
3. Does other related factors, nicotine use variables and MA use variables are associated with LINE-1s methylation pattern.
4. What is the relationships between LINE-1s methylation pattern in each group of in nicotine, MA, nicotine&MA, and healthy control groups.

1.3 Research objectives

1. To study the patterns of LINE-1s methylation in nicotine experienced/ non-experienced nicotine smokers, heavy MA/non-heavy MA use and control groups.
2. To compare LINE-1s methylation levels between nicotine experienced/ non-experienced nicotine smokers, heavy MA/non-heavy MA use and control groups.
3. To study association of other related factors, nicotine use variables and MA-use variables with the patterns of LINE-1s methylation
4. To study the relationships between LINE-1s methylation pattern in each group of in nicotine experienced/ non-experienced nicotine smokers, heavy MA/non-heavy MA use and control groups.

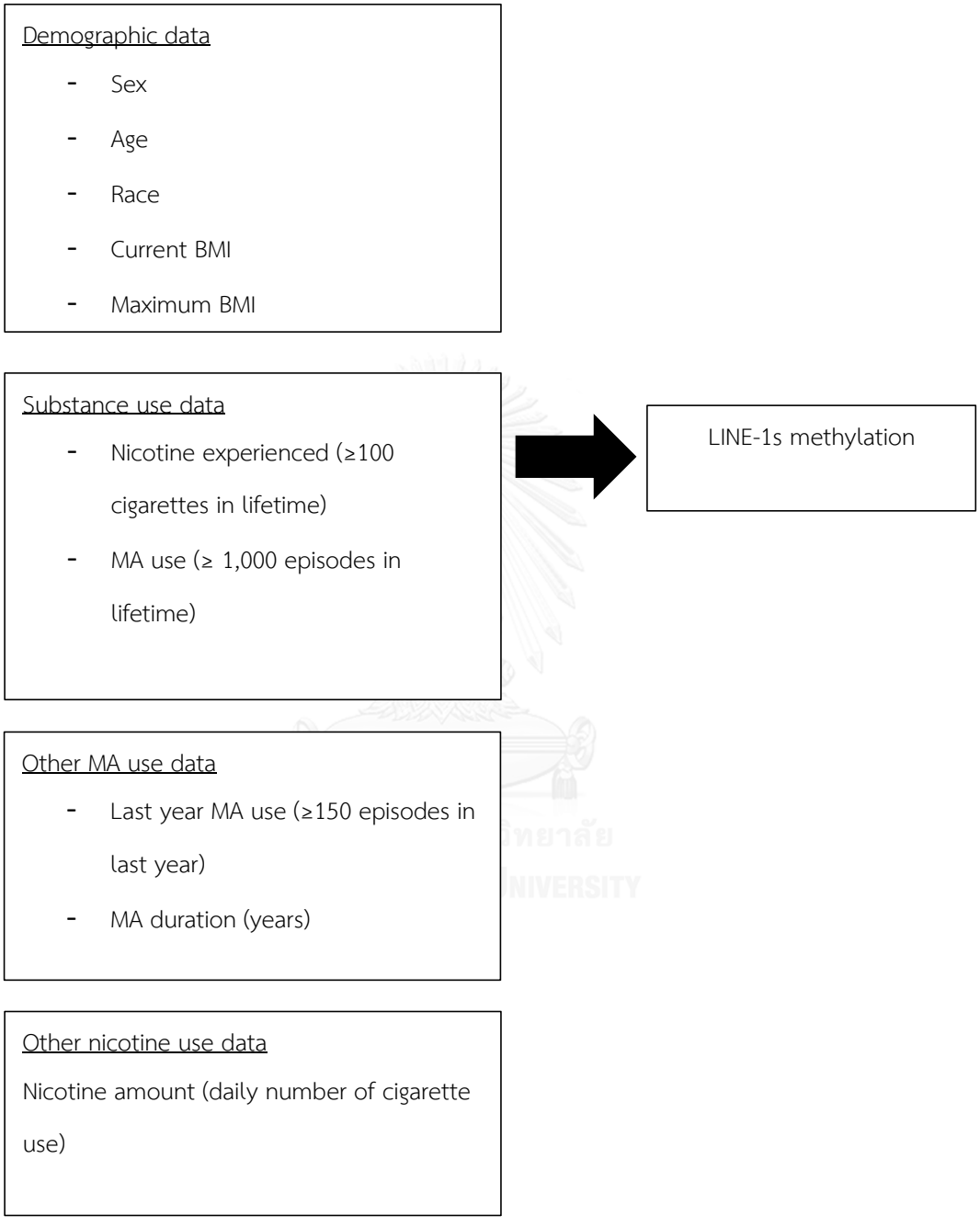
1.4 Hypothesis

Nicotine and/or MA use induces the hypomethylation patterns of LINE-1 in peripheral blood.

1.5 Conceptual Framework

Independent variables

Dependent variables



1.6 Expected beneficial

This research will let us understand the basic knowledge of global methylation pattern of LINE-1s of the most common illegal psychostimulant use in the Thai population. Identifying the different patterns of LINE-1s methylation between nicotine smoking, MA use, and healthy controls may be used for evaluation the association between substance use and alteration of the methylation pattern that might mediate the mechanism of the occurrence of the substance-induced medical and other neuropsychiatric illness that further study is warranted.

CHAPTER II

Review literature

In this chapter, the review of the literature includes 3 parts

2.1 Addiction

2.1.1 Clinical features of addiction

2.1.2 Prevalence and consequence of nicotine use and MA use

2.1.3 Neuroscience of addiction

2.2 Nicotine and methamphetamine

2.3 Epigenetics : DNA methylation and LINE-1s methylation

2.1 Addiction

2.1.1 Clinical features of addiction

Addiction is a chronic relapsing brain disorder that characterized by [17] :

1. Loss of control : the person takes the substance and cannot stop intake.
2. Withdrawal symptom: there are craving, feeling depress, may increased insomnia in some case withdrawal can promote violence, seizures, hallucinations.
3. Become tolerance: the person need to use more of the subsance to get the same effects.
4. Continues use despite knowing the harmful effects: they taking substance regularly, even though they have some illnesses direct linked to it.
5. Social and/or recreaional sacrifies: the person have abandoned activitiest that used to enjoy such as hobbies, socializing.
6. Spend a lot of time: to tinking about the drug, how to take more, and recovering the substance effectes.

Substance addiction based on the DSM-IV criteria is defined as “a maladaptive pattern of substance use leading to clinically significant impairment or distress, as manifested by three (or more) of the following, occurring any time in the same 12-month period:”

1. Tolerance, as defined by either of the following:
   (a) A need for markedly increased amounts of the substance to achieve intoxication or the desired effect or
   (b) Markedly diminished effect with continued use of the same amount of the substance.

2. Withdrawal, as manifested by either of the following:

   (a) The characteristic withdrawal syndrome for the substance

   (b) The same (or closely related) substance is taken to relieve or avoid withdrawal symptoms.

3. The substance is often taken in larger amounts or over a longer period than intended.

4. There is a persistent desire or unsuccessful efforts to cut down or control substance use.

5. A great deal of time is spent in activities necessary to obtain the substance (such as visiting multiple doctors or driving long distances), use the substance (for example, chain-smoking), or recover from its effects.

6. Important social, occupational, or recreational activities are given up or reduced because of substance use.

7. The substance use is continued despite knowledge of having a persistent physical or psychological problem that is likely to have been caused or exacerbated by the substance (for example, current cocaine use despite recognition of cocaine-induced depression or continued drinking despite recognition that an ulcer was made worse by alcohol consumption).

2.1.2 Prevalence and consequence of nicotine use and MA use

Primary data sources included the websites of the World Health Organization (WHO), the United Nations Office on Drugs and Crime (UNODC), and the Alberta Gambling Research Institute. The study of 2015 revealed that 22.5% of the global adult population (1 billion people; 32% of men, 7% of women) smoke tobacco which caused an estimated about 11% of deaths in men and 6% of deaths in women annually[18]. Regarding in 2015 the smoking situation in Thailand, about 23% (approximately 12.3 million; 44% of men and 3% of women) of Thai adult population smoked tobacco. In the year 2025, around 20% of the Thai population (about 11.8 millions) are estimated to be smokers. The highest rate of smoking among men was seen in the age-group 40 - 54; and among women in the age-group 70 up [19].According to the United Nations Office on Drugs and Crime (UNODC), 14 to 54 million users of amphetamines worldwide were estimated at 2013 [20].

Generally, there have many reasons postulated why people start to use substance including to make oneself feel good or better, to decrease or alleviate negative feelings [21]. Substance stimulates feeling of pleasure. In addition, people who are suffering from depression or stress may begin to use substance which lead to continuing the use and unable to stop using the substance despite they want to. Nevertheless, vulnerability to substance addiction may differ from person to person. Specifically, person with more risk factors will have a greater chance to take more substance and more likely to have an addiction. Risk factors may be either environmental or biological [22].

Environmental factors

1. Violence behavior in childhood
2. Have no parental care
3. Poor social skills
4. Substance experimentation
5. Easy to available substances at school or communities.

Biological factors

Genetic materials account for 40 to 60 percent of a person's vulnerability to addiction Adolescents and persons with psychiatric disorder have a greater risk for using the substance and addiction than normal healthy population. Furthermore, the next question is why do some people become addicted, while some people do not? To address this question we focus on the epigenetic aspect, because the epigenetic changes differ from person to person. While DNA sequence of human is mostly the same.

2.1.3 Neuroscience of addiction

Substance addiction is a chronic, relapsing neurological disease that characterized by loss of control to seek or taking the substance [23]. Currently the addiction studies are mostly focused on neuronal mechanisms. Neuronal mechanism of addiction is correlated with the neuronal reward system, the system that the related neurotransmitter, dopamine, is originated from the dopaminergic neurons in the ventral tegmental area (VTA) and projects in many brain areas including the nucleus accumbens (NAc), striatum, amygdala, hippocampus and the prefrontal cortex [24], [25]. All of the classes of substance of abuse increase the dopamine transmission from the VTA to the NAc or other parts of the limbic system [25]. In addition, glutamatergic system is involved in the brain reward circuit via controlling the above brain regions on the dopamine pathway [26].

2.2 Nicotine and methamphetamine (MA)

Nicotine is the major chemical component of cigarette, which is produced from many types of plants, including tobacco. After smoking a cigarette, nicotine enters into the lungs and absorbed into the pulmonary venous circulation, then pass to arterial circulation and crosses the blood brain barrier to bind to the nicotinic acetylcholine receptors (nAChRs). Stimulating nAChRs causes the influx of sodium or calcium ions into neurons. The entry of these ions, allowing more calcium to enter, which resulting in the release of the neurotransmitter including dopamine [27]. Dopamine transmission from dopaminergic neurons in ventral tegmental area (VTA) to mesocorticolimbic system such as frontal cortex, hippocampus, amygdala, nucleus accumbens. The addictive behavior of nicotine may controlled by nAChRs the most nAChRs that widely expressed in the mesocorticolimbic system are $\alpha_4\alpha_5\beta_2$ (with and without the α_6 subunit) and α_7 nAChRs in the VTA but $\alpha_4\beta_2/\alpha_6\beta_2\beta_3$ (with and without the α_4 subunit) in the NAc [28]. Nicotine promotes desensitization of $\alpha_4\beta_2$ receptors of GABAergic neurons to enhance the dopamine transmission in the NAc. Moreover, nicotine involved in activation of α_7 nAChRs of glutamateric neurons that activated dopaminergic neurons via N-methyl-D-aspartate (NMDA)-type glutamate receptors located on the cell body of dopaminergic neurons [29]. The combination of desensitization and activation of nAChRs are modulated in the mesocorticolimbic system[28].

MA is a powerful psychostimulant on the central nervous system. The chemical structure of the MA are composed of phynyl ring connected to the amino group ($-NH_2$) by a two –carbon side chain with a methyl group ($-CH_3$) [30]. MA induced the release of dopamine that could be explained by the exchange diffusion model. The influx of MA to substitute and exchange for dopamine is operated by the dopamine transporter (DAT), leading to an increase in dopamine level in the brain synapse [31].The molecular alterations of gene induced by substance are necessary to describe the abnormal of brain function and behavioral response. The DNA methyltransferase (DNMT) is involve in DNA replication and some research suggest that MA regulate the DNMT1 expression in the NAc of rat [32].

Moreover, MA lead to transient phosphorylation of methyl CpG binding protein 2 (MeCP2) to decreases the inhibitory effect of MeCP2[33], MeCP2 also known to modulate brain derived neurotrophic factor (*bdnf*) that necsessary for neuronal plasticity [34]. In non-phosphorylated MeCP2 mice show increased seft-administation of MA and decreased the MA-triggered locomotor sensitization [35] . Moreover, MA also inhibits the reuptake of dopamine from the synapse leading to increased dopamine activity. Increasing of DA appears to be responsible for locomotive, stimulating, and euphoric response and other related addictive behaviors [36]. The MA addiction also produces molecular alteration of glutamarteric synapse in striatum including downregulation of GluA1 , GluA2 AMPAR and GluN1 NMDAR subunit [37].In this study we will focus on one of the factors that may influence addiction including biological aspect specifically epigenetics of nicotine and MA use

2.3 Epigenetics: DNA methylation and LINE-1s methylation

Epigenetics is a process that refers to changes in gene expression via control of the structure of chromatin or DNA sequence by non –mutation mechanism [38]. Recent years, at least 4 major mechanism of the epigenetic modification including histone tail modification,DNA methylation, gene priming and micro RNA has been explored [24]. In this study we focus on the DNA methylation which occurs at the 5' position of cytosine bases in mammalians. Almost all of the DNA methylation occur in 5'-CpG-3' islands, which are concentrated in the promoter regions [39]. The process is catalysed by DNA methyltransferase (DNMT) [40].DNA methylation does not change in DNA sequence because normal cytosine bases or 5'methylated cytosine bases still bind to guanine. Normally, methylated CpG promotes the recruitment of methylated DNA binding domain such as DNA methyl CpG binding protein 2 (MeCP2) that obstruct the transcription factors to bind to the promoter regions. Obstruction of the binding of transcription factors to the promoter regions disturbs the process of DNA transcription[41].

In the human genome, we have interspersed repetitive DNA also called transposons that are capable to insert themselves into numerous locations of our genome [(39)].These insertions of transposons alter the neuronal transcription. For example, the mutation of *MeCP2* gene exhibit LINE-1s retrotransposition and correlated with Rett syndrome. However, the effect of LINE-1s retrotransposition on the pathology of Rett syndrome is unclear [(42)]. Transposons are classified into 2 groups including 1) DNA transposons, which insert themselves directly into a DNA sequence (cut-and-paste) and 2) retrotransoposons, which duplicate themselves and transpose into genome (copy-and–paste)[(43)]. Long Interspersed Nuclear element-1s (LINE-1s) is a retrotransposon element that disperses in mammalian genomes. There are more than 500,000 copies of LINE-1s. Approximately at 12,000 copies of LINE-1s have full length and 40-60 percents of these full-length LINE-1s are expected to be active[(44)]. A full-length LINE-1s (~6.1 kilobase, kb) consists of 5'untranslated region (5'UTR) within the promoter, two open reading frames (ORF1 and ORF2) and 3'untranslated region (3'UTR) end of LINE-1s that contains an AATAAA polyadenylation signal and poly A tail [(39)]. LINE-1s are reverse transcribed and then inserted into the genome by Target-primed reverse transcription (TPRT)[(43)]. To our knowledge, some previous studies showed how LINE-1s hypomethylation promote genomic instability in cancer. First, LINE-1s retrotransposition usually produces DNA rearrangement [(45)]. For example, insertion of these LINE-1s into a coding region will disrupt the normal coding sequence [(46)] resulting to missense or nonsense mutations. Other, LINE-1s hypomethylation also down-regulates DNA repair genes such as *PPP2R2B* [(47)] (function is to activate nuclear ATM protein [(48)] that is critical to stimulation of the DNA damage checkpoint and depletion of ATM protein may promotes genomic instability[(49)]), Thereby, LINE-1s hypomethylation may be one of mechanism that indirectly promote genomic instability of our genome. Methylation of the CpG region in the 5' UTR promoter may silence the LINE-1s retrotransposition by recruiting co-repressor complex to bind at the promoter and cause the repression of LINE-1s activity [(50)].

CHAPTER III

METERIALS AND METHODS

The study is a cross-sectional descriptive study. This study was approved by the Human Ethics Committee of the Faculty of Medicine, Chulalongkorn University (IRB 417/57).

3.1 Population and sample size

3.1.1 Population

Population: Individuals with nicotine or methamphetamine (MA) use

Target population: Individuals with nicotine or MA use receiving the treatment for MA use at the Thanyarak Institute between the years 2007 to 2011

Sample population: Individuals with nicotine or MA use receiving the treatment for MA use at the Thanyarak Institute between the years 2007 to 2011 who were enrolled in the study of genetics of MA-induced paranoia.The inclusion criteria of the study population were having life-time use of MA > 10 instances. The exclusion criteria in the study were having a history of primary psychotic disorders or history of other brain diseases (i.e. epilepcy, stroke, brain trauma) [51]

Sample: Individuals with nicotine or MA use receiving the treatment for MA use at the Thanyarak Institute between the years 2007 to 2011 who were enrolled in the study of genetics of MA-induced paranoia [51] and not meet the exclusion criteria including.

Exclusion criteria:

1) No existing DNA samples
2) use another substance (i.e. met the DSM-IV criteria for alcohol dependence or use cannabis, opioids, or inhalants at least 100 instances in the lifetime)
3) the methylation level is higher than 1.5 standard deviation (SD)

As a result, 664 out of 991 DNA sample were available for the study. Of 664 samples, 331 were included in the study for further sampling based on the above exclusion criteria of the current study.

3.1.2 Sample size calculation

$$n/group = 2(Z\alpha_{/2} + Z\beta)^2\sigma^2/ (X_1 - X_2)$$

$\overline{X_1}$ = average of LINE-1s methylation control group = 69

$\overline{X_2}$ = average of LINE-1s methylation substance use = 67

σ^2 = Pool variance

$$= \frac{(n_1-1)S_1^2+(n_2-1)S_2^2}{n_1+ n_2- 2}$$

$= 3.5$

$n/group = 2(1.96+1.28)^2(6)/(69-67)^2$

$= 20$

The number of participants of each group = 20 samples.

3.1.3 Measurement

3.1.3.1. Questionnaire 1: SSADDA and MEQ

Diagnostic assessments were performed by using the Thai version of the Semi-Structured Assessment for Drug Dependence and Alcoholism (SSADDA) from the parent study [52]. In this study, demographics and substance use data including smoking initiation (>100 instances of cigarette smoking), experienced MA use (> 1000instances of lifetime MA use), MA duration, amount or frequency during period of heaviest MA use) were used to determine the independent variables. Methamphetamine Experience Questionnaire (MEQ) was used to measure MA-induced paranoia (MIP) [53]. We categorized participants into 4 groups depending on the history of nicotine and MA exposure

1. MA heavy use and experienced nicotine smokers (n = 181)
2. MA heavy use and non-experienced nicotine smokers (n = 25)
3. non-MA heavy use and experienced nicotine smokers (n = 102)
4. non-MA heavy use and non-experienced nicotine smokers (n = 23)

Sampling: We selected to match gender and age of the first three groups with the last group resulting to estimate 20 samples per group.

3.2 DNA extraction and Bisulfite modification [10]

3.2.1 DNA extraction

Blood samples were collected from participants in the Thanyarak Institute. Blood samples were centrifuged at 1,000 g for 10 minutes to collect peripheral blood mononuclear cells (PBMCs) and then stored at -80 ° C before performed DNA extraction.From whole blood was extracted by adding lysis buffer with 10 % SDS and protinase K and then incubated overnight at 50 °C and after that purify by phenol - chloroform and centrifuged at 4 °C with 14000 g for 15 minutes, and precipitated DNA pellet by using 10 M ammonium acetate and absolute EtOH then DNA pellet was washed by 70% EtOH and finally DNA pellet was dried and dissolved by Tris-EDTA. A total of 1 µg DNA was performed in the bisulfite treatments.

3.2.2 Bisulfite modification

Basically, after treating with bisulphite reaction it will convert unmethylated cytosine to uracil, while methylated cytosine cannot be changed. Bisulphite DNA was performed by using EZ-DNA methylation kit and specific primers is LINE-1s-F (5'GTTAAAGAAAGGGGTGA YGGT-3') and LINE-1s-R (5' AATACRCCRTTTCTTAAACC RATCTA -3') at 95 °C denature for 15 minutes, 50 °C annealing for 35 cycles and 72 °C final extension. Then the LINE-1s were digested with *Taq*I and *Tas*I at 65 °C overnight. And separated DNA products by polyacrylamide gel electrophoresis and stained with SYBR. We used water as a negative control and HeLa, Daudi, and Jurkat as positive control.

3.2.3 Combined Bisulfite Restriction Analysis (COBRA) LINE-1s

We classified LINE-1s into 4 groups depending on 2 CpG dinucleotides:2 unmethylated CpGs ($^{u}C^{u}C$), 2 methylated CpGs ($^{m}C^{m}C$), 5' unmethylated with 3' methylated CpGs ($^{u}C^{m}C$) and 5' methylated with 3' unmethylated CpGs ($^{m}C^{u}C$). After we digested with enzyme, the LINE-1s were digested and separated into 5 strands depending on their length including, 92, 60, 50, 42, and 32 bp

The CpGs of the 92 bp were derived from $^{m}C^{u}C$

The CpGs of the 60 bp were derived from $^{u}C^{u}C$

The CpGs of the 50 bp were derived from $^{m}C^{m}C$ and $^{u}C^{m}C$

The CpGs of the 42 bp were derived from $^{u}C^{u}C$ and $^{u}C^{m}C$

We calculated the intensity of each band by following formula:

% 92/92 = A

% 60/56 = B

% 50/48 = C

% 42/40 = D

% 32/28 = E

((D + E) – (B + C))/2 = F

% Methylation = ((A + 2C +F) x 100) / (2A +2 B +2C + 2F)

% ($^{m}C^{m}C$) hypermethylation = ((C/2)*100) / ((C/2) + A + B + F)

% ($^{u}C^{m}C$) partial methylated = (F * 100) / ((C/2) + A + B + F)

% ($^{m}C^{u}C$) partial methylated = (A * 100) / ((C/2) + A + B + F)

% ($^{u}C^{u}C$) hypomethylation = (B * 100) / ((C/2) + A + B

3.3 Statistical Analysis

1. Descriptive statistics including percentages, standard deviation, linear correlation to describe patterns of LINE-1s methylation in the four groups including nicotine only, MA only, nicotine&MA, and healthy control group
2. One-way analysis of variance (ANOVA) to compare % of methylation levels between the four groups all *p*-values less than 0.05 were considered to significant
3. *Post-hoc t*-Test analysis was performed to compare % methylation pattern between each pairs of the four groups all *p*-values less than 0.05 were considered to significant.
4. ANOVA and/or t-test analysis were performed to compare % methylation pattern of related variables and nicotine use and MA use variables in each of the four groups

CHAPTER IV

RESULTS

The result of this study will be presented in 2 parts as following

Part I: Demographics, nicotine & MA use variables, and patterns of LINE-1s methylation in people with nicotine experienced/non-experienced smokers, MA heavy/non-heavy use, and control group

1.1 Demographics and nicotine and MA use variables

1.2. % patterns of LINE-1s methylation of people with nicotine experienced/non-experienced smokers, MA heavy/non-heavy use, and control group

Part II: Association of the pattern of LINE-1 methylation and other nicotine and MA use variables

Part I: Demographics, nicotine & MA use variables, and patterns of LINE-1s methylation in people with nicotine experienced/non-experienced smokers, MA heavy/non-heavy use and control group

In this part, demographics, nicotine & MA use variables, and pattern of LINE-1s methylation of the four groups including, MA heavy use and nicotine experienced smokers (MA heavy & nicotine exp; n = 181, matched case; n = 24), MA heavy use and non- experienced nicotine smokers (MA heavy & non- exp nicotine; n= 23, matched case; n = 21) , non-MA heavy use and nicotine experienced smokers (non- MA heavy & nicotine exp; n = 102, matched case; n = 21) and non-MA heavy use and non-experienced nicotine smokers (non- MA heavy & non-nicotine exp; n = 23, matched case; n = 21) are shown.

1.1 Demographics and nicotine and MA use variables

Table 1 Demographics and nicotine and MA use variables in MA heavy/non-MA heavy, Nicotine experienced smokers/ non-experienced nicotine smokers (group 1, 2 and 3) and non-MA heavy and non-experienced nicotine smokers (group 4). (All cases)

	Samples				Univariate analyses		
Total	Group 1	Group 2	Group 3	Group 4	X^2	df	*p* value
Male Female	67 (37.01%) 114 (62.99%)	7 (28.00%) 18 (72.00%)	31 (30.39%) 71 (69.61%)	9 (39.13%) 14 (60.87%)	1.956	3	0.5817[a]
Age (years) (mean±SD)	26.45 ± 7.20	28.92 ± 7.14	24.46 ± 7.21	28.91 ± 7.22	-	-	0.0010[b]
BMI (kg/m^2) (mean±SD)	22.86 ± 3.37	23.88 ± 3.44	22. 38 ± 6.09	23.62 ± 3.81	-	-	0.7524[b]

[a] *p* value was calculated from Chi-square test

[b] *p* value was calculated from ANOVA

1.1 Demographics and nicotine and MA use variables

Table 2 Demographics and nicotine and MA use variables in MA heavy/non-MA heavy, Nicotine experienced smokers/ non-experienced nicotine smokers (group 1, 2 and 3) and non-MA heavy and non-experienced nicotine smokers (group 4). (Matched cases)

	Samples				Univariate analyses		
Total	Group 1	Group 2	Group 3	Group 4	X^2	df	*p* value
Male Female	8 (33.33%) 16 (66.67%)	7 (31.81%) 15 (69.19%)	7 (33.33%) 14 (66.67%)	8 (38.09%) 13 (61.91%)	0.212	3	0.9756[a]
Age (years) (mean±SD)	27.41 ± 7.27	28.95 ± 7.20	27.42 ± 7.25	27.47 ± 7.19	-	-	0.7687[b]
BMI (kg/m^2) (mean±SD)	23.27 ± 6.15	23.62 ± 3.81	22.56 ± 3.97	22.33 ± 4.35	-	-	0.6941[b]

[a] *p* value was calculated from Chi-square test

[b] *p* value was calculated from ANOVA

From table 1and 2 the demographic variables (age, sex and BMI) did not differ between all groups (Matched case). Except, we found the age were more likely to differences between four groups (all cases).

Table 3 Demographics and nicotine use variables of the subjects.

Nicotine smoking variables (in the group 1 and group 3)	Number of subjects			
	MA heavy & nicotine exp (group 1) (all: matched)	MA heavy & non- exp nicotine (group 2) (all: matched)	non- MA heavy & nicotine exp (group 3) (all: matched)	Non-MA heavy & non-exp nicotine (group 4) (all: matched)
1.Nicotine lifetime (>100 cigarettes in lifetime)	181 : 23	0 : 0	102 : 21	0 : 0
2.Nicotine frequency (day per week of smoking)	7 : 7	0 : 0	7 : 6	0 : 0
3.Nicotine amount (daily number of cigarette use)	16 : 16	0 : 0	9 : 12	0 : 0

Table 4 Demographics and MA use variables of the subjects.

MA use variables (in all groups)	Number of subjects			
	MA heavy & nicotine exp (group 1) (all:matched)	MA heavy & non- exp nicotine (group 2) (all:matched)	Non-MA heavy & nicotine exp (group 3) (all:matched)	Non-MA heavy & non- exp nicotine (group 4) (all:matched)
1.Lifetime MA use (≥1000 episodes per lifetime)	181 : 23	25 : 21	0 : 0	0 : 0
2.Past year MA use (≥150 episodes in the past year)	149 : 21	16 : 15	0 : 0	0 : 0
3.MA duration (years)	8 : 9	10 : 10	5 : 6	5 : 5

From table 3and 4 the substance use variables (nicotine lifetime, frequency, daily number of cigarette use, MA lifetime, past year MA use and MA duration these data did not differ between all groups.

1.2 Patterns of LINE-1s methylation

1.2.1 % patterns of LINE-1s methylation of people with and without nicotine and MA use

Table 5 % of LINE-1s methylation of MA and nicotine use (all cases)

	Substance use (N = 331)				
	MA heavy & nicotine exp (n =181) (gr.1)	MA heavy & non-exp nicotine (n = 25) (gr.2)	non- MA heavy & nicotine exp (n = 102) (gr.3)	non- MA heavy & non-nicotine exp (n =23) (gr.4)	*P* Values
% ^{m}C	67.42 ± 3.48	65.41 ± 3.78	66.11 ± 3.79	68.61 ± 3.75	< 0.0001***
% $^{m}C^{m}C$	34.26 ± 4.56	32.11 ± 4.56	32. 58± 4.56	34.78 ± 4.54	=0.0007 ***
% $^{m}C^{u}C$	19.98 ± 2.40	20.21 ± 2.41	21.11 ± 2.40	20.56 ± 2.42	=0.0034**
% $^{u}C^{m}C$	24.23 ± 4.04	24.36 ± 3.93	24.14 ± 4.05	25.37 ± 4.06	=0.6644
% $^{u}C^{u}C$	21.50 ± 3.45	23.30 ± 3.38	22.16 ± 3.46	19.28 ± 3.43	< 0.0001***

ANOVA was used to compared the percentage of LINE-1s ***$p < 0.0001$: ** $p < 0.001$: * $p < 0.05$

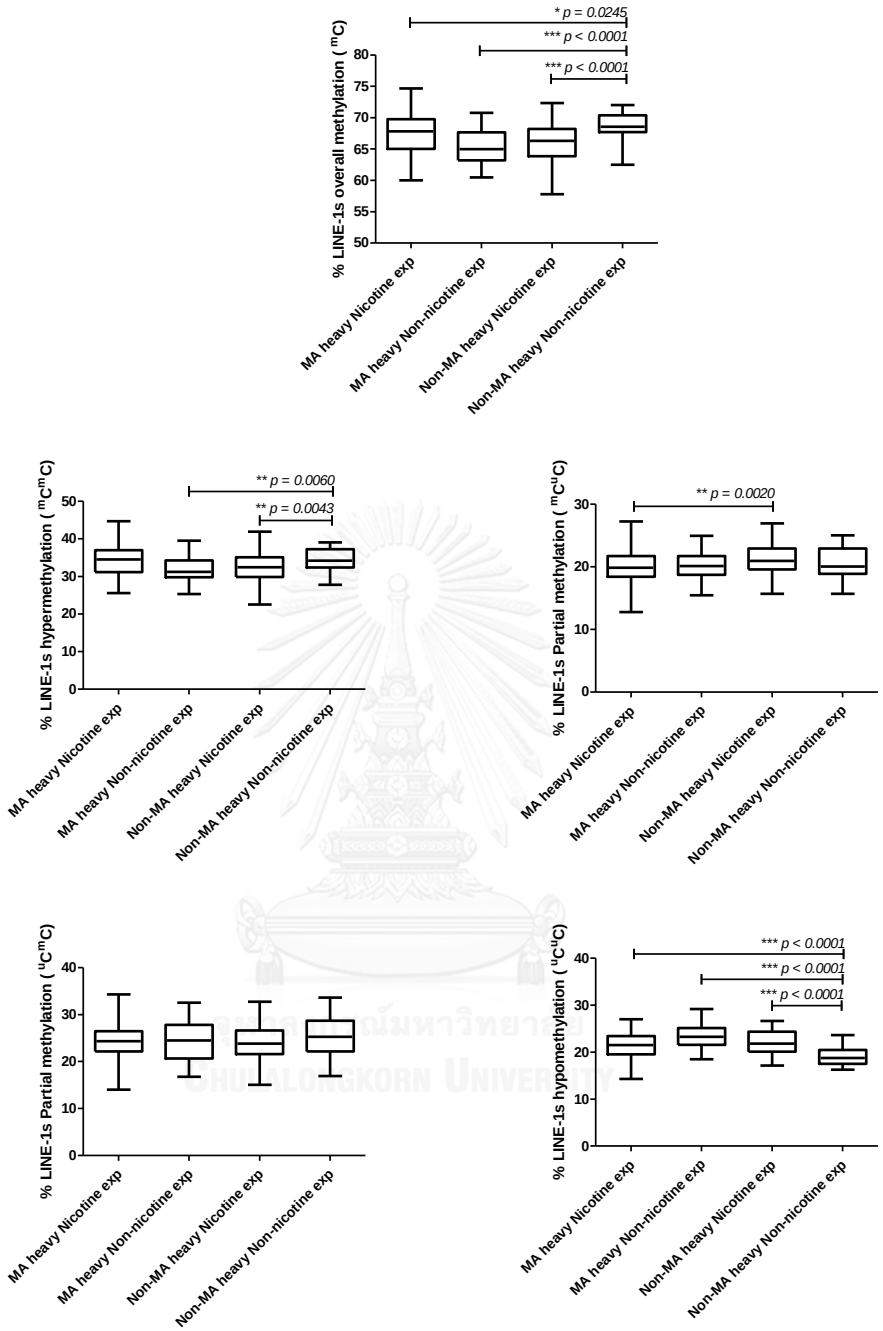


Figure 1 The percentage of LINE-1s methylation pattern in PBMCs among non-nicotine experienced/non-MA heavy use and the other substance use in all cases. Represent as box-whisker plot of the LINE-1s methylation levels observed in the substance use subjects. Horizontal bars represent the median (IQR).

From Table 5 and Figure 1, we compared the pattern of LINE-1s methylation among non-nicotine experienced/non-MA use (control, group 4) and people with nicotine experienced/non-experienced smokers and MA heavy/non-heavy use (substance use, group 1, 2 and 3) in all cases. The control group had significantly higher % overall methylation (^{m}C) and hypermethylation ($^{m}C^{m}C$) whereas lower % hypomethylation than substance use. However, we did not find any significant partial in methylation type $^{m}C^{u}C$ and $^{u}C^{m}C$.

Table 6 % of LINE-1s methylation of MA and nicotine use (matched case)

	Substance use (N = 331)				
	MA heavy & nicotine exp (n =23) (gr.1)	MA heavy & non-exp nicotine (n = 22) (gr.2)	non- MA heavy & nicotine exp (n = 21) (gr.3)	non- MA heavy & non-nicotine exp (n =21) (gr.4)	*P* Values
% ^{m}C	66.09 ± 3.82	65.43 ± 3.80	65.36 ± 3.83	68.70 ± 3.76	= 0.0005***
% $^{m}C^{m}C$	32.90 ± 4.60	32.13 ± 4.58	31. 76 ± 4.61	34.85 ± 4.55	= 0.0236*
% $^{m}C^{u}C$	19.52 ± 2.41	20.26 ± 2.42	21.45 ± 2.40	20.24 ± 2.46	= 0.0425*
% $^{u}C^{m}C$	24.72 ± 4.00	24.32 ± 3.92	23.91 ± 4.02	25.76 ± 4.07	= 0.5526
% $^{u}C^{u}C$	22.83 ± 3.47	23.27 ± 3.40	22.86 ± 3.48	19.13 ± 3.44	< 0.0001***

ANOVA was used to compared the percentage of LINE-1s ***$p < 0.0001$: ** $p < 0.001$: * $p < 0.05$

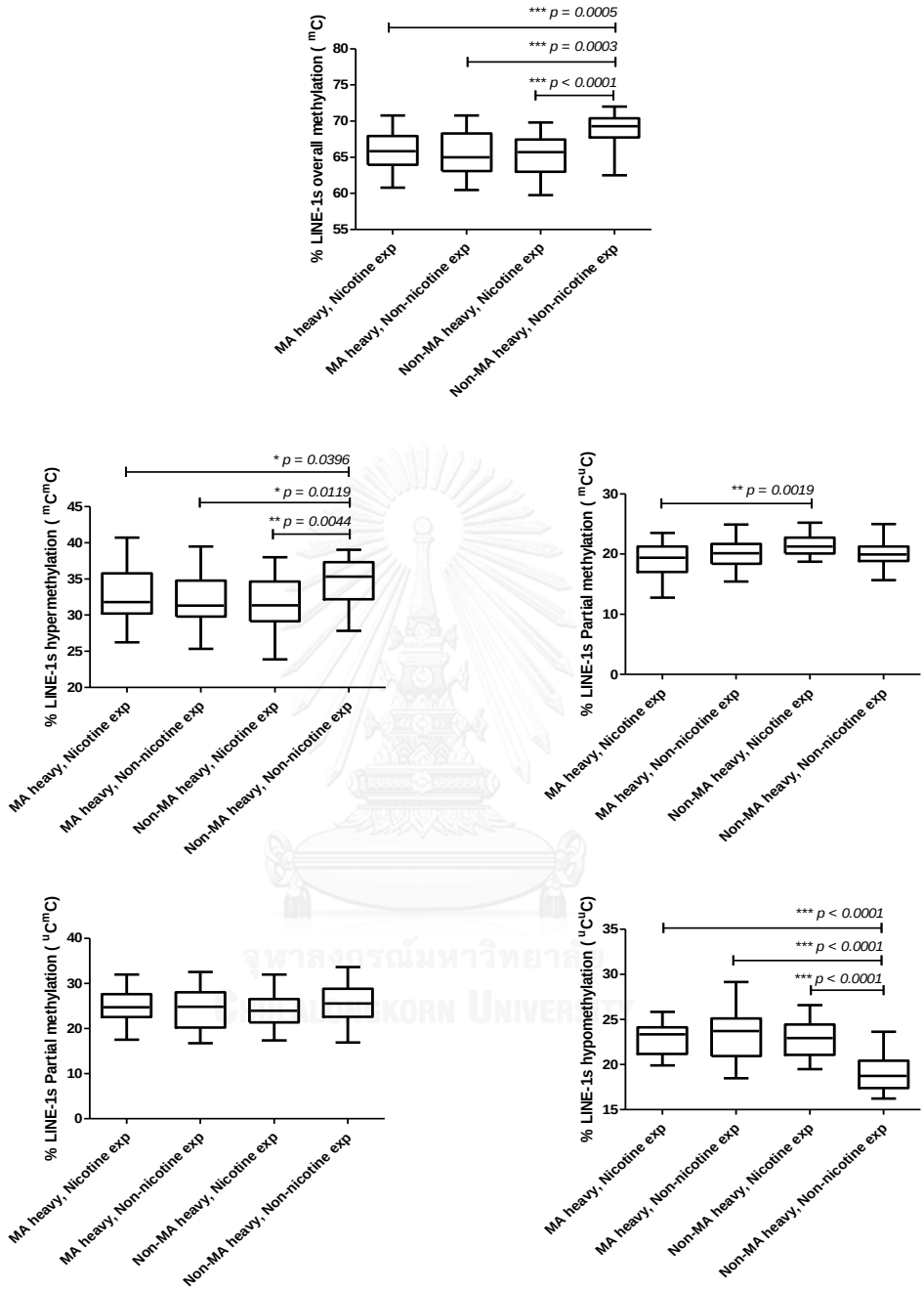


Figure 2 The percentage of LINE-1s methylation pattern in PBMCs among non-nicotine experienced/non-MA use and the other substance use. We selected to match gender and age. Represent as box-whisker plot of the LINE-1s methylation levels observed in the substance use subjects. Horizontal bars represent the median (IQR).

From Table 6 and Figure 2, we compared the pattern of LINE-1s methylation among non-nicotine experienced/non-MA use (control, group 4) and people with nicotine experienced/non-experienced smokers and MA heavy/non-heavy use (substance use, group 1, 2 and 3) and we evaluated the influenced of gender and age on LINE-1s methylation pattern, no signification between gender and age in all matched case groups. We found significant higher in % overall methylation (^{m}C) and hypermethylation ($^{m}C^{m}C$) in the control group. Whereas lower % hypomethylation than substance use group. However, we did not find any significant partial in methylation type $^{m}C^{u}C$ and $^{u}C^{m}C$ similary in all cases (table 5 and figure 1).

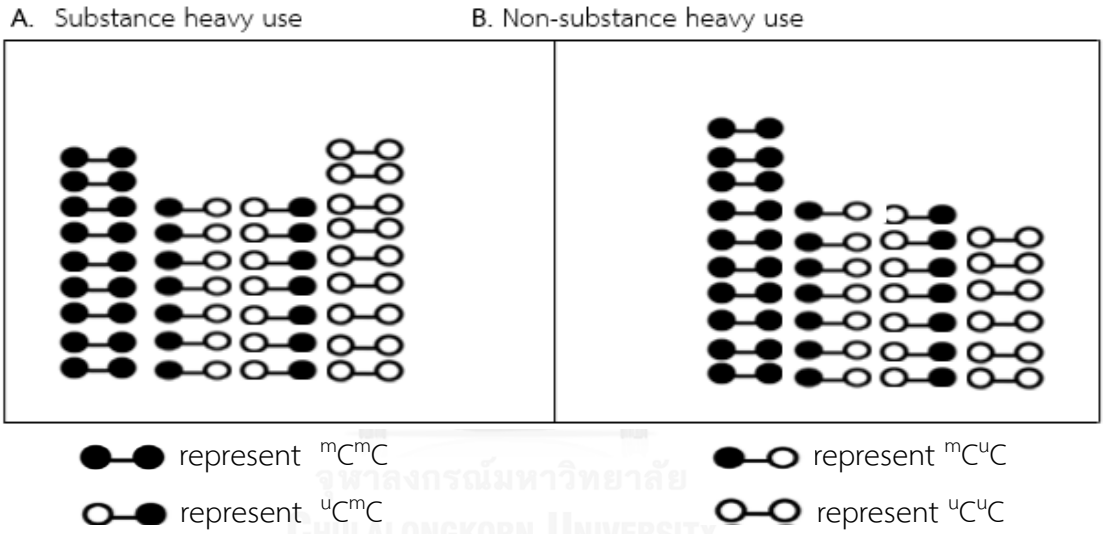


Figure 3 The influence of substance heavy use on LINE-1s methylation pattern we found the number of $^{m}C^{m}C$ in substance heavy use were decreased.Wheseas, the number of $^{u}C^{u}C$ were increased. While, we did not find any significant in partial methylation pattern $^{m}C^{u}C$ and $^{u}C^{m}C$.

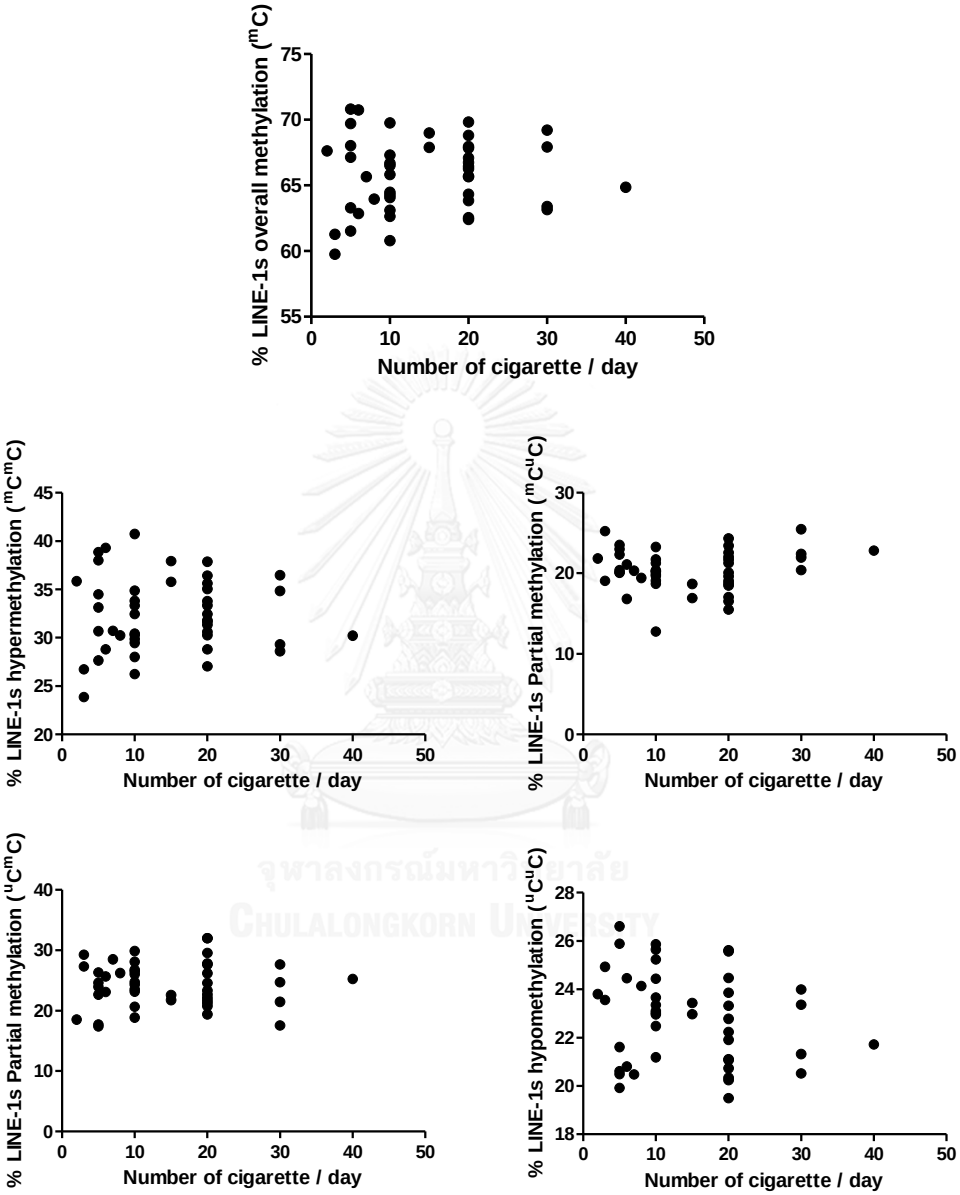
Part II Association of the pattern of LINE-1 methylation and other nicotine and MA use variables

In this part we investigated the association between LINE-1s pattern and other variable such as number of cigarette smoked per day, MA use duration.

2.1 Association between numbers of daily cigarette and the pattern of LINE-1s methylation (n= 45, group 1+group 3); (n=21, only group 3)

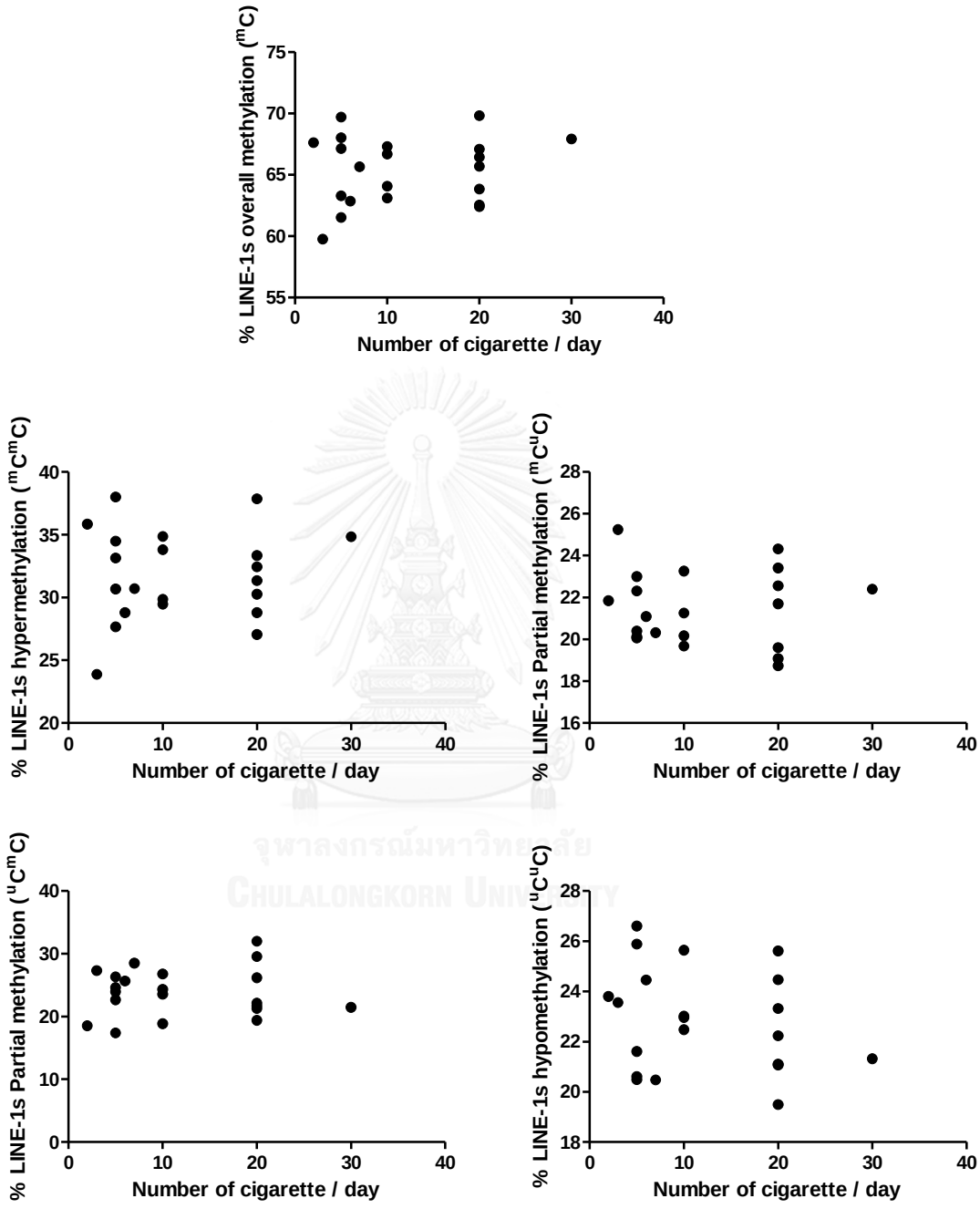
This study we selected to match gender and age to investigate the association between LINE-1s pattern and the amount of cigarette use per day.

Figure 4 The scatter plot indicates the number of cigarette use per day and % of overall LINE-1s methylation and other LINE-1s methylation pattern (group 1 and group 3)



From figure 4 we did not find any correlation between LINE-1s methylation pattern and number of cigarette use per day, number of cigarette and ^{m}C (r = 0.07951, *p* value = 0.6036; 95% confidence interval = -0.2192 to 0.3646), number of cigarette and $^{m}C^{m}C$ (r = 0.01734, *p* value = 0.9100 ; 95% confidence interval = -0.2777 to 0.3094), number of cigarette and $^{m}C^{u}C$ (r = 0.1043, *p* value = 0.4954; 95% confidence interval = -0.1953 to 0.3861), number of cigarette and $^{u}C^{m}C$ (r = 0.01925, *p* value = 0.9001; 95% confidence interval = -0.2759 to 0.3111), number of cigarette and $^{u}C^{u}C$ (r = -0.2119, *p* value = 0.1624; 95% confidence interval = -0.4759 to 0.08715).

Figure 5 The scatter plot indicates the number of cigarette use per day and % of overall LINE-1s methylation and other LINE-1s methylation pattern (group 3 only)



From figure 5 we did not find any correlation between LINE-1s methylation pattern and number of cigarette use per day, number of cigarette and ^{m}C (r = 0.1589, *p* value = 0.4915; 95% confidence interval = -0.2930 to 0.5528), number of cigarette and $^{m}C^{m}C$ (r = 0.1052, *p* value = 0.6500 ; 95% confidence interval = -0.3421 to 0.5136), number of cigarette and $^{m}C^{u}C$ (r = -0.02435 , *p* value = 0.9166; 95% confidence interval = -0.4514 to 0.4118), number of cigarette and $^{u}C^{m}C$ (r = 0.03404, *p* value = 0.8835; 95% confidence interval = -0.4037 to 0.4591), number of cigarette and $^{u}C^{u}C$ (r = -0.2282, *p* value = 0.3199; 95% confidence interval = -0.6008 to 0.2259). These results suggest that number of cigarette per day did not correlate with the % of LINE-1s methylation.

2.2 Comparative of frequency of cigarette smoking and LINE-1s methylation (n= 45, group 1+group 3); (n=21, only group 3)

In this study we evaluated the frequency of cigarette use in two groups

1. 2-3 days per week
2. = 7 days per week

Figure 6 The graph shows the comparative smoking < 7 days per week and smoking = 7 days per week in % of LINE-1s methylation (n=45).

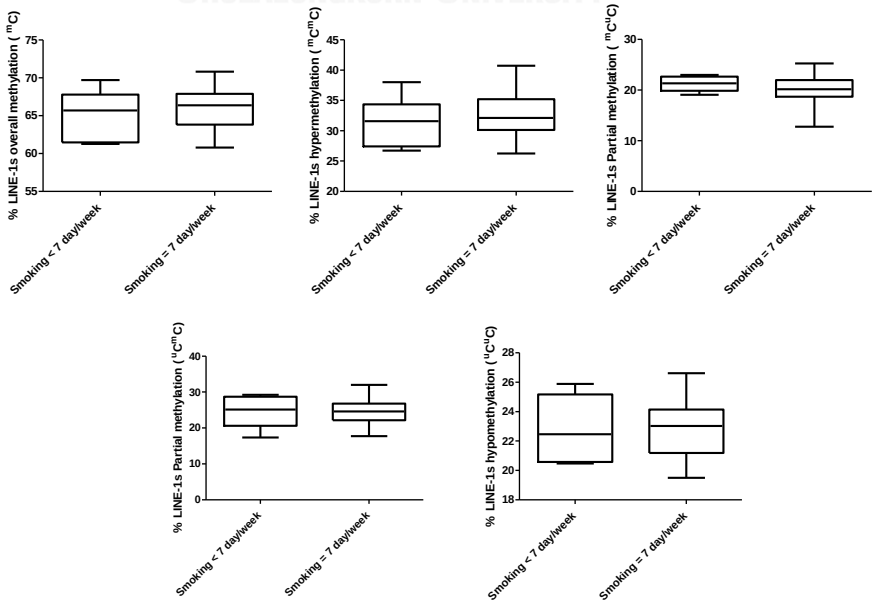
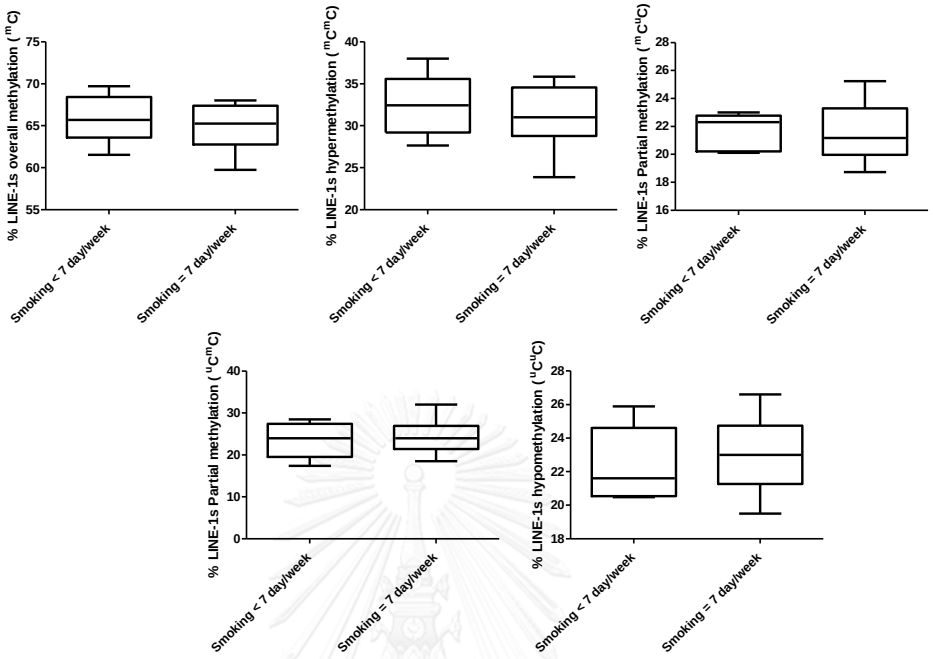


Figure 7 The graph shows the comparative smoking < 7 days per week and smoking = 7 days per week in % of LINE-1s methylation (n=21).

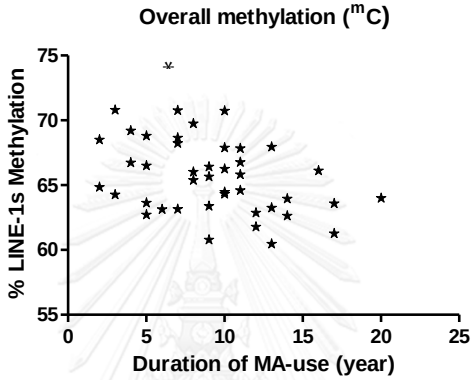


From figure 6 and 7. We did not find any significant differences in smoking less than 7 days per week and smoking all week. The results from 2.1 and 2.2 demonstrates that the effect of nicotine experienced on LINE-1s methylation may occur, no matter how frequency or amount of cigarette use.

2.3 Association between duration of MA use and LINE-1s methylation (n = 46)

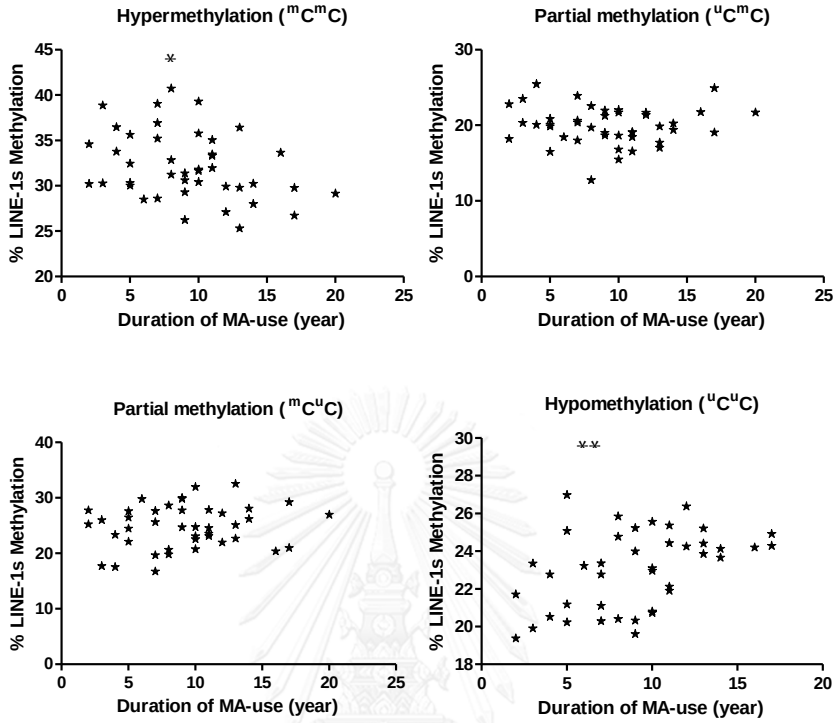
In this part we evaluated the association of period of heavy MA use (group 1 and group 2) and period of non-MA heavy use (group 3 and group 4) on LINE-1s methylation pattern.

Figure 8 The scatter plot shows the effect of duration of MA heavy use (group 1 and group 2) and % of overall LINE-1s methylation



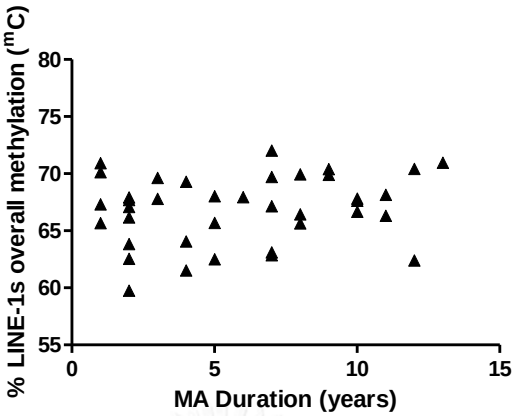
From figure 8 implies that decreased of overall methylation (^{m}C) is associated with the increased period of time of MA use r = -0.3911, **p* value = 0.0104; 95% confidence interval = -0.6212 to -0.09880.

Figure 9 The scatter plot shows the effect of duration of MA heavy use (group 1 and group 2) and % of other pattern of LINE-1s methylation



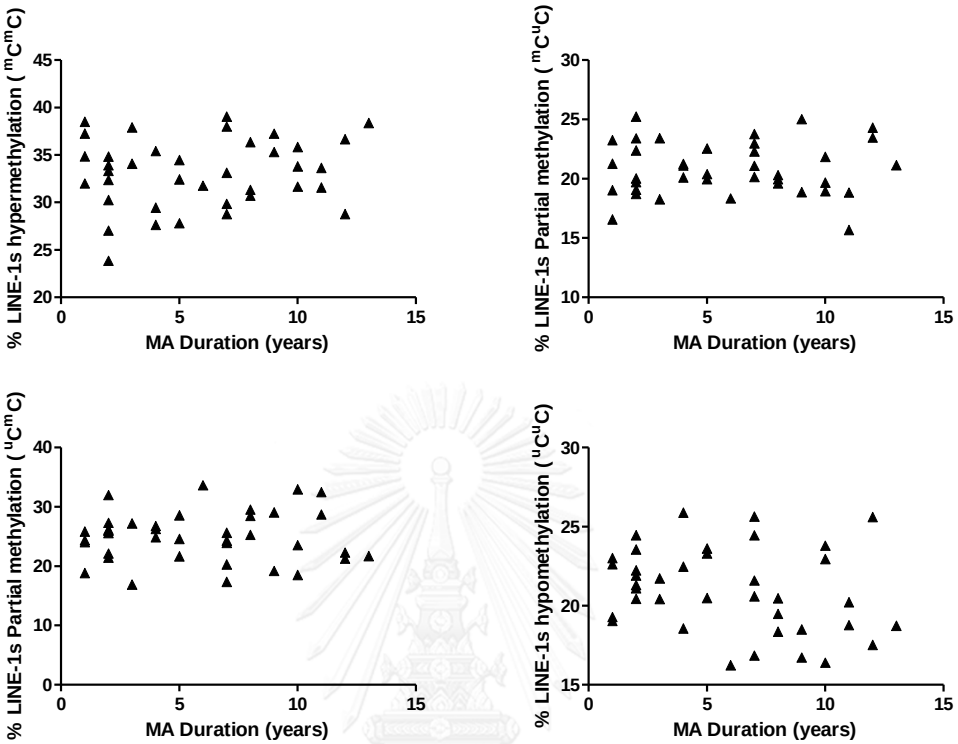
From figure 9 we found the association between period of MA use and LINE-1s methylation pattern. Negative correlation between period of MA use and hypermethylation ($^{m}C^{m}C$) (r = -0.3357, **p* value = 0.0297; (95% confidence interval = -0.5805 to -0.03530)), positive correlation between period of MA use and hypomethylation ($^{u}C^{u}C$) (r = 0.4326, ***p* value = 0.0047; (95% confidence interval = 0.1440 to 0.6533)). Nevertheless, we did not found the correlation in partial methylation $^{u}C^{m}C$ (r = -0.02984, *p* value = 0.8512; (95% confidence interval = -0.3308 to 0.2767)), and partial methylation $^{m}C^{u}C$ (r = 0.1485, p value = 0.3481; (95% confidence interval = -0.1629 to 0.4329)).

Figure 10 The scatter plot shows the effect of duration of non-MA heavy use (group 3 and group 4) and % of overall LINE-1s methylation



From figure 10 we did not find any correlation between non-MA heavy and overall methylation (^{m}C) (r = 0.1977, *p* value = 0.2341; 95% confidence interval = -0.1303 to 0.4867).

Figure 11 The scatter plot shows the effect of duration of MA heavy use (group 1 and group 2) and % of other pattern of LINE-1s methylation

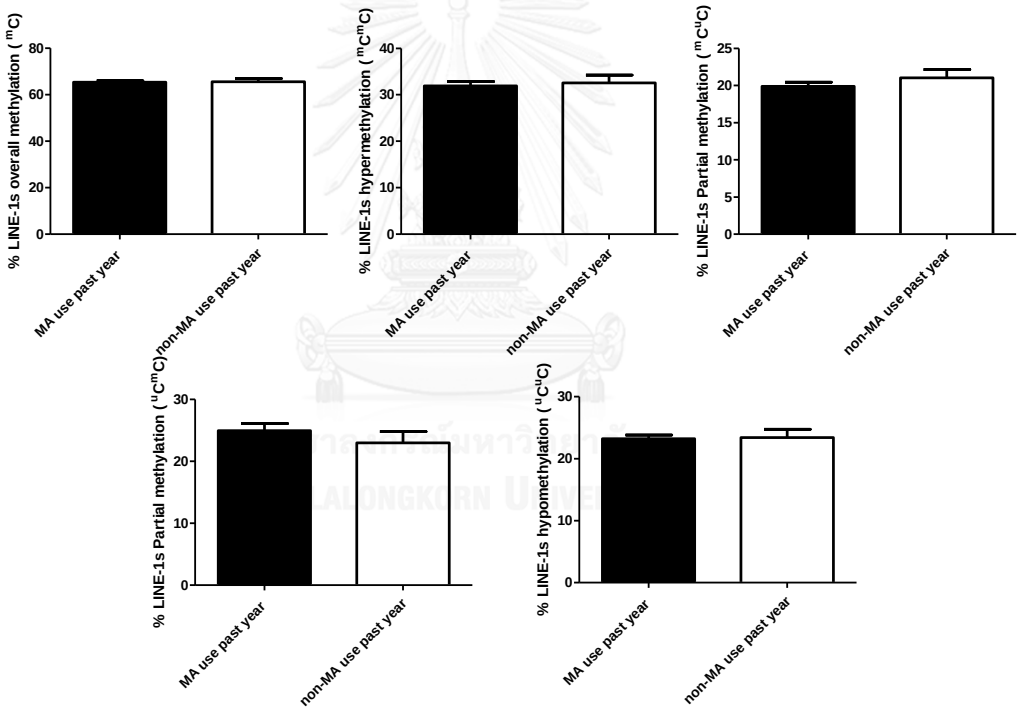


From figure 11 we not found the association between period of non-MA heavy duration use and LINE-1s methylation pattern. Non-MA heavy use duration and $^{m}C^{m}C$ (r = 0.1422, *p* value = 0.3943; (95% confidence interval = -0.1860 to 0.4419)),$^{m}C^{u}C$ (r = 0.02368, *p* value =0.8878; (95% confidence interval =-0.2983 to 0.3409)), $^{u}C^{m}C$ (r = 0.02779, *p* value = 0.8685; (95% confidence interval = -0.2946 to 0.3445)),and non-MA heavy use duration and $^{u}C^{u}C$ (r = -0.2577, *p* value = 0.1183 ; (95% confidence interval = -0.5335 to 0.06764)). These results indicate that long time of MA heavy use may correlate with increase hypomethylation ($^{u}C^{u}C$). Whereas, long time use of non-MA heavy use may not associate with increase hypomethylation ($^{u}C^{u}C$).

2.4 Comparative percentage of LINE-1s methylation among past year MA use (≥ 150 episodes in past year) and non-MA use past year (150 < episodes in past year), (n = 21, past year MA use = 15; non- MA use past year = 6)

In this part we determined the effects of past year MA use compared to non-MA use in the past year. Because we try to study the percentage of LINE-1s methylation in continuing MA heavy use (≥ 150 episodes) compared to use less than 150 episodes at least 1 year.

Figure 12 The graph shows the percentage of LINE-1s methylation in past year MA use and non-past year MA use.



From figure 12 we have not found the any differences of the percentage of LINE-1s methylation in all pattern. These results may imply that even though use MA less than 150 episodes at least 1 year did not turn LINE-1s methylation to normal level, the effect of MA heavy use still persisted.

CHAPTER V

CONCLUSION AND DISCUSSION

The alteration of gene expression after chronic substance exposure may be a key role in the development of substance dependence. Recent studies indicate that epigenetic alterations contribute to substance induced changes in patterns of gene expression and behavioral responses to dependence [7, 8]. DNA methylation is the part of epigenetic that convert cytosine to 5'methylcytosine (5mc) that catalysed by DNA methyltransferases (DNMTs) [9] while unmethylated cytosine will convert to uracil this alters the DNA sequence that lead to arrange a new target site for restriction enzyme that reflect differences in a methylation pattern[54].In order to investigate whether nicotine smokers or the MA use dysregulate DNA methylation of global DNA, repetitive elements such as LINE-1s, because LINE-1s have been widely dispersed among mammalian genome (~ 20 % of the human genome or estimate 500,000 copies) almost of LINE-1s are truncated; approximately 10% of LINE-1s have full length and 40-60% of these are able to be transcripted [44] . We classified the methylation pattern into 4 groups according to LINE-1s methylation status including, 2 methylated CpGs or hypermethylation ($^{m}C^{m}C$), 2 unmethylated CpGs or hypomethylation ($^{u}C^{u}C$), 5' methylated with 3' unmethylated CpGs ($^{m}C^{u}C$) and 5' unmethylated with 3' methylated CpGs ($^{u}C^{m}C$)[10]. Recent study indicates that MA or cocaine activate LINE-1s retrotransposition in neuronal cell lines [13]. Chronic MA also reduces the DNA methylation levels in the CpG region of the five immediate early genes (IEGs) in prefrontal cortex[14]. Cigarette smoking also reduce the methylation levels at many loci in the brain[15]. Changes in LINE-1s methylation of the oral mucosa of cigarette smokers were found from Dr. Apiwat Mutirangura laboratory. Specifically they found an increase of % hypermethylation ($^{m}C^{m}C$), % hypomethylation ($^{u}C^{u}C$) and decrease in partial methylation $^{m}C^{u}C$ without change in partial methylation $^{u}C^{m}C$ [10]. In our study we selected to match gender and age of all groups and did not find differences in

demographic, substance use variable in all groups. Next, we explored the effects of nicotine or MA in people with MA heavy/non-heavy and nicotine experienced/non-experienced on LINE-1s methylation pattern and found that MA heavy or nicotine experienced induced hypomethylation ($^{u}C^{u}C$) in LINE-1s element on the other hand decreased hypermethylation ($^{m}C^{m}C$) and overall methylation (^{m}C). The differences result may depend on difference in tissue samples, type of substance abuse (MA or Nicotine) and sample sizes. And then, we evaluated the effect of amount of cigarette use per day on LINE-1s methylation, we did not find any correlation between number of cigarette use per day and LINE-1s methylation. Morover, we also examined the effect of cigarette smoking on frequency of use, we subdivided into two groups 1. At least once per week 2. All of a week. We found a similar effect of all pattern of LINE-1s methylation in two group. These two results may refer that the effect of nicotine experienced (nicotine experienced ≥ 100 instances in lifetime) on LINE-1s methylation pattern will exist, it does not matter how much of cigarette smoking or what frequency of use. Next, we clarified the association between periods of MA use and LINE-1s methylation and found that long time use of heavy MA may promote increased in hypomethylation ($^{u}C^{u}C$) of LINE-1s while, no correlation between time of non-heavy MA use and LINE-1s methylation pattern. Furthermore, we clarified the percentage of LINE-1s methylation in MA heavy use (≥ 150 episodes) compared to use MA less than 150 episodes at least 1 year, we did not find any significant in all pattern these results suggests that the alteration of LINE-1s after MA heavy use cannot unchanged to normal in only one year, even though use less than 150 episodes at least 1 year. The alteration of LINE-1s methylation pattern specifically, hypomethylation ($^{u}C^{u}C$) of LINE-1s promote many events includes retrotransposition, endogenous DNA double-strand break (EDSB), and the abnormalities of DNA repairing genes that associated genomic instability [39]. This retrotranposition generally induces DNA rearrangement that promotes chromosomal instability [45]. Nevertheless, the direct correlation between MA use and nicotine smoking to LINE-1s retrotransposition are also needs to investigation in the next experiment. In conclusion, from our study we found that the MA heavy use and nicotine experienced altered the LINE-1s methylation (increased in number of hypomethylation ($^{u}C^{u}C$) while decreased hypermethylation ($^{m}C^{m}C$) and overall

methylation (^{m}C). The mechanism that causes loss or raise LINE-1s methylation are different. Global hypomethylation ($^{u}C^{u}C$) of LINE-1s may promote genomic instability of central nervous system in MA heavy use or nicotine experienced smokers.

Limitation

1. Blood samples in this study were collected from participants and keep in freezer for a long time, that may affect the integrity of DNA or LINE-1s.
2. In this study we did not use healthy control (have no substance use history) to compare with substance heavy use.

Expected beneficial and future direction

1. This study may use to predict the trend of nicotine or MA addicted to identifying new method for prevention of nicotine or MA use.
2. To study the correlation or association between nicotine of MA use with other neuropsychiatic disorders.
3. This information may essential for further study to integrate the association of methylation and the expression of other genes that involves in substance addiction.

รายการอ้างอิง

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ภาคผนวก

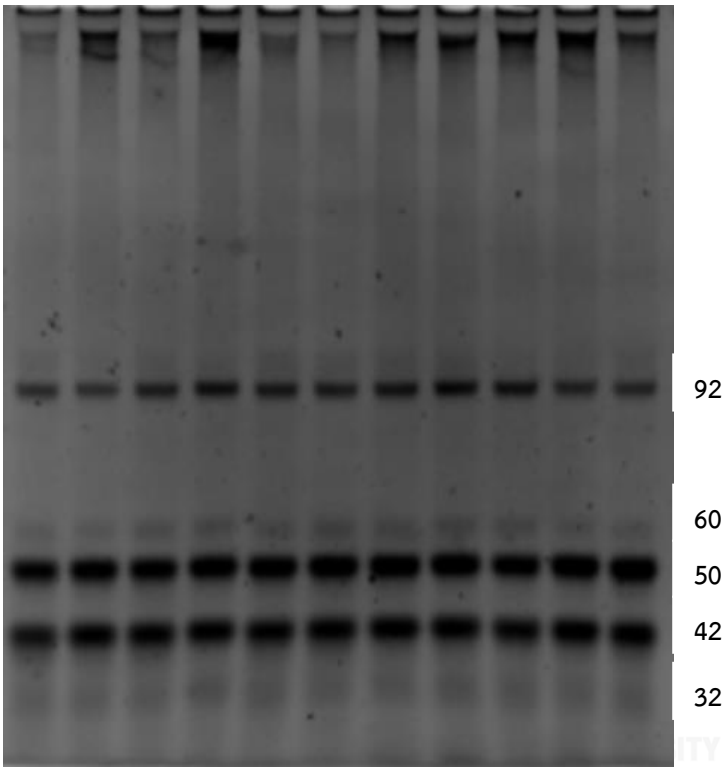
LINE-1 X primer

GTTAAAGAAAGGGGTGACGGTCGTATTTGGAAAATCGGGTTATTTTTATTCGAATATTGCGT
TTTTTAGATCGGTTTAAGAAACGGCGTATT

new CobraL-1 Forward 5’ GTTAAAGAAAGGGGTGAYGGT 3’

new CobraL-1 Reverse 5’-AATACRCCRTTTCTTAAACCRATCTA-3’

Gel example



Percentage of LINE-1s calculation

The CpGs of the 92 bp were derived from $^{m}C^{u}C$

The CpGs of the 60 bp were derived from $^{u}C^{u}C$

The CpGs of the 50 bp were derived from $^{m}C^{m}C$ and $^{u}C^{m}C$

The CpGs of the 42 bp were derived from $^{u}C^{u}C$ and $^{u}C^{m}C$

we calculated the intensity of each band by following formula:

% 92/92 = A

% 60/56 = B

% 50/48 = C

% 42/40 = D

% 32/28 = E

((D + E) – (B + C))/2 = F

% Methylation = ((A + 2C +F) x 100) / (2A +2 B +2C + 2F)

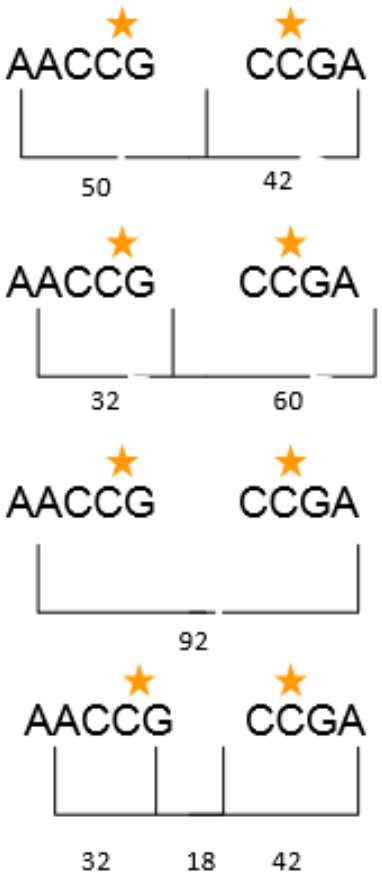
% ($^{m}C^{m}C$) hypermethylation = ((C/2)*100) / ((C/2) + A + B + F)

% ($^{u}C^{m}C$) partial methylated = (F * 100) / ((C/2) + A + B + F)

% ($^{m}C^{u}C$) partial methylated = (A * 100) / ((C/2) + A + B + F)

% ($^{u}C^{u}C$) hypomethylation = (B * 100) / ((C/2) + A + B

LINE-1s amplicons



ประวัติผู้เขียนวิทยานิพนธ์

BIOGRAPHY

Mr. Korakot Kraijak graduated with Bachelor degree of Science in program Biology from Faculty of Science, Chulalongkorn University in 2012. His research focuses on the LINE-1 s Methylation and substance dependence such as methamphetamine or nicotine

Poster Presentation

1.Kraijak, K., Mutirangura, A., Kalayasiri R. Association between LINE-1s and methamphetamine use. The 11th Asia-Pacific Microscopy Conference 33rd Microscopy Society/39th Anatomy Association- of Thailand Annual Conference on May 23-27, 2016, Phuket, Thailand.