การศึกษาสภาวะดีเอ็นเอเมทิลเลชั่นของส่วนเบสซ้ำชนิด ไลด์1, อลู, เอชอีอาร์วี-อี และ เอชอีอาร์วี-เค ในเซลล์นิวโตรฟิลของผู้ป่วยโรคเอสเอลอีและผู้มีสุขภาพดี

นายปรเมษฐ์ พรหมณเรศ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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THE STUDY OF DNA METHYLATION STATUS OF THE INTERSPERSED REPETITIVE SEQUENCES TYPE LINE-1, ALU, HERV-E AND HERV-K IN NEUTROPHIL OF SYSTEMIC LUPUS ERYTHEMATOSUS PATIENTS AND HEALTHY CONTROLS

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	The study of DNA methylation status of the interspersed repetitive sequences type LINE-1, ALU, HERV-E and HERV-K in neutrophil of systemic lupus erythematosus patients and healthy controls
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Field of Study	Medical Sciences
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ปรเมษฐ์ พรหมณเรศ : การศึกษาสภาวะดีเอ็นเอเมทิลเลชั่นของส่วนเบสซ้ำชนิดไลด์1, อลู, เอชอีอาร์วี-อี และเอชอีอาร์วี-เค ในเซลล์นิวโตรฟิลของผู้ป่วยเอสเอลอีและผู้มีสุขภาพดี (The study of DNA methylation status of the interspersed repetitive sequences type LINE-1, ALU, HERV-E AND HERV-K in neutrophil of systemic lupus erythematosus patients and healthy controls) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.พญ. ดร.ณัฏฐิยา หิรัญกาญจน์, 117 หน้า.

สาเหตุที่แท้จริงของการเกิดโรคเอสเอลอียังคงคลุมเครือ จากรายงานหลายฉบับเชื่อว่าภาวะเหนือพันธุกรรม น่าจะมีบทบาทสำคัญที่อาจก่อให้เกิดโรคเอสเอลอี หนึ่งในกลไกสำคัญของภาวะเหนือพันธุกรรม คือ ดีเอ็นเอเมทิลเลชั่น ซึ่งมีความเป็นไปได้ว่าจะเป็นสาเหตุสำคัญของโรค โดยมีรายงานถึงความผิดปกติของระดับดีเอ็นเอเมทิลเลชั่นในเซลล์เม็ดเลือดขาวชนิด ลิวโคไซค์, พีบีเอ็มซี, ทีเซลล์และซีดีโฟทีเซลล์ในระบบภูมิคุ้มกันของผู้ป่วยเอสเอลอี ซึ่งพบว่ามีระดับดีเอ็นเอเมทิลเลชั่นต่ำในผู้ป่วยเมื่อเปรียบเทียบกับผู้มีสุขภาพดี นอกจากนี้ยังพบว่าความสัมพันธ์ของการเกิดโรคเอสเอลอีนั้น ไม่ได้พบเฉพาะในเซลล์เม็ดเลือดขาวชนิดลิวโคไซค์, พีบีเอ็มซี, ทีเซลล์และซีดีโฟทีเซลล์ เท่านั้นแต่ยังรวมถึงเซลล์เม็ดเลือดขาวชนิดนิวโตรฟิลด้วย เนื่องจากมีรายงานหลายฉบับรายงานถึงความผิดปกติของกระบวนการตายที่เกิดขึ้นในเซลล์นิวโตรฟิลของผู้ป่วยเอสเอลอีเมื่อเปรียบเทียบกับผู้มีสุขภาพดี กลไกการเกิดดีเอ็นเอเมทิลเลชั่นเกี่ยวข้องกับการเติมหมู่เมทิลที่เบสไซโตซีนที่อยู่ติดกับเบสกวานีนนั้น ไม่ได้เกิดขึ้นเฉพาะในส่วนโปรโมเตอร์ของยีนเท่านั้นแต่ยังสามารถเกิดขึ้นในส่วนเบสซ้ำด้วย ซึ่งมีรายงานการศึกษาเกี่ยวกับระดับเมทิลเลชั่นของลำดับเบสซ้ำในเซลล์เม็ดเลือดขาวชนิดโมโนนิวเคลียสและลิมโฟไซค์ แต่อย่างไรก็ตามยังไม่มีการศึกษาในเซลล์เม็ดเลือดขาวชนิดนิวโตรฟิล การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาระดับและรูปแบบของดีเอ็นเอเมทิลเลชั่นในส่วนเบสซ้ำชนิดไลด์1, อลู, เอชอีอาร์วี-อี และเอชอีอาร์วี-เค ในเซลล์เม็ดเลือดขาวชนิดนิวโตรฟิลของกลุ่มผู้ป่วยเอสเอลอีเปรียบเทียบกับกลุ่มผู้มีสุขภาพดีและศึกษาความสัมพันธ์ระหว่างระดับและรูปแบบของดีเอ็นเอเมทิลเลชั่นกับอายุและข้อมูลทางคลินิกของผู้ป่วย จากการทดลองพบความแตกต่างอย่างมีนัยสำคัญทางสถิติของระดับและรูปแบบ (mC) และ (mCmC) ของดีเอ็นเอเมทิลเลชั่นในส่วนเบสซ้ำชนิด ไลด์1 ในเซลล์เม็ดเลือดขาวชนิดนิวโตรฟิลของผู้ป่วยเอสเอลอีต่ำกว่าผู้มีสุขภาพดี (p-value<0.0001, p-value<0.0001 ตามลำดับ) และพบความแตกต่างอย่างมีนัยสำคัญทางสถิติของระดับและรูปแบบ ((uCuC)) และ (mCuC) ของดีเอ็นเอเมทิลเลชั่นในส่วนเบสซ้ำชนิด ไลด์1 ในเซลล์เม็ดเลือดขาวชนิดนิวโตรฟิลของผู้ป่วยเอสเอลอีสูงกว่าผู้มีสุขภาพดี (P-value$=0.0028$, p-value<0.0001 ตามลำดับ) แต่อย่างไรก็ตามไม่พบความสัมพันธ์อย่างมีนัยสำคัญทางสถิติระหว่างระดับและรูปแบบของดีเอ็นเอเมทิลเลชั่นในส่วนเบสซ้ำชนิด อลู, เอชอีอาร์วี-อี และเอชอีอาร์วี-เค ยิ่งไปกว่านั้นเราได้ทำการค้นหาความสัมพันธ์ระหว่าง ไลด์1 ที่แทรกอยู่ในยีน กับข้อมูลการแสดงออกของยีนในเซลล์เม็ดเลือดขาวชนิดนิวโตรฟิลเปรียบเทียบกับกลุ่มควบคุม พบว่ายีนที่มี ไลด์1 แทรกอยู่ ส่งเสริมให้มีการแสดงออกของยีนในเซลล์เม็ดเลือดขาวชนิดนิวโตรฟิลเพิ่มสูงขึ้น (p-value$= 7.74\text{X}10^{-3}$; OR $= 1.28$) นอกจากนี้เมื่อแยกวิเคราะห์ในแต่ละกลุ่มของไลด์1 พบว่ายีนที่มี ไลด์1 ชนิดแอนไทเซนแทรกอยู่ ส่งเสริมให้มีการแสดงออกของยีนในเซลล์เม็ดเลือดขาวชนิดนิวโตรฟิลเพิ่มสูงขึ้นด้วย (p-value$= 6.22\text{X}10^{-3}$; OR $= 1.38$) จากหลักฐานที่กล่าวมาแสดงให้เห็นว่าการเคลื่อนไหวของระดับและรูปแบบของดีเอ็นเอเมททิลเลชั่นในส่วนเบสซ้ำชนิด ไลน์-1 ในเซลล์เม็ดเลือดขาวชนิดนิวโตรฟิลของผู้ป่วยเอสเอลอีอาจส่งผลกระทบต่อการเกิดโรคเอสเอลอี แต่อย่างไรก็ตามการศึกษานี้เป็นหลักฐานแรกที่อาจนำไปสู่องค์ความรู้ใหม่ซึ่งเกี่ยวข้องกับสาเหตุของการเกิดโรคเอสเอลอี

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

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KEYWORDS: DNA METHYLATION/ SYSTEMIC LUPUS ERYTHEMATOSUS / NEUTROPHIL / LINE-1 / ALU / HERV-E / HERV-K

PARAMATE PROMNARATE : THE STUDY OF DNA METHYLATION STATUS OF THE INTERSPERSED REPETITIVE SEQUENCES TYPE LINE-1, ALU, HERV-E AND HERV-K IN NEUTROPHIL OF SYSTEMIC LUPUS ERYTHEMATOSUS PATIENTS AND HEALTHY CONTROLS. ADVISOR: ASSOC. PROF. NATTIYA HIRANKARN, M.D.,Ph.D., 117 pp.

The exact etiology of Systemic Lupus Erythematosus (SLE) is still obscure. Various report believe that the epigenetic, it important role that may cause SLE. One of the major mechanisms of epigenetic be DNA methylation, it is possible an important cause of the disease. Several papers report that abnormalities of DNA methylation level in some important immune cells and SLE-relate genes of SLE patients. It was found that leukocytes, PBMC, T-cell and $CD4^+$ T-cell DNA of SLE patients are DNA hypomethylation comparing to healthy donor. Not only are PBMC and lymphocyte is those involve with pathogenesis of SLE but have also been neutrophil. Several papers reported that abnormalities in cell-death processes of SLE neutrophils. DNA methylation that involved the addition of a methyl group to cytosine within CpG pairs. That does not only occur in genes but also occur on the IRSs in human genome. Previous studies report that methylation levels of IRSs in PBMC and Lymphocyte of SLE's patients but limited in Neutrophils. In this study we evaluated methylation level and patterns of LINE-1, ALU, HERV-E and HERV-K in SLE's patient compared to healthy controls and examined the association between methylation level and patterns according to age and some clinical data of SLE patients. We observed that some methylation level and pattern of LINE-1 was significant difference, precise methylation (mC), hypermethylated (mCmC) in neutrophils from SLE patients were significantly lower than healthy controls. (p-value<0.0001, p-value<0.0001 respectively) and found that hypomethylation (uCuC) and %mCuC pattern in neutrophils from SLE patients were significantly higher than healthy controls. (P-value=0.0028, p-value<0.0001 respectively). However, the methylation level and pattern of Alu, HERV-E and HERV-K in neutrophils from SLE patients include active and inactive group compared to healthy control were not significantly different. Moreover, we investigate the correlation between intragenic LINE-1 and differential expressions in SLE neutrophils expression array. We explored higher prevalence of up-regulated of genes containing LINE-1s (p-value= $7.74X10^{-3}$; OR = 1.28). Moreover, genes with antisense LINE-1s were higher prevalence of up-regulated genes containing LINE-1s (p-value= $6.22X10^{-3}$; OR = 1.38). We also provided an evidence to propose the dynamics of DNA methylation levels and pattern of LINE-1 in Neutrophils might affect to pathogenesis of the SLE. However, this study is the first evidence that could lead to new knowledge related to the etiology of SLE.

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

Academic Year: 2012 Advisor's Signature .....

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COBRA	Combined Bisulfite Restriction Analysis
CU-DREAM	Connection Up- or Down-Regulation Expression Analysis of Microarray
CU-DREAM X	Connection Up- or Down-Regulation Expression Analysis of Microarray Extension program
DNMTs	DNA Methyltransferases
GEO	Gene Expression Omnibus
HERVs	Human Endogenous Retroviruses
IRSs	Interspersed Repetitive Sequences
LINE-1	Long Interspersed Nuclear Element type 1
SINE-1	Short Interspersed Nuclear Element type 1
LTRs	Long Terminal Repeats
PBMCs	Peripheral Blood Mononuclear Cells
PMN	Polymorphoneuclear Cells
RA	Rheumatoid arthritis
Bp	Base pair
IFN	Interferon
IL	Interleukin
MHC	Major Histocompatibility Complex
PCR	Polymerase Chain Reaction
SLE	Systemic Lupus Erythematosus
SLEDAI-2K	Systemic Lupus Erythematosus Disease Activity 2000

CHAPTER I

INTRODUCTION

Systemic lupus erythematosus (SLE) is a complex autoimmune disease. SLE is associated with impairments in immune system, which is a response of the immune system against its own tissues in almost every organs such as skin, nervous system, heart, kidney, blood components, cells and cell components. The landmark of SLE is the production of autoantibody against self-antigen, especially against nuclear antigen e.g. intact chromatin, single-strand DNA, double-strand DNA, nucleosomes collectively called antinuclear autoantibodies (ANAs). These autoantibodies combined with a self-antigen into immune complex which accumulate to tissues and vessels with high turbulence flow causing tissue, and organ damage via inflammatory response. This process results in various clinical manifestations such as glomerulonephritis, arthritis, serositis, vasculitis and neurological disorders (1). Currently, the exact etiology of SLE is still unclear. We believe that the etiology of SLE is caused by several factors, composite genetic factors and environmental factors and on abnormalities of immune system. These factors contribute to the pathogenesis of SLE (2). In addition, several previous studies demonstrated the importance of epigenetic in human pathologies such as inflammatory response and malignancies. Especially, dysregulation of DNA methylation processes. This evidence proposed as a potentially increasing in frequency of autoimmune and cancer (Table 3) (3). The epigenetic refers to the study of heritable changes in gene expression that do not involve sequence change in DNA. There are believes that epigenetic is a link between genetic and environmental factors and affects the immune responses abnormalities such as immune regulation, apoptosis of immune cells and clearance of immune complex or apoptotic cells (3, 4). (Figure 1) These abnormalities which occurred may predispose to the development of autoimmune diseases.

In SLE, there are many reports that DNA hypomethylation was found in leukocytes (5), PBMCs (6, 7), T-cell (30) and $CD4^+$ T-cell DNA (8-11) of SLE patients comparing to healthy control. For example, there are report that hypomethylation levels of the CpG islands in the IL-4 and IL-6 promoters were found in T cell of SLE patients (13) as well as the hypomethylation levels of IL10 and IL13 promoter in $CD4^+$ T-cell of SLE patient (14). The hypomethylation of the HTR1A promoter was reported in PBMC of SLE patient (15). In addition, DNA hypomethylation is important to initiate apoptotic DNA to induce SLE-like autoimmune disease in non-susceptible mice (12). These results suggested that DNA methylation is another major etiology of the SLE. One of heritable epigenetic modifications is DNA methylation that involves the addition of a methyl group to cytosines within CpG (cytosine/guanine) pairs. Methylation does not only occur only in the gene promoter but also occur at the interspersed repetitive sequences (IRSs) in human genome (16). In human genome, the IRSs was found at approximately 45% of whole genome, (17) which were found to be CpG-rich sequences (18, 19). Furthermore there was report that the changes in the methylation status of different IRSs loci may lead to the differences in cellular phenotypes depending on their location under normal condition (20, 21). The IRSs can be divided into two major groups according to the motion that they can move from site to site between the genome e.g. DNA transposons found about 2.8% it is generally excised themselves as DNA from one genome site and inserted into new position by a “cut and paste” mechanism. While, retrotransposons found about 42.2% it is transcripted into RNA and reversed by using reverse transcriptase and inserted at new location by a “copy and paste” mechanism (22, 95). They can be classified as retrotransposons according to the structure based on the presence or absence of long terminal repeats (LTRs) into two groups as a non-LTR retroelements and LTR retroelements (22). The majority and high copy of non-LTR retroelements include long interspersed nuclear element (LINES) such as an LINE-1 found about 16.9%. Following is short interspersed nuclear element (SINEs) such as an Alu found about 10.6% (Figure 7a) (17). Meanwhile, the majority and high copy of LTR

retroelements in human genome is Human endogenous retroviruses (HERVs) found about 8.3 % (Figure 7a) such as HERV-E and HERV-K (17). In the past the IRSs have often been referred to as parasitic or junk DNA sequence (23). However, current studies about the functional role of many IRSs in human genome, suggested that they can affect the human genome from generating insertion mutations and genomic instability by serving as new promoter, new enhancer, new exon and polyadenylation signal to neighboring genes. As a result, it can change gene expression and contribute to genetic innovation (24-26). The methylation levels of various IRSs element have been studied, such as LINE-1, ALU and some types of HERV sequences respectively (23). The hypomethylation of LINE-1, ALU, HERV-E and HERV-K have been found in various condition such as cancers (27) embryogenesis (28, 29), aging (30, 31), congenital malformation (32), exposure to certain environments (33), nutrition (34), and autoimmune diseases (35). Interestingly, in aging cells have hypomethylation of Alu element and HERV-K, but not LINE-1. Therefore, loss of genome-wide methylation in IRSs is type-specific in some conditions (36). Moreover, LINE-1 hypomethylation was found in many other conditions (25). The regulated gene expressions in cis by LINE-1 were reported in at least two studies. Both mechanisms are dependent on the transcriptional activity of the LINE-1 promoter. The first mechanism is that LINE-1-mediated control of gene expression is through the production of unique RNA sequences. Second mechanism is that intragenic LINE-1 RNAs repress host gene expression via the nuclear RNA-induced silencing complex (23). The consequences of LINE-1 and other IRSs hypomethylation that regulate the adjacent cellular gene and may encourage to disease. Several studies have reported an association between IRSs and autoimmune diseases. For example, LINE-1 hypomethylation found in $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, and B lymphocytes of SLE patient and found in lymphocyte of synovial fibroblast of rheumatoid arthritis (37, 38). Besides the association with hypomethylation, the HERV-E and HERV-K gag gene were increased expression in PBMC of SLE patients and rheumatoid arthritis patients respectively (39-41). In addition, when treating PBMC with 5-azacytidine causing

alteration methylation levels, the expression of HERV-E gag transcript and HERV-K gag protein were seen, respectively (39, 42). These results may suggest that HERV-E and HERV-K up-regulation in lymphocyte of SLE patient's effect from methylation losing. Moreover, they are report that Alu element insertion in an intron of Fas gene resulted in Fas gene mutations in autoimmune lympho proliferative syndrome (ALPS) which caused splicing errors or missense changes (43). Not only PBMC and lymphocyte that were involve with pathogenesis of SLE but neutrophil is another important cell. Neutrophil is the most abundant and constitute ~40–60% of the white blood cell population and is critical to the pathogenesis of SLE (44). Several studies have reported an association between irregular neutrophil function and SLE. For example, there are the increases in the percentage of apoptotic neutrophil from patients with SLE (45-48). SLE neutrophil apoptosis correlated positively with lupus disease activity and may contribute to autoantigen excess including dsDNA (45). The apoptotic neutrophils and macrophages is increased in SLE patients and the ability of macrophages to phagocytosis apoptotic bodies is decreased from an impaired clearance mechanism (46). SLE neutrophil secondary necrosis levels significantly higher than neutrophil necrosis levels from control (46). Furthermore, in 2004 paper has new evidence in SLE neutrophils. Increased neutrophil turnover correlates with interferon levels, suggesting a link between neutrophil activation and chronic plasmacytoid dendritic cell (pDCs) activation, leads to increased IFN-α production in SLE (49). Moreover, in 2011 a key of puzzling issues aim to NETosis in SLE. The researchers found that extracellular DNA might frequently be present owing to the formation of NETosis or NET which, NETs are abundant released by neutrophils in patients with SLE (50-52). Possibility is that enhanced abnormalities SLE neutrophil through cell-death process, apoptosis and NETosis including synthesizes increased amounts of type I IFNs. Dysfunction of neutrophil and increased apoptosis and NETosis in SLE might be important in the promotion of autoimmunity and the development of organ damage in SLE. Another interesting evidences, from the analysis of the genes expression in leukocytes of SLE patient by oligonucleotide microarray showed

the overexpression of granulopoiesis-related and interferon (IFN)-induced genes by comparison between SLE and healthy patients. Therefore, Interferon and granulopoiesis signatures in leukocytes of SLE, reveals that immature granulocytes may be involved in SLE pathogenesis (53). Moreover, using methylation array to investigate the existence of gene-specific DNA methylation changes associated with the development of the disease in white blood cells (WBCs) of human monozygotic (MZ) twins discordant for SLE, shows that significantly difference in a global decrease in the 5-methylcytosine content of 49 genes between SLE and healthy MZ twins (5). As mention earlier, a large fraction of WBCs (leukocytes) and granulocyte is neutrophils. However, there are few studies about the relationship between DNA methylation and neutrophils. One reported a correlation between DNA methylation and gene expression levels with polycythemia rubra vera-1 (PRV-1) gene in neutrophils and found that overexpression of the PRV-1 gene in patients with polycythemia vera (PV) and essential thrombocythemia (ET) is associated with a decrease in methylation of this gene. Therefore, DNA methylation might control PRV-1 expression in neutrophils under physiologic and pathologic conditions (54). So far these are no information about DNA methylation level of LINE-1, ALU, HERV-E and HERV-K in neutrophils of SLE.

In this study, we determined and compared DNA methylation level and different methylation patterns of LINE-1, Alu, HERV-E and HERV-K in neutrophils from normal control, SLE patients, inactive SLE patients and active SLE patients by COBRA method. The LINE-1 methylation patterns were classified to hypermethylated (mCmC), hypomethylated (uCuC) and 2 partially methylated loci (mCuC and uCmC). Then, we analyzed the association between LINE-1, Alu, HERV-E and HERV-K methylation patterns according to age and some clinical data of SLE patients. Moreover, using bioinformatics analyzed the correlation between intragenic LINE-1and differential expressions in SLE neutrophils expression array (GSE27427) by the CU-DREAM X program. Our results may reveal role for neutrophils in

SLE pathogenesis and identify a novel link between the methylation level and some important IRSs in this disease.

Research question

How is the methylation level and patterns of LINE-1, Alu, HERV-E and HERV-K in neutrophil of SLE patients and is it correlated with disease activity?

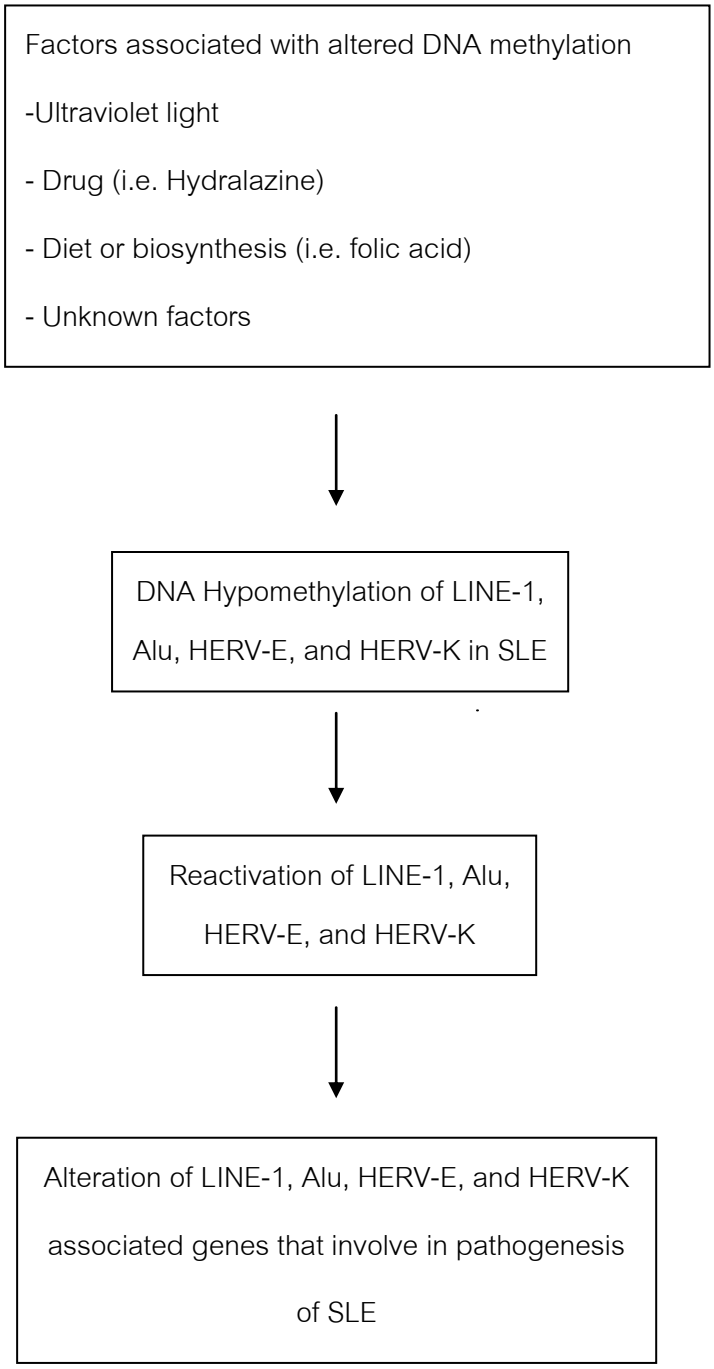
Hypothesis

DNA hypomethylation at LINE-1, ALU, HERV-E and HERV-K sequences occurs in neutrophil of SLE patients is different from normal control and correlate with SLE disease activity.

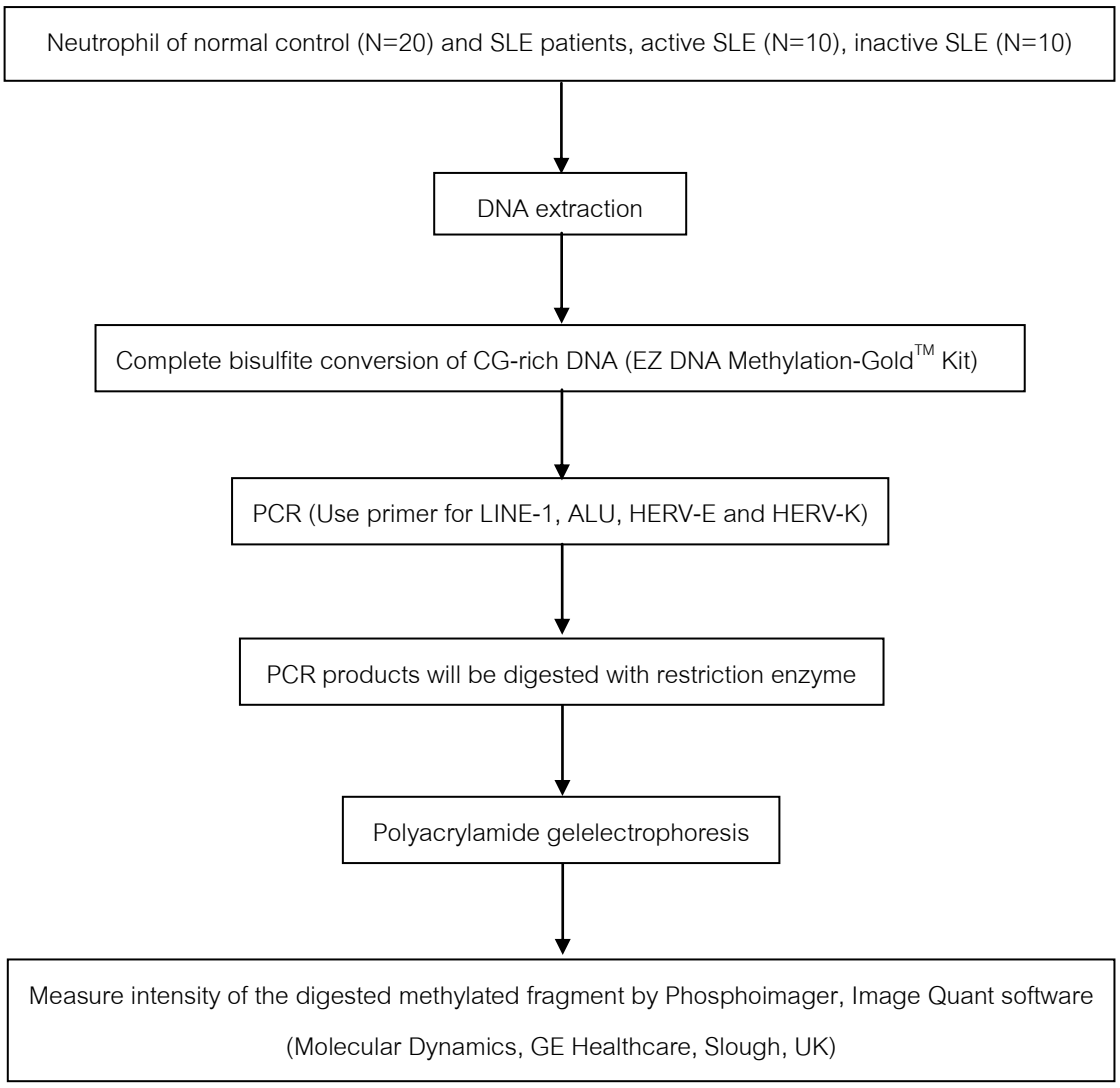
The objectives of this thesis study were:

1. To determine and compare the methylation level and patterns at LINE-1, ALU, HERV-E and HERV-K sequences in neutrophil from normal control, SLE patients, inactive SLE patients and active SLE patients.
2. To analyze the association between LINE-1, Alu, HERV-E and HERV-K methylation level and patterns according to age and some clinical data of SLE patients.
3. To investigate the correlation between intragenic LINE-1and differential expressions in SLE neutrophils expression array (GSE27427).

Conceptual framework



Research framework



CHAPTER II

LITERATURE REVIEW

1. Systemic lupus erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a complex autoimmune disease. SLE is associated with impairments in immune system, which lead to the loss of tolerance of self-antigens. SLE patient displays response of the immune system against its own tissues in multiorgan involvement such as skin, nervous system, heart, kidneys, and blood components. The landmark of SLE is the production of autoantibody against self-antigen, especially against nuclear antigen i.e. intact chromatin, single-strand DNA, double-strand DNA, nucleosomes collectively called antinuclear autoantibodies (ANAs). These autoantibodies combined with a self-antigen into immune complex. These immune complexes deposite in tissue and vessels with high turbulence flow causing tissue and organ damage via inflammatory response, result in several clinical manifestations such as glomerulonephritis, arthritis, serositis, vasculitis and neurological disorders (1). The disease severity and the progression of the disease were different between the patients. The American college of Rheumatology (ACR) has established diagnostic criteria for SLE (Table 1) (55, 56). The patients with at least 4 of the American College of Rheumatology (ACR) criteria were classification to be SLE. Moreover, the activity of the disease was classified by the systemic lupus erythematosus disease activity index 2000 (SLEDAI-2K) (Table 2) (57). The patients with SLEDAI-2K score more than 6 points or more are classified as patients with active disease whereas the patients with SLEDAI-2K score less than or equal to 6 points are classified as patients with inactive disease (58).

Table 1. The American College of Rheumatology's criteria for classification of SLE, revised in 1982 and 1997 (55, 56).

Criterion	Definition
Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
Discoid rash	Erythematosus raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by a physician
Arthritis	Nonerosive arthritis involving 2 or more peripheral joints, characterized by tenderness, swelling or effusion
Serositis	a) Pleuritis (convincing history of pleuritic pain or rub heard by a physician or evidence of pleural effusion) OR b) Pericarditis (documented by ECG or rub or evidence of pericardial effusion)
Renal disorder	a) Persistent proteinurea > 0.5 g/day, or greater than 3+ if quantification not performed OR b) Cellular casts: red cell, hemoglobin, granular, tubular or mixed
Neurologic disorder	a) Seizures (in the absence of offending drugs or known metabolic derangements) OR b) Psychosis (in the absence of offending drugs or known metabolic derangements)
Haematologic disorder	a) Haemolytic anaemia with reticulocytosis OR b) Leukopenia (<4000/mm^3 on 2 or more occasions) OR c) Lymphopenia (<1500/mm^3 on 2 or more occasions) OR d) Thrombocytopenia (<100,000/mm^3 in the absence of offending drugs)
Immunologic disorder	a) Antibody to native DNA in abnormal titre OR b) Antibody to Sm nuclear antigen OR c) Antiphospholipid antibodies - based on 1) abnormal serum level of IgG or IgM anticardiolipin antibodies, 2) positive test result for lupus anticoagulant using a standard method, or 3) a false-positive serologic test for syphilis for at least 6 months confirmed by Treponema pallidum immobilization or fluorescent treponemal antibody absorption test
Anti-nuclear antibodies	Abnormal titre of antinuclear antibody by immunofluorescence or equivalent assay, in the absence of drugs known to be associated with 'drug-induced lupus' syndrome

Table 2. SLEDAI– 2K data collection form (57).

Study No. : _____ Patient Name: _____ Visit Date: _____

(Enter weight to SLEDAI Score column if descriptor is present at the time of the visit or in the preceding 10 days.)

Weight	SLEDAI SCORE	Descriptor	Definition
8		Seizure	Recent onset. Exclude metabolic, infectious or drug cause
8		Psychosis	Altered ability to function in normal activity due to severe disturbance in the perception of reality. Include hallucinations, incoherence, marked loose associations, thinking, bizarre, disorganized, or catatonic behavior. Excluded uremiaimpoverished thought content, marked illogical and drug causes.
8		Organic Brain Syndrome	Altered mental function with impaired orientation, memory or other intelligent function, with rapid onset fluctuating clinical features. Include clouding of consciousness with reduced capacity to focus, and inability to sustain attention to environment, plus at least two of the following: perceptual disturbance, incoherent speech, insomnia or daytime perceptual disturbance, incoherent speech, insomnia or daytime drowsiness, or increased or decreased psychomotor activity. Exclude metabolic, infectious or drug causes.
8		Visual disturbance	Retinal changes of SLE. Include cytoid bodies, retinal hemorrhages, serious exodate or hemorrhages in the choroids, or optic neuritis. Exclude hypertension, infection, or drug causes.
8		Cranial Nerve Disorder	New onset of sensory or motor neuropathy involving cranial nerves.
8		Lupus Headache	Severe persistent headache: may be migrainous, but must be nonresponsive to narcotic analgesia.
8		CVA	New onset of cerebrovascular accident(s). Exclude arteriosclerosis
8		Vasculitis	Ulceration, gangrene, tender finger nodules, periungual, infarction, splinter hemorrhages, or biopsy or angiogram proof of vasculitis
4		Arthritis	More than 2 joints with pain and signs of inflammation (i.e. tenderness, swelling, or effusion).
4		Myositis	Proximal muscle aching/weakness, associated with elevated creatine phosphokinase/adolase or electromyogram changes or a biopsy showing myositis.
4		Urinary Casts	Heme-granular or red blood cell casts
4		Hematuria	>5 red blood cells/high power field. Exclude stone, infection or other cause.
4		Proteinuria	>0.5 gm/24 hours. New onset or recent increase of more than 0.5 gm/24 hours.
4		Pyuria	>5 white blood cells/high power field. Exclude infection.
2		Rash	New onset or recurrence of inflammatory type rash.
2		Alopecia	New onset or recurrence of abnormal, patchy or diffuse loss of hair.
2		Mucosal Ulcers	New onset or recurrence of oral or nasal ulcerations
2		Pleurisy	Pleuritic chest pain with pleural rub or effusion, or pleural thickening.

2	Pericarditis	Pericardial pain with at least 1 of the following: rub, effusion, or electrocardiogram confirmation.
2	Low Complement	Decrease in CH50, C3, or C4 below the lower limit of normal for testing laboratory.
2	Increased DNA binding	>25% binding by Farr assay or above normal range for testing laboratory.
1	Fever	>38 °C. Exclude infectious cause
1	Thrombocytopenia	<100,000 platelets/mm3
1	Leukopenia	<3,000 White blood cell/mm3. Exclude drug causes.

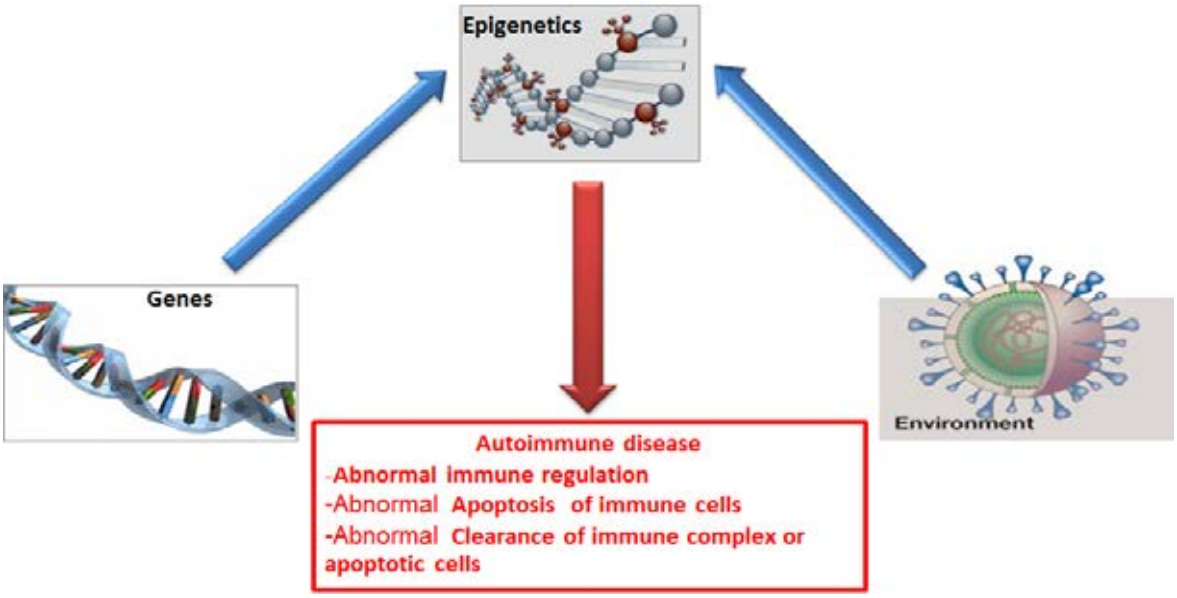
2. Epidemiology of SLE

SLE can be found worldwide in almost all ages. The incidence rates range from approximately 1 to 10 per 100,000 person-years. The prevalence rate range of SLE is from 20 to 70 per 100,000 depending on racial and geographic background (59). Gender is also a risk factor for SLE. The prevalence rates for men to women are approximately 1:10 (59). There is a possible role of hormones particularly the estrogens (59). The nationality of African-American, Asian and Hispanic have more prevalence of SLE and severely affected than Caucasian (60). According to a study in Asia the Hong Kong population, the prevalence of SLE patients were found 58.8 per 100,000 persons and had SLE with renal involvement up to 50%. While, only 30-39% of the Caucasian patients have lupus nephritis (61, 62). Moreover, as high as 78% of Thai SLE had renal involvement, suggesting that Thai patients may have more severe disease than Hong Kong and the Caucasian (63).

3. Etiology of SLE

The past studies found that the etiology of SLE is caused by genetic factors and environmental factors which lead to the abnormalities of immune system, particularly the loss of tolerance of self-antigens. However, the exact etiology of SLE is still obscure (2). Various evidences suggest a role of epigenetics in human pathologies such as inflammatory response and malignancies, especially, the dysregulation of DNA methylation processes. We believe that epigenetic, especially DNA methylation is a linker between genetic and environmental factors (3, 4). As show in Figure 1

Figure 1: Predisposing factors in SLE



3.1 The genetic factors predisposition to SLE

SLE are complex autoimmune diseases with strong genetic factors. The important evidences come from the familial aggregation and twin studies. From familial aggregation studies, forty-two SLE patients had 2 or more relatives with an autoimmune disease and a λ sibling of 5.8 and 29.0 for SLE, when comparing with their reported high or intermediate population prevalence (64). Moreover, twin studies show the 2-5% and 24–57% disease concordance in dizygotic and monozygotic twin, respectively (65, 66). Interestingly, the gene-specific DNA methylation changes associated with the development of the SLE was found in monozygotic twins, showing significantly global decrease in the 5-methylcytosine content of 49 genes between SLE and healthy monozygotic twins (5). In addition, there are reports about a number of genetic risk factors for SLE. Those identified candidate genes including *HLA, FCGR, PTPN22*, *STAT4, BLK, BANK1, ITGAM, IRF5*, *ITPR3 and TNXB*, which have been studies in Caucasian and Asia patients with SLE by genome wide association studies (GWAS) (54-59). These data support the role of genetics factors in SLE.

3.2 The environmental factors predisposition to SLE

One more factor that is important to the pathogenesis of SLE is the environmental factors including ultraviolet light. The SLE patients around 73% have been reported to have photosensitivity symptom (67). The chemicals or other compounds including environmental pollutants or drugs such as hydralazine and procainamide can cause lupus-like disease. Most likely the mechanism is by inhibiting T cell DNA methylation (68, 69). Other environment factors are smoking and diet are behavioral factors that associated with risk of SLE if exposure to high levels (70, 71). As for the infectious agents, infected virus could lead to the loss of self-tolerance by several mechanisms, including molecular mimicry, protein changes or exposition of cryptic antigens. Moreover, DNA and RNA viruses can stimulate intracellular toll-like receptors (TLR) in plasmocytoid dendritic cells (pDCs) to produce interferon (INF-α), which stimulates the adaptive immune system and development to autoimmunity (4). For example, the Epstein-Barr Virus (EBV) effect by viral antigens cross-reactivity with self-antigens, which lead to self-reactive B cells imperishable (72-75). These data support the role of environmental factors in SLE.

3.3 Abnormalities of immune system in SLE

Pathophysiology of SLE can be defined by congruence of clinical and laboratory criteria and presents variety of immune system dysregulation in both adaptive and innate immunity. The key of the pathophysiology and therapy of SLE has focused on abnormalities of adaptive immune system, T lymphocytes and B lymphocytes (2). Recently, focus has shifted and gained attention to the role of innate immune system because accumulating data support abnormalities in innate immunity, which might be involved in the regulation of cell death, presentation of putative autoantigens and synthesis of type I interferons (IFNs). These defects appear in innate immune cell such as monocytes, macrophages, dendritic cells (DCs). However the role of neutrophils in the pathogenesis of SLE had not been well characterized. Here we review normal neutrophil biology and some abnormalities of

neutrophils that discovered in SLE, and purposed mechanism of how neutrophil could promote to SLE pathogenesis (76).

3.3.1 Normal neutrophil biology

Neutrophils or polymorphonuclear cells (PMN) are normally found approximately ~40-60% of all white blood cells (leukocytes) and they are the most abundant types of leukocytes in the human body and an essential part of the innate immune system. However, they have a short lifespan and their homeostasis is maintained by the bone marrow. Neutrophils are crucial component in the first line of defense against invading pathogens. Normally resting neutrophils become primed by agents of bacterial products and cytokines or chemokines, such as TNF-α, GM-CSF, IL-8 and IFN-γ. These priming substances will activate transcription factors that trigger the receptors and cytokines expression, which increase neutrophil function or lifespan. Then activated neutrophils are migrating towards the site of infection or inflammation within minutes. They are controlled by interactions between L-selectin expressed on the surface of neutrophils allowing loose tethering to ligands expressed on the surface of vascular endothelial cells such as E- and P-selectin, and P-selectin glycoprotein ligand-1 (PSGL-1)] and very late antigen-4 (VLA-4). These interaction result in the conformational changes in integrin adhesion molecules on the surface of endothelial cells, which composed of intercellular adhesion molecule (ICAM)-1 and -2, vascular cell-adhesion molecule-1 (VCAM-1) and mucosal vascular cell-adhesion molecule-1 (MADCAM-1). Then the rolling arrest is mediated by high-affinity ligand binding and strong adherence of neutrophil receptors and endothelium surface ligand. Neutrophils then transmigrate via the junctions between neighbouring endothelial cells (paracellular) into tissue, which the surface area presents expression of ligands including ICAM-2, PECAM-1 (platelet endothelial-cell adhesion molecule-1) and proteins of the junctional adhesion molecule (JAM) family. (Figure 2) Then neutrophils diapedese from peripheral blood passing through endothelium and chemotaxis to the sites of inflammation. The

chemoattractants induces neutrophils to migrate towards inflamed tissue along a chemotactic gradient such as N-formylmethionyl-leucyl-phenylalanine (fMLP) and complement component 5a (C5a). Elimination of pathogen at the infected site, complement proteins and immunoglobulins recognize and bind opsonized pathogen leading to the formation of pseudopodia and destruction within the intracellular phagosome. Neutrophils rapidly kill phagocytosed pathogens by generating reactive oxygen species (ROS) via the respiratory burst, the release of microbicidal substances from cytoplasmic granules and the formation of neutrophil extracellular traps (NETs), which contain a backbone of DNA and nuclear histones as well as many granular antimicrobial peptides. Indeed these toxic molecules can also damage host tissue. The inappropriate activation of neutrophils might be the induction of autoimmunity and the development of organ damage in patients with SLE (44, 76). Intracellular granules of neutrophil are mobilized upon priming of the cell, enabling the sequential release of secretory vesicle, followed by gelatinase granules, specific granules and finally azurophilic granules into the extracellular environment (44). (Figure 3)

Figure 2: Neutrophil diapedesis (44). (E: endothelial cell; BM: basement membrane)

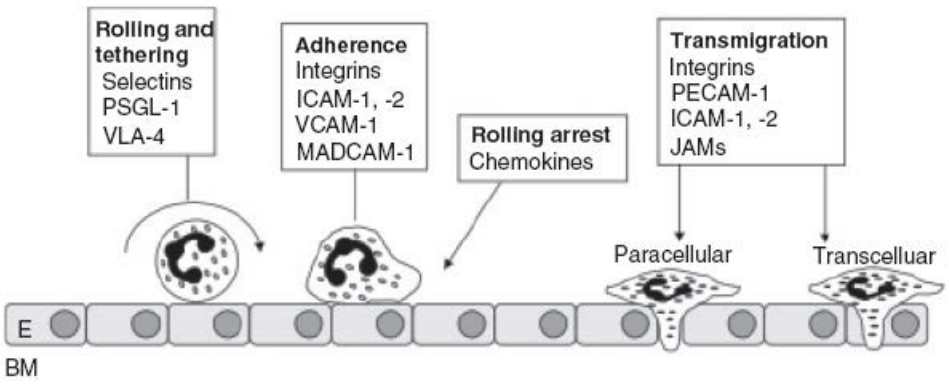
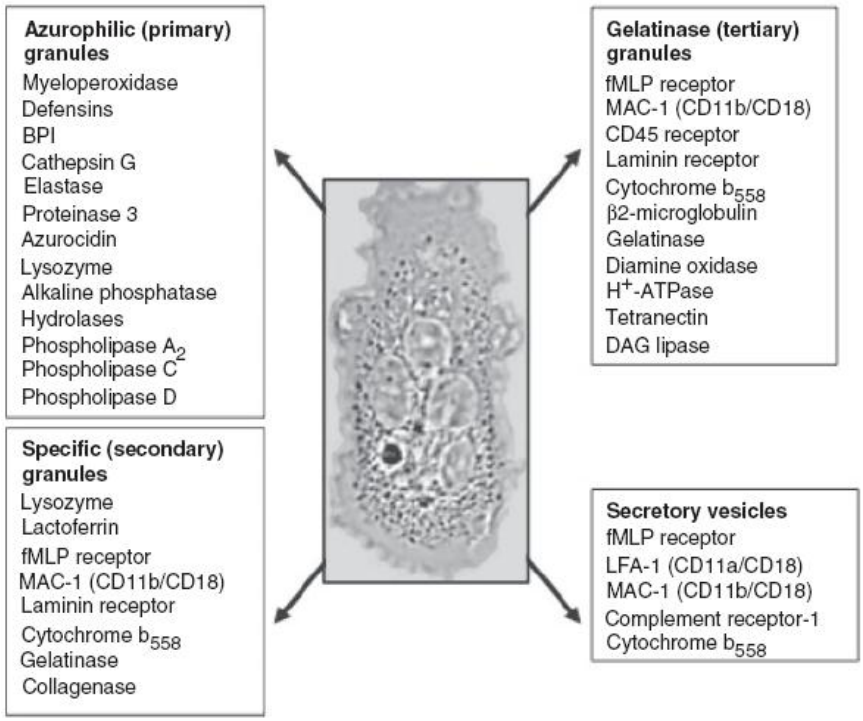


Figure 3: Summary of neutrophil granules and their contents (44).



3.3.2 Abnormalities in SLE neutrophil function

Abnormalities in SLE neutrophil phenotype and function have been discovered. Crucially, several recent papers reported that abnormalities in cell-death process of SLE neutrophils. There were increased levels of apoptotic neutrophils from patients with SLE, compared with neutrophils from healthy donors. In addition the aberrant clearance of apoptotic bodies are positively correlated with lupus disease activity (45-47, 48) as well as SLE neutrophil secondary necrosis levels significantly higher than neutrophil necrosis levels from control (46). Usually neutrophils are the most abundant types and short lifespan compared with other blood cell types so neutrophils represent an abundant source of antigenic material and can contribute to excess production of SLE-related autoantibodies, including those against double-stranded DNA (dsDNA) and nucleosomes (77). These sources of self-antigens will be taken up by antigen-presenting cells and present peptides

to T cells. Then stimulating T cells and B cells to produce autoantibodies to self-antigen. The consequences of increased loss of neutrophils or low number of circulating neutrophils are neutropenia which is a feature of the disease in a numerous proportion of patients with SLE (78). The mechanisms that drive the progression of neutropenia in patients with SLE including neutrophil-reactive autoantibody, neutralizing autoantibodies against growth factors such as G-CSF, bone marrow suppression and increased neutrophil death by apoptosis, necrosis or by NETosis (79). One possibility is that neutropenia and enhanced neutrophil induced tissue damage in patients with SLE occurred by infiltration of neutrophils to organs are actively undergoing NETosis or apoptosis. These mechanisms lead to neutropenia but also to organ damage and immune dysregulation. However, the exact roles of abnormalities in neutrophils that promoted SLE pathogenesis and organ damage remains obscure.

In 2004 paper reported the new evidence in SLE neutrophils. Increased neutrophil turnover correlates with interferon levels, suggesting a link between neutrophil activation and chronic plasmacytoid dendritic cell (pDCs) activation, leading to increased IFN-α production in SLE (49). Moreover, self-DNA in immune complexes and LL-37 autoantibodies as well as human neutrophil peptide (HNP) was detected in sera from patients with SLE which was contrast to control sera. These immunogenic complex help promoting the formation of NETs by protected DNA degradation from nucleases and can trigger Toll-like receptor (TLR) 7 and TLR9 activation on pDCs as well as interaction with Fcγ receptor IIa (FcγRIIa) on pDCs. In addition, LL-37–DNA complexes stimulate pDCs to synthesize IFN-α and might also trigger B-cell stimulation, suggesting that NETs might promote activated B-cell and development of antibodies against antimicrobial peptides (50). These evidences support the concept of SLE neutrophil-dependent pathway dysregulation and driving chronic pDC activation as well as autoimmunity in SLE.

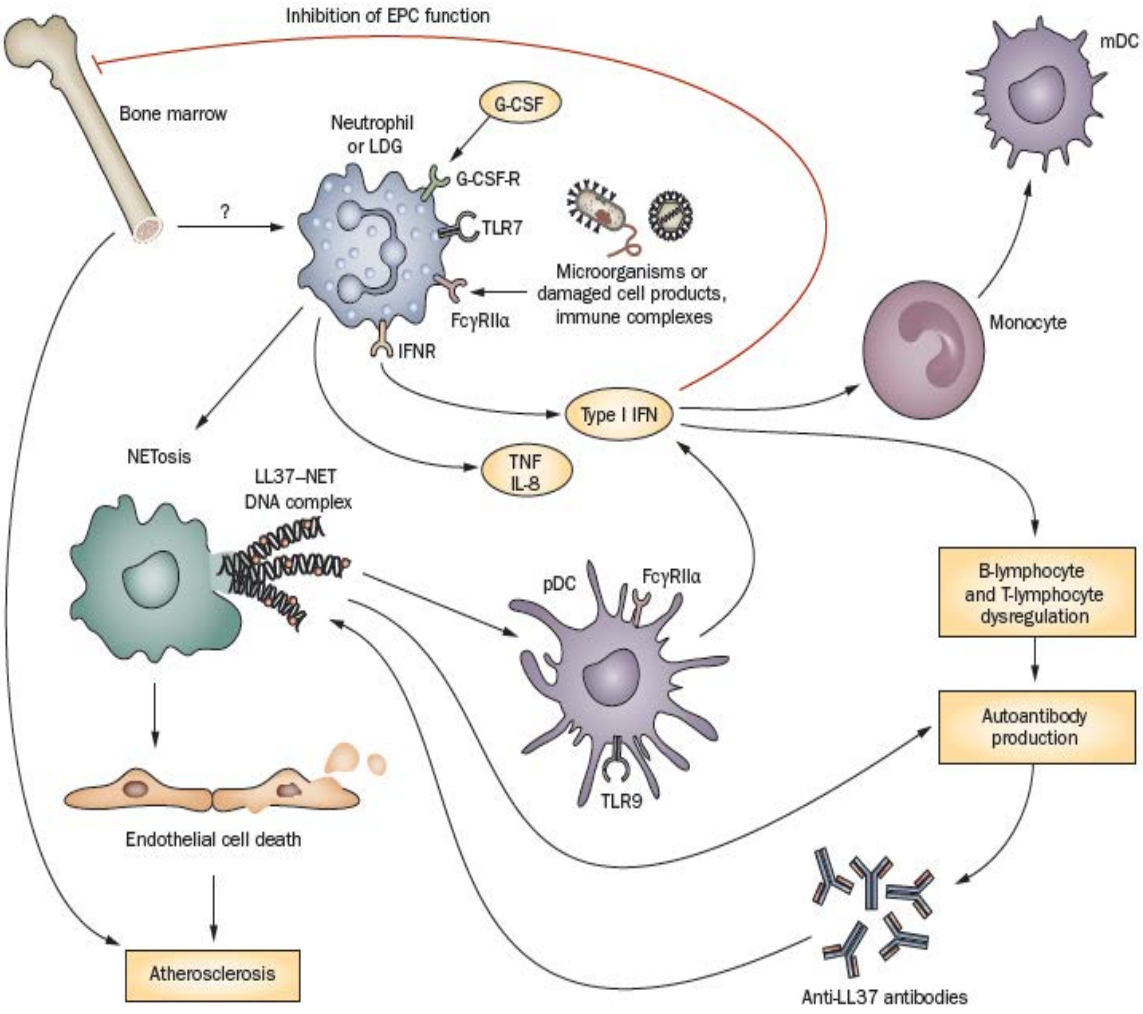
In 2011, the researchers found that extracellular DNA might frequently be present owing to the formation of NETosis or NET which, produced from neutrophils upon response

to microbial infections. Moreover, NETs are abundantly released by neutrophils in patients with SLE (50-52). The term NETosis is cell death that occurred upon encountering various stimuli including extracellular pathogen, proinflammatory cytokines, and activated platelets or endothelial cells. NETosis associated with reactive oxygen species generation, as well as by translocation of neutrophil granule components to the cytoplasm and nucleus, encouraging to chromatin decondensation. The chromatin fibers were tightly packed extrusion and affect to nuclear membrane breakdown and elimination neutrophil extracellular trap (NET). NETs consists of DNA backbone and histones as well as neutrophil granules that contain many granules of antimicrobial peptides and some cytoplasmic proteins, which initiate the trap and promote the clearance of extracellular pathogen (76). The investigation of NETs production by neutrophils in the skin of patients with SLE was similar to the findings in mouse model SLE disease. Skin injury leads to the leukocyte infiltration and stimulating pDCs to synthesize IFN-α and production of NETs by neutrophils. In summary, the abnormalities in neutrophil phenotype and function in patients with SLE are the enhanced neutrophil death through apoptosis and NETosis, which can increase the production of type I IFNs. Therefore, neutrophil dysfunction and increased apoptosis and NETosis in SLE are important in the promotion of autoimmunity and the development of organ damage in SLE (76),(80). (Figure 4)

Figure 4: Role of neutrophils or LDG in the pathogenesis of SLE and associated organ damage (76).

NETosis occurred upon exposure to micro-organisms, damaged cell products, immune complexes and other as yet uninvestigated stimuli. NETs consists of externalize bactericidal immunostimulatory peptides such as LL-37, autoantigens including dsDNA. The formation of LL-37-DNA complexes will stimulate pDCs to synthesize IFN-α. Moreover, upon stimulation with G-CSF lead to neutrophils and LDGs synthesis of type I IFNs. Consequences to increased levels of type I IFNs stimulation by LL-37-DNA complexes and

G-CSF which can promote monocyte differentiate to mDCs as well as B-cell and T-cell dysregulated and synthesis of autoantibodies and detrimental effect on bone marrow EPCs. In addition, LDGs also promote endothelial cell death through a NET-mediated effect (76).



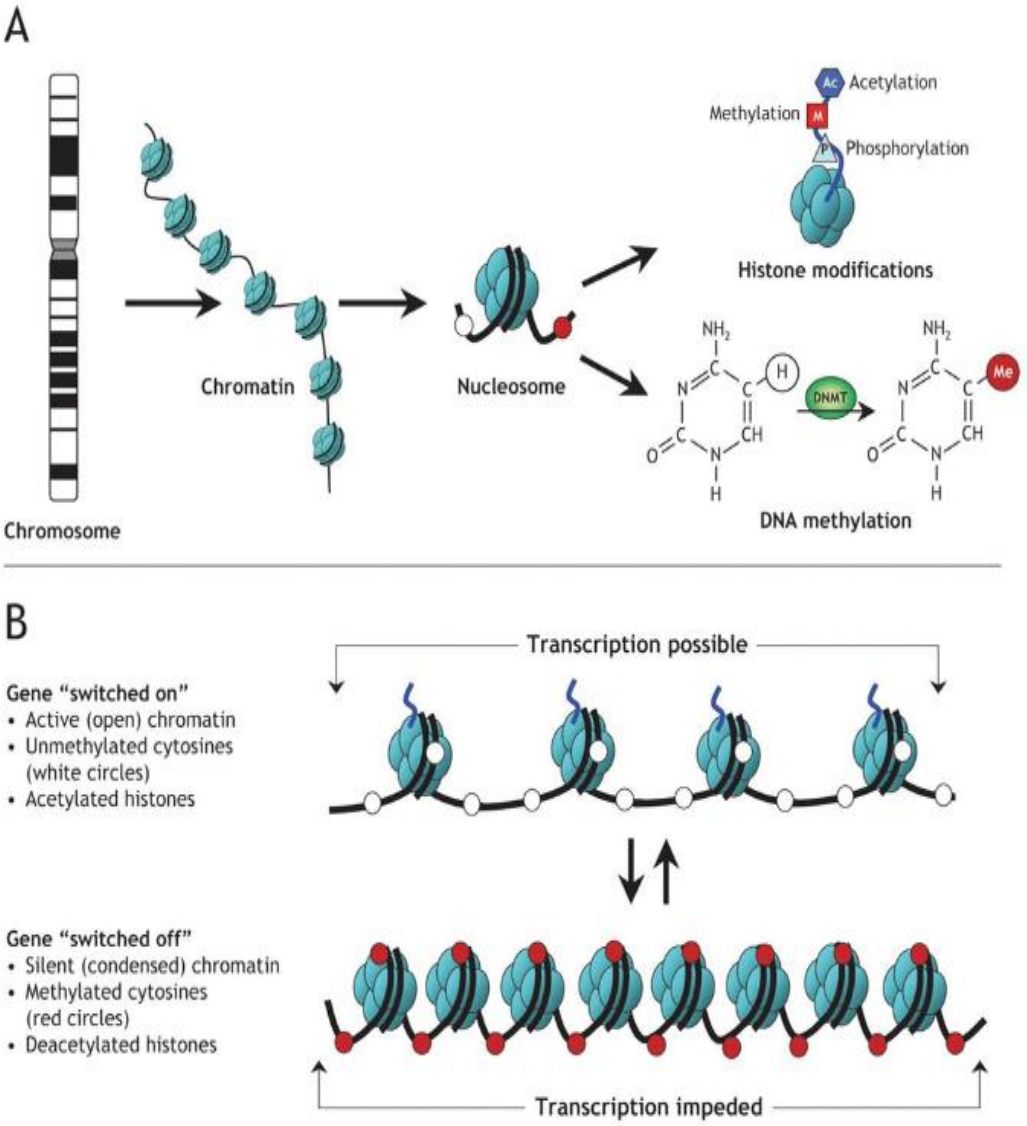
Abbreviations: EPCs, endothelial progenitor cells; FcγRIIα, low affinity immunoglobulin-γ Fc region receptor II-α, G-CSF, granulocyte-colony stimulating factor; G-CSF-R, G-CSF receptor; IFNR, interferon receptor; LDGs, low-density granulocytes; mDC, myeloid dendritic cell; NET, neutrophil extracellular trap; pDC, plasmacytoid dendritic cell; TLR, Toll-like receptor (76).

4. Epigenetics

Epigenetic refers to the heritable changes in gene expression that do not involve sequence change in DNA. This mechanism helps initiation and maintenance of cells differentiation. It was normally found early during cellular development and differentiation (81, 82). The scope of epigenetic mechanisms consists of three main stations exist in a normally regulated genome, 1) DNA methylation, 2) histone modification in chromatin and 3) micro-RNA associated with silencing. The gene expression of cells that must be expressed in specific cells at precise times depends on nucleosomes formation because DNA stands are wrapped around histone octamers proteins. These nucleosomes are organized into chromatin. (Figure 5A) The modifications of chromatin structure can affect gene expression. During gene expression (switched on), the transcription factors (TFs) are attached to binding site on DNA promoters when chromatin is open (active). In contrast, the way to inactivate gene (switched off) is the inability of TFs to bind to DNA, when chromatin is condensed (silent). (Figure 5B) The dynamic of chromatin states between active and silent chromatin are controlled by reversible epigenetic patterns of DNA methylation and histone modifications (3, 83).

Figure 5: Epigenetic modification (3).

DNA strands are wrapped around histone octamers to nucleosomes formation. Polynucleosomes are organized into chromatin. (A) The dynamic chromatin state is controls by DNA methylation involve addition of methyl group to cytosine within CpG and histone modification via acetylation, methylation and phosphorylation. (B) The dynamic reversible chromatin organization that alter gene expression by activation or silencing chromatin.

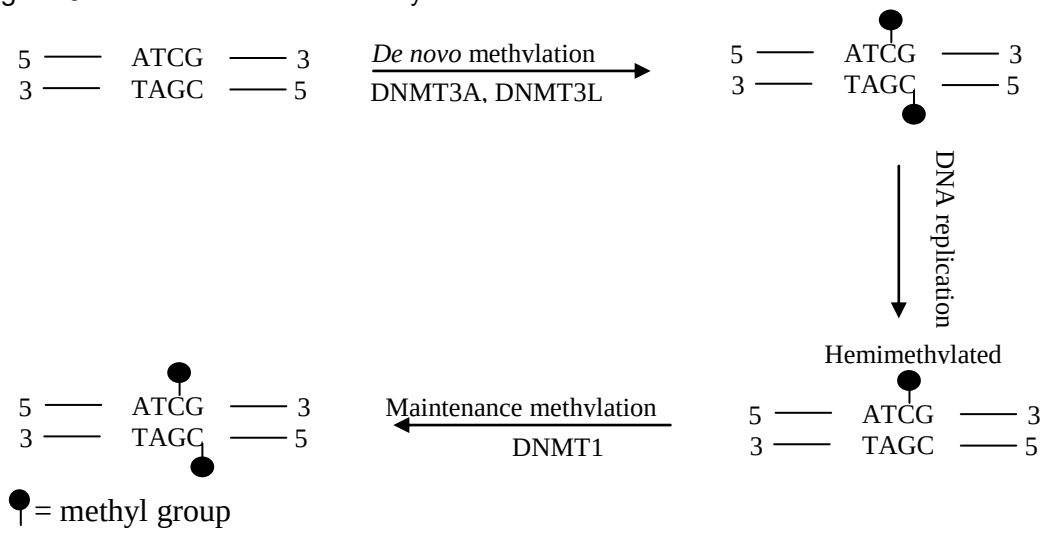


4.1 DNA methylation

DNA methylation involved the addition of a methyl group to the 5' carbon position of cytosines within CpG (cytosine/guanosine) pairs. CpGs are enriched in short stretches of genome called CpG island (CGIs), which are targets for transcription factor proteins that bind to unmethylated CpGs and initiate gene transcription. The majority of CGIs are located on 5' end regions of promoters and first exon (3). The criteria to determine CGIs is a contiguous C+G content more than 55% of DNA, at least 500 bp in length and an observed

CpG over expected CpG ratio in excess of 0.65 (84). DNA methylation process involved DNA methyltransferases (DNMTs) which, served by transfering the methyl groups from S-adenosyl methionine to cytosine residues in DNA. Distinct DNMTs include DNMT1, DNMT2, DNMT3a, DNMT3b, and DNMT3L are encoded by at least three different genes called *DNMT1*, *DNMT3A* and *DNMT3L*. Functional DNMT1 have been known as maintenance DNA methylation. This enzyme preferentially methylates hemimethylated DNA to create the methylation pattern for newly replicated daughter strands during mitosis, based on that of the parent strand (85). In addition, functional DNMT3A and DNMT3L have been known as addition of methyl group to the unmethylated CpG (*De novo* methylation), have an effect to formation of new hemimethylation and fully methylated CpG (86). (Figure 6) DNA promoters of active genes and tissue-specific genes are generally hypomethylated. In contrast, DNA promoters of inactive genes are silenced by methylation. This dynamic of transcriptional suppression is mediated by cytosine methylation that directly interfere TFs binding DNA. Moreover, cytosine methylation can be indirectly interfered by recruiting methyl binding domains and co-repressors proteins to methylated CpG to prevent the binding of TFs to DNA (87).

Figure 6: Mechanism of DNA methylation



4.2 Histone modifications

The modifications of histone include acetylation, methylation, ubiquitination, phosphorylation, sumoylation, deimination/citrullination, ADP-ribosylation and proline isomerization. Each mechanism of histone modification serves a specific purpose. The acetylation and deacetylation of histones participate in the control of transcription. Histone acetylation involving the addition of an acetyl group (Ac) by histone acetyl transferase to the N-terminal tail of histones is enough to decondense the chromatin. Therefore transcription factor can bind to DNA, and influent the transcription. In contrast, histone deacetylase involves with removing acetyl groups from histones leading to chromatin condensation and gene silencing (83).

4.3 Micro-RNA

Micro-RNA (miRNAs) consists of RNA length 21-23 bps and function as post-translational regulators of gene expression. The operation of miRNAs that involves with the role of RNA silencing has been associated with epigenetic controls. The researcher found that one-third of the human transcriptome is regulated by 1,000 miRNAs. Some of them interact with transcripts of genes that alter DNA methylation and histone modification. In contrast, miRNA expression can be affected by DNA methylation and histone accommodation (83).

5. DNA methylation and SLE

Various evidences suggest a key important role for epigenetics, in human pathologies such as inflammatory response and malignancies. Particularly the dysregulation of DNA methylation processes was found to be increasing in frequency in various autoimmune and cancer patients (Table 3) (49). Several papers report the abnormalities of DNA methylation level in some important immune cells of SLE patients. It was found that leukocytes (5), PBMCs (6, 7), T-cell (35) and CD4+ T-cell DNA (8-11) of SLE patients were

DNA hypomethylation comparing to healthy donor. This hypomethylation was associated with decreasing of DNMT1 mRNA levels (11). In addition, DNA hypomethylation T cells are important to initiate apoptotic DNA to induce SLE-like autoimmune disease in nonsusceptible mice (12). The evidence supporting the role of demethylation comes from studies with demethylating agents such as procainamide, hydralazine, or 5-azacytidine. These agents are cytosine analog that contains a nitrogen atom at the 5' position of the pyrimidine ring and are incorporated into newly synthesized DNA. Treatment with these agents causes global hypomethylation. This mechanism have been proved in vitro by treating T cell with demethylating agents, it can induces major histocompatibility complex–specific T cell autoreactivity (88-92). Moreover, the adoptive transfers of 5-azacytidine or procainamide-treated T cells into syngeneic recipient mice caused a lupus-like disease (93, 88). Therefore, hypomethylation of CD4+ T cell may contribute the development of SLE. In addition, overexpression and demethylation of CD40 ligand (TNFSF5) was found only in CD4+ T cell of female SLE. This evidence suggested that demethylation in X inactivation chromosome may be an important factor result in highly incidence of SLE in female than male (94). Recent study reported the abnormalities of DNA methylation level in some important immune cells and SLE-relate genes of SLE patients. The hypomethylation levels of the CpG islands in the IL-4 and IL-6 promoters were found in T cell of SLE patients (13). The hypomethylation levels of IL10 and IL13 promoter were found in CD4+ T-cell of SLE patients (14). And hypomethylation of the HTR1A promoter was found in PBMC of SLE patient (15).

Table 3: Associations between epigenetic modifications and human diseases (3).

Disease/condition	Gene	Biological process	Disease/condition	Gene	Biological process
Cancer			Neurologic		
Bladder	Multiple genes	Hypermethylation[20]	Schizophrenia	*RELN*	Hypermethylation[46,47]
Brain (glioma)	*RASSF1A*	Hypermethylation[28,29]	Bipolar disorder	*11p?*	Unknown[48]
Brain (glioblast)	*MGMT*	Hypermethylation[30]	Memory formation	Multiple genes	Hypo-, hypermethylation[49]
Breast	*BRCA1*	Hypermethylation[31]	Lupus	Retroviral DNA	Hypomethylation[50]
Breast	Multiple genes	Hypermethylation[32,33]	Cardiovascular		
Cervix	*p16*	Hypermethylation[34]	Atherosclerosis	Multiple genes	Hypo-, hypermethylation[19,51]
Colon	Multiple genes	Hypermethylation[20]	Homocysteinemia	Multiple genes	Hypomethylation[52]
Colorectal	L1 repeats	Hypomethylation[35]	Vascular endothelium	*eNOS*	Hypomethylation[53]
Esophagus	*CDH1*	Hypermethylation[20]	Imprinting and pediatric syndromes		
Head/neck	*p16, MGMT*	Hypermethylation[20]	PWS or AS	15q11-q13	Imprinting[54]
Kidney	*TIMP-3*	Hypermethylation[20]	BWS	11p15	Imprinting[55]
Leukemia	p15	Hypermethylation[20]	SRS	Chromosome 7	Imprinting[56]
Liver	Multiple genes	Hypermethylation[36]	UPD14	14q23-q32	Imprinting[57]
Lung	*p16, p73*	Hypermethylation[20]	PHP, AHO, MAS	20q13.2	Imprinting[58]
Lymphoma	*DAPK*	Hypermethylation[20]	Rett syndrome	*MECP2*	Mutation[59]
Myeloma	*DAPK*	Hypermethylation[37]	ICF syndrome	*DNMT3B*	Mutation[60]
Ovary	*BRCA1*	Hypermethylation[38]	ATRX	*ATRX*	Chromatin structure[61]
Ovary	*Sat2*	Hypomethylation[39]	FraX	Triplet repeat	Silencing[62]
Pancreas	*APC*	Hypermethylation[20]	FSHD	3.3 kb repeat	Chromatin structure[63]
Pancreas	Multiple genes	Hypomethylation[40]	Reproductive		
Prostate	*BRCA2*	Hypermethylation[20,41]	Ovarian teratoma	No paternal genome	Imprinting[64]
Rhabdomyosarcoma	*PAX3*	Hypermethylation[42]	CHM	No maternal genome	Imprinting[65]
Stomach	*Cyclin D2*	Hypomethylation[43]	BiCHM	Maternal genome	Imprinting[65]
Thymus	*POMC*	Hypomethylation[44]	Aging	Chromatin	Hypo-, hypermethylation[66]
Urothelial	Satellite DNA	Hypomethylation[45]			
Uterus	*hMLH1*	Hypermethylation[20]			

6. Interspersed repetitive sequences (IRSs)

In human genome, interspersed repetitive sequences (IRSs) was found approximately 45% of whole genome, which can be divided into two major groups include DNA transposons and retrotransposons. Each group was divided according to the motion that they are able to move from site to site between the genome. DNA transposons were found about 2.8%. It is generally excised themselves as DNA from one genome site and

inserted into new position by a “cut and paste” mechanism. While, retrotransposons were found about 42.2%. It is transcribed into RNA and reversed by using reverse transcriptase and inserted at new location by a “copy and paste” mechanism (22, 95). Moreover, we can classify retrotransposons according to the structure based on the presence or absence of long terminal repeats (LTRs) into two groups as a non-LTR retroelements and LTR retroelements (22). The highest copies of non-LTR retroelements is the long interspersed nuclear element (LINES) such as an LINE-1 (L1) found about 16.9% (Figure 7a). There are >500,000 L1 copies composed of full-length L1 element which is approximately 6 kb long and L1 structural consists of a 5′ UTR containing an internal RNA polymerase II (RNAPII) promoter, two open reading frames (oRF1 and oRF2) and a 3′ uTR containing a polyadenylation signal ending with an oligo (dA)-rich tail of variable length. (Figure 7b) The second most common non-LTR retroelement is the short interspersed nuclear element (SINEs) called Alu found about 10.6% (Figure 7a). There are >1 million Alu copies composed of full-length ALU element which is approximately 300 bp long and Alu structural consists of two related monomers separated by an A-rich linker region. The left monomer contains A and B boxes (blue boxes), which are transcriptional promoters for RNA polymerase III. The element ends with an oligo (dA)-rich tail (AAA) that can be up to 100 bp long (Figure 7b) (87). In addition, Alu requires activities of enzymatic assistance from LINE-1 or the host for their mobility. Therefore, LINE-1 and Alu are the abundant non-LTR retrotransposons in human genome (96-99). Meanwhile, another high copy number of LTR retroelements in human genome is Human endogenous retroviruses (HERVs) which was found about 8.3 % such as HERV-E and HERV-K (17). A typical size of HERVs is about 6-7 kb and HERVs structural consists of four internal universal genes, including gag, pro, pol and env, which are flanked by two LTRs as exogenous retrovirus (Figure 8). Moreover, HERVs contain numerous deleterious mutations such as smaller deletions, frame shifts or stop codons and hypermethylation of promoters. These mechanisms occur in order to maintain the inactivation the expression of functional proteins from proviral genes (100).

Most HERVs were remained in the human genome as solitary LTRs due to recombination of the two LTRs (101).

Figure 7: The human genome content of transposable elements and structural of LINE-1 (*L1*) and *Alu* (87).

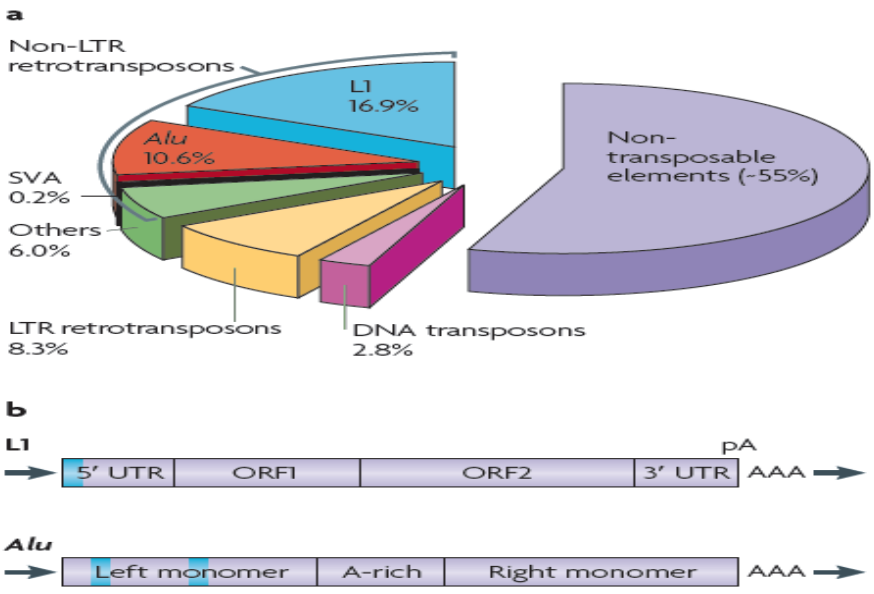
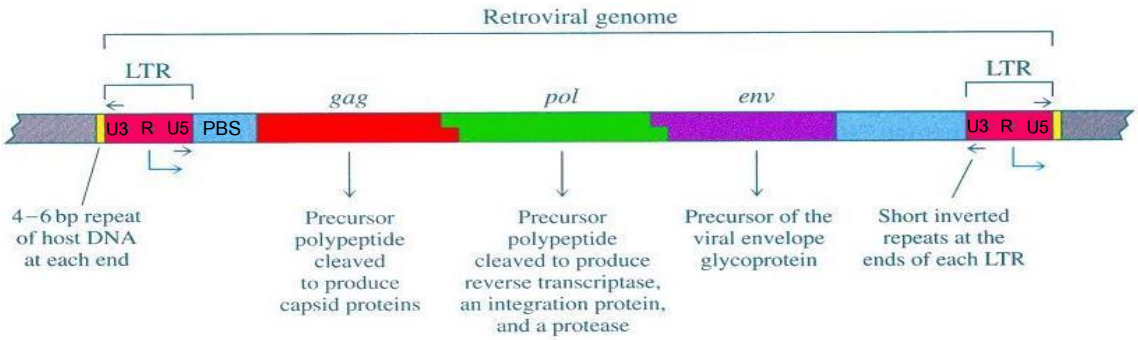


Figure 8: Structure of the retroviral genome (100).

LTR: long terminal repeat, U3: unique 3' sequence, R: repeat sequence; U5: unique 5' sequence; PBS: primer binding site; gag: group-specific antigen; pro: protease; pol: RNA-dependent DNA polymerase, env: envelope.



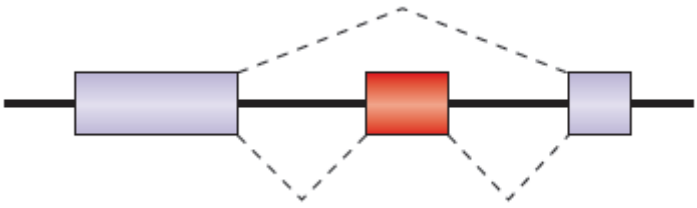
7. The impact of interspersed repetitive sequences on human genome

In the past, the IRSs have often been referred to as parasitic or junk DNA sequence (23). However, current studies reported the functional role of many IRSs studies in human genome, suggested that some important IRSs might alter gene expression and contributing to genetic innovation in human genome (24-26). Interspersed repetitive sequences (IRSs) including LINE-1, Alu, HERVs can integrate into the human genome and affect gene transcription of human genomes via various mechanisms such as exonization or alternative splicing, transcription elongation defects, modulation of gene expression, alternative promoter, RNA editing and epigenetic control (26, 102-106).

Figure 9: The impact of interspersed repetitive sequences on human gene expression (26).

(A) Exonization and alternative splicing

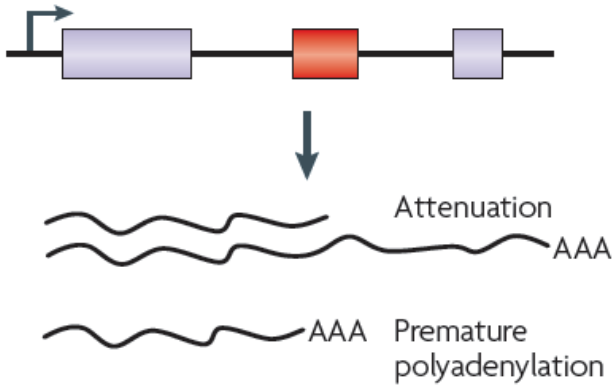
This mechanism occurs 40-60% in human genes and associates with alternative splicing (dashed lines). Retrotransposon sequences (red box) such as LINE-1, Alu and HERVs can be inserted into an intron, and then sometimes they can be integrate into a gene act as exons (made up here of two exons, grey boxes) in a process termed exonization. A single gene produces more than one type of mRNA and contributes to human proteome variation include promoting a new exon and splice sites.



(B) Transcription elongation defects

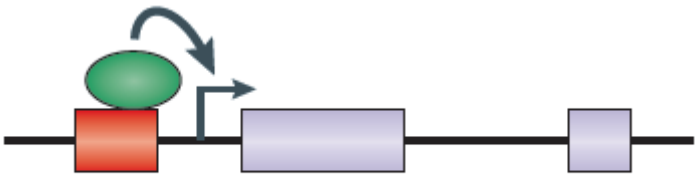
Retrotransposon sequences presence in the intron of a gene (Grey boxes which represent intron), such as intronic L1 elements (Red box which represent exons) can disturb

transcriptional elongation of the host gene by promoting polyadenylation signals. Moreover, HERV-LTR sequences can provide polyadenylation signals that induce transcription elongation defects. The result of transcription elongation defects cause to induce the end of gene transcription including attenuation or premature polyadenylation.



(C) Modulation of gene expression

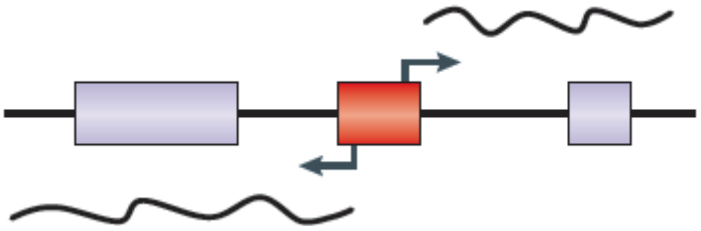
Some Retrotransposons such as Alu elements carry transcription factor-binding sites (red box) that might modulate gene expression. A transcription factor (green oval) carried by a retrotransposon can upregulate or downregulate (curved arrow) the expression (horizontal arrow) of nearby genes (grey boxes).



(D) Alternative promoter

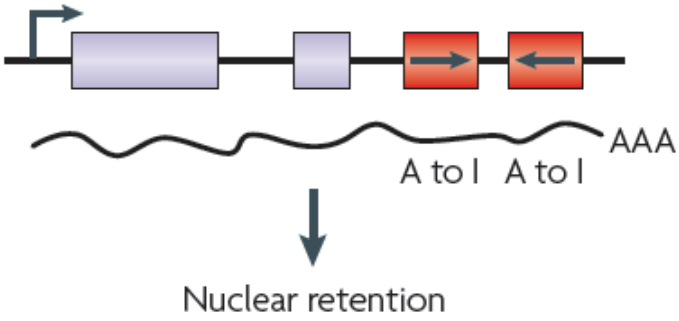
Retrotransposons such as LINE-1, Alu and HERVs element carries sequences similar to functional promoters (red box). This promoter has a function to initiate sense or

antisense transcription (Horizontal arrows) through nearby genes (grey boxes) by controlling gene expression that can initiate downstream and upstream transcription.



(E) RNA editing

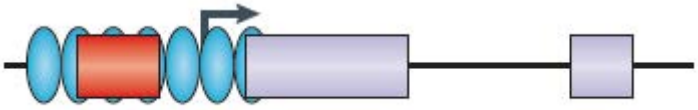
RNA editing is a process by which RNA sequences provide post transcriptional modification by conversion of adenosine (A) to inosine (I) in dsRNA such as two Alu elements appear in the opposite orientation in gene transcripts (red boxes) and lead to A to I editing. This mechanism calls RNA editing, which can result in suppression of expression through nuclear retention of edited RNA transcripts.



(F) Epigenetic controls

Epigenetic regulation by retrotransposon activity was mediated through highly methylated at CpG sites of retrotransposon such as LINE-1, Alu and HERVs. (red box) DNA methylation process is important to defend the cell. Because retrotransposons can be methylated and frequently found near genes, which may initiate and spread the

heterochromatin formed at retrotransposons (blue ovals) therefore altering the expression (horizontal arrow) of neighbouring genes (grey boxes).



8. Role of DNA methylation on Interspersed repetitive sequences and human diseases.

One of the heritable epigenetic modifications is DNA methylation that does not only occur in genes but also occur on the interspersed repetitive sequences (IRSs) in human genome (16). Some IRSs element such as LINE-1, Alu and HERVs have been found to be hypomethylated in various condition such as cancers (27) embryogenesis (28, 29), aging (30, 31), congenital malformation (32), exposure to certain environments (33), nutrition (34), and autoimmune diseases (35). Since 1983, a relationship between DNA methylation and cancer was estabilised. It was shown that global hypomethylation of malignancies was cause by the loss of DNA methylation from repetitive regions in the genome. Demethylation of repetitive transposon promoters might also result in irregular gene regulation in cancer by interfere gene transcription or produce antisense transcripts. LINE-1 from oral rinses of oral squamous cell carcinoma patients was observed to be global hypomethylation (107). Moreover, the researcher reported that distinction of DNA hypermethylation and hypomethylation are independent processes and play different role in colorectal tumor progression (108). LINE-1 and Alu hypomethylation were found in well-differentiated neuroendocrine tumors (pancreatic endocrine tumors and carcinoid tumors). These tumors sample were found less methylated of LINE-1 and Alu than normal tissue (109). In addition, DNA repetitive elements, LINE-1 and Alu were significantly more hypomethylated in chronic myeloid leukemia (CML) progression (110). Interestingly, in aging cells have hypomethylation of Alu element and HERV-K, but not LINE-1. Therefore, loss of genome-wide methylation in IRSs is type-specific in some conditions (36). However, LINE-1

hypomethylation was found more often in various conditions, most likely because LINE-1 methylation was regulated by the 5' UTR methylation level (25). Recent year, the regulated gene expressions in cis by LINE-1 at least by two mechanisms were reported. Both mechanisms are dependent on the transcriptional activity of the LINE-1 promoter. The first mechanism is that LINE-1-mediated control of gene expression through the production of unique RNA sequences. Second mechanism is that intragenic LINE-1 RNAs repress host gene expression via the nuclear RNA-induced silencing complex (23). The consequences of LINE-1 and other IRSs hypomethylation that regulate the adjacent cellular gene may contribute to disease pathogenesis. Several papers have reported association between IRSs and autoimmune diseases. LINE-1 hypomethylation was found in $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, and B lymphocytes of SLE patient and found in lymphocyte of synovial fibroblast of rheumatoid arthritis (37, 38). Besides, the association between LINE-1 and autoimmune diseases, the association between HERVs and autoimmune diseases was reported. The HERV-E and HERV-K gag gene expression were increased in PBMC of SLE patients and rheumatoid arthritis patients respectively (38-40). In addition, when treating PBMC with 5-azacytidine causing alteration methylation levels, increase expression of HERV-E gag transcript and HERV-K gag protein was observed, respectively (39, 42). These evidences suggest that HERV-E and HERV-K up-regulation in lymphocyte of SLE patient's is an effect from methylation dynamic losing.

CHAPTER III

MATERIALS AND METHODS

Subjects

Forty SLE subjects were recruited in the study.Twentyfemale patients with SLE were recruited from KingChulalongkorn Memorial Hospital, which were diagnosed as SLE by theAmerican College of Rheumatology (ACR) criteria. All patients with SLE were classifiedaccording tothe severityof the disease by the Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) (Table 3)(56).Inactive disease was defined as SLEDAI-2K score $\leq$ 6 (10 Inactive SLE patients) whereas active disease had to have SLEDAI-2K score > 6 (10 active SLE patients)(57). Thedemographic data of patients with SLE was shown in Table 4.Twenty healthy female volunteers with no history of any autoimmune disease and cancer served as normal controls. Informed consent was obtained from each subject before entering the study. The trial was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

Patient criteria

Inclusion criteria:

- All patients included in the study were diagnosed as SLE with at least 4 of the American College of Rheumatology (ACR) criteria.
- All patients included female only.
- All patients included Thai or Thai Chinese.
- All patients included all severity.
- All patients included all age.

- All patients included in the study were consent by signing the Informed consent.

Exclusion criteria:

- All patients with other autoimmune diseases and malignancies were excluded from the study.

Healthy control criteria

Inclusion criteria:

- All healthy volunteersincluded in the study were no history of autoimmune disease and cancer.
- All healthy volunteers included in the study were no history ofexposure toLupus-induced drugs.
- All healthy volunteers included in the study were no family history of autoimmune disease.
- All healthy volunteers included female only.
- All healthy volunteers included Thai or Thai Chinese.
- All healthy volunteers included all age.
- All healthy volunteers included in the study were consent by signing the Informed consent.

Exclusion criteria:

- All healthy volunteerswith autoimmune disease and malignancies were excluded from the study.
- All healthy volunteerswithhistory ofexposure toLupus-induced drugs such aswere excluded from the study.
- All healthy volunteerswithfamily history of autoimmune diseasewere excluded from the study.

Sample sizes

We calculated a sample sizes (two independent groups) by this formula for the study.

$N/group = 2(Z_{a/2}+Z_b)^2\sigma^2 / (\overline{X}_1-\overline{X}_2)^2$

σ^2= Pooled variance = $\{(n_1-1)S_1^2 + (n_2-1)S_2^2\} / (n_1+n_2-2)$

α= 0.05

β = 0.10

$Z_{\alpha/2}$ = $Z_{0.05/2}$ = 1.96 (two-tail)

Z_β = $Z_{0.10}$ = 1.28

Calculated a sample sizes from preliminary test

SLE neutrophils n_1 = 11

Normal neutrophils n_2 = 5

$\overline{X}_1$= 35.38

$\overline{X}_2$= 31.38

$\sigma^2 = \{(11-1)(1.721)^2 + (5-1)(2.563)^2\} / (11+5-2) = 3.99$

$N/group = 2(1.96+1.28)^2(3.99)/ (35.38-31.38)^2$

= 83.71/16.00 = 5.23

= ~6 samples/group

The calculation of a sample sizesin this study required6samples/group but to increases the reliability of the study. Therefore,we increase the sample size to20 samples /group, which can be divided into twentypatients with SLE and20healthy controls.

Cell isolation

The polymorphonuclear granulocytes (PMN)or Neutrophil were isolated into two steps from heparinized venous blood by gradient centrifugation. First step were to isolate peripheral blood mononuclear cells (PBMCs) by Ficoll-Hypaque (Amersham-Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. In brief, the

collectedheparinized blood was diluted in RPMI by ratio 1:1. The diluted blood was layered on Ficoll-Hypaque. The mononuclear cell fractionobtained from gradient centrifugationwas collected and washed twice with RPMI. PBMCs were counted using a hematocytometer. Then PBMCs were stored at -80 ^{o}C.Second step were isolate PMNfrom blood remaining in the first step by polymorphprep(AXIS-SHIELD-PoC.com, Oslo, Norway) density gradient centrifugation. In brief, the blood remaining in the first step was diluted in RPMI by ratio 1:1. The diluted blood was carefully layered over the polymorphprep. The PMN fraction obtained from gradient centrifugation was collected and washed once with RPMI and then using hypotonic lysisbuffer shatter red blood cells. PMN were counted using a hemacytometer. The purity of PMN was confirm by cytospin method and evaluated by flow cytometry. PMN were stored at -80 ^{o}C until procedure.

Cell lines

Six cell lines of carcinomas and lymphomas were used forinter-assay variation normalizationincluding cervical (HeLa, and CaSki), hepatocellular (HepG2) and leukemia (K-562, Daudi and Jurkat).We used DNA template of Hela, Daudi and Jurkat as a control forinterassay-variation in each experiments of Line-1, Alu and HERV-E. We used DNA template of K562, HepG2 and CaSkias a control forinter-assay variation in each experiments of HERV-K. The methylation levels of each cell line were standardized. Therefore, all experiments were adjusted with the same control methylation levels.All cell lines were cultured in Dulbecco's modified Eagle's medium or RPMI1640 (Gibco BRL, Life Technologies, Pairly, UK), supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St Louis, MO, USA) and incubated at 37^{o}C in 5% CO_2.

DNA extraction

DNA was isolated from PMN collected with heparinized blood tube, using a salting out method (111).In brief, in pellet tube were added with200 µl nuclei lysis buffer (NLB) and

50 µl 10% SDS. Using pipette tip to mix the pellet and vortexing until tiny flakeswere obtained. Then, 10 µl of proteinase K (10 mg/ml in H_2O stored frozen) was added. This solution was incubation at 65 ºC for 2 hours. After that, 175 µl of 5.3 M NaCl was added for precipitation proteins was obtained and then centrifuged at 10,000-12,000 rpm for 15 minutes in micro centrifuge. After centrifugation, precipitated DNA was suspended by add 1 ml cold absolute ethanol. Invert 6-10 times to precipitate DNA. Then, centrifuged at 10,000-12,000 rpm for 10 minutes and discarded supernatant from the pellet. This pellet was suspended in 1 ml of cold 70% ethanol. Then centrifugation 1-2 minutes at 10,000-12,000 rpm and the supernatant was discarded to obtain the pellet. After that, the pellet was dried at 37 ºC with the cap open to evaporate the ethanol. This pellet was dissolved in 200 µl of sterile distilled water, followed by incubation at 65 ºC for 15 minutes and using gentle vortexing to resuspend. If clumps of undissolved DNA are present, it was incubated at 65 ºC until completely resuspended.

DNA preparation and combined bisulfite restriction analysis (COBRA)

Bisulfite conversion of DNA was performed using the EZ DNA methylation Kit™ (Zymo Research, Orange, CA, USA), according to the manufacturer protocol. In brief, 20 µl of 500 ng of DNA was dissolved in 130 µl of the reagent mixture of bisulfite solution and then was performed in thermal cycler under the following conditions: 98 ºC for 10 min, 64 ºC for 2.5 hours and 4 ºC storage up to 20 hours. Then, Zymo-Spin™IC Column was used to isolate the bisulfite-treated DNA. The DNA was eluted with 10 µl of M-Elution Buffer. The bisulfite-treated DNA samples were stored at -20 ºC until procedure.

Combined Bisulfite -Restriction analysis (COBRA),COBRA-LINE-1 and COBRA-Alu

The technique for detection of methylation in LINE-1 and Alu in the study is COBRA-LINE-1 and COBRA-Alu, whichhas been validated previously with pyrosequencing to be an accurate and reliable technique (27, 36). This techniquedesigned for detection

ofmethylation of thousands of CpG loci using one set of conserved primers in each IRS. For COBRA-LINE-1 and COBRA-Alu, bisulfited DNA were amplifiedusing primers sequence as follows respectively:LINE-1 (GenBank: M80343) forward 5'- ccgtaaggggttagg gag tttt -3' and LINE-1 reward5'- (ag)taaaa ccc tcc (ag)aaccaaatataaa -3'. Alu forward 5'- gg(t/c) g(c/t)g gtggtt ta(c/t) gtttgtaa -3' and Alu reward 5'- cac cat attaaccaa act aat ccc ga -3'. The Alu sequence primers were related to the nucleotides of the AluSx subfamily sequence (36). The PCR product of COBRA-LINE-1 was performed under the cycle conditions includingpre-denature at 95 ^{o}C for 15 min and thendenaturingat95 ^{o}C for1 min, and annealing at 50 ^{o}C for 1 min, andextension at 72 ^{o}C for 1 min for 35 cycles and final extension at 72 ^{o}C for 7 min. The PCR product of COBRA-Aluwas performed under the cycle conditions includepre-denature at95 ^{o}C for 15 minand then denature at 95 ^{o}C for 45 sec, and annealing at 63^{o}C for 45 sec, and extension at 72 ^{o}C for 45 sec for 45 cycles and final extension at 72 ^{o}C for 7 min.After amplification, PCR products of LINE-1 (160bp in length) were digested with the restriction enzyme 2 U *TaqI*(Fermentas International Inc., Burlington, Canada) and 2 U *TasI*(Fermentas International Inc., Burlington, Canada). The PCR products of Alu (133bp in length) were digested with the restriction enzyme 2 U *TaqI*(Fermentas International Inc., Burlington, Canada). Eachreactionwill be incubated overnight at 65 ^{o}C and then DNA fragments will be separated in8% polyacrylamide gels. After that, the gel was stained using the SYBR green nucleic-acidstain(Invitrogen). The intensity of DNA fragments was measured with Phosphoimager using ImageQuant software (Molecular Dynamics, GE Healthcare, Slough, UK)COBRA LINE-1amplicons were 160 bp and then after digestion, the LINE-1 methylated bands were 80 bp and LINE-1 unmethylatedbands were 98 and 62 bp. COBRA-Aluamplicons were 133 bp and then after digestion, the Alu methylated bands were 58, 43 and 32 bp and Aluunmethylated bands were 133 bp.Forinter-assay variation normalizationbetween each experiment, we used DNA templates from HeLa, Jurkat and Daudicell line as a control for COBRA-LINE-1 and COBRA-Alu. The methylation levels of

each cell linewere standardized. Therefore, all experiments were adjusted with the same control methylation levels.

LINE-1 and Alumethylation analysis

COBRA, LINE-1 and Aluloci were categorized into four groups based onthe status of methylation at 2 CpGdinucleotides in the 5' and 3' of LINE-1 and Alusequence. Thesefour groups consisted of: (26) twounmethylatedCpGs (uCuC) at LINE-1 and Alu loci; (112)two methylatedCpGs (mCmC) at LINE-1 and Aluloci; (113)5'-methylated and 3'-unmethylatedCpGs (mCuC) at LINE-1 and Aluloci; and (114)5'-unmethylated and 3'-methylated CpGs (uCmC) LINE-1 and Alu loci. We calculatethe LINE-1 and Alu methylation level and percentage of LINE-1 and Aluloci from each group using thepercentage of intensity of COBRA-digested LINE-1 and Aluproducts. Phosphorimager and theImageQuant Software (Molecular Dynamics, GE Healthcare, Slough, UK) were used toquantitate the intensities of the COBRA-LINE-1 and Alu bands. DNA fragments from enzymaticdigestion for COBRA LINE-1 and Aluwere separated into 5 fragments and 6 fragments respectively including 160, 98, 80, 62, 18 bp and 133, 90, 75, 58, 43, 32bprespectively. Which, each bands of LINE-1 were represented different methylation states. ThemCuCrepresented the methylation state of the 160 bp fragments. The uCuCrepresented the methylation state of the 98 bp fragment. The mCmC and uCmCmixturerepresented the methylation states of the 80 bp fragments. The uCuC and uCmCmixture represented the methylation states of the 62 bp fragments. At last, uCmCrepresented the methylation states of the 18bp fragments.In case of Alu, each bands represented different methylation states.TheuCuCrepresented the methylation state of the 133 bp fragments. The mCuCrepresented the methylation state of the 90 bp fragments. The uCmCrepresented the methylation state of the 75 bp fragments. The mCmC and uCmCmixturerepresented the methylation states of the 58bp fragments.The mCmC and mCuCmixturerepresented the methylation states of the 43bp fragments.At last, mCmC represented the methylation state of the 32 bp fragments. The number

ofCpGdinucleotides was calculated from the band intensity and dividing the intensity of each band by the number of double-stranded bp of DNA sequence. LINE-1 as following: A = intensity of 160 bp fragment divided by 160; B = intensity of 98 bp fragmentdivided by 94; C = intensity of 80 bp fragment divided by 79;and D = intensity of 62 bp fragment divided by 62. After calculating the number of CpGdinucleotidesthen substitute in the formula, LINE-1 methylation levels were calculated according to the following formulas: LINE-1methylation level percentage (%mC) = 100×(C+A)/(C+A+A+B+D); percentage of mCuC loci(%mCuC) = 100×(A)/(((C-D+B)/2)+A+D); percentage of uCmC loci (%uCmC) = 100×(D-B)/((CD+B)/2)+A+D; percentage of uCuC loci (%uCuC) = 100×B/(((C-D+B)/2)+A+D); andpercentage of mCmC loci (%mCmC) = 100×((C-D+B)/2)/(((C- D+B)/2)+D+A) (115, 116). The number ofCpGdinucleotides of Aluwas calculated similar to LINE-1. Alu as follows: A = intensityof 133bp fragment divided by 133; B = intensityof 58 bp fragment divided by 58; C = intensity of 75 bp fragment divided by 75; D = intensity of 90 bp fragment divided by 90; E = intensity of 43 bp fragment divided by 43; and F = intensity of 32 bp fragment divided by 32. After calculating the number of CpGdinucleotidesthen substitute in the formula, Alu methylation levels were calculated according to the following formulas: Alu methylation level percentage (%mC) = 100×(E+B)/(2A+E+B+C+D); percentage of mCmC loci (%mCmC) =100×F/(A+C+D+F); percentage of uCmC loci (%uCmC) =100×C/(A+C+D+F); percentage of mCuC loci (%mCuC) =100×D/(A+C+D+F); and percentage of uCuC loci (%uCuC) =100×A/(A+C+D+F) (115, 116).

Combined Bisulfite -Restriction analysis (COBRA), COBRA-HERV-E and COBRA-HERV-K

The technique for detection of methylation in HERV-E and HERV-K used in the study is COBRA-HERV-E and COBRA-HERV-K, which has been validated previously with pyrosequencing to be an accurate and reliable technique (37).This techniqueis similar to COBRA-LINE-1 and ALU but for doing COBRA-HERV-E and COBRA-HERV-K, bisulfitedDNA were amplified by using primers sequence as following respectively: HERV-E

(Genbank: M10976) forward 5'- TTT TGT TAG TTG ATG T(A/G)(G/T) GTA- 3' and HERV-E reverse 5'- CCC CAA AAA AAA AAT TC(C/T) TAA CC-3'.HERV-K(GenBank: M14123)forward 5'-ATA TTA AGG GAA TTT AGA GGT TGG-3', and HERV-K reverse 5'-CCC CTA CAC ACC TAT AAA TAT TTC-3'.The PCR product of COBRA-HERV-E was performed under the cycle conditions include pre-denature at 95°C for 5 min and then denature at 95°C for 1 min, and annealing at 58°C for 1 min, andextension at 72°C for 1 min for 35 cycles and final extension at 72°C for 7 min. The PCR product of COBRA-HERV-K was performed under the cycle conditions are similar to COBRA-HERV-E but, there aredifferent in the annealing step as follows: 1 min at 60ºC of HERV-K primer. After amplification, PCR products of HERV-E (126bpin length) and HERV-K (156 bp in length) were digested with the restriction enzyme 2 U of *Tai*I (Fermentas International Inc., Burlington, Canada).Eachreactionwas incubated overnight at 65°C and then DNA fragments were separated in8%non-denaturing polyacrylamide gels. After that, the gel was stained using the SYBR green nucleic-acidstain(Invitrogen). The intensity of DNA fragments was measured with Phosphoimager, using ImageQuant software (Molecular Dynamics, GE Healthcare, Slough, UK). COBRA-HERV-E amplicons were 126 bp and then after digestion, the HERV-Emethylated bands were 91, 82, 72 and 53bp fragments and HERV-Eunmethylated bands were 126 bp. COBRA-HERV-K amplicons were 156bp and then after digestion, the HERV-K methylated bands were 112 and 44 bp fragments and HERV-K unmethylated bands were 156bp.Forinter-assay variation normalizationbetween each experiment, we used DNA templates from HeLa, Daudi and Jurkatcell line as a control for COBRA-HERV-E andused DNA templates from K562, HepG2 and CaSkicell line as a control for COBRA-HERV-K.The methylation levels of each cell line were standardized. Therefore, all experiments were adjusted with the same control methylation levels (37).

HERV-E and HERV-K methylation analysis

We calculatethe HERV-E and HERV-K methylation level and percentage of HERV-E and HERV-K loci from each amplicons yielded. The HERV-E methylated bands were 91, 82, 72 and 53bp fragments and HERV-E unmethylated bands were 126 bp. COBRA-HERV-K amplicons were 156 bp and then after digestion, the HERV-K methylated bands were 112 and 44 bp fragments and HERV-K unmethylated bands were 156 bp.The methylation level of both was calculated as a percentage as following: (%Methylation = the intensity of the digested methylated fragment and multiplied by one-hundred and divided by the sum of the undigested and digested-amplicons)(37).

$$\%\text{Methylation} = \frac{\text{Intensity of the digested methylated fragment} \times 100}{\text{Intensity of undigested + digested-amplicons}}$$

CU-DREAM X analysis

Connection Up- or Down- Regulation Expression Analysis of Microarrays X (CU-DREAM X) program was used to measure the correlation between gene-containing LINE-1 and differential expression in SLE expression array for identify LINE-1 regulated genes that differently expressed in neutrophils of SLE patients (25). The program was accessiblein web page(http://pioneer.netserv.chula.ac.th/~achatcha/cu-dream/) (25,117).Gene containing LINE-1 data was reported in the L1base (http://l1base.molgen.mpg.de) (118).The extracted microarray data available from Gene Expression Omnibus (GEO) dataset (http://www.ncbi.nlm.nih.gov/geo),a public repository that archives and freely distributes microarray data submitted by the scientific community(119, 120).We performed the correlation between SLE neutrophils expression libraries microarray from GEO dataset GSE27427 (52) with genes containing LINE-1 data. The numbers of genes in each subset were compared with genes containing LINE-1 or not by using a chi-square test (Table 5). The significant *P*-value <0.01 indicated the specific correlation between gene-containing

LINE-1 and differential expression array. Theodds ratios > 1 indicatedthat the intragenic LINE-1 regulated genes in one study were also regulated in another study. Whereas, if odds ratios< 1 indicate that the event of interest (up or down) inhibit the mechanism (s) that altered gene expression in another experiment(117).

Functional analysis

We performed gene functional classification analysis by DAVID Bioinformatics'tool (http://david.abcc.ncifcrf.gov/home.jsp) (121, 122). Gene list was performed with DAVID functional annotation chart to get EASE scores, a modified Fisher Exact P-Value, for each enriched annotation terms and calculate geometric mean of EASE scores of those terms involved in this gene group. Minus log transformation is applied on the geometric mean to emphasize that the geometric mean is a relative score instead of an absolute P value. For example, enrichment scores 0.05 is equivalent to 1.3 in minus log scale. The Ranks of the biological significant of gene groups are based on overall EASE scores (enrichment score) of all enriched annotation terms. Therefore, a higher enrichment score for a group indicates that the group members are involved in more important roles (121).Furthermore, pathway analysis was performed by DAVID Bioinformatics'tool (http://david.abcc.ncifcrf.gov/home.jsp)

Statistical analyses

We applied an independent or dependent sample t-test(sig 2-tailed)for compared the methylation levels between groups. Statistical significance was P value of < 0.05. Pearson's correlation coefficient was used to examine the relationship between two continuous variables. All analyses were performedusing the SPSS software for windows version 15.0 (SPSS Inc., Chicago, IL, USA)

Table 4. Patient clinical data and medications

Patient	Group	Age	Sex	SLEDAI-2K	Medications
1	Inactive	18	F	5	Oral Prednisolone 5 mg/day, Immuran 50 mg/day
2	Active	20	F	10	Oral Prednisolone 5 mg/day, Cellcept 2.0 g/day, Immuran 50 mg/day
3	Inactive	17	F	0	None
4	Active	24	F	16	Oral Prednisolone 10 mg/day, Cellcept 2.0 g/day
5	Inactive	37	F	0	Oral Prednisolone 2.5 mg/day, Cellcept 0.5 g/day
6	Active	39	F	12	Oral Prednisolone 20 mg/day
7	Inactive	38	F	5	Oral Prednisolone 10 mg/day
8	Inactive	29	F	4	Oral Prednisolone 5 mg/day, Cellcept 2.0 g/day
9	Inactive	39	F	4	Oral Prednisolone 2.5 mg/day
10	Inactive	49	F	6	Oral Prednisolone 2.5 mg/day, Immuran 25 mg/day
11	Active	29	F	14	Oral Prednisolone 5 mg/day, Immuran 100 mg/day
12	Active	21	F	14	Oral Prednisolone 40 mg/day
13	Active	28	F	10	Oral Prednisolone 10 mg/day, Immuran 75 mg/day
14	Inactive	25	F	5	Oral Prednisolone 5 mg/day

15	Active	40	F	16	Oral Prednisolone 15 mg/day, Endoxan 500 mg/day, Immuran 50 mg/day
16	Inactive	30	F	4	Oral Prednisolone 2.5 mg/day, Immuran 50 mg/day
17	Active	46	F	10	Oral Prednisolone 20 mg/day
18	Inactive	31	F	2	None
19	Active	19	F	10	Oral Prednisolone 15 mg/day
20	Active	29	F	16	Oral Prednisolone 2.5 mg/day

CHAPTER IV

RESULTS

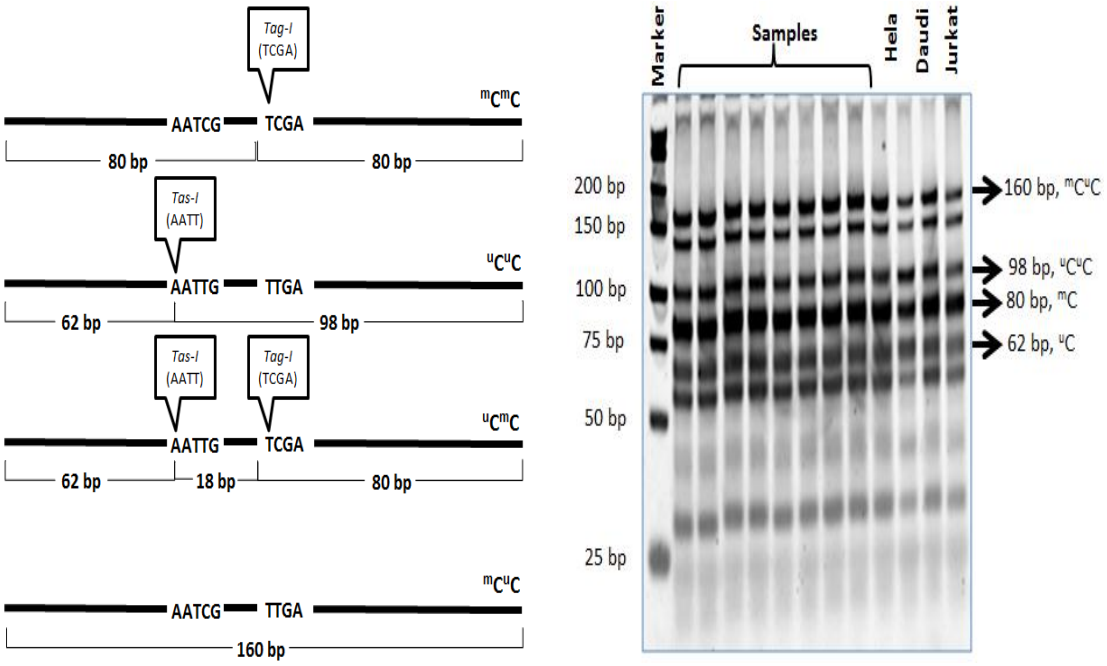
Combined Bisulfite -Restriction analysis (COBRA) for interspersed repetitive elements, COBRA-LINE-1, COBRA-Alu, COBRA-HERV-E and COBRA-HERV-K

We performed COBRA technique for detection of methylation in LINE-1, Alu, HERV-E and HERV-K as shown in Figure 10. The PCR product size of LINE-1, Alu, HERV-E and HERV-K were 160, 133, 126 and 156 bp, respectively. The percentage of the intensity of the digested bands were measured and calculated to methylation levels.

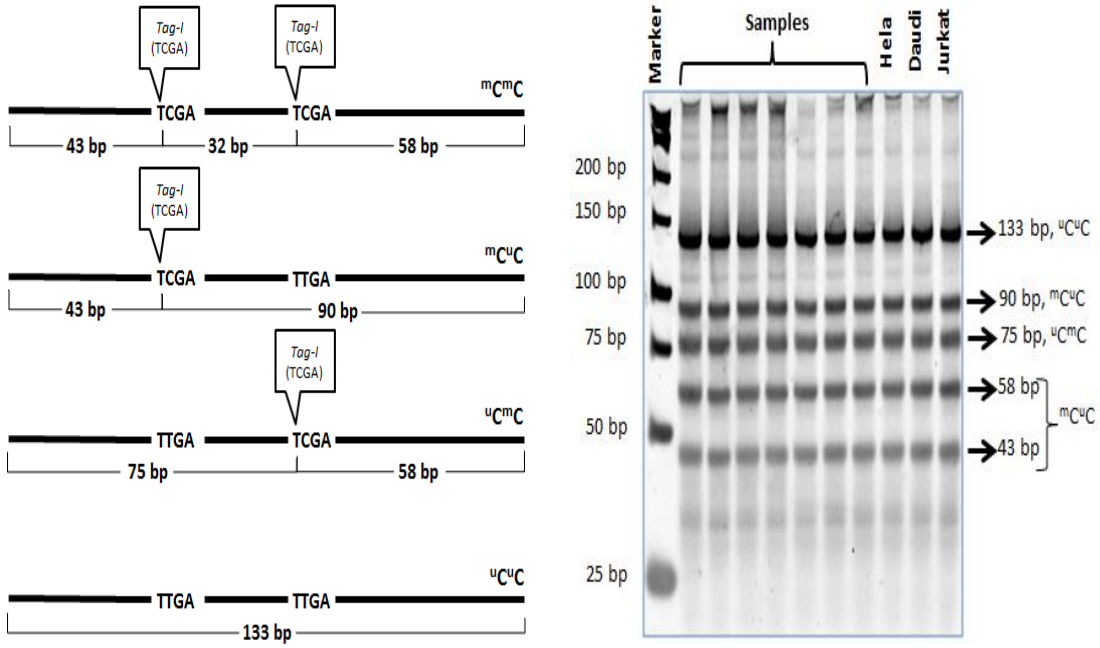
Figure 10: COBRA technique for LINE-1, Alu, HERV-E and HERV-K

(A) LINE-1 (B) Alu (C) HERV-E and (D) HERV-K amplicons sizes are 160, 133, 126 and 156 bp, respectively. The products of Line-1 and Alu were digested by restriction enzymes (TaqI and TasI) while, the products of HERV-E and HERV-K were digested by restriction enzymes (TaiI). The LINE-1 amplicons were 160, 98, 80 and 62 bp. Alu amplicons were 133, 90, 75, 58 and 43 bp. HERV-E amplicons were 126, 91, 82, 73 and 53 bp. And HERV-K amplicons were 156 and 112 bp. The methylation levels were calculated as a percentage of the intensity of the digested methylation fragment divided by the sum of the undigested and digested products. Marker is Low range DNA ladder.

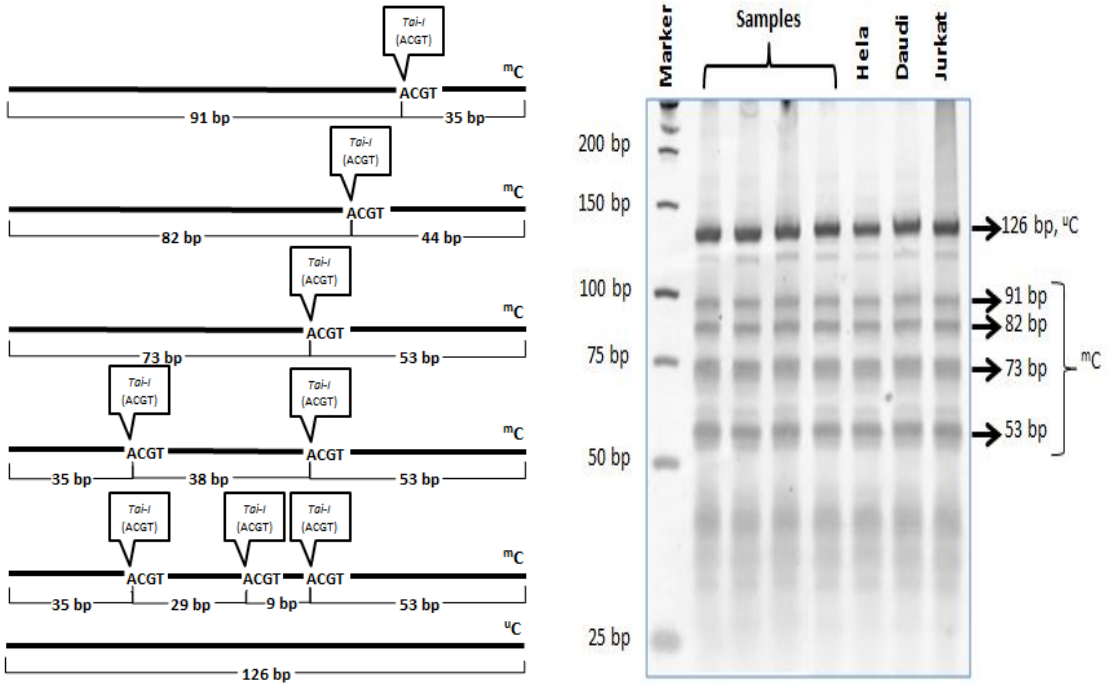
(A) COBRA-Line-1 methylation analysis



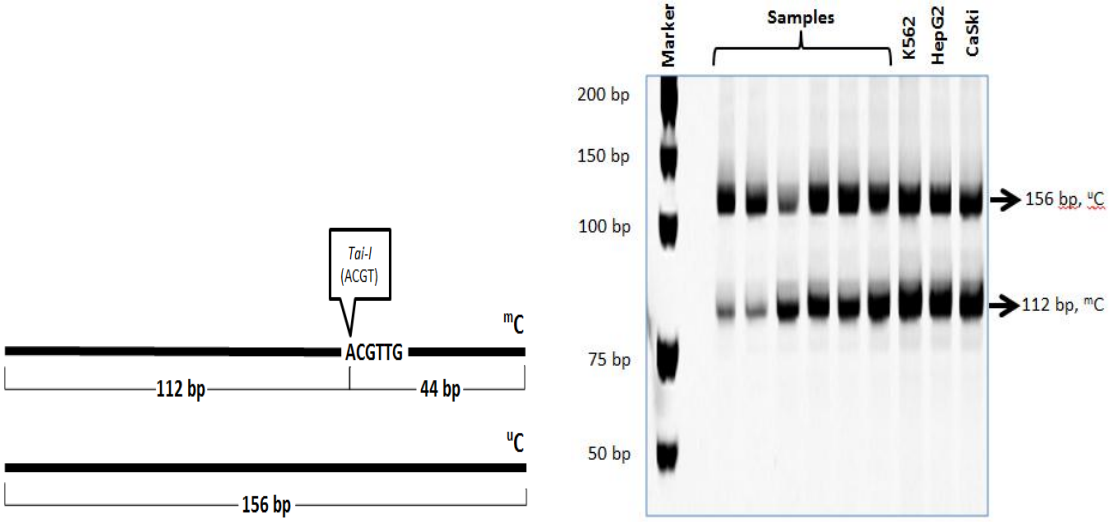
(B) COBRA-Alu methylation analysis



(C) COBRA-HERV-E methylation analysis



(D) COBRA-HERV-K methylation analysis



Differences in DNA methylation levels of LINE-1 between Neutrophil cells from SLE patients versus healthy donors

DNA methylation levels of LINE-1 in neutrophils from patients with SLE and healthy donors were compared. By COBRA-LINE-1 analysis, there were differences in LINE-1 methylation levels and pattern in neutrophils from patients with SLE (n=20) and healthy donors (n=20). We found a significant difference of methylation levels between SLE and healthy donors. The number of precise methylation loci (mC) of LINE-1 of neutrophils from patients with SLE (n=20) was significantly lower than neutrophils from healthy donors (n=20) p-value<0.0001. Percent precise methylation in SLE neutrophils compared to methylation in normal neutrophils are 47.95% and 51.38% respectively. If we look at each pattern, the number of hypermethylated loci (mCmC) of LINE-1 from patients with SLE (n=20) was significantly lower than neutrophils from healthy donors (n=20) p-value<0.0001. Percent hypermethylation in SLE neutrophils compared to methylation in normal neutrophils are 27.49% and 32.01% respectively. Moreover, the number of hypomethylated loci (uCuC) of LINE-1 from patients with SLE (n=20) was significantly higher than neutrophils from healthy donors (n=20) p-value=0.0028. Percent hypomethylation in SLE neutrophils compared to methylation in normal neutrophils are 31.58% and 29.24%, respectively. As for the percent mCuC pattern of LINE-1, the value from patients with SLE (n=20) was significantly higher than value from neutrophils from healthy donors (n=20) p-value<0.0001. Percent mCuC methylation in SLE neutrophils compared to methylation in normal neutrophils are 22.01% and 19.29% respectively. However, there was no significant difference in the percent uCmC and percent partial methylation of LINE-1 between patients with SLE (n=20) and healthy donors (n=20) p-value=0.6368, p-value=0.0520. Percent uCmC and partial methylation in SLE neutrophils compared to methylation in normal neutrophils are 18.91% and 19.44%, 40.92% and 38.74% respectively). (Figure 11) The data were shown in Table 5.

Figure 11: Each pattern of LINE-1 methylation levels in SLE neutrophils compared to normal neutrophils was shown in Mean ± SEM. P-value calculated by unpaired t-test two-tailed analysis.

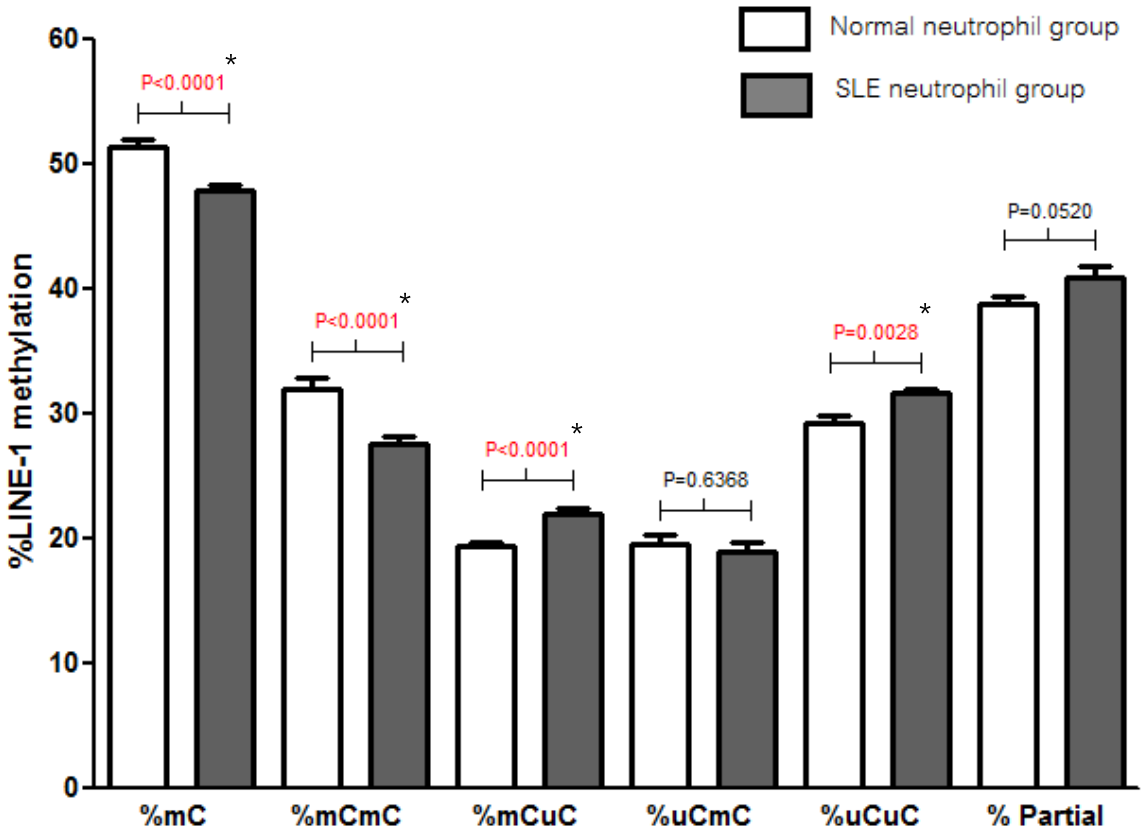


Table 5. Changes in DNA methylation of LINE-1, the data was shown in Mean ± SEM. P-value calculated by unpaired t-test two-tailed analysis.

LINE-1 methylation	Normal Neutrophils (n=20)	SLE Neutrophils (n=20)
^{m}C	51.38 ±2.82	47.95 ±1.41
$^{m}C^{m}C$	32.01 ±3.51	27.49 ±2.93
$^{m}C^{u}C$	19.29 ±1.7	22.01 ±1.65
$^{u}C^{m}C$	19.44 ±3.6	18.91 ±3.48
$^{u}C^{u}C$	29.24 ±2.88	31.58 ±1.55
Partial	38.74 ±3.08	40.92 ±3.74

P-value $<0.0001^{*}$ (mC normal neutrophils compared to SLE neutrophils)

P-value $<0.0001^{*}$ (mCmC normal neutrophils compared to SLE neutrophils)

P-value $<0.0001^{*}$ (mCuC normal neutrophils compared to SLE neutrophils)

P-value = 0.6368 (uCmC normal neutrophils compared to SLE neutrophils)

P-value = 0.0028^{*} (uCuC normal neutrophils compared to SLE neutrophils)

P-value = 0.0520 (Partial methylation normal neutrophils compared to SLE neutrophils)

Differences in DNA methylation levels of Alu between Neutrophil cells from SLE patients versus healthy donors

DNA methylation levels of Alu in neutrophils from patients with SLE and healthy donors were compared. By COBRA-Alu analysis, there were no significant difference in Alu methylation levels and pattern of neutrophils from patients with SLE (n=20) and healthy donors (n=20). The number of precise methylation loci (mC) of Alu of neutrophils from patients with SLE (n=20) was lower than neutrophils from healthy donors (n=20) p-value=0.0567. Percent precise methylation in SLE neutrophils compared to methylation in normal neutrophils are 79.90% and 81.04% respectively. If we look at each pattern, the number of hypermethylated loci (mCmC) of Alu from patients with SLE (n=20) was lower than neutrophils from healthy donors (n=20) p-value=0.0744, Percent hypermethylation in SLE neutrophils compared to methylation in normal neutrophils are 45.88% and 48.70% respectively. If we look at each pattern, the number of hypomethylated loci (uCuC) of Alu from patients with SLE (n=20) was higher than neutrophils from healthy donors (n=20) p-value=0.0567. Percent hypomethylation in SLE neutrophils compared to methylation in normal neutrophils are 20.10% and 18.96% respectively. If we look at each pattern, the percent mCuC pattern of Alu from patients with SLE (n=20) was higher than neutrophils from healthy donors (n=20) p-value=0.4209. Percent mCuC methylation in SLE neutrophils compared to methylation in normal neutrophils are 15.51% and 14.94% respectively. If we look at each pattern, the percent uCmC pattern of Alu from patients with SLE (n=20) was higher than neutrophils from healthy donors (n=20) p-value=0.2433. Percent uCmC methylation in SLE neutrophils compared to methylation in normal neutrophils are 18.50% and 17.40% respectively. In summary, there was no significant difference in Alu methylation levels and pattern but some pattern of Alu methylation levels including mC and uCuC has a trend of hypomethylation in neutrophils of patients with SLE compared to healthy donors. (Figure 12) The data were shown in Table 6.

Figure 12: Each pattern of Alu methylation levels in SLE neutrophils compared to normal neutrophils was shown in Mean ± SEM. P-value calculated by unpaired t-test two-tailed analysis.

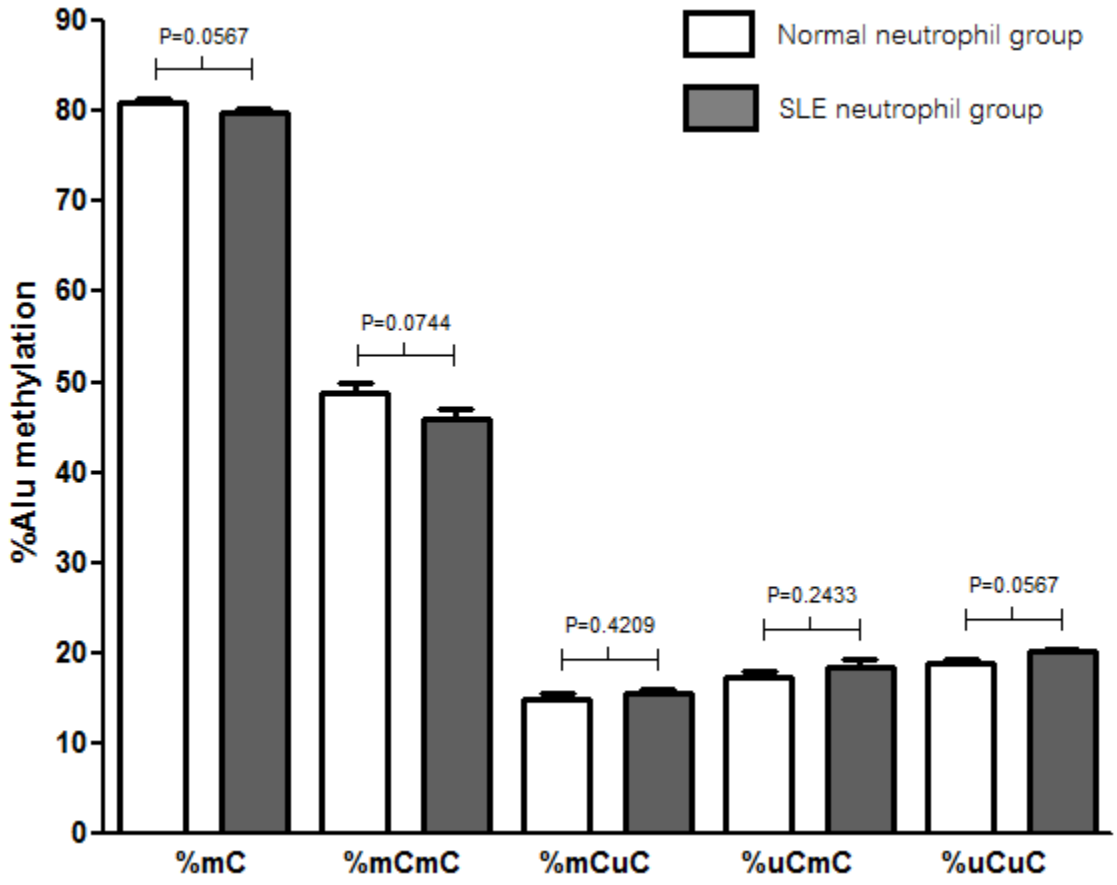


Table 6. Changes in DNA methylation of Alu, the data was shown in Mean ± SEM. P-value calculated by unpaired t-test two-tailed analysis.

Alu methylation	Normal Neutrophils (n=20)	SLE Neutrophils (n=20)
^{m}C	81.04 ±2.04	79.90 ±1.58
$^{m}C^{m}C$	48.70 ±5.00	45.88 ±4.68
$^{m}C^{u}C$	14.94 ±2.44	15.51 ±1.96
$^{u}C^{m}C$	17.40 ±2.37	18.50 ±3.42
$^{u}C^{u}C$	18.96 ±2.04	20.10 ±1.58

P-value = 0.0567 (mC normal neutrophils compared to SLE neutrophils)

P-value = 0.0744 (mCmC normal neutrophils compared to SLE neutrophils)

P-value = 0.4209 (mCuC normal neutrophils compared to SLE neutrophils)

P-value = 0.2433 (uCmC normal neutrophils compared to SLE neutrophils)

P-value = 0.0567 (uCuC normal neutrophils compared to SLE neutrophils)

Differences in DNA methylation levels of HERV-E and HERV-K between Neutrophil cells from SLE patients versus healthy donor

DNA methylation levels of HERV-E and HERV-K in neutrophils from patients with SLE and healthy donors were compared. By COBRA-HERV-E analysis, there were no significant difference in HERV-E and HERV-K methylation levels of neutrophils from patients with SLE (n=20) and healthy donors (n=20). We found that percent methylation of HERV-E of neutrophils from patients with SLE (n=20) was higher than percent methylation in neutrophils from healthy donors (n=20) p-value=0.4834. Percent methylation in SLE neutrophils compared to methylation in normal neutrophils are 72.71% and 72.07% respectively. (Figure 13) The data were shown in Table 7. Meanwhile, we found that percent methylation of HERV-K of neutrophils from patients with SLE (n=20) was lower than neutrophils from healthy donors (n=20) p-value = 0.6993. Percent methylation in SLE neutrophils compared to methylation in normal neutrophils are 52.81% and 53.75% respectively. (Figure 14) The data were shown in Table 8.

Figure 13: DNA Methylation levels in SLE neutrophil compared to normal control of HERV-E was shown in Mean ± SEM. P-value calculated by unpaired t-test two-tailed analysis.

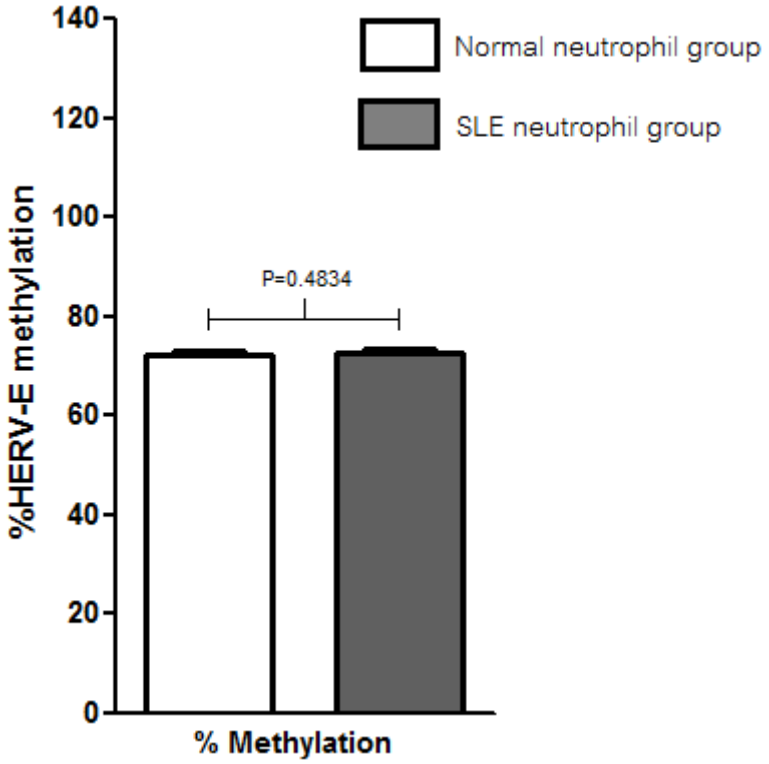


Table 7. Changes in DNA methylation of HERV-E, the data was shown in Mean ± SEM. P-value calculated by unpaired t-test two-tailed analysis.

HERV-E methylation	Normal control Neutrophils (n=20)	SLE Neutrophils (n=20)
% Methylation	72.07 ±3.27	72.71 ±2.36

P-value = 0.4834 (normal neutrophils compared to SLE neutrophils)

Figure 14: DNA Methylation levels in SLE neutrophil compared to normal control of HERV-K was shown in Mean ± SEM. P-value calculated by unpaired t-test two-tailed analysis.

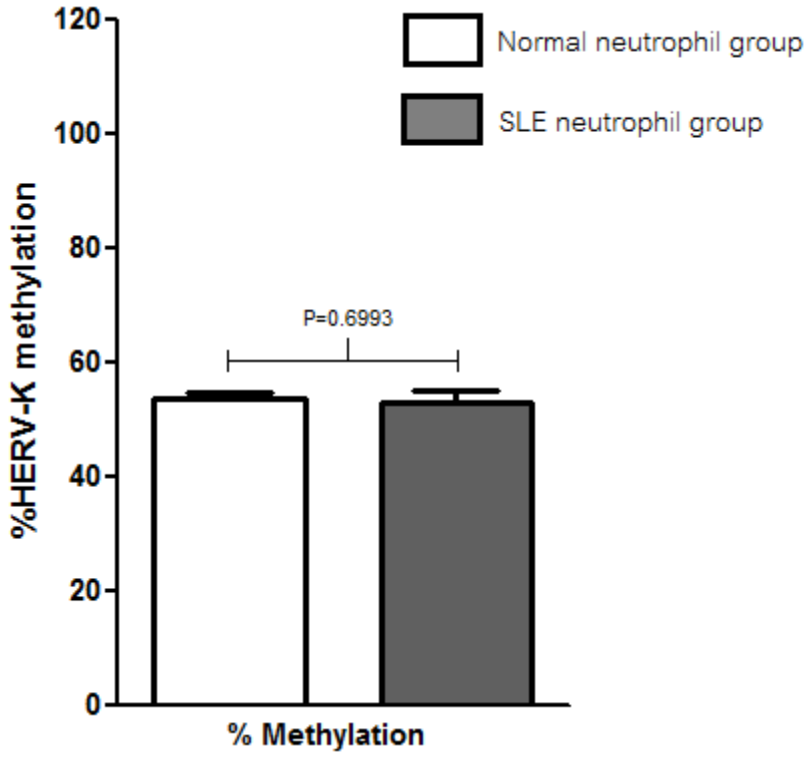


Table 8. Changes in DNA methylation of HERV-K, The data was shown in Mean ± SEM. P-value calculated by unpaired t-test two-tailed analysis.

HERV-K methylation	Normal control Neutrophils (n=20)	SLE Neutrophils (n=20)
% Methylation	53.75 ±4.97	52.81 ±9.56

P-value = 0.6993 (normal neutrophils compared to SLE neutrophils)

Association of LINE-1 methylation with age and some clinical data in SLE

We analyzed the association between each pattern of LINE-1 methylation patterns and levels with some clinical data of patients with SLE including age, SLEDAI score and neutrophils count. The data were shown in Table 9. We found no significant correlation between each patterns of LINE-1 methylation levels with age (Figure 15), SLEDAI score. (Figure 16), and neutrophils count (Figure 17).

DNA methylation levels of LINE-1 in neutrophils from patient with inactive and active SLE and healthy group were compared. The SLE patients were classified according to severity of the disease, which divided into active and inactive groups based on their SLEDAI-2K score. We analyzed the association of LINE-1 methylation pattern and disease activity (10 inactive SLE vs. 10 active SLE and 20 healthy donors). Interestingly, the % mC and % mCmC pattern of LINE-1 were significant lower in both groups compared to healthy control (mC: p- value=0.0005 and p-value=0.0029, respectively) (mCmC: p- value=0.0008 and p-value=0.0034, respectively) (% methylation of mC pattern in inactive and active SLE vs. healthy control = 47.63 vs. 51.38 and 48.27 vs. 51.38, respectively) (% methylation of mCmC pattern in inactive and active SLE vs. healthy control = 27.02 vs. 32.01 and 27.96 vs. 32.01, respectively). Moreover, the % mCuC and % uCuC pattern of LINE-1 were significant higher in both groups compared to healthy control (mCuC: p- value$<$0.0001 and p-value=0.0012, respectively) (uCuC: p- value=0.0134 and p-value=0.0412, respectively) (% methylation of mCuC pattern in inactive and active SLE vs. healthy control = 22.19 vs. 19.29 and 21.82 vs. 19.29, respectively) (% methylation of uCuC pattern in inactive and active SLE vs. healthy control = 31.74 vs. 29.24 and 31.42 vs. 29.24, respectively). However, there were no significant difference in the % uCmC pattern and % partial methylation of LINE-1 between in both groups and healthy control (uCmC: p- value=0.7711 and p-value=0.6419, respectively) (partial: p- value=0.0686 and p-value=0.1552, respectively) (% methylation of uCmC pattern in inactive and active SLE vs. healthy control = 19.04 vs. 19.44 and 18.77 vs. 19.44, respectively) (% methylation of partial pattern in inactive and active SLE vs. healthy

control = 41.23 vs. 38.74 and 40.6 vs. 38.74, respectively). In summary, all pattern of LINE-1 methylation level has no significant differences between inactive compared to active SLE. (Figure 18) The data were shown in Table 10.

Table 9. Correlation between each pattern of LINE-1 methylation levels with age and some clinical data.

LINE-1 methylation	Data					
	Age		SLEDAI-2K		% Neutrophils	
	SLE neutrophils		SLE neutrophils		SLE neutrophils	
	r	P-value	r	P-value	r	P-value
^{m}C	0.067	0.270	0.019	0.561	0.003	0.817
$^{m}C^{m}C$	0.121	0.133	0.030	0.468	0.009	0.685
$^{m}C^{u}C$	0.000	0.956	0.026	0.493	0.001	0.874
$^{u}C^{m}C$	0.145	0.097	0.010	0.673	0.051	0.340
$^{u}C^{u}C$	0.034	0.435	0.005	0.758	0.080	0.227
Partial	0.122	0.132	0.027	0.486	0.037	0.415

* indicate significant differences by Pearson's correlation (r) coefficient test.

Numbers in the parentheses indicate mean ± SEM.

Figure 15. Correlation between each pattern of LINE-1 methylation levels with age in neutrophils of SLE patients was show. (A) mC, (B) mCmC, (C) mCuC, (D) uCmC, (E) uCuC and (F) partial pattern. Each dot represents an individual patient. P-value calculated by Pearson's correlation coefficient test.

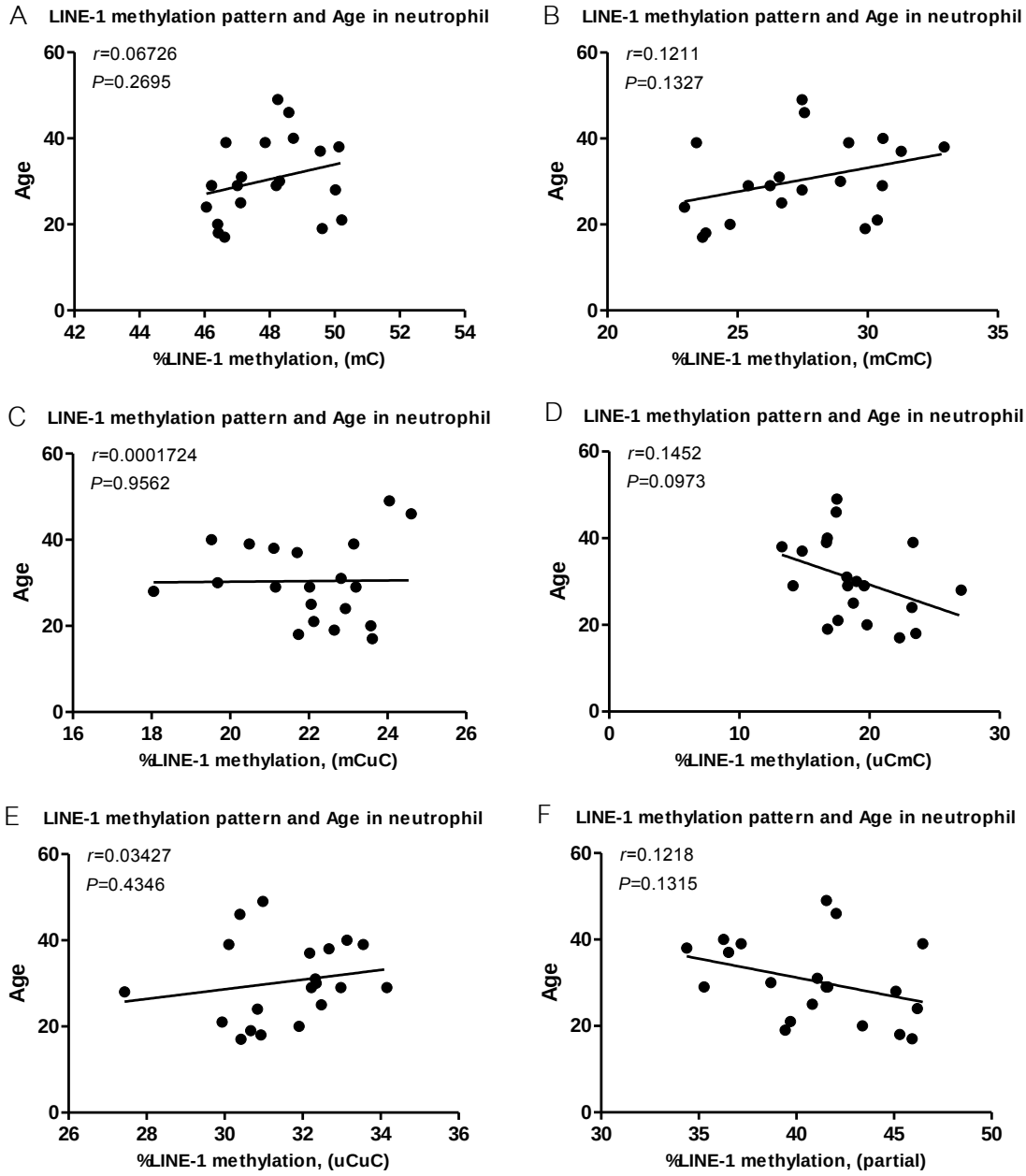


Figure 16. Correlation between each pattern of LINE-1 methylation levels with SLEDAI score in neutrophils of SLE patients was show. (A) mC, (B) mCmC, (C) mCuC, (D) uCmC, (E) uCuC and (F) partial pattern. Each dot represents an individual patient. P-value calculated by Pearson's correlation coefficient test.

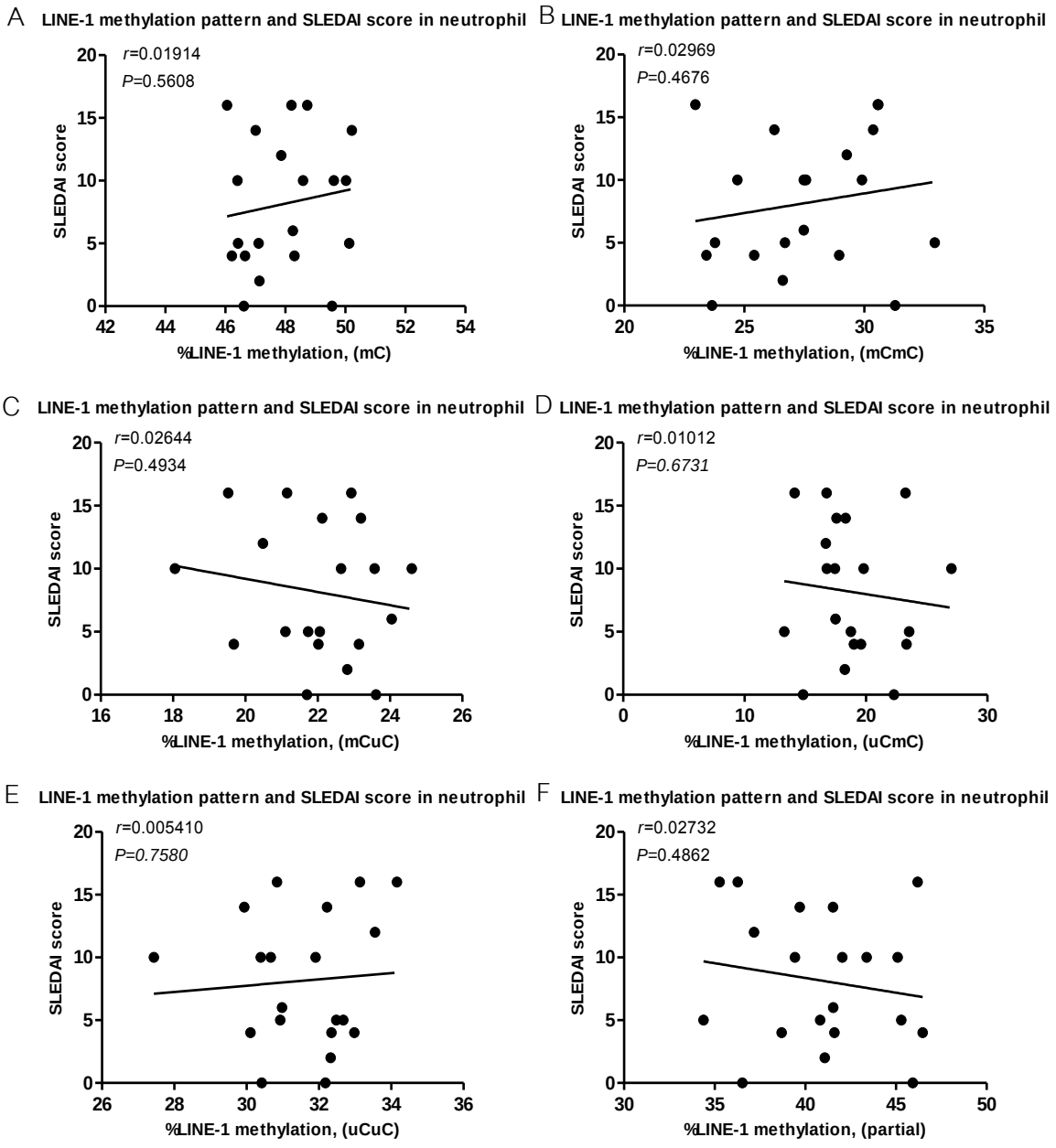


Figure 17. Correlation between each pattern of LINE-1 methylation levels with %neutrophils in SLE patients was show. (A) mC, (B) mCmC, (C) mCuC, (D) uCmC, (E) uCuC and (F) partial pattern. Each dot represents an individual patient. P-value calculated by Pearson's correlation coefficient test.

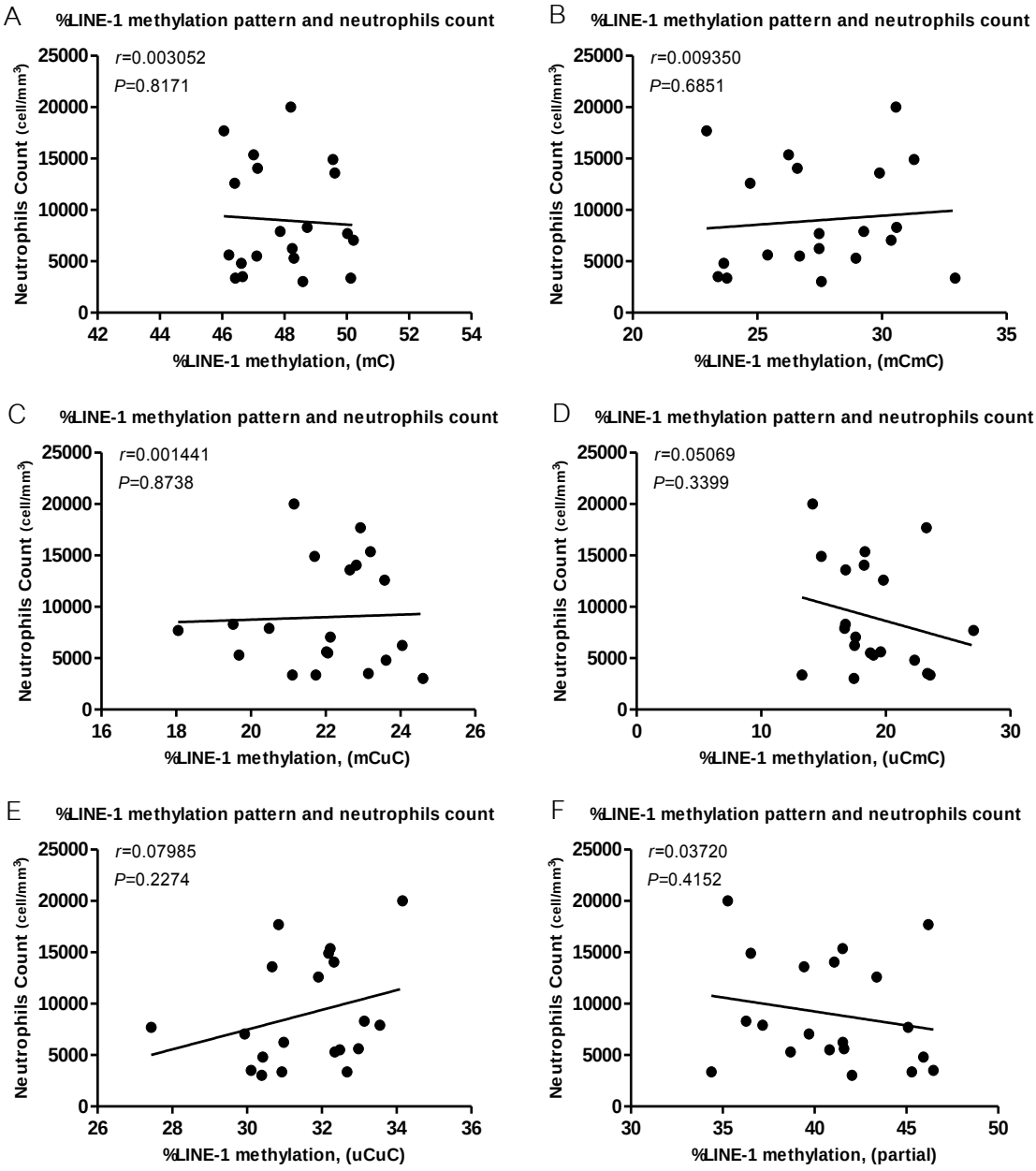


Figure18. Each pattern of LINE-1 methylation levels in different SLE disease activity was shown in Mean ± SEM. P-value calculated by unpaired t-test two-tailed analysis.

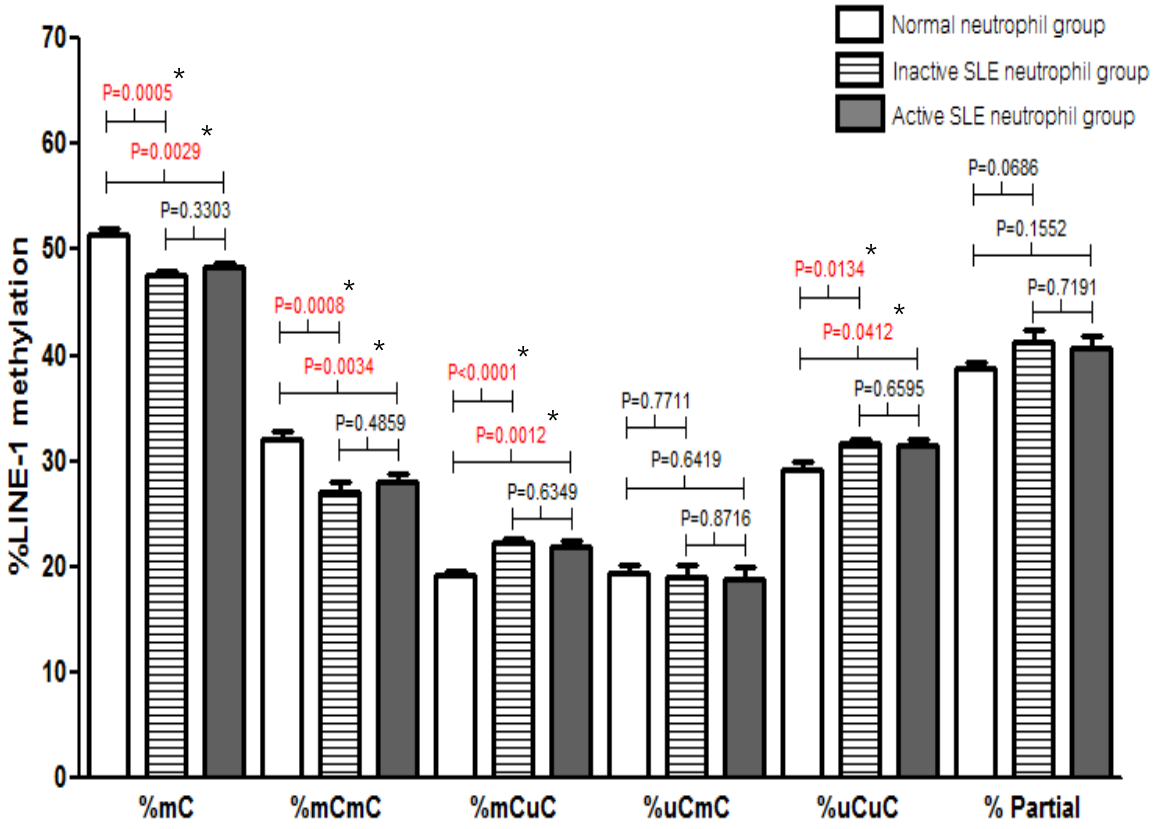


Table 10: Alteration of LINE-1 methylation in disease activity (SLE patients were divided into active and inactive groups based on their SLEDAI-2K score)

LINE-1 methylation	Normal control Neutrophils (n=20)	Inactive SLE Neutrophils (n=10)	Active SLE Neutrophils (n=10)
^{m}C	51.38 ±2.82	47.63 ±1.36	48.27 ±1.45
$^{m}C^{m}C$	32.01 ±3.51	27.02 ±3.24	27.96 ±2.66
$^{m}C^{u}C$	19.29 ±1.7	22.19 ±1.27	21.82 ±2.01
$^{u}C^{m}C$	19.44 ±3.6	19.04 ±3.39	18.77 ±3.75
$^{u}C^{u}C$	29.24 ±2.88	31.74 ±1.02	31.42 ±1.99
Partial	38.74 ±3.08	41.23 ±3.96	40.6 ±3.69

*P-value = 0.0005 (mC, normal control compared to inactive SLE)

*P-value = 0.0029 (mC, normal control compared to active SLE)

*P-value = 0.0008 (mCmC, normal control compared to inactive SLE)

*P-value = 0.0034 (mCmC, normal control compared to active SLE)

*P-value <0.0001 (mCuC, normal control compared to inactive SLE)

*P-value = 0.0012 (mCuC, normal control compared to active SLE)

P-value = 0.7711 (uCmC, normal control compared to inactive SLE)

P-value = 0.6419 (uCmC, normal control compared to active SLE)

*P-value = 0.0134 (uCuC, normal control compared to inactive SLE)

*P-value = 0.0412 (uCuC, normal control compared to active SLE)

P-value = 0.0686 (Partial, normal control compared to inactive SLE)

P-value = 0.1552 (Partial, normal control compared to inactive SLE)

P-value = 0.3303 (mC, inactive SLE compared to active SLE)

P-value = 0.4859 (mCmC, inactive SLE compared to active SLE)

P-value = 0.6349 (mCuC, inactive SLE compared to active SLE)

P-value = 0.8716 (uCmC, inactive SLE compared to active SLE)

P-value = 0.6595 (uCuC, inactive SLE compared to active SLE)

P-value = 0.7191 (Partial, inactive SLE compared to active SLE)

Association of Alu methylation with age and some clinical data in SLE

We analyzed the association between each pattern of Alu methylation patterns and levels with some clinical data of patients with SLE including age, SLEDAI score and neutrophils count. The data were shown in Table 11. Surprisingly, mCmC and uCmC of Alu methylation levels were significant positive and negative correlation with age respectively. (P-value = 0.0149, r = 0.2872 and P-value = 0.0111, r = 0.3070, respectively). (Figure 19) We found no significant correlations between each patterns of Alu methylation levels with SLEDAI score (Figure 20) and neutrophils count. (Figure 21)

DNA methylation levels of Alu in neutrophils from patient with inactive and active SLE and healthy group were compared. There were no significant difference in Alu methylation pattern and disease activity of neutrophils (10 inactive SLE vs. 10 active SLE and 20 healthy donors). The precise %methylation (mC) and % mCmC pattern of Alu were no significant lower in both groups compared to healthy control (mC: p- value=0.0773 and p-value=0.2300, respectively) (mCmC: p- value=0.2427 and p-value=0.0923, respectively) (% methylation of mC pattern in inactive and active SLE vs. healthy control = 79.66 vs. 81.04and 80.14 vs. 81.04, respectively) (% methylation of mCmC pattern in inactive and active SLE vs. healthy control = 46.45 vs. 48.70 and 45.32 vs. 48.70, respectively). Moreover, the % mCuC, % uCmC and % uCuC pattern of Alu were no significant higher in both groups compared to healthy control (mCuC: p- value=0.5845 and p-value=0.4807, respectively) (uCmC: p- value=0.7227 and p-value=0.0926, respectively) (uCuC: p-value=0.0773 and p-value=0.2300, respectively) (% methylation of mCuC pattern in inactive and active SLE vs. healthy control = 15.42 vs. 14.94 and 15.60 vs. 14.94, respectively) (% methylation of uCmC pattern in inactive and active SLE vs. healthy control = 17.79 vs. 17.40 and 19.22 vs. 17.40, respectively) (% methylation of uCuC pattern in inactive and active SLE vs. healthy control = 20.34 vs. 18.96 and 19.86 vs. 18.96, respectively). In summary, all pattern of Alu methylation level has no significant difference in inactive SLE compared to active SLE. (Figure 22) The data were shown in Table 12.

Table 11. Correlation between each pattern of Alu methylation levels with age and some clinical data.

Alu methylation	Data					
	Age		SLEDAI-2K		% Neutrophils	
	SLE neutrophils		SLE neutrophils		SLE neutrophils	
	r	P-value	r	P-value	r	P-value
^{m}C	0.007	0.727	0.045	0.371	0.020	0.557
$^{m}C^{m}C$	0.287	0.015*	0.002	0.853	0.140	0.104
$^{m}C^{u}C$	0.142	0.102	0.002	0.860	0.004	0.801
$^{u}C^{m}C$	0.308	0.011*	0.033	0.441	0.170	0.071
$^{u}C^{u}C$	0.007	0.727	0.045	0.371	0.020	0.557

* indicate significant differences by Pearson's correlation (r) coefficient test.

Numbers in the parentheses indicate mean ± SEM.

Figure 19. Correlation between each pattern of Alu methylation levels with age in neutrophils of SLE patients was show. (A) mC, (B) mCmC, (C) mCuC, (D) uCmC and (E) uCuC. Each dot represents an individual patient. P-value calculated by Pearson's correlation coefficient test.

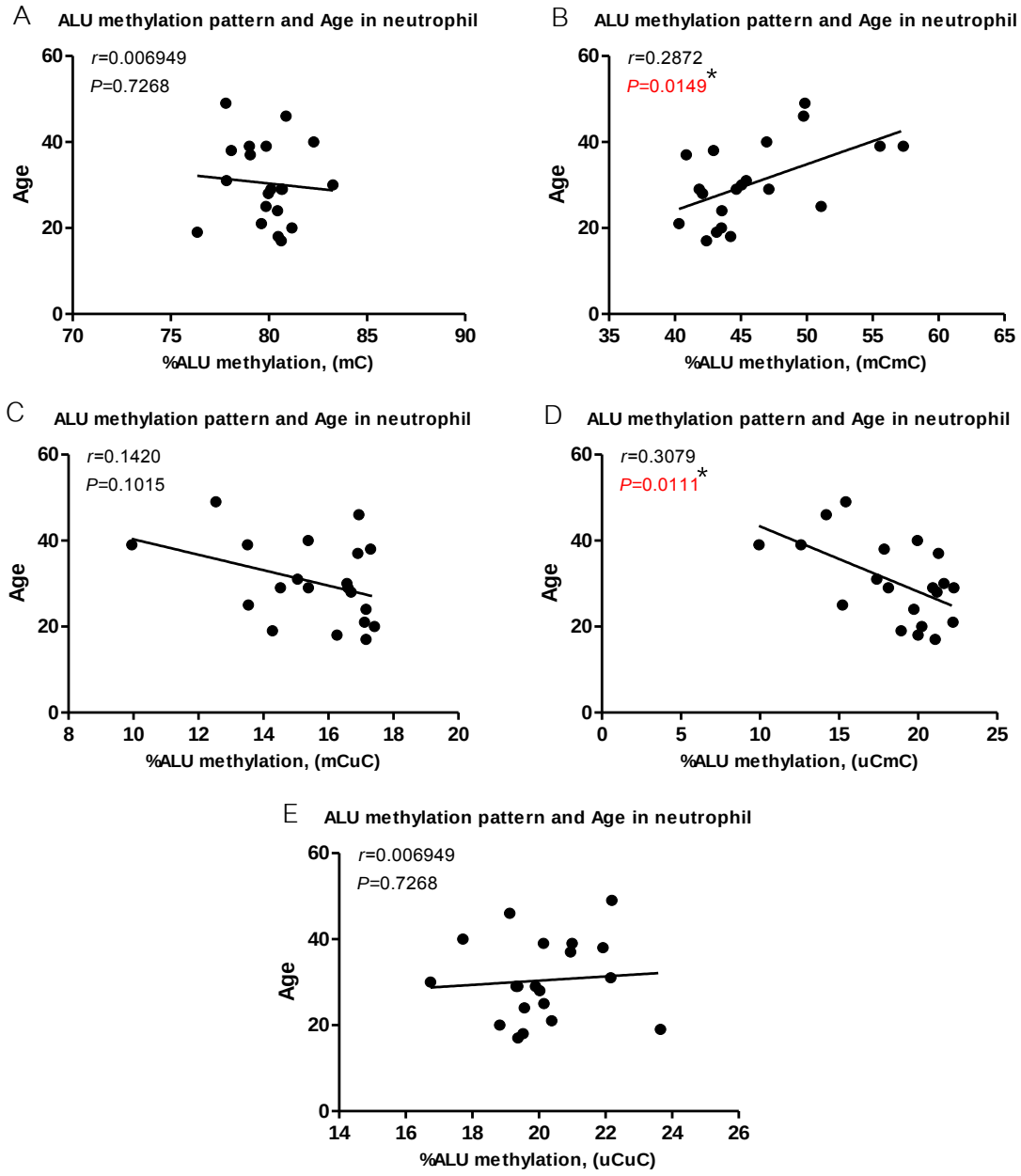


Figure 20. Correlation between each pattern of Alu methylation levels with SLEDAI score in neutrophils of SLE patients was show. (A) mC, (B) mCmC, (C) mCuC, (D) uCmC and (E) uCuC. Each dot represents an individual patient. P-value calculated by Pearson's correlation coefficient test.

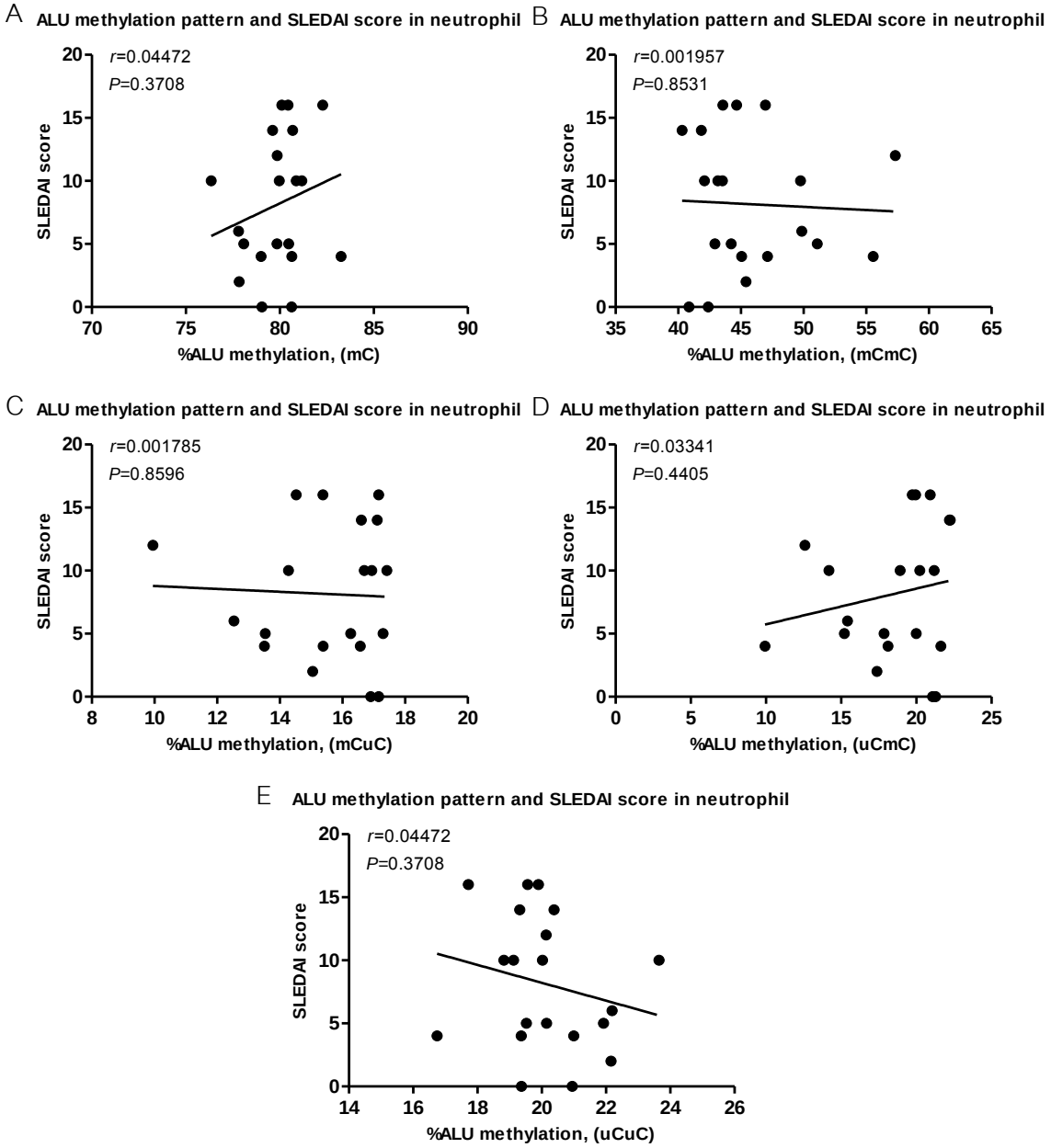


Figure 21. Correlation between each pattern of Alu methylation levels with %neutrophils in SLE patients was show. (A) mC, (B) mCmC, (C) mCuC, (D) uCmC and (E) uCuC. Each dot represents an individual patient. P-value calculated by Pearson's correlation coefficient test.

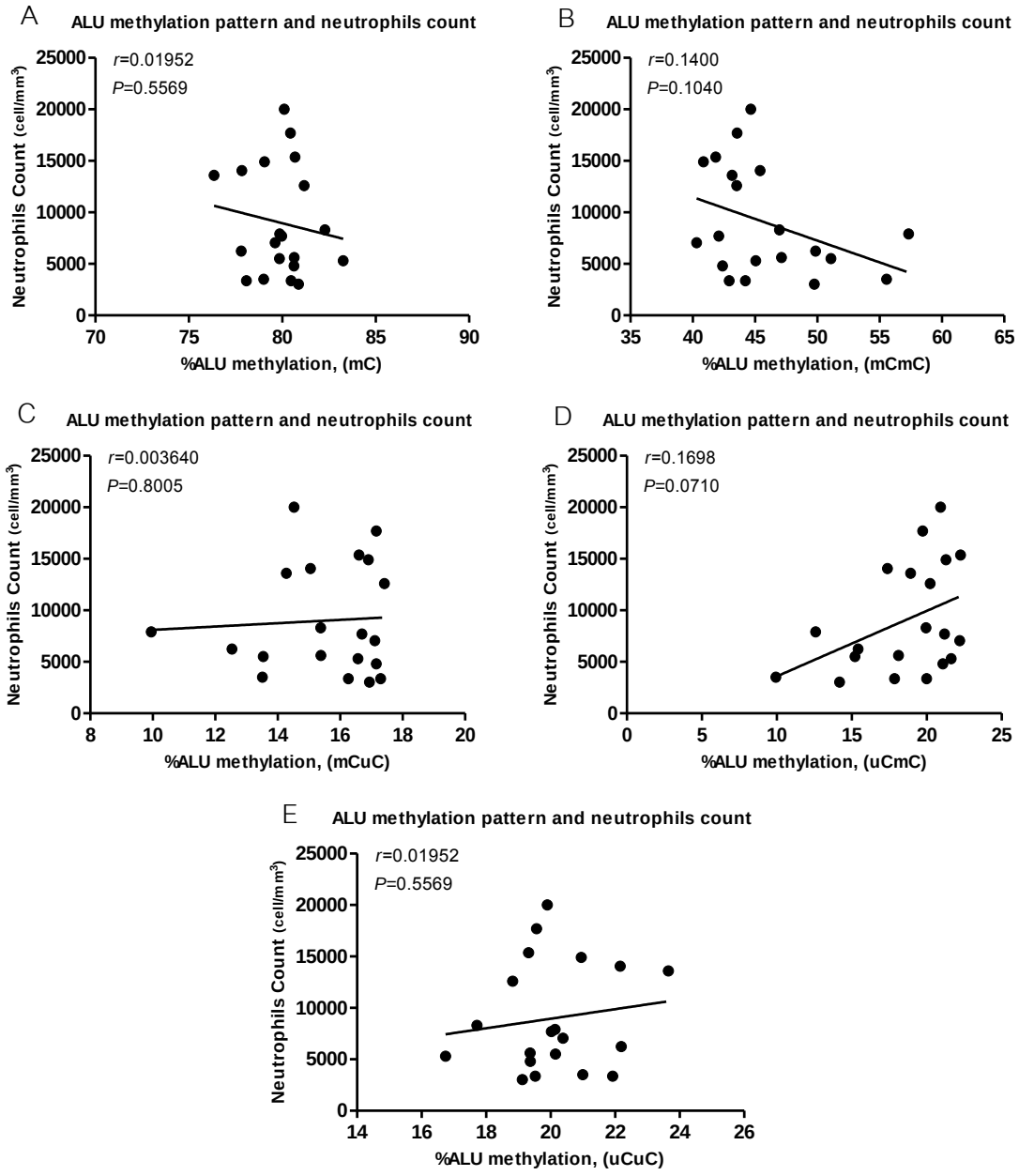


Figure22. Each pattern of Alu methylation levels in different SLE disease activity was shown in Mean ± SEM. P-value calculated by unpaired t-test two-tailed analysis.

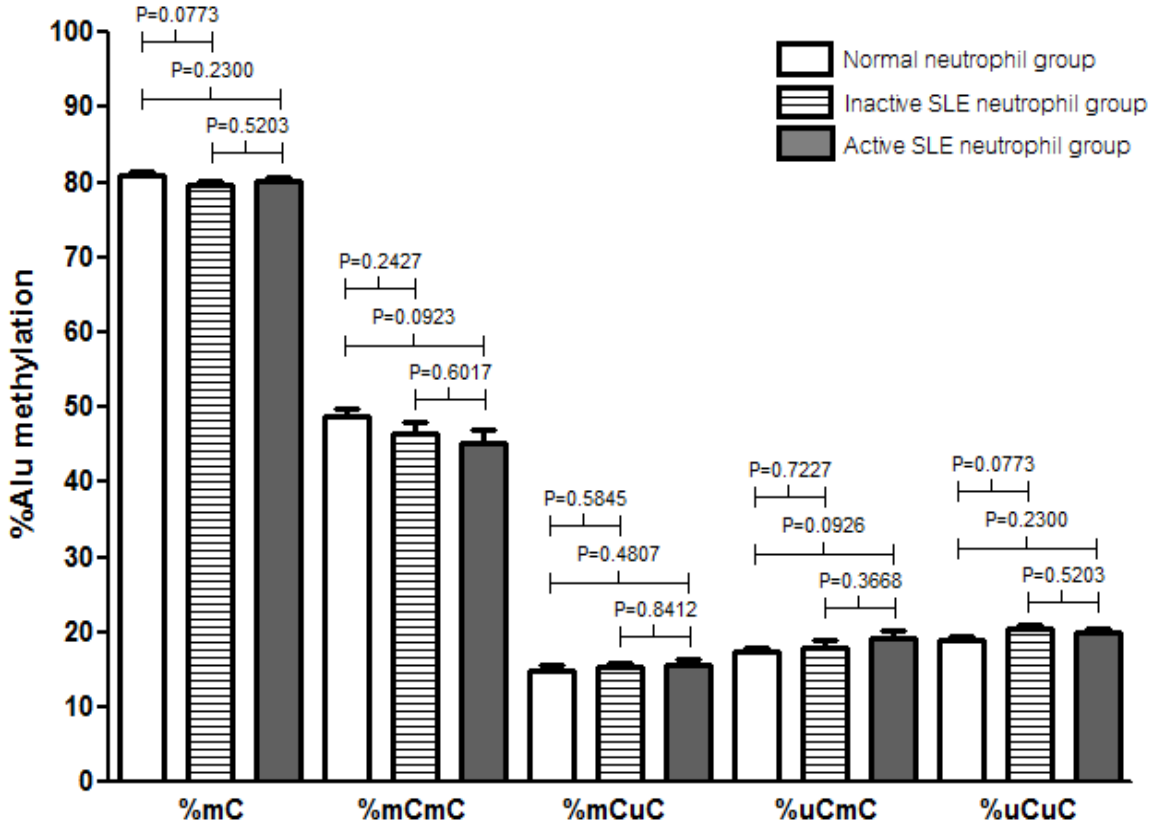


Table 12: Alteration of Alu methylation in disease activity (SLE patients were divided into active and inactive groups based on their SLEDAI-2K score)

Alu methylation	Normal control Neutrophils (n=20)	Inactive SLE Neutrophils (n=10)	Active SLE Neutrophils (n=10)
^{m}C	81.04 ±2.04	79.66 ±1.68	80.14 ±1.53
$^{m}C^{m}C$	48.70 ±5.00	46.45 ±4.53	45.32 ±5.00
$^{m}C^{u}C$	14.94 ±2.44	15.42 ±1.71	15.60 ±2.28
$^{u}C^{m}C$	17.40 ±2.37	17.79 ±3.60	19.22 ±3.26
$^{u}C^{u}C$	18.96 ±2.04	20.34 ±1.68	19.86 ±1.53

P-value = 0.0773 (mC, normal control compared to inactive SLE)
P-value = 0.2300 (mC, normal control compared to active SLE)
P-value = 0.2427 (mCmC, normal control compared to inactive SLE)
P-value = 0.0923 (mCmC, normal control compared to active SLE)
P-value = 0.5845 (mCuC, normal control compared to inactive SLE)
P-value = 0.4807 (mCuC, normal control compared to active SLE)
P-value = 0.7227 (uCmC, normal control compared to inactive SLE)
P-value = 0.0926 (uCmC, normal control compared to active SLE)
P-value = 0.0773 (uCuC, normal control compared to inactive SLE)
P-value = 0.2300 (uCuC, normal control compared to active SLE)
P-value = 0.5203 (mC, inactive SLE compared to active SLE)
P-value = 0.6017 (mCmC, inactive SLE compared to active SLE)
P-value = 0.8412 (mCuC, inactive SLE compared to active SLE)
P-value = 0.3668 (uCmC, inactive SLE compared to active SLE)
P-value = 0.5203 (uCuC, inactive SLE compared to active SLE)

Association of HERV-E and HERV-K methylation with age and some clinical data in SLE

We analyzed the association between HERV-E and HERV-K methylation levels with some clinical data from patients with SLE including age, SLEDAI score and neutrophils count. The data were shown in Table 13 (Figure 23) and Table 14 (Figure 24) respectively. We found no significant correlation between HERV-E and HERV-K methylation levels with age, SLEDAI score, and neutrophils count.

We analyzed the association of HERV-E and HERV-K methylation and severity of diseases. There were no significant difference in HERV-E and HERV-K methylation and disease activity of neutrophils from 10 inactive SLE and 10 active SLE and 20 healthy donors. We found that percent methylation of HERV-E and HERV-K in neutrophils from both group was higher than healthy donors (HERV-E: p-value=0.5651and p-value=0.6150, respectively) (% methylation of HERV-E in inactive and active SLE neutrophils vs. normal neutrophils are 72.74 vs. 72.07 and 72.67 vs. 72.07, respectively) (HERV-K: p-value= 0.2943 and p-value= 0.1410, respectively) (% methylation of HERV-K in inactive and active SLE neutrophils vs. normal neutrophils are 56.17 vs. 53.75 and 49.46 vs. 53.75, respectively). In summary, HERV-E and HERV-K methylation level has not significantly different in inactive SLE compared to active SLE (HERV-E: p- value=0.9507) (% methylation of HERV-E in inactive SLE vs. active SLE are 72.74 vs. 72.67). (Figure 25) The data were shown in Table 15. (HERV-K: p- value= 0.1190) (% methylation of HERV-K in inactive SLE vs. active SLE are 56.17 vs. 49.46). (Figure 26) The data were shown in Table 16.

Table 13. Correlation between HERV-E methylation levels with age and some clinical data.

HERV-E methylation	Data					
	Age		SLEDAI-2K		% Neutrophils	
	SLE neutrophils		SLE neutrophils		SLE neutrophils	
	r	P-value	r	P-value	r	P-value
% Methylation	0.062	0.292	0.008	0.709	0.035	0.430

* indicate significant differences by Pearson's correlation (r) coefficient test.

Numbers in the parentheses indicate mean ± SEM.

Figure 23. Correlation between HERV-E methylation levels with age (A), SLEDAI score (B) and %neutrophils (C) in neutrophils of SLE patients was show. Each dot represents an individual patient. P-value calculated by Pearson's correlation coefficient test.

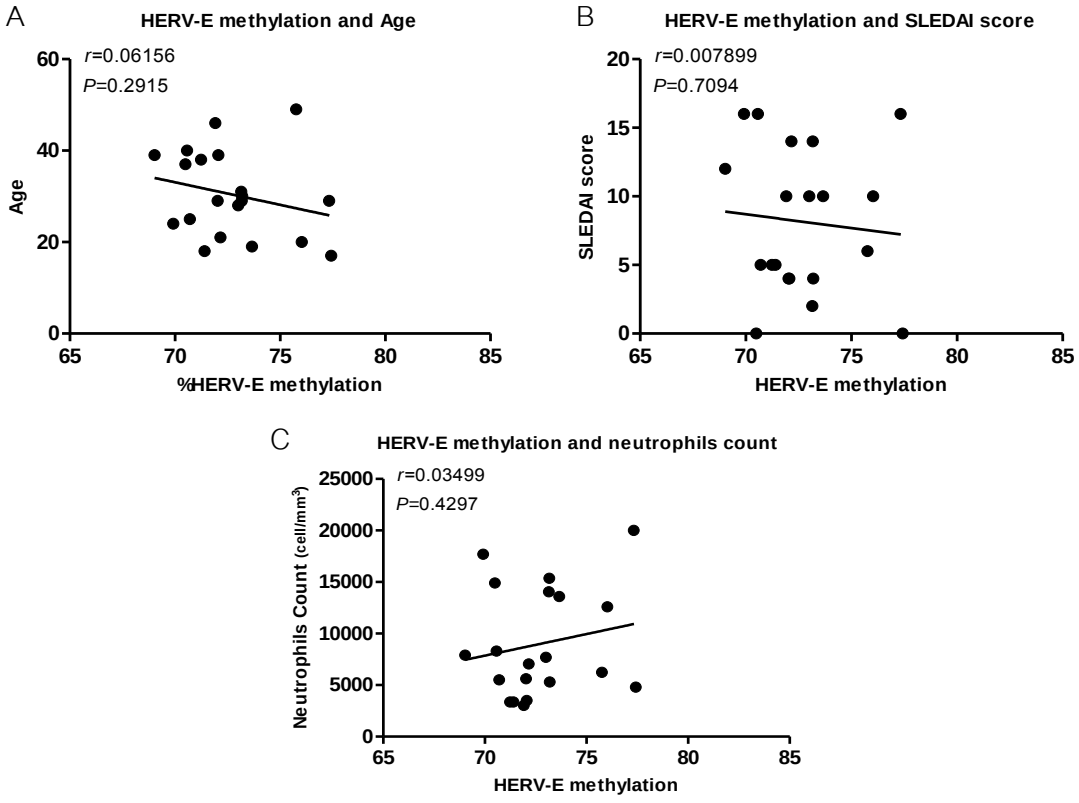


Table 14. Correlation between HERV-K methylation levels with age and some clinical data

HERV-K methylation	Data					
	Age		SLEDAI-2K		% Neutrophils	
	SLE neutrophils		SLE neutrophils		SLE neutrophils	
	r	P-value	r	P-value	r	P-value
% Methylation	0.051	0.339	0.133	0.114	0.094	0.188

* indicate significant differences by Pearson's correlation (r) coefficient test.

Numbers in the parentheses indicate mean ± SEM.

Figure 24. Correlation between HERV-K methylation levels with age (A), SLEDAI score (B) and %neutrophils (C) in neutrophils of SLE patients was show. Each dot represents an individual patient. P-value calculated by Pearson's correlation coefficient test.

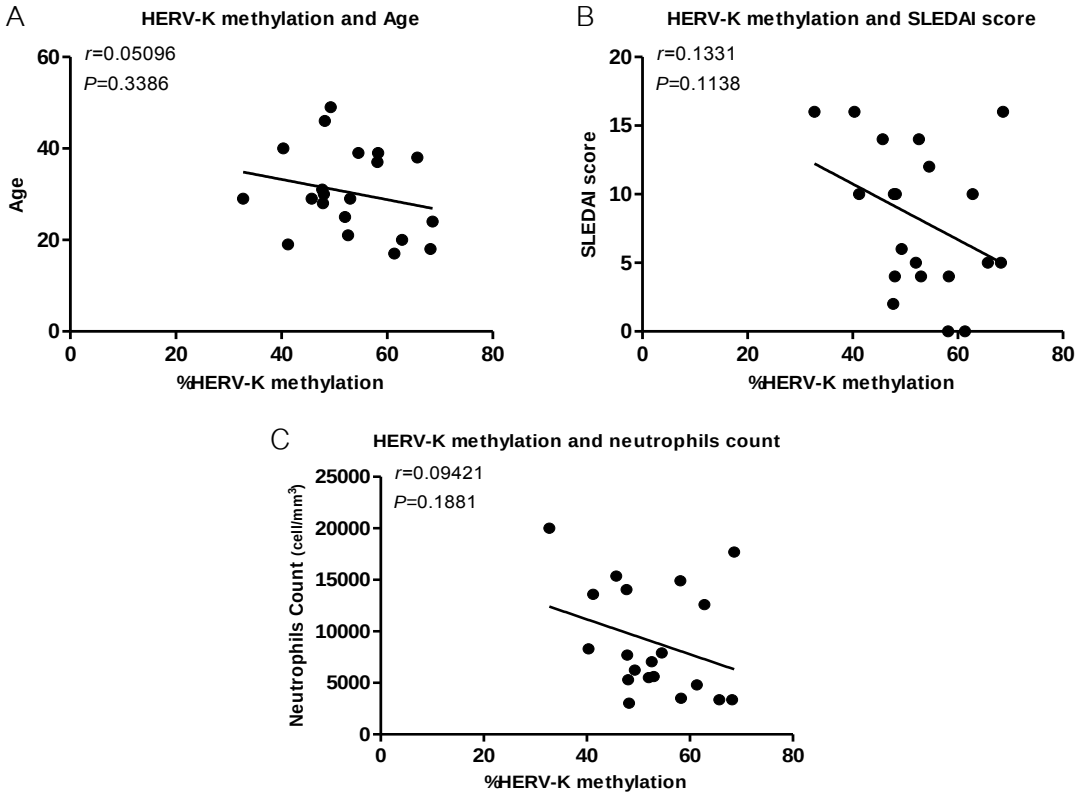


Figure 25. HERV-E methylation levels in different SLE disease activity was shown in Mean ± SEM. P-value calculated by unpaired t-test two-tailed analysis.

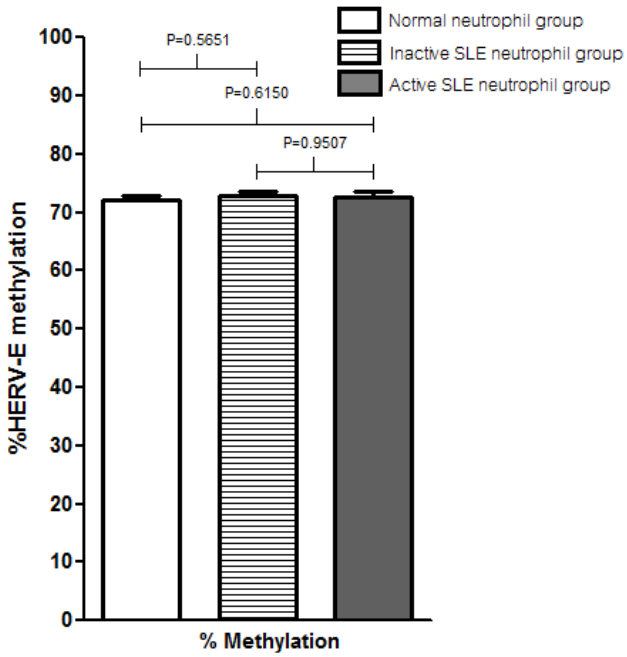


Table 15: Alteration of HERV-E methylation in disease activity (SLE patients were divided into active and inactive groups based on their SLEDAI-2K score)

HERV-E methylation	Normal control Neutrophils (n=20)	Inactive SLE Neutrophils (n=10)	Active SLE Neutrophils (n=10)
% Methylation	72.07 ±3.27	72.74 ±2.25	72.67 ±2.58

P-value = 0.5651 (normal control compared to inactive SLE)

P-value = 0.6150 (normal control compared to active SLE)

P-value = 0.9507 (inactive SLE compared to active SLE)

Figure26. HERV-K methylation levels in different SLE disease activity was shown in Mean ± SEM. P-value calculated by unpaired t-test two-tailed analysis.

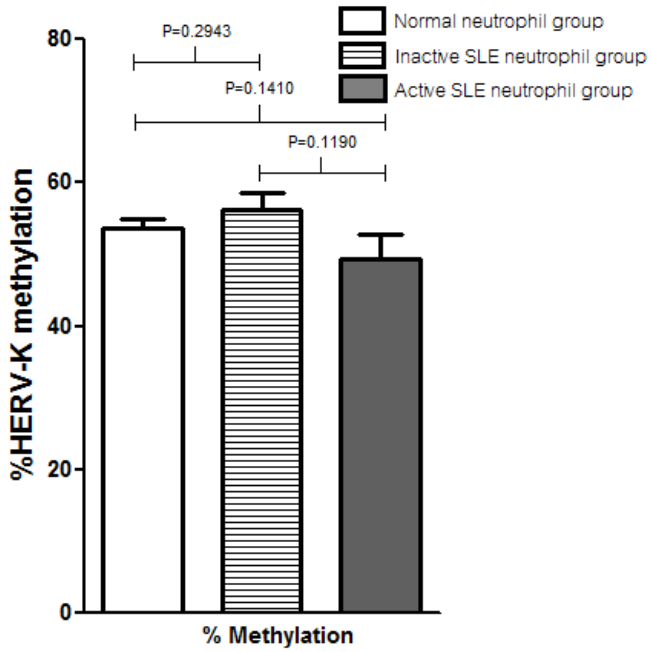


Table 16: Alteration of HERV-K methylation in disease activity (SLE patients were divided into active and inactive groups based on their SLEDAI-2K score)

HERV-K methylation	Normal control Neutrophils (n=20)	Inactive SLE Neutrophils (n=10)	Active SLE Neutrophils (n=10)
% Methylation	53.75 ±4.97	56.17 ±7.33	49.46 ±10.68

P-value = 0.5651 (normal control compared to inactive SLE)

P-value = 0.6150 (normal control compared to active SLE)

P-value = 0.9507 (inactive SLE compared to active SLE)

The correlation between intragenic LINE-1 and differential expressions in SLE neutrophils expression array

Several studies have proved that the intragenic LINE-1 (gene containing LINE-1) hypomethylation in cancer cells can regulate host gene expressions in cis (23). It was proposed that intragenic LINE-1 RNAs repress host gene expression via the nuclear RNA-induced silencing complex (RISC) (23). Therefore, we would like to investigate whether intragenic LINE-1 hypomethylation correlate with gene expression in SLE neutrophil. Genes processing LINE-1 were determined by L1base (118) and listed by Aporntrwan et al (23). CU-DREAMX program was used to evaluate the association of genes containing intragenic LINE-1 and level of gene expression. Genes containing LINE-1 were classified into four groups depending on the regulation of gene and the presence of intragenic LINE-1 1) genes containing L1s 1454 genes, 2) genes with sense L1s 336 genes, 3) genes with antisense L1s 832 genes and 4) genes with both sense and antisense L1s 286 genes. The Chi-square test to evaluate intragenic LINE-1 influence SLE neutrophils expression by the intersection between expression microarray data available from GEO, GSE27427 and gene-containing LINE-1 (4 groups) were analyzed (Table 17). The result showed significant association between the up-regulated genes in SLE neutrophils compared to normal neutrophils and genes containing LINE-1s (p-value GSE27427 = $7.74X10^{-3}$; OR = 1.28). Interestingly, the significant association is mainly among genes with antisense LINE-1s (p-value GSE27427 = $6.22X10^{-3}$; OR = 1.38) (Table 18).

Table 17: 2x2 table of Chi-square test to evaluate intragenic LINE-1 can control the expression of genes in neutrophils of SLE patients. Gene- containing LINE-1 was classified into four groups depending on the regulation of genetic and the presence of intragenic LINE-1 (A: Genes containing L1s 1454 genes, B: Genes with sense L1s 336 genes, C: Genes with antisense L1s 832 genes and D: Genes with both sense and antisense L1s 286 genes).The down-or up-regulation of a gene were denoted by "Up" and "Down" and this

experiment determined by paired t-test (P-value threshold is set at 0.01). While, genes containing or without intragenic LINE-1 were denoted by LINE-1 and No LINE-1, respectively. The p-values of 2x2 tables were obtained from Chi-square distribution.

A: 2x2 table of Chi-square test to evaluate intragenic LINE-1 (Genes containing L1s 1454 genes) influence methylation levels in neutrophils of SLE patients.

	GSE27427 SLE neutrophil			
	Down (P<0.01)	Not-Down	Up (P<0.01)	Not-Up
LINE-1	67	1,179	137	1,109
No LINE-1	2,284	21,467	2,088	21,663
	P value = 0.000000582		P value = 0.007743	
	Odds ratio = 0.53		Odds ratio = 1.28	
	Lower 95% CI = 0.42		Lower 95% CI = 1.07	
	Upper 95% CI = 0.69		Upper 95% CI = 1.54	

B: 2x2 table of Chi-square test to evaluate intragenic LINE-1 (Genes with sense L1s 336 genes) influence methylation levels in neutrophils of SLE patients.

	GSE27427 SLE neutrophil			
	Down (P<0.01)	Not-Down	Up (P<0.01)	Not-Up
LINE-1	22	261	27	256
No LINE-1	2,329	22,385	2,198	22,516
	P value = 0.344406		P value = 0.703951	
	Odds ratio = 0.81		Odds ratio = 1.08	
	Lower 95% CI = 0.52		Lower 95% CI = 0.72	
	Upper 95% CI = 1.25		Upper 95% CI = 1.61	

C: 2x2 table of Chi-square test to evaluate intragenic LINE-1 (Genes with antisense L1s 832 genes) influence methylation levels in neutrophils of SLE patients.

	GSE27427 SLE neutrophil			
	Down (P<0.01)	Not-Down	Up (P<0.01)	Not-Up
LINE-1	37	686	85	638
No LINE-1	2,314	21,960	2,140	22,134
	P value = 0.0000612		P value = 0.006215	
	Odds ratio = 0.51		Odds ratio = 1.38	
	Lower 95% CI = 0.37		Lower 95% CI = 1.09	
	Upper 95% CI = 0.71		Upper 95% CI = 1.74	

D: 2x2 table of Chi-square test to evaluate intragenic LINE-1 (Genes with both sense and antisense L1s 286 genes) influence methylation levels in neutrophils of SLE patients.

	GSE27427 SLE neutrophil			
	Down (P<0.01)	Not-Down	Up (P<0.01)	Not-Up
LINE-1	8	232	25	215
No LINE-1	2,343	22,414	2,200	22,557
	P value = 0.001203		P value = 0.407372	
	Odds ratio = 0.33		Odds ratio = 1.19	
	Lower 95% CI = 0.16		Lower 95% CI = 0.79	
	Upper 95% CI = 0.67		Upper 95% CI = 1.81	

Table 18: The expression status of genes containing LINE-1s compared between SLE neutrophils vs. normal neutrophils.

	Genes containing L1s (1454 genes)		Genes with sense L1s (336 genes)		Genes with antisense L1s (832 genes)		Genes with both sense and antisense L1s (286 genes)	
	P-value	OR	P-value	OR	P-value	OR	P-value	OR
GSE27427 SLE neutrophil VS. Normal neutrophils Up-regulation (p<0.01)	7.74E-03*	1.28	0.7	1.08	6.22E-03*	1.38	0.41	1.19
GSE27427 SLE neutrophil VS. Normal neurophils Down-regulation (p<0.01)	5.82E-07	0.53	0.34	0.81	6.13E-05	0.51	0.0012	0.33

Bioinformatics analysis of the up or down-regulated genes containing LINE-1s that involved in the biological processes

To identify functional networks, genes with intragenic LINE-1 that associated with the expression of genes in neutrophils of SLE were performed gene category p-value analysis using the online available DAVID bioinformatics resources. The functional annotation chart with a group P-value less than or equal to 0.05 were analyzed. From 67 down-regulation genes and 137 up-regulation genes (Table 17A), 22 down-regulation genes and 27 up-regulation genes (Table 17B), 37 down-regulation genes and 85 up-regulation genes (Table 17C), 8 down-regulation genes and 25 up-regulation genes (Table 17D). (Table 19) Interestingly, the up-regulated genes containing LINE-1s involved in the biological processes of cell apoptosis and induction of programmed cell death (Table 19 B). The down-regulated genes containing LINE-1s were found to be innate immune response, lymphocyte mediated immunity and leukocyte mediated immunity (Table 19 A). Furthermore, from 137 up-regulation genes (Table 17A in appendix B), the significant p-value was cell death process genes that involve in the calcium signaling pathway were present in this category (Figure 27). Meanwhile, 67 down-regulation genes (Table17A in appendix B), the significant p-value was innate immune response, lymphocyte mediated immunity and leukocyte mediated immunity genes that involve in the complement and coagulation cascades were present in this category (Figure 28).

Table 19. Functional annotation chart identified for up or down-regulation genes from genes containing L1s group by DAVID Functional Classification Tool. Official full name of these genes were showed in Appendix B

A: SLE down-regulated genes with genes containing L1s

Category	Phenotype function	P-value	LINE-1 down-regulated gene in SLE neutrophils
GOTERM_BP_FAT	innate immune response	0.014	CD46, CR1, IL1RL2, LYST
GOTERM_BP_FAT	lymphocyte mediated immunity	0.028	CD46, CR1, LYST
GOTERM_BP_FAT	leukocyte mediated immunity	0.04	CD46, CR1, LYST

B: SLE up-regulated genes with genes containing L1s

Category	Phenotype function	P-value	LINE-1 up-regulated gene in SLE neutrophils
GOTERM_BP_FAT	induction of apoptosis	0.026	AKAP13, CD38, GCH1, JAK2, C6, PTEN, VAV3
GOTERM_BP_FAT	induction of programmed cell death	0.027	AKAP13, CD38, GCH1, JAK2, C6, PTEN, VAV3
GOTERM_BP_FAT	positive regulation of apoptosis	0.033	AKAP13, CD38, GCH1, JAK2, C6, PTEN, STK3, VAV3
GOTERM_BP_FAT	positive regulation of programmed cell death	0.034	AKAP13, CD38, GCH1, JAK2, C6, PTEN, STK3, VAV3
GOTERM_BP_FAT	positive regulation of cell death	0.035	AKAP13, CD38, GCH1, JAK2, C6, PTEN, STK3, VAV3
GOTERM_BP_FAT	cellular calcium ion homeostasis	0.041	ATP7B, CD38, JAK2, CYSLTR1, PLCE1
GOTERM_BP_FAT	elevation of cytosolic calcium ion concentration	0.043	CD38, JAK2, CYSLTR1, PLCE1
GOTERM_BP_FAT	calcium ion homeostasis	0.045	ATP7B, CD38, JAK2, CYSLTR1, PLCE1
GOTERM_BP_FAT	cytosolic calcium ion homeostasis	0.052	CD38, JAK2, CYSLTR1, PLCE1

C: SLE down-regulated genes with genes sense L1s

Category	Phenotype function	P-value	LINE-1 down-regulated gene in SLE neutrophils
GOTERM_BP_FAT	cytoskeleton-dependent intracellular transport	0.067	DST, MYO5A
GOTERM_BP_FAT	macromolecule catabolic process	0.082	FAF1, GAN, PYGB, PSMA1

D: SLE up-regulated genes with genes sense L1s were identified functional annotation chart by DAVID Functional Classification Tool. The result showed that zero chart records.

E: SLE down-regulated genes with genes antisense L1s

Category	Phenotype function	P-value	LINE-1 down-regulated gene in SLE neutrophils
GOTERM_BP_FAT	innate immune response	0.027	CD46, IL1RL2, LYST
GOTERM_BP_FAT	sexual reproduction	0.051	CD46, FANCC, MICALCL, DNAH9

F: SLE up-regulated genes with genes antisense L1s

Category	Phenotype function	P-value	LINE-1 up-regulated gene in SLE neutrophils
GOTERM_BP_FAT	defense response	0.016	ABCC9, CLEC5A, DDX58, GCH1, C6, ITGB1, IL15, PLD1
GOTERM_BP_FAT	regulation of defense response to virus by host	0.038	DDX58, IL15
GOTERM_BP_FAT	regulation of hydrolase activity	0.056	RABGAP1L, RICTOR, CHRM2, C6, PLCE1
GOTERM_BP_FAT	response to zinc ion	0.07	ATP7B, PTEN
GOTERM_BP_FAT	regulation of protein kinase B signaling cascade	0.07	RICTOR, PTEN
GOTERM_BP_FAT	trans membrane receptor protein tyrosine kinase signaling	0.071	EPHB1, EPS15, PTEN, PLCE1

	pathway		
GOTERM_BP_FAT	immune response	0.074	OAS3, ABCC9, CLEC5A, DDX58, GCH1, C6, IL15
GOTERM_BP_FAT	hemostasis	0.078	AP3B1, HMCN1, TFPI

G: SLE down-regulated genes with genes both sense and antisense L1s were identified functional annotation chart by DAVID Functional Classification Tool. The result showed that zero chart records.

H: SLE up-regulated genes with genes both sense and antisense L1s

Category	Phenotype function	P-value	LINE-1 up-regulated gene in SLE neutrophils
GOTERM_BP_FAT	cell adhesion	0.0079	DSCAM, COL4A6, COL24A1, CNTNAP5, FNDC3A
GOTERM_BP_FAT	biological adhesion	0.0079	DSCAM, COL4A6, COL24A1, CNTNAP5, FNDC3A
GOTERM_BP_FAT	regulation of apoptosis	0.066	AKAP13, STK3, TEX11, VAV3
GOTERM_BP_FAT	regulation of programmed cell death	0.067	AKAP13, STK3, TEX11, VAV3
GOTERM_BP_FAT	regulation of cell death	0.068	AKAP13, STK3, TEX11, VAV3
GOTERM_BP_FAT	positive regulation of apoptosis	0.09	AKAP13, STK3, VAV3
GOTERM_BP_FAT	positive regulation of programmed cell death	0.091	AKAP13, STK3, VAV3
GOTERM_BP_FAT	positive regulation of cell death	0.092	AKAP13, STK3, VAV3

Figure 27. Calcium signaling pathway. Genes that are up-regulated are shown in red star.

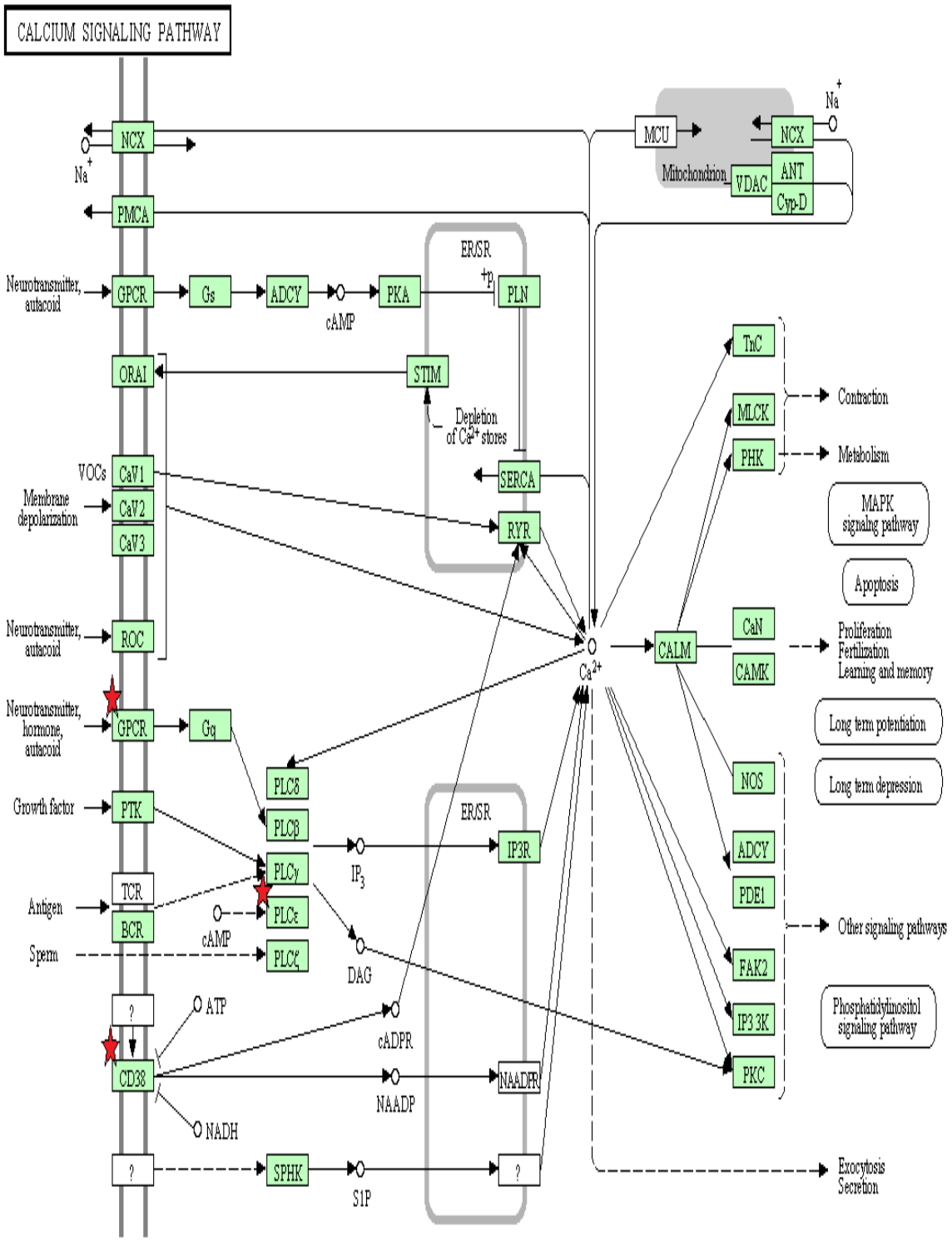
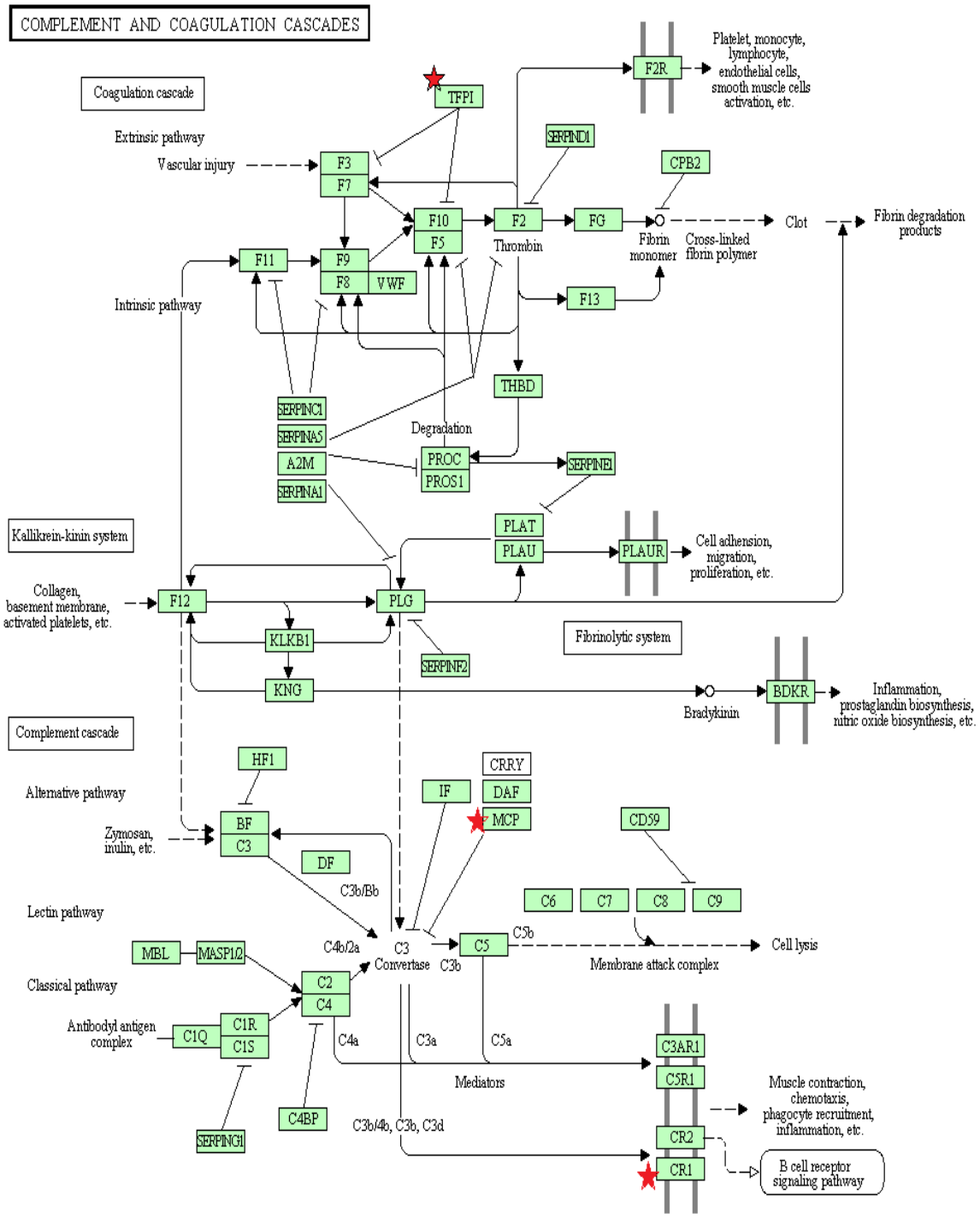


Figure 28. Complement and coagulation cascades. Genes that are down-regulated are shown in red star.



CHAPTER V

DISCUSSION AND CONCLUSION

Currently, the exact etiology of SLE is still obscure. However, various evidences suggest a key important role for epigenetics in human pathologies, especially, dysregulation of DNA methylation processes. Several papers reported the abnormalities of DNA methylation level in some important immune cells of SLE patients by independent studies which, involved demethylation processes. They found that leukocytes (5), PBMCs (6, 7), T-cell (30) and $CD4^+$ T-cell DNA (8-11) of SLE patients have DNA hypomethylation comparing to healthy donor. Moreover, several studies suggested the potential role of IRSs to regulate cellular functions. Previous studies showed that treating normal lymphocyte with 5-azacytidine can reactivate IRSs transcript (HERV) (39). In addition, some IRS, LINE-1 and HERV-E transcripts were detected in patients with SLE (123, 124). Not only PBMC and lymphocyte are those involve with pathogenesis of SLE, but neutrophils also play an important role. Neutrophil are the large fraction of the immune cells which is critical to the pathogenesis of SLE (44). Specifically, several recent papers reported the abnormalities in cell-death process of SLE neutrophils (45-48). However, there has never been studied on methylation in neutrophils of SLE patients. Similar to previous finding in lymphocytes, we hypothesized that DNA methylation status of LINE-1, ALU, HERV-E, and HERV-K in neutrophil of SLE patients might be significant difference from normal control and significant correlated with SLE disease activity. This study aimed to determine and compared the methylation level and patterns at LINE-1, ALU, HERV-E, and HERV-K sequences in neutrophil from healthy control, inactive and active SLE patients. We also analyzed the association between methylation level and patterns of LINE-1, Alu, HERV-E, and HERV-K and some clinical data of SLE patients. Furthermore, we used the bioinformatics tool to

investigate the correlation between intragenic LINE-1 and differential gene expressions in SLE neutrophils using expression array (GSE27427).

First, we determined and compared the methylation level and patterns at LINE-1, ALU, HERV-E, and HERV-K sequences in neutrophil from healthy control, inactive and active SLE patients. Our experiments showed that some methylation level and pattern of LINE-1 was significant difference (precise methylation (mC) and hypermethylated (mCmC)) in neutrophils from SLE patients. The neutrophils from SLE patients included both active and inactive group have significantly lower LINE-1 methylation than healthy control. To confirm this, hypomethylation (uCuC) and %mCuC pattern in neutrophils from SLE patients were significantly higher than healthy control. This finding is similar with previous studied in $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, and B lymphocytes of SLE patient as well as in lymphocyte of synovial fibroblast of rheumatoid arthritis patients (37, 38). Furthermore, previous studied showed hypomethylation of LINE-1 but not Alu in keratinocyte from psoriasis (37). These evidences suggested that the aberration of the methylation level of LINE-1 might be one key feature in autoimmune diseases in various cell types. These hypomethylation of LINE-1 may affect cellular gene expression and contribute to the pathogenesis of SLE, which remain to be further explored.

However, the methylation level and pattern of Alu, HERV-E and HERV-K in neutrophils from SLE patients include active and inactive group compared to healthy control were not significantly different. This finding is differ from some previous studies that reported the significant differences of methylation indices between $CD4^+$T cell and $CD8^+$T cell or non-$CD4^+$ T cell of HERV-E and HERV-K (5, 6). In lymphocytes, there is one reported of the significant differences of methylation patterns of HERV-E and HERV-K between T and B cells (66). Interestingly, in aging cells the hypomethylation of Alu element and HERV-K, but not LINE-1 were reported (36). Therefore, it seems that distinction of methylation levels of Alu, HERV-E and HERV-K is cell type and disease-specific.

Second, we also analyzed the association between methylation level and patterns of LINE-1, Alu, HERV-E, and HERV-K and some clinical data of SLE patients. Interestingly, we did not observed any significant correlation between methylation level of IRSs with disease activity or neutrophil count at all. However, our result showed that some methylation level and pattern of Alu, mCmC and uCmC pattern has significant positive and negative correlation with age respectively. This finding is differ from previous studies that found a negatively associated between age and methylation levels of Alu in PBMC of healthy donor. Hypomethylation of Alu element occurred during ages 34–68 year (36). Our result showed that association between methylation levels and patterns of Alu and age of neutrophil and PBMC is different. Therefore, we suggest that Alu element mechanisms is cell type and disease/condition-specific and may be involve with aging process.

In summary, we only observed a significant hypomethylation of LINE-1 in neutrophil of SLE patients. Next, we would like to explore whether these LINE-1 hypomethylation has any functional significance or not. Therefore, we investigated the correlation between intragenic LINE-1 and differential expressions in SLE neutrophils expression array (GSE27427) whether intragenic LINE-1 control host genes in SLE. The gene expression in SLE neutrophil was compared with genes possessing intragenic LINE-1. We observed the higher prevalence of up-regulated of genes containing LINE-1s in SLE neutrophils compared to normal neutrophils by CU-DREAM X (25, 117). In addition, we analyzed the association with different type of LINE-1. Interestingly, only antisense LINE-1s have higher prevalence of up-regulated genes containing LINE-1s. Recent year, Aporntrwan et al. reported that the consequences of intragenic LINE-1 (gene containing LINE-1) hypomethylation in cancer cells can regulate host gene expressions in cis by LINE-1. They found that intragenic LINE-1 RNAs repress host gene expression via the nuclear RNA-induced silencing complex (RISC) (23). Therefore, we suggest that the effect of hypomethylation of intragenic antisense LINE-1 in SLE neutrophils may be different from the

mechanism in cancer. Other disease related factors should be responsible for this difference.

We hypothesized that some genes that hypomethylation of LINE-1 might influent the expression of some genes in close proximity of LINE-1 especially the genes that related to SLE pathogenesis. Therefore, we explored the phenotypic data of up-regulated of genes containing LINE-1s by online available DAVID Bioinformatics Resources, bioinformatics analysis. The high prevalence of up-regulated genes containing LINE-1s involved in the biological processes of cell including induction of apoptosis and induction of programmed cell death. That may be involved in pathogenesis of SLE through cell death processes. (Table 19 B). The down-regulated genes containing LINE-1s involved with innate immune response, lymphocyte mediated immunity and leukocyte mediated immunity. That may be involved in pathogenesis of SLE through neutrophil cells (Table 19 A). Furthermore, from 137 up-regulation genes (Table 17A in appendix B), the significant p-value was cell death process genes that involve in the calcium signaling pathway (Figure 27). It has long been known that $Ca2^{+}$ signals control an essential cell functions and are necessary for cell survival. Recently it has become clear that cellular $Ca2^{+}$ overload, or interference of intracellular $Ca2^{+}$ compartmentalization, can cause cytotoxicity and trigger either apoptotic or necrotic cell death (125). For the 67 down-regulation genes (Table17A in appendix B), the significant p-value was innate immune response, lymphocyte mediated immunity and leukocyte mediated immunity genes. These phenotypes involve with the complement and coagulation cascades (Figure 28). The 67 down-regulated genes contain LINE-1s may influent inflammation of innate immune cell especially, neutrophils through complement and coagulation cascades. The dysregulation of the cascade activities or functions of inhibitors in these systems can result in clinical manifestations of disease, such as systemic lupus erythematosus, or ischemia–reperfusion injury, with critical thrombotic and inflammatory complications (126). The relationship between complement and coagulation cascades may involve with complement-associated inflammatory responses in SLE neutrophils. However,

this is only a result from association study and bioinformatics data, further functional study are required to prove whether these genes above are in fact regulated by LINE-1 hypomethylation or not.

In conclusion, the hypomethylation (uCuC) in neutrophils of SLE patients was interspersed repetitive sequences (IRSs) specific, mainly found in LINE-1 rather than Alu, HERV-E and HERV-K. We proposed consequence mechanism of LINE-1 hypomethylation in SLE that LINE-1 transcripts might have an effect in *cis* to up-regulate some cell death processes genes. Further study to find out the functional role of IRSs hypomethylation might lead to the discovery of novel pathogenesis pathway in SLE neutrophils.

Table 20: Association between the dynamic of DNA methylation levels of interspersed repetitive sequences (IRSs) and human diseases or condition.

IRSs	Disease/Condition													
	Cancer			SLE										Aging
	Cancer cells	PBMC	Leukocyte	B cell		$CD3^+$ T cell		$CD4^+$ T cell		$CD8^+$ T cell		Neutrophil		PBMC
				Active	Inactive	Active	Inactive	Active	Inactive	Active	Inactive	Active	Inactive	
LINE-1	Hypo-	Hypo-	Hypo-	Hypo-	Hypo-	–	–	Hypo-	–	Hypo-	–	Hypo-	Hypo-	–
Alu	Hypo-	–	–	–	–	–	–	–	–	–	Hypo-	–	–	Hypo-
HERV-E	Hypo-	–	–	–	–	Hypo-	–	Hypo-	–	–	–	–	–	–
HERV-K	Hypo-	–	–	–	–	–	Hypo-	–	Hypo-	–	–	–	–	Hypo-

Abbreviations: Hypo-: Hypomethylation, **–**: Unaltered

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APPENDICES

APPENDIX A

BUFFER AND REAGENT

1. Nuclei Lysis Buffer (NLB)

1 M Tris (pH 8.0)	10	ml
5 M NaCl	0.5	ml
0.5 M EDTA (pH 8.0)	0.4	ml

Adjust volume to 100 ml with distilled water. Adjust pH to 7.2. Keep refrigerated. Shelf life is approximately 6 months.

2. 1 M Tris

Tris base	12.11	g
Distilled water	100	g

Adjust volume to 100 ml with distilled water. Adjust pH to 7.2. The solution was mixed and sterilizes by autoclaving at 121 ºC for 15 minutes.

3. 5 M NaCl

NaCl	29.22	g
Distilled water	100	ml

Adjust volume to 100 ml with distilled water. The solution was mixed and sterilized by autoclaving at 121 ºC for 15 minutes.

4. EDTA

EDTA	37.22	g
Distilled water	200	ml

Adjust volume to 200 ml with distilled water. The solution was mixed and sterilizes by autoclaving at 121 ºC for 15 minutes. Keep refrigerated.

5. 5.3 M NaCl

NaCl	15.5	g
Distilled water	50	ml

Adjust volume to 50 ml with distilled water. The solution was mixed and sterilizes by autoclaving at 121 ºC for 15 minutes. Keep refrigerated.

6. Proteinase K 10 mg/ml

Proteinase K	100	mg
Distilled water	10	ml

Mix the solution and store at -20 ºC

7. 10% SDS

SDS	10	g
Distilled water	100	ml

Adjust volume to 100 ml with distilled water. The solution was mixed and sterilizes by autoclaving at 121 ºC for 15 minutes.

8. 10X Tris-boric acid buffer (TBE) 1000 ml

Tris base	108.0	g
Boric acid	55	g
0.5 m EDTA Ph 8.0	40	ml

Adjust volume to 1 liter with distilled water. The solution was mixed and sterilized by autoclaving at 121 ºC for 15 min.

APPENDIX B

Table 1A: Up and down-regulated genes with L1s insertion group in the neutrophils of SLE patient

Gene symbol	Full Name
CD46	CD46 molecule, complement regulatory protein
CR1	complement component (3b/4b) receptor 1 (Knops blood group)
IL1RL2	interleukin 1 receptor-like 2
LYST	lysosomal trafficking regulator
AKAP13	A kinase (PRKA) anchor protein 13
CD38	CD38 molecule
GCH1	GTP cyclohydrolase 1
JAK2	Janus kinase 2
C6	complement component 6
PTEN	phosphatase and tensin homolog; phosphatase and tensin homolog pseudogene 1
STK3	serine/threonine kinase 3 (STE20 homolog, yeast)
VAV3	vav 3 guanine nucleotide exchange factor
ATP7B	ATPase, Cu++ transporting, beta polypeptide
CYSLTR1	cysteinyl leukotriene receptor 1
PLCE1	phospholipase C, epsilon 1
DST	dystonin
MYO5A	myosin VA (heavy chain 12, myoxin)
FAF1	Fas (TNFRSF6) associated factor 1
GAN	gigaxonin
PYGB	phosphorylase, glycogen; brain
PSMA1	proteasome (prosome, macropain) subunit, alpha type, 1
FANCC	Fanconi anemia, complementation group C

MICALCL	MICAL C-terminal like
DNAH9	dynein, axonemal, heavy chain 9
ABCC9	ATP-binding cassette, sub-family C (CFTR/MRP), member 9
CLEC5A	C-type lectin domain family 5, member A
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58
ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)
IL15	interleukin 15
PLD1	phospholipase D1, phosphatidylcholine-specific
RABGAP1L	RAB GTPase activating protein 1-like
RICTOR	RPTOR independent companion of MTOR, complex 2
CHRM2	cholinergic receptor, muscarinic 2
EPHB1	EPH receptor B1
EPS15	epidermal growth factor receptor pathway substrate 15
OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa
AP3B1	adaptor-related protein complex 3, beta 1 subunit
HMCN1	hemicentin 1
TFPI	tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)
DSCAM	Down syndrome cell adhesion molecule
COL4A6	collagen, type IV, alpha 6
COL24A1	collagen, type XXIV, alpha 1
CNTNAP5	contactin associated protein-like 5
FNDC3A	fibronectin type III domain containing 3A
TEX11	testis expressed 11

BIOGRAPHY

Mr. Paramate Promnarate was born on December13th in 1978 at Nakhonratchasima, Thailand. He graduated in the Bachelor degree of Science (Microbiology) from Ubonratchatani University in 2001. After graduated, he worked at the Armed forces Research Institute of Medical Sciences in the position of Laboratory Technician until the present. In 2012, he graduated in Master degree of Medical Sciences program, Faculty of Medicine Chulalongkorn University.