รายงานวิจัยฉบับสมบูรณ์

โครงการ ความสัมพันธ์ระหว่างระดับการเกิดเมธิลเลชั่นในจีโนม และความชราภาพของเซลล์

โดย ดร. พรรัศมี์ จินตฤทธิ์

วันสิ้นสุดโครงการ พฤษภาคม 2553

สัญญาเลขที่ MRG5180072

รายงานวิจัยฉบับสมบูรณ์

โครงการ ความสัมพันธ์ระหว่างระดับการเกิดเมธิลเลชั่นในจีโนม และความชราภาพของเซลล์

คณะผู้วิจัย	สังกัด
1. ดร. พรรัศมิ์ จินตฤทธิ์	คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล
2. ศาสตราจารย์ ดร. นายแพทย์อภิวัฒน์ มุทิรางกูร	คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สนับสนุนโดยสำนักงานคณะกรรมการการอุดมศึกษา และสำนักงานกองทุนสนับสนุนการวิจัย
(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และ สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

Project Code: MRG5180072

(รหัสโครงการ)

Project Title: ความสัมพันธ์ระหว่างระดับการเกิดเมธิลเลชั่นในจีโนมและความชราภาพของเซลล์

(ชื่อโครงการ)

Investigator:

(คณะผู้วิจัย)

1. ดร. พรรัศมิ์ จินตฤทธิ์ คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล

E-mail Address: tmpjt@mahidol.ac.th

2. ศาสตราจารย์ ดร. นายแพทย์อภิวัฒน์ มุทิรางกูร คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

E-mail Address: mapiwat@chula.ac.th

Project Period: 15 พฤษภาคม 2551 ถึง 14 พฤษภาคม 2553

(ระยะเวลาโครงการ)

บทคัดย่อ

รูปแบบความแตกต่างการลดลงตามอายุของการเติมหมู่เมททิลบนชนิดตำแหน่งดีเอนเอที่มีลำดับเบสซ้ำ

พรรัศมิ์ จินตฤทธิ์[1] และอภิวัฒน์ มุทิรางกูร[2]

[1] ภาควิชาโภชนศาสตร์เขตร้อนและวิทยาศาสตร์อาหาร คณะเวชศาสตร์เขตร้อน มหาวิทยาลัยมหิดล, [2] หน่วยมนุษยพันธุศาสตร์ ภาควิชากายวิภาคศาสตร์ คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

ตำแหน่งดีเอนเอที่มีลำดับเบสซ้ำกัน (IRS) จะอยู่กระจายเป็นส่วนใหญ่ตามขนาดของจีโนมและอาจส่งผลต่อการทำหน้าที่ของเซล ซึ่งสามารถแบ่งได้ตามขนาดและหน้าที่เชิงโครงสร้างได้เป็น บริเวณที่มีลำดับเบสซ้ำขนาดสั้น (short interspersed elements), บริเวณที่มีลำดับเบสซ้ำขนาดยาว (long interspersed elements), บริเวณที่มีลำดับเบสซ้ำที่สามารถเคลื่อนไปมาจากตำแหน่งหนึ่งไปอีกตำแหน่งหนึ่งได้โดยตรง (DNA transposon) และบริเวณที่มีลำดับเบสซ้ำส่วนปลายที่สามารถเคลื่อนไปมาจากตำแหน่งหนึ่งไปอีกตำแหน่งโดยใช้ RNA (LTR-retrotransposon) มีหลายชนิดของตำแหน่งเหล่านี้อาจผลิต RNA และควบคุมยีนโดยอาศัยกลไกที่หลากหลาย การเติมหมู่เมททิลบนลำดับดีเอนเอส่วนใหญ่แล้วเกิดในบริเวณ IRS และเชื่อว่าทำให้กดการทำงานของ IRS การลดลงของการเติมหมู่เมททิลทั่วทั้งหมด (Global hypomethylation) หรือการบกพร่องของการเติมหมู่เมททิลในจีโนม (Loss of genome-wide methylation) เป็นสภาวะเหนือพันธุกรรมที่พบบ่อยทั้งในเซลล์ที่ชราภาพและเซลมะเร็ง การลดลงของการเติมหมู่เมททิลบนลำดับดีเอนเอที่ตำแหน่งของไลน์-1 (LINE-1) มีการศึกษาอย่างกว้างขวางในเซลมะเร็งเกือบทุกชนิด ดังนั้นการศึกษานี้คณะผู้วิจัยจึงศึกษาการเติมหมู่เมททิลบริเวณตำแหน่งไลน์-1 (LINE-1), อะลู (Alu) และโปรไวรัส K (Human endogenous retrovirus, HERV-K) ในเซลเม็ดเลือดขาวชนิด mononuclear cell ของอาสาสมัครปกติจำนวน 177 ราย ช่วงอายุระหว่าง 20-88 ปี พบว่าอายุมีความสัมพันธ์ในเชิงลบกับระดับเมธิลเลชั่นที่ตำแหน่งอะลู และโปรไวรัส K อย่างมีนัยสำคัญทางสถิติ (r_{Alu} = -0.452, $P<10^{-3}$, r_{HERV-K} = -0.326, $P<10^{-3}$) แต่ไม่พบความสัมพันธ์ในตำแหน่งไลน์-1 (r_{LINE-1}=0.145, P=0.055) การบกพร่องของการเติมหมู่เมททิลที่ตำแหน่งอะลูพบในช่วงอายุ 34-68 ปี และที่ตำแหน่งโปรไวรัส K พบในช่วงอายุ 40-63 ปี และ 64-83 ปี และเป็นที่น่าสนใจอย่างยิ่งพบว่ามีความสัมพันธ์ในเชิงบวกของการเติมหมู่เมททิลของตำแหน่งอะลูและไลน์-1 ในช่วงอายุมากกว่าหรือเท่ากับ 49 ปี (r=0.49, $P<10^{-3}$) จากผลการศึกษานี้แสดงว่าการบกพร่องตามอายุของการเติมหมู่เมททิลบนลำดับดีเอนเอของชนิดตำแหน่งที่มีลำดับเบสซ้ำกันนั้นต่างกันและพบในบางตำแหน่งและบางช่วงจำเพาะของอายุเท่านั้น การบกพร่องตามอายุของการเติมหมู่เมททิลที่ตำแหน่งอะลูและตำแหน่งของโปรไวรัส K แตกต่างกัน และอาจมี

กลไก 2-3 ชนิด ที่เกี่ยวข้องกับการเติมหมู่เมททิลทั่วทั้งหมดในจีโนม ซึ่งกลไกเหล่านี้ไม่ใช่ทั้งหมดที่ขึ้นกับอายุ การศึกษาครั้งนี้อาจนำไปสู่ความเข้าใจที่ดีขึ้นทั้งสาเหตุทางชีววิทยาระดับเซลล์และผลกระทบของการลดลงการเติมหมู่เมททิลทั่วทั้งหมดในจีโนม ตลอดจนบทบาทของชนิดตำแหน่งดีเอนเอที่มีลำดับเบสซ้ำกันในขั้นตอนที่เกี่ยวข้องกับความชราภาพ

คำสำคัญ: การลดลงของการเติมหมู่เมททิล, การเติมหมู่เมททิลในจีโนม, ความชราภาพ, ชนิดตำแหน่งดีเอนเอที่มีลำดับเบสซ้ำกัน

Abstract

Distinctive patterns of age-dependent hypomethylation in interspersed repetitive sequences

Pornrutsami Jintaridth[1], Apiwat Mutirangura[2]

[1]Department of Tropical Nutrition and Food Science, Faculty of Tropical Medicine, Mahidol University; [2]Center for Excellence in Molecular Genetics of Cancer and Human Diseases, Department of Anatomy, Faculty of Medicine, Chulalongkorn University

Interspersed repetitive sequences (IRSs) are a major contributor to genome size and may contribute to cellular functions. IRSs are subdivided according to size and functionally related structures into short interspersed elements (SINEs), long interspersed elements (LINEs), DNA transposons and LTR-retrotransposons. Many IRSs may produce RNA and regulate genes by a variety of mechanisms. The majority of DNA methylation occurs in IRSs and is believed to suppress IRS activities. Global hypomethylation, or the loss of genome-wide methylation, is a common epigenetic event not only in senescent cells but also in cancer cells. Loss of LINE-1 methylation has been characterized in many cancers. Here, we evaluated the methylation levels of LINE-1, Alu and human endogenous retrovirus (HERV-K) in 177 samples obtained from volunteers between 20 and 88 years of age. Age was negatively associated with methylation levels of Alu ($r=-0.452$, $p<10^{-3}$) and HERV-K ($r=-0.326$, $p<10^{-3}$) but not LINE-1 ($r=0.145$, $p=0.055$). Loss of methylation of Alu occurred during ages 34-68 yr, and loss of methylation of HERV-K occurred during ages 40-63 and again during ages 64-83 yr. Interestingly, methylation of Alu and LINE-1 are directly associated, particularly at ages 49 yr and older ($r=0.49$, $p<10^{-3}$). Therefore, only some types of IRSs lose methylation at certain ages. Moreover, Alu and HERV-K become hypomethylated differently. Finally, there may be several mechanisms of global methylation. However, not all of these mechanisms are age-dependent. This finding may lead to a better understanding of not only the biological causes and consequences of genome-wide hypomethylation but also the role of IRSs in the aging process.

Key words: global hypomethylation; genome-wide methylation; aging; interspersed repetitive sequences

สรุปข้อเสนอโครงการ

(Executive Summary)

1. ความสำคัญและที่มาของปัญหา

Global hypomethylation, or the decreasing of genome-wide DNA methylation, is a common epigenetic alteration in senescent cells (23, 56, 57). Overall, total deoxymethylcytosine levels tend to decrease with aging in most vertebrate tissues, including the brain, liver, small intestine mucosa, heart and spleens of salmon, mice, rats, cows and cattle and aged human peripheral blood cells (7, 22, 23, 25, 36, 48, 53, 54, 57). Interestingly, although global hypomethylation is related to the replicative senescence associated with aging (56), it is also a common feature of cells with limitless replication potential, including stem cells (62) and cancer cells (8). Age-dependent global hypomethylation has been shown to be associated with many diseases (20, 27, 49, 55). Moreover, in cancer, global hypomethylation is associated with more advance stages and poor prognosis (30, 31, 36, 39, 40, 43, 50, 51). Therefore, it is important to understand how the same epigenetic characteristic is associated with a variety of cellular phenotypes.

Interspersed repetitive sequences (IRSs) are a major contributor to genome size, accounting for approximately 45% of human genomic DNA (35, 45). Under normal circumstances, IRSs are methylated to variable degrees depending on their location(46). Therefore, loss of IRS methylation was believed to be the major contributor of global hypomethylation (8). IRSs can be subdivided by size and the presence of transposable elements. Commonly known IRSs are short interspersed elements (SINEs) being less than 500 bp, long interspersed elements (LINEs) being 6-8 Kb, LTR-retrotransposons and DNA transposons (14). SINEs, LINEs and LTR-retrotransposons possess transcriptional activity, and several reports have suggested they are cis regulatory elements (28, 34, 52, 61). Therefore, changes in SINE, LINE or LTR-retrotransposon methylation should alter cellular functions. Because cellular biology of cancer and aging are different, we hypothesized that the age-dependent pattern of methylation loss in SINEs, LINEs and LTRs should be different.

Alu is the most abundant SINE(4, 14). Human endogenous retroviruses (HERVs) are a major subset of LTR-retrotransposons(5, 14, 47). LINE-1, or L1, is the most abundant and well-known LINE(14, 29). The methylation status of Alu, LINE-1 and HERV-K, a well-known member of HERV, has been reported for many cancers (5, 8, 16, 19, 50, 51, 55, 60). Recently, Bollati et al. reported an age-related methylation of peripheral blood mononuclear cells (PBMC) of Alu and LINE-1. They found a significant association between age and Alu hypomethylation, but the significance of the association between age and LINE-1 hypomethylation was borderline (6). In the present study, we describe patterns of age-dependent methylation alternations in Alu, LINE-1 and HERV-K. Our analysis suggests that IRSs lose methylation at a certain age. Moreover, different IRSs lose methylation differently. Finally, there may be several global methylation mechanisms and not all of them are dependent on age.

2. วัตถุประสงค์

The aim of this study is

2.1 To elucidate the mechanisms of global methylation

2.2 To evaluate the differences in IRSs methylation between adults of different ages and between age intervals in PBMC by using modified COBRA-PCR and pyrosequencing

2.3 To analyze the association between IRS methylation and age

2.4 To determine the pair-wise associations between methylation of IRSs in different age intervals

2.5 To understand the biological cause and consequence of genome-wide hypomethylation in aging process

2.6 To understand the role of IRSs in aging process

3. ระเบียบวิธีวิจัย

Materials and Methods

Subjects

Our study population consisted of 42 males and 135 females aged 20-90 years old. Healthy volunteer subjects in the urban area of Bangkok were recruited for sample collection according to the following criteria: I) agreed to participate in the study and gave informed consent, II) no history of chronic or genetic diseases, III) had a Body Mass Index <30 Kg/m^2, IV) no history of smoking and alcohol drinking 10 years prior to sample collection, V) no supplementation with vitamins for three months before study, VI) had normal living and eating habits. This study was approved by the ethics committee of the Faculty of Tropical Medicine, Mahidol University.

Blood collection

Heparinized blood was collected, and PBMC were prepared by Ficoll density gradient centrifugation according to published methods (3). Blood hematological parameters and blood chemistries of participants were analyzed. Volunteers with normal hematological parameters and blood chemistries were chosen to participate. DNA methylation in PBMC from healthy subjects of different ages was measured. Hematological data and the amount of PBMC in whole blood were not significantly different between individual.

DNA extraction and modified combined bisulfite restriction analysis (COBRA)

DNA was extracted from PBMC using QiAmp DNA blood kits (Qiagen, Hilden, Germany). A total of 200 ng DNA (concentration 4 ng/μl) was used for bisulfite treatment. The combined bisulfite restriction analysis (COBRA) consisted of a standard sodium bisulfite PCR treatment followed by restriction digestion and quantitation (58). Bisulfite modification of genomic DNA was performed using previously published methods (8). In brief, 200 ng of DNA was dissolved in 50 μl distilled water and then denatured in 5.5 μl of 2 M NaOH for 10-30 min at 37°C. Next, 30 μl of freshly prepared 10 mM hydroquinone (Sigma) and 520 μl of 3 M bisulfite (pH 5.0) were added and mixed. The samples were incubated at 50°C for 16 h.

The bisulfite-treated DNA was isolated using the Wizard DNA Clean-up System. The DNA was eluted with 50 μl of warm water and desulfonated with 5.5 μl of 3 M NaOH for 5 min. The DNA was precipitated (NH_4OAC-EtOH) using glycogen as a carrier and resuspended in 20 μl of water. Bisulfite-treated DNA was stored at -20°C until ready for use.

COBRA-IRS

COBRA is a standard technique for measuring methylation levels of IRSs (2, 8, 9, 25, 32, 38, 41-43, 50, 51, 59). A schematic representation and examples of COBRA of LINE-1, Alu and HERV-K is shown in Figure 1. The primer sequences that correspond to the nucleotides in the regulatory region of the LINE-1 sequence (GenBank: M80343) are F, 5'-CGT AAG GGC TTA GGG AGT TTT T -3' and R, 5'-(AG)TA AAA CCC TCC (AG)AA CCA AAT ATA AA 3' (8). The PCR reactions consisted of 35 cycles of 95°C for 1 min, 53°C for 1 min and 72°C for 1 min. The PCR products were subsequently digested with 2 U of *TaqI* (MBI Fermentas) and 2 U of *TasI* (MBI Fermentas) in TE buffer 3 (Biolab) at 65°C overnight and were then run on an 8% nondenaturing polyacrylamide gel. The gel was stained with SyBr Green, and band intensities were measured by PhosphoImager using Image Quant software (Molecular Dynamics). The Alu primer sequences, which correspond to the nucleotides of the Alu Sx subfamily sequence (4), are F, 5'-GG(T/C) G(C/T)G GTG GTT TA(C/T) GTT TGT AA-3' and R, 5'-CAC CAT ATT AAC CAA ACT AAT CCC GA3'. Alu PCR conditions and restriction digestion conditions were similar to those of LINE-1. The HERV-K primer sequences (Genbank accession number AF394944.1), which correspond to the nucleotides in the regulatory region of the HERV-K sequence, are F, 5'-TGG GAA GGG AAA GAT TTG AT -3' and R, 5'-ACA AAA AAC AAA TAC CTT CCT CTT- 3'. The PCR reactions consisted of 30 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec. The PCR products were then digested with 2 U of *TaqI* (MBI Fermentas) and in TE buffer 3 (Biolab) at 65°C overnight and were run on an 8% nondenaturing polyacrylamide gel. The gel was stained with SyBr Green, and band intensities were measured by PhosphoImager using Image Quant software (Molecular Dynamics). COBRA LINE-1, Alu and HERV-K amplicons were 160, 99 and 126 bp, respectively. After digestion, the LINE-1, Alu and HERV-K methylated bands were 80, 57 and 94 bp, respectively. Unmethylated bands of LINE-1 were 97 and 63 bp, and the unmethylated band of Alu was 78 bp. Methylation levels were calculated as the intensity of methylated bands divided by sum of the methylated and unmethylated bands. Each COBRA-IRS was performed two to four times. To ensure that measurements of IRS methylation from band intensities of samples reduced inter-assay variation, we used Daudi, Jurkat, Hela and Molt4 cell lines from the same stocks as a control to validate the interassay variation. IRS methylation levels of the stock cell lines was standardized and IRS methylation in each experiment was adjusted so that all experiments had the same control IRS methylation levels. The average differences in IRS methylation of the LINE-1, Alu and HERV-K repetitive elements between adults of different ages were evaluated. IRS methylation of LINE-1, Alu and HERV-K repetitive elements were also analyzed for associations with age intervals. Associations between IRS methylation of LINE-1, Alu and HERV-K were also determined.

4. การวิเคราะห์ทางสถิติ

Statistical analysis

Data were analyzed using SPSS statistical software. The Mann-Whitney U test was used to make non-parametric comparisons of the median methylation levels of LINE-1, Alu and HERV-K between males and females. To analyze different age intervals, ages were divided into 20 years intervals, and the age interval of each group overlapped adjacent intervals by 15 years. Some groups were not normally distributed. The averages of methylation levels of LINE-1, Alu and HERV-K in each group were determined as the median value and the Kruskal-Wallis test was used to make non-parametric comparisons between the groups. To evaluate the correlations between ages and IRS methylation levels, Pearson and Spearman correlations were used to determine associated 95% confidence intervals. The associations between LINE-1, Alu and HERV-K relative methylation levels in the population and groups of age intervals were examined while controlling for confounding variable such as age. Partial correlation was used to determine 95% confidence intervals. All tests were two-tailed analyses.

5. ผลการทดลอง

Results

Association between age and methylation of LINE-1, Alu and HERV-K

This study consisted of PBMC of 177 study subjects aged between 20 and 90 years old (mean age 52.92 years, standard error (SE) =1.19). Methylation levels of LINE-1, Alu and HERV-K were measured by COBRA (2, 8, 9, 25, 32, 38, 41-43, 50, 51, 59). Examples of COBRA-IRS were demonstrated (Figure 1A-F). Our test produced limited result deviations (supplementary information figure 1).

First, we evaluated the relationship between IRS methylation levels and age. LINE-1 methylation was not associated with age (r=0.145, p=0.055) (Figure 2A). However, DNA methylation of both Alu and HERV-K was inversely correlated with age (r=-0.452, $p<10^{-3}$ for Alu; r=-0.326, $p<10^{-3}$ for HERV-K) (Figure 2B and C). Therefore, age-related genomic hypomethylation is occured at only specific types of IRSs, i.e., Alu and HERV-K, but not LINE-1. We then evaluated whether sex influences IRS methylation. Similar to a previous report (8), there were no differences in the methylation of LINE-1, Alu or HERV-K between males and females (Figure 3A-C).

Association between LINE-1, Alu and HERV-K methylation

We evaluated the correlations of methylation levels between IRSs. Because only Alu and HERV-K are demethylated with age, we expected a positive correlation exclusively between Alu and HERV-K. In contrast, we found a significant association between LINE-1 and Alu (r=0.290, $p<10^{-4}$) (Figure 4A). There was no association between LINE-1 and HERV-K methylation (r=-0.058, p=0.45) (Figure 4B). Unexpectedly, Alu and HERV-K methylation were also not correlated (r=0.018, p=0.81) (Figure 4C).

Changes in LINE-1, Alu and HERV-K methylation levels in different age intervals

To determine the certain age at which hypomethylation occurs, we grouped samples according to age into 20-year intervals, with the intervals of group overlapping each adjacent interval by 15 years (Figure 5, 6 and 7). In Figure 5A, B and C, the median, minimum and maximun of LINE-1, Alu and HERV-K methylation, respectively, of each interval are shown. Figure 6 shows the r values of the Pearson correlation between IRS methylation and each interval. No significant LINE-1 loss of methylation was observed in any age, but levels of LINE-1 methylation might increase during ages 40-59 (r=0.32, p=0.02) (Figure 6A). An increase of LINE-1 methylation has been seen previously in placentas (44) and in some specific loci in cancer (46). More importantly, we discovered that age-related hypomethylation was found at certain ages. Age-dependent Alu loss of methylation was significant from 34 to 68 years of age (Figure 5B, 6B and Supporting Table S1). The shape of the association between Alu methylation and age interval 34-68 yr had significant linear and curvilinear trends (Supporting Fig S2, Supporting Table S2). HERV-K lost methylation twice, during the 40-63 and 64-83 age intervals (Figure 5C, 6C and Supporting Table S1). The shape of association between HERV-K methylation and the age interval 40-83 yr did not fit with both linear and nonlinear models due to errors from curve fit having nonnormal distribution (Supporting Fig S3, Supporting Table S3). These data support the previous correlation results that the age-related hypomethylations of Alu and HERV-K do not occur via the same process.

Associations between LINE-1, Alu and HERV-K methylation levels in different age intervals

We analyzed the association between Alu and LINE-1 methylation to study how LINE-1 was not demethylated by age despite its methylation levels being directly correlated with Alu. Alu and LINE-1 methylation were not correlated at younger ages, but were significant correlated at ages 49 and older (r=0.49, $p<10^{-3}$) (Figure 7A and Supporting Table S4). These data support the previous conclusion that Alu methylation may be classified as both age-dependent and -independent. Moreover, the age-independent process may be the same mechanism as that for LINE-1. Alu and HERV-K methylation were directly and significantly correlated during the 40-59 age interval (Figure 7B and Supporting Table S4). Interestingly, LINE-1 and HERV-K were inversely correlated during the 44-68 age interval (Figure 7C and Supporting Table S4). Therefore, there may be a link between LINE-1 hypermethylation and HERV-K hypomethylation during these ages. Finally, there was direct association between LINE-1 and HERV-K during the 69-88 yr age interval (Figure 7C and Supporting Table S4). This may be similar to the association between Alu and LINE-1 at advanced ages.

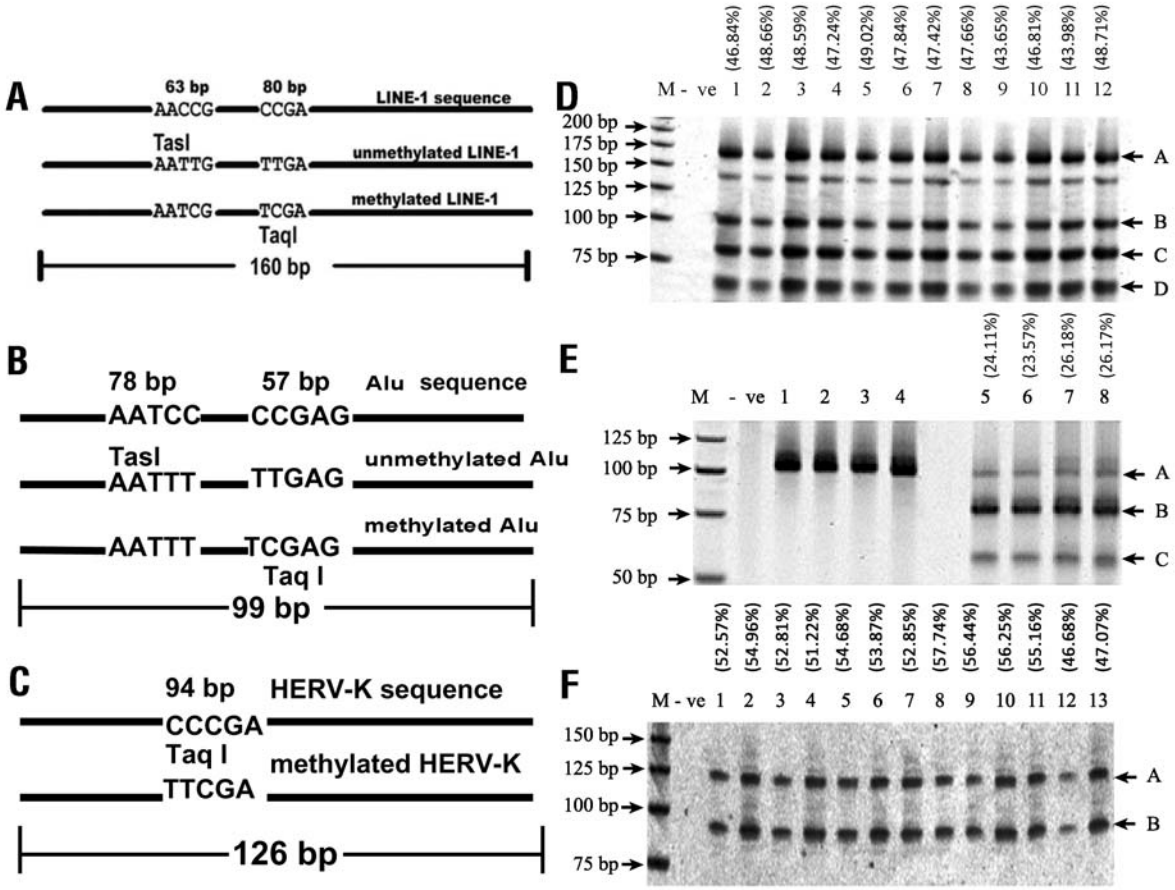
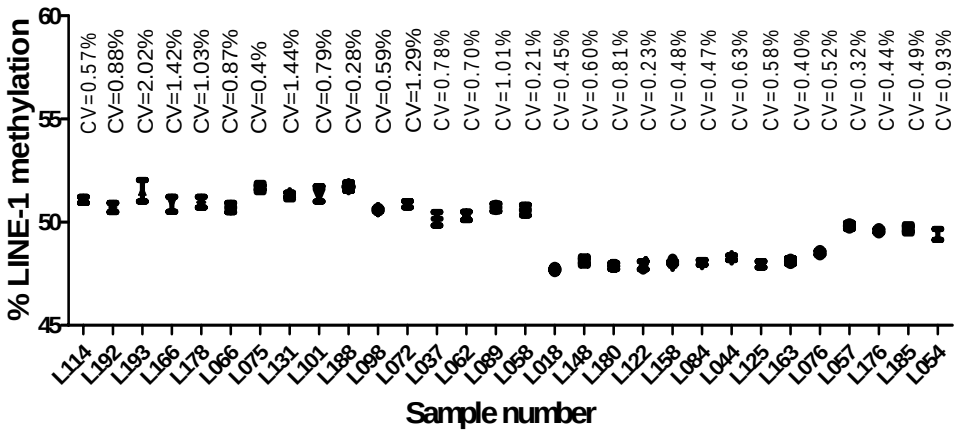
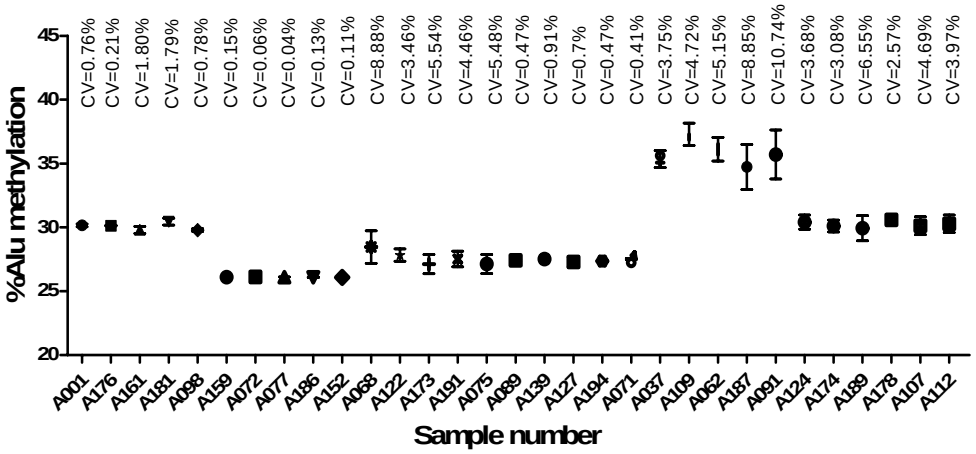


Figure 1 COBRA (Combined Bisulfite Restriction Analysis)-IRS of sodium bisulfite-treated PBMC DNA of healthy subjects. (A) and (B) COBRA of LINE-1. Unmethylated CpGs within AACCG sequences and methylated CpGs within CCGA sequences create *TasI*-specific AATT(G) and *TaqI*-specific TCGA sites, respectively, after sodium bisulfite treatment. Methylation levels can be assessed by *TasI*-*TaqI* double digestion within the 160-bp amplicon. The presence of an 80-bp fragment was yielded from *TaqI* digestion, whereas *TasI* digestion yielded 63- and 97-bp products. **(C) and (D) COBRA of Alu.** *TasI*-specific unmethylated AATTTT and *TaqI*-specific methylated TCGAG sites were created from the original AATCC and CCGAG sequences, respectively, of Alu after sodium bisulfite treatment. A 99-bp amplicon was cut and a 78-bp methylation-specific band was generated in the presence of *TasI,* and a 57-bp band was present with *TaqI* digestion when the CpG of the AATCC was methylated. **(E) and (F) COBRA of HERV-K.** A 126-bp amplicon was cut, resulting in 94- and 32-bp fragments when the CCGA sequences of the original bisulfite-treated HERV-K sequences were methylated and converted to TCGA sequences. M represents a DNA marker and –ve stands for negative (water and no template) control. Percentage of methylation is listed above each test.

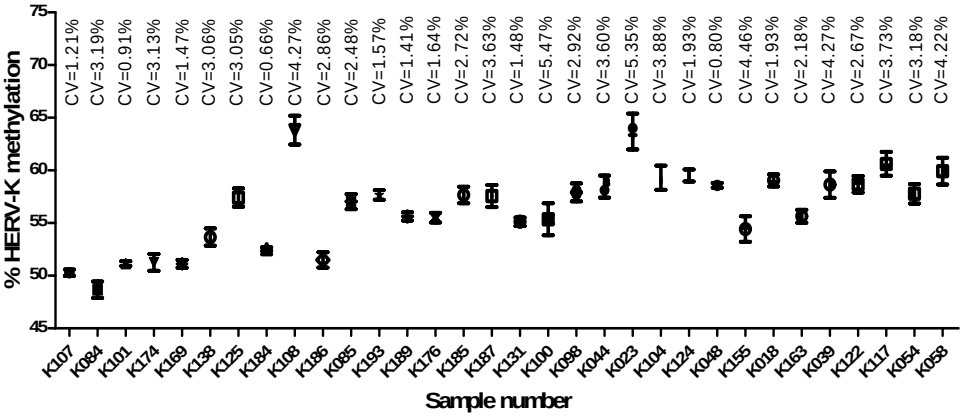
A



B



C



Supporting fig S1: Mean (±SE) methylation levels of LINE-1, Alu and HERV-K and coefficients of variance (%CV) between those measurements 31 cases were measured methylation levels of LINE-1 (A), Alu (C) and HERV-K (E) in four times and coefficients of variance (%CV) between those measurements were also analyzed. They were shown in figure A, B and C, respectively. Mean CV was 0.71% in LINE-1 (CV_{max}=2.02%), 3.16% in Alu (CV_{max}=10.74%) and 2.79% in HERV-K (CV_{max}= 5.47 %).

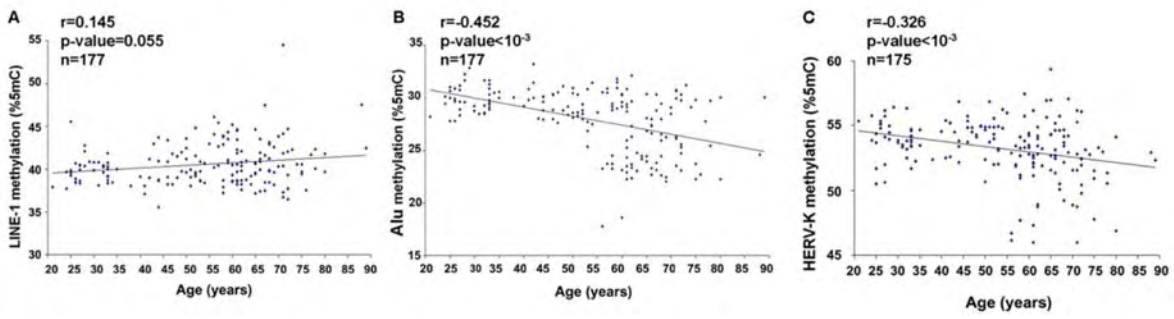


Figure 2 Association between age and IRS methylation of LINE-1 (A), Alu (B) and HERV-K (C). Significance of correlation coefficients (r) between age and IRS methylation was set at $p<0.05$. %5 mC is %5-methylcytosine.

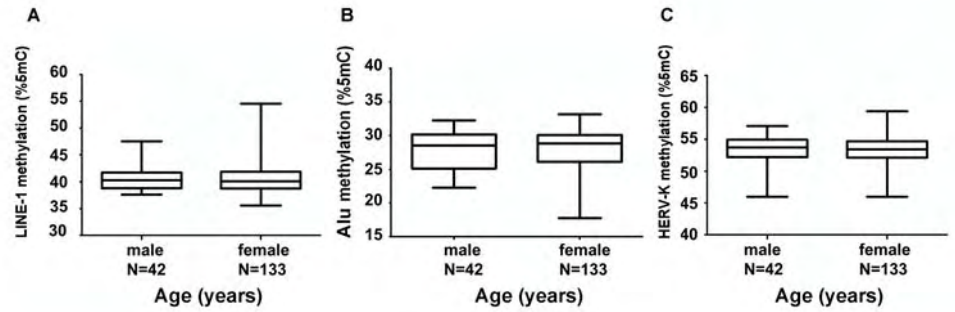


Figure 3 Methylation differences of LINE-1 (A), Alu (B) and HERV-K (C) in males and females. Comparisons of methylation levels were made between males and females. Error bars represent 95% confidence intervals. %5 mC is %5-methylcytosine.

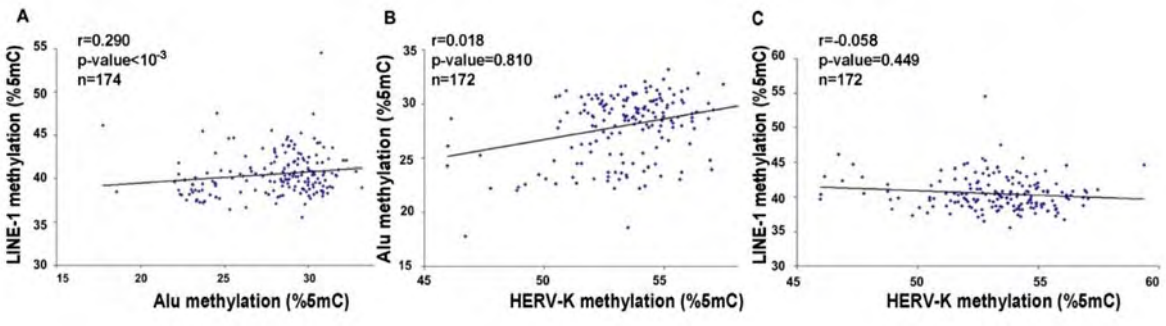


Figure 4 Association between methylation of repetitive elements. Each plot represents methylation levels of individuals, and error bars represent 95% confidence intervals. Pearson's correlation coefficients (r) with *p*-values are indicated. Alu and LINE-1 methylation levels (A) are significantly correlated ($p<0.0001$). In contrast, no correlation was observed either for Alu and HERV-K methylation levels (B) or for LINE-1 and HERV-K methylation levels (C). %5 mC is %5-methylcytosine.

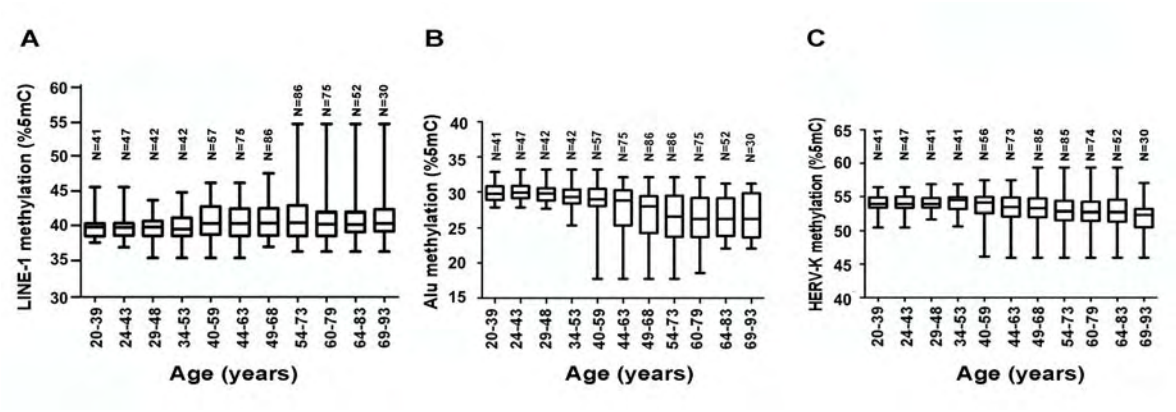


Figure 5 Changes in LINE-1 (A), Alu (B) and HERV-K (C) methylation levels in different age intervals. Error bars indicate ± SD, which was calculated using the averaged methylation level of all individuals in each group. %5 mC is %5-methylcytosine.

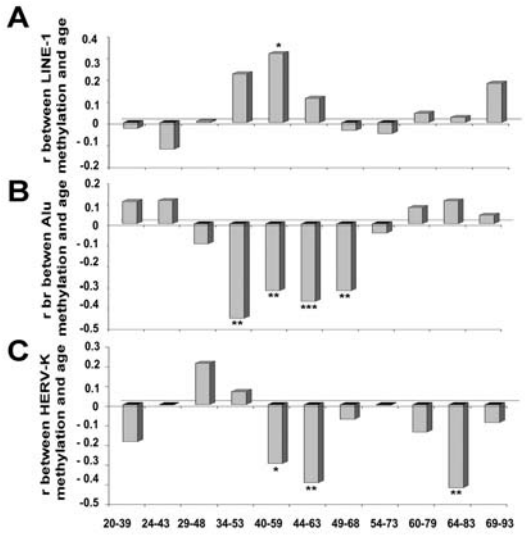


Figure 6 Association between age and IRS methylation (LINE-1 (A), Alu (B) and HERV-K (C)) in different age intervals. Different levels of significances are denoted by asterisks. *,p<0.05, **p<0.01 and ***p<0.001

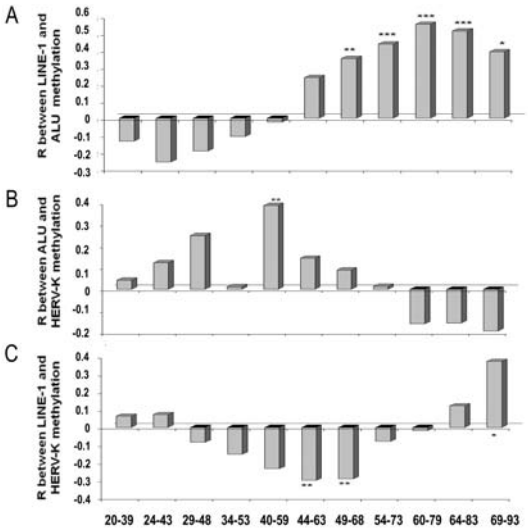


Figure 7 Pairwise association of methylation of IRS in different age intervals. LINE-1 and Alu (A), Alu and HERV-K (B), LINE-1 and HERV-K (C). Asterisks are used to denote different levels of significance; p<0.05 (*), 0.01 (**) and 0.001 (***).

Supporting Table S1: Pearson' correlation (r) between Alu methylation-age and HERV-K methylation-age in different age intervals

	Pearson'correlation (r)	p-value	Number of cases (n)
Alu methylation-age			
20-48 yrs	-0.046	0.727	61
34-68 yrs	-0.477	$<10^{-3***}$	110
54-93 yrs	-0.032	0.756	98
HERV-K methylation-age			
20-53 yrs	0.033	0.773	78
40-63 yrs	-0.363	0.001^{**}	80
49-79 yrs	-0.196	0.040	111
64-83 yrs	-0.423	0.002^{**}	52
69-93 yrs	-0.091	0.634	30

*** indicate significant statistics**

Supporting Table S2: Comparison R, R^2, and coefficient, constant (b_0, b_1, and …..) and significant of T-test in linear and curvilinear trends between Alu methylation and age range 34-68 yrs

	First order polynomial (straight line)	Second order polynomial (quadratic)	Third order polynomial (cubic)	Logarith	Inverse	Compound	Power	S	Growth	Lgstic	Exponent
Best-fit values			Ambiguous								
B_0	36.93±1.671	34.84±9.23	~ -10.62±45.97	62.07±6.19	19.83±1.46	38.82±2.52	99.12±23.8	3.02±0.06	3.66±0.07	0.03±0.002	38.82±2.52
B_1	-0.17±0.03	-0.09±0.35	~ 2.63±2.72	-8.59±1.54	424.24±78.36	0.99±0.001	-0.32±0.06	15.82±3.04	-0.006±0.001	1.01±0.001	-0.01±0.001
B_2		-0.001±0.003	~ -0.05±0.05								
B_3			~ 0.0003±0.0003								
Signif of t											
B_0	$<10^{-3}$	0.0003	0.0003	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
B_1	$<10^{-3}$	NS	NS	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	0.0001	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
B_2		NS	NS								
B_3			NS								
Goodness of Fit											
Degrees of Freedom	108	107	106	108	108	108	108	108	108	108	108
R square	0.228	0.228	0.235	0.223	0.214	0.214	0.2097	0.2	0.214	0.214	0.214
R	0.477	0.477	0.477	0.472	0.462	0.462	0.458	0.448	0.462	0.462	0.462
errors from curve fit	normal distribution	normal distribution	normal distribution	normal distribution	normal distribution	normal distribution	normal distribution	normal distribution	normal distribution	normal distribution	normal distribution

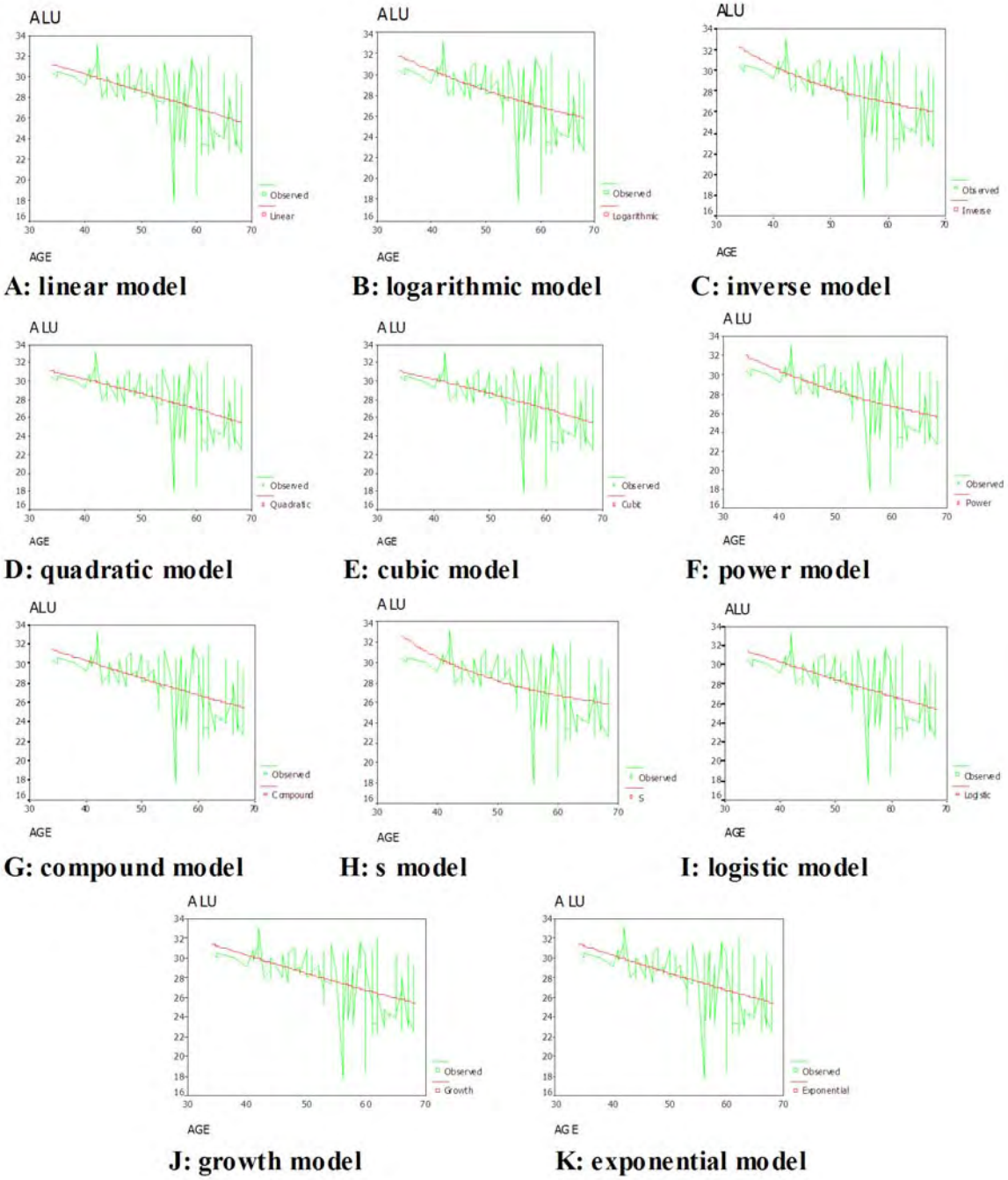
Supporting Table S3: Comparison R, R^2, coefficient, constant (b_0, b_1, and …..) and significance of T-test in linear and curvilinear trends between HERV-K methylation and age range 40-83 yrs

Curvilinear Regression	First order polynomial (straight line)	Second order polynomial (quadratic)	Third order polynomial (cubic)	Logarith	Inverse	Compound	Power	S	Growth	Lgstic	Exponent
Best-fit values											
B0	57.29±1.23	59.71±5.63	58.92±3.82	70.04±4.83	49.05±1.16	57.47±1.36	73.39±6.84	3.89±0.02	4.05±0.02	0.02±0.0004	57.47±1.36
B1	-0.07±0.02	-0.15±0.19	-0.11±0.09	-4.15±1.18	235.42±67.33	0.999±0.003	-0.08±0.02	4.52±1.3	-0.001±0.0003	1.001±0.0003	-0.001±0.0003
B2		0.0007±0.002	-1.35±-0.01								
B3			3.63E-06±8.04E-06								
Signif of t											
B0	0.0007	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	<10-^{3}	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
B1	$<10^{-3}$	NS	NS	$<10^{-3}$	0.001	$<10^{-3}$	0.001	0.001	0.001	$<10^{-3}$	0.001
B2		NS	NS								
B3			NS								
Goodness of Fit											
Degrees of Freedom	132	131	131	132	132	132	132	132	132	132	132
R square	0.09	0.09	0.09	0.09	0.09	0.09	0.09	0.084	0.083	0.083	0.083
R	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3		
errors from curve fit	non-normal distribution	non-normal distribution	non-normal distribution	non-normal distribution	non-normal distribution	non-normal distribution	non-normal distribution	non-normal distribution	non-normal distribution	non-normal distribution	non-normal distribution

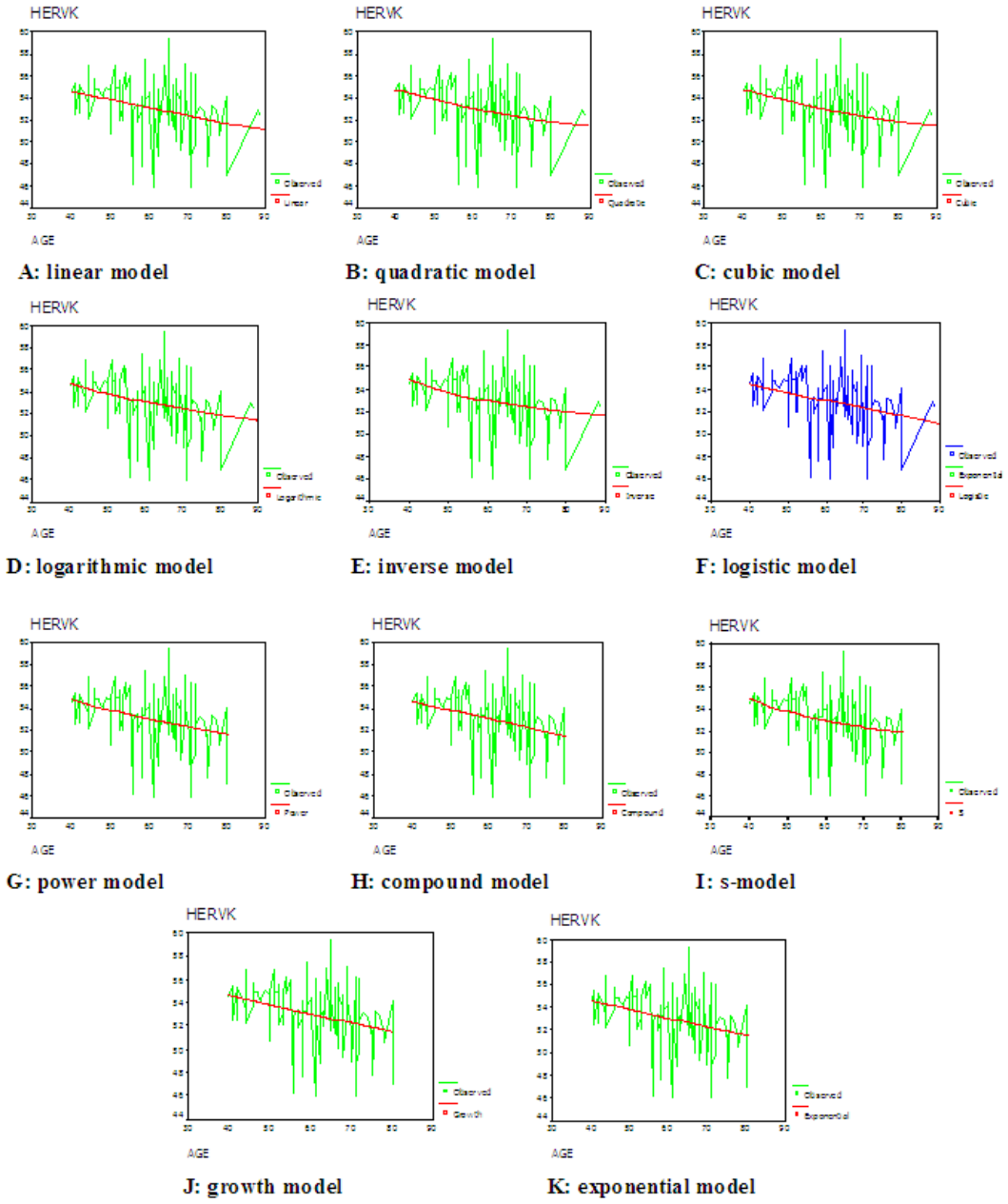
Supporting Table S4: The relationship between LINE-1-Alu methylation, Alu-HERV-K methylation and LINE-1-HERV-K methylation in various range of ages

	Pearson' correlation (r)	p-value	Number of cases (n)
LINE-1-Alu methylation			
20-59 yrs	-0.0887	0.388	95
20-63 yrs	0.1571	0.084	120
49-93 yrs	0.3793	$<10^{-3***}$	113
Alu-HERV-K methylation			
20-53 yrs	0.0133	0.909	75
40-59 yrs	0.3881	0.003**	53
44-93 yrs	0.0027	0.976	124
LINE-1-HERV-K methylation			
20-59 yrs	-0.191	0.062	94
44-68 yrs	-0.3070	0.002**	94
54-83 yrs	-0.0637	0.542	92
69-93 yrs	0.3750	0.045*	27

*** indicate significant statistics**



Supporting Fig S2: Comparison curve fits in each curvilinear models between Alu methylation and age range 34-68 yrs. Regression is able to reveal that there is a significant linear and curvilinear trends.



Supporting Fig S3: Comparison curve fit in each curvilinear models between HERV-K methylation and age range 40-83 yrs. The shape of association between HERV-K methylation and the age interval 40-83 yr did not fit with both linear and nonlinear models.

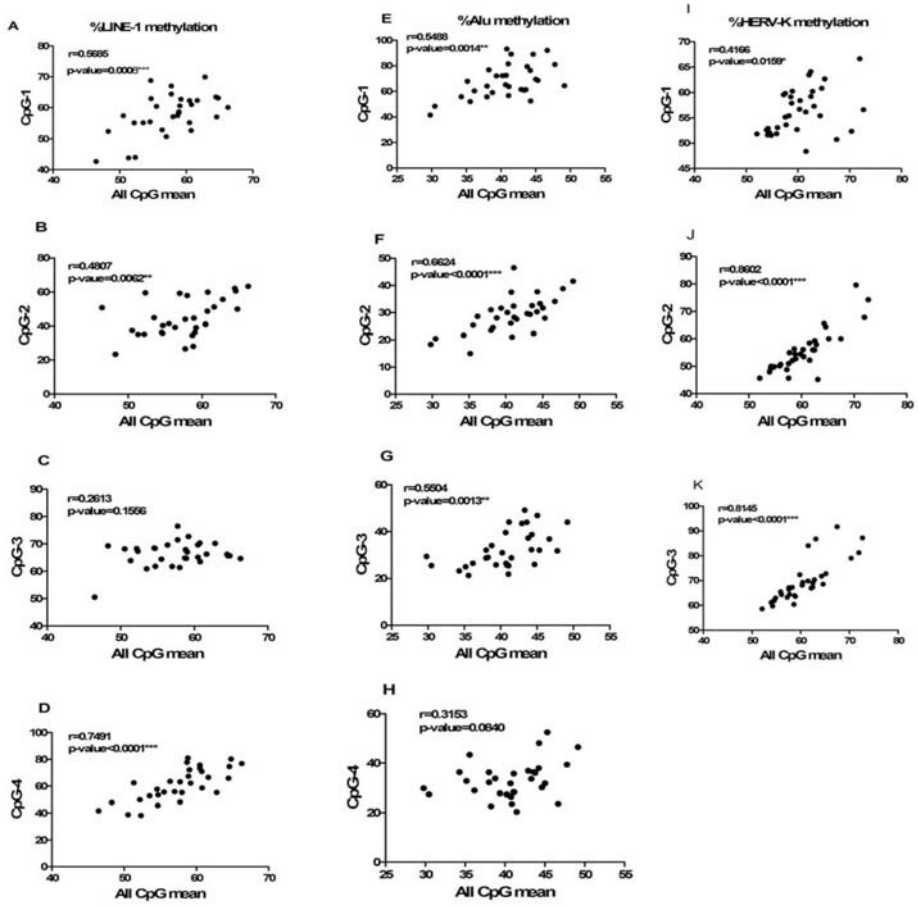
Discussion

COBRA-IRS

There is a premise that COBRA-IRS should have been more subject to error. COBRA detects methylation of only one CpG dinucleotide. If there is a mutation, for example C→T, the methylation status will be misinterpreted. COBRA-IRS; however, detects thousands of loci. Consequently, if a mutation does occur, it will not interfere with the interpretation. Moreover, methylation levels of each LINE-1 CpG dinucleotide is in positive linear correlation with the others on the same locus (46). Moreover, pyrosequencing demonstrated frequent positive linear correlations between methylation levels of means and each IRS CpG dinucleotides including TaqI CpG sites of COBRA-Alu, LINE-1 and HERV-K (Supporting Fig S4). Therefore, COBRA-IRS is an accurate and reliable technique. Up to now, there are several IRS methylation studies. Nonetheless, there has been no conflict result yielded because of different techniques (6, 8, 10, 11, 13, 15, 24, 25, 31, 39, 40, 43, 46, 49-51, 55). It is important to note that IRS methylation studies will not display absolute levels. Therefore, methylation levels between different IRS types or PCR protocols cannot be compared. IRS sequences vary. Therefore, each PCR protocol will target different number of IRS templates. Consequently, IRS methylation levels are relative numbers and the levels between samples can be compared only by the same detection protocol. As a result, we do not compare Alu (Fig 3B) or LINE-1 methylation levels in this study and Bollati et al (6).

Age- and cancer-related global hypomethylation

In the past, IRSs were wrongly called “junk DNA” because they were thought to not possess any physiological role (33). Consequently, it has been expected that, in general, the level of methylation at each IRS should represent genome-wide levels. Here, our data suggest that different types of IRSs lose methylation via different age-dependent mechanisms. While age-dependent hypomethylation of Alu and HERV-K was observed, hypomethylation of LINE-1 was not. This data does not conflict with previous findings. Although Bollati et al. reported a trend of LINE-1 hypomethylation with aging (6), they observed only borderline significant levels, unlike Alu. Several other reports did not identify an age-associated hypomethylation of LINE-1 (8, 18, 25). Instead of age-associated hypomethylation, LINE-1 hypomethylation is common in cancer cells (8, 10, 11, 13, 15, 19, 24, 25, 31, 39, 40, 43, 46, 49-51, 55). Interestingly, head and neck squamous cell carcinoma risk factors such as smoking could lower levels of LINE-1 methylation in peripheral blood cells (25). Finally, hypomethylation of both Alu or HERV-K and LINE-1 elements often coexist in cancer cells (10, 13, 19). Therefore, age- and cancer-related global hypomethylation are two distinctive processes.



Supporting Fig S4

The correlation between LINE-1 (A-D), Alu (E-H) and HERV-K (I-K) methylation levels of each CpG nucleotides and mean methylation levels of all CpG by pyrosequencing. D, E and J are TaqI COBRA-IRS sites and means methylation levels. The IRS methylation were quantitated by Pyro-sequencing using the primers and conditions as follows: LINE-1 primers were 10 pmol of forward primer 5'-GGTTAGGGAGTTTTTTTTTTGAG-3' and 10 pmol of reverse biotinylated primer 5'-TCAACGAAATT CCGTAAACGTA-3'. Alu primers were 10 pmol of forward primer 5'- GGcgcgGTGGtTtAcgttTGTAA-3', 10 pmol of reverse biotinylated primer 5'- CACCATATTAACCAAACTAATC***C***CgA-3'. HERV-K primers were 10 pmol of forward primer 5'-TGGGAAGGGAAAGATTTGAT-3' and 10 pmol of reverse biotinylated primer 5'-ACAAAAAACAAATACCTTCCTCTT-3'. The LINE-1, Alu and HERV-K in a 50 μl PCR reaction were quantitated using the PSQ HS96 Pyrosequencing System. The ratio of C to T nucleotides was evaluated for LINE-1, Alu and HERV-K methylation. Pyrosequencing analysis of bisulfite-converted genomic DNA encompassing subsets of Alu, LINE-1 and HERV-K loci were chosen by sampling from 31 cases with different age groups. Mean of all CpG-mean LINE-1 methylation was 57.51±4.897%, mean of all CpG-mean Alu methylation was 40.697±4.61%, and mean of all CpG-mean HERV-K methylation was 60.4±5.18%. All values were represented by multiply the peak height methylated by 100 and dividing by sum peak height methylated and peak height non-methylated. The methylation levels of each CpG nucleotides, including COBRA TaqI representative nucleotides of LINE1, Alu, and HERV-K, were commonly in direct correlation with the mean methylation levels.

Age-dependent and -independent mechanisms of IRS methylation

Age-dependent and -independent mechanisms of IRS methylation are complex. Because Alu and HERV-K losses of methylation are not occurred simultaneously, age-dependent hypomethylation of Alu and HERV-K may be independent events. Moreover, there is IRS methylation that is not age dependent. While hypomethylation of Alu and HERV-K is age-dependent, hypomethylation of LINE-1 is not. We found that Alu and HERV-K methylation was directly associated with LINE-1 methylation at older ages, which may be after age-related IRS loss of methylation has ended. Therefore, Alu methylation levels may be divided into two classes. The first reduced methylation in relation to age. The other persists and directly correlated with age independent LINE-1 methylation. Because LINE-1 hypomethylation is commonly associated with cancer (8, 10, 11, 13, 15, 19, 24, 25, 31, 39, 40, 43, 46, 49-51, 55), it is intriguing to hypothesize whether age-independent mechanisms of IRS methylation may prevent cancer development. It is possible that the same IRS element, such as Alu or HERV-K, may be methylated by both age-dependent and -independent mechanisms. Recently, we reported the methylation statuses of several LINE-1 loci and found some were resistant to cancer hypomethylation processes (46).

IRS methylation as epigenetic marks of aging

Several physiological and anatomical alterations occur during aging. These changes occur and progress at different rates at certain ages. We have demonstrated that Alu methylation decreased during ages 34-68 and HERV-K decreased during ages 40-63 yr and again during ages 64-83 yr. Both were also correlated within age intervals. This may be due to the loss of methylation processes of both IRSs occurring commonly at the period. Because genomic methylation was proposed to be related to several diseases (55), it would be interesting to determine whether hypomethylation of SINE and LTR is associated with age-related disabilities. At present, IRSs are no longer considered “junk DNA”. Instead, several lines of evidence suggest that SINEs, LINEs and LTRs are gene cis regulatory elements (12, 17, 26, 44, 45, and 47). Because SINEs, LINEs and HERVs produce RNA, DNA methylation should alter their gene regulation activities. In addition, IRS hypomethylation patterns associated with cancer and aging are different. Therefore, global hypomethylation in cancer and aging should lead to different cellular and physiological consequences. Finally, modifying epigenetic marks that contribute to disease development has been proposed as a novel therapeutic approach for disease treatment and/or prevention (1, 21, 37). This study may provide crucial information on future therapeutic targets for the prevention of aging or age-associated disabilities.

Acknowledgements

We thank all personals in Center for Excellence in Molecular Genetics of Cancer and Human Diseases, Department of Anatomy, Faculty of Medicine, Chulalongkorn University

Grants

This work was supported by the Thailand Research Fund (TRF) – MRG 5180072 and a TRF Senior Research Scholar grant.

References

1. Altucci L, and Minucci S. Epigenetic therapies in haematological malignancies: searching for true targets. *Eur J Cancer* 45: 1137-1145, 2009.
2. Asada K, Kotake Y, Asada R, Saunders D, Broyles RH, Towner RA, Fukui H, and Floyd RA. LINE-1 hypomethylation in a Choline-Deficiency-Induced Liver Cancer in Rats: Dependence on Feeding period. *J Biomed Biotechnol* 2006: 17142, 2006.
3. Baser K, Sunguroglu A, Bokesoy I, and Uysal A. Spontaneous micronuclei in cytokinesis-blocked bone marrow and peripheral blood lymphocytes of CML patients. *Tr J of Med Sci* 29: 125-128, 1999.
4. Batzer MA, and Deininger PL. Alu repeats and human genomic diversity. *Nature Reviews* 3: 370-380, 2002.
5. Belgnaoui SM, Gosden RG, Semmes OJ, and Haoudi A. Human LINE-1 retrotransposon induces DNA damage and apoptosis in cancer cells. *Cancer Cell International* 6: 1-10, 2006.
6. Bollati V, Schwatz J, Wright R, Litonjua A, Taratini L, Suh H, Sparrow D, Vokonas P, and Baccarelli A. Decline in genomic DNA methylation through aging in a cohort of elderly subjects *Mechanisms of Aging and Development* 130: 234-239, 2009.
7. Castro R, Rivera I, Struys EA, Jansen EEW, Ravasca P, Camilo ME, Blom HJ, Jakobs C, and Almeida ITD. Increased homocysteine and S-adenosylhomocysteine concentrations and DNA hypometylation in vascular disease. *Clinical Chemistry* 49: 1292-1296, 2003.
8. Chalitchagorn K, Shuangshoti S, Hourpai N, Kongruttanachok N, Tangkijvanich P, Thong-ngam D, Voravud N, Sriuranpong V, and Mutirangura A. Distinctive pattern of LINE-1 methylation level in normal tissues and the association with carcinogenesis. *Oncogene* 23: 8841-8846, 2004.
9. Cho NY, Kim BH, Choi M, Yoo EJ, Moon KC, Cho YM, Kim D, and Kang GH. Hypermethylation of CpG island loci and hypomethylation of LINE-1 and Alu repeats in prostate adenocarcinoma and their relationship to clinicopathological features. *J Pathol* 211: 269-277, 2007.
10. Choi IS, Estecio MRH, Nagano Y, Kim DH, White JA, Yao JC, Issa JPJ, and Rashid A. Hypomethylation of LINE-1 and Alu in well-differentiated neuroendocrine tumors (pancreatic endocrine tumors and carcinoid tumors). *Modern Pathology* 20: 802-810, 2007.
11. Choi SH, Worswick S, Byun HM, Shear T, Soussa JC, Wolff EM, Douer D, Garcia-Manero G, Liang GN, and Yang AS. Changes in DNA methylation of tandem DNA repeats are different from interspersed repeats in cancer. *International Journal of Cancer* 125: 723-729, 2009.
12. Conley AB, Piriyapongsa J, and Jordan IK. Retroviral promoters in the human genome. *Bioinformatics* 24: 1563-1567, 2008.
13. Daskalos A, Nikolaidis G, Xinarianos G, Savvari P, Cassidy A, Zakopoulou R, Kotsinas A, Gorgoulis V, Field JK, and Liloglou T. Hypomethylation of retrotransposable elements correlates with genomic instability in non-small cell lung cancer. *International Journal of Cancer* 124: 81-87, 2009.
14. Deininger PL, Moran JV, Batzer MA, and Kazazian JHH. Mobile elements and mammalian genome evolution. *Current Opinion in Genetics&development* 13: 651-658, 2003.
15. Ehrlich M. Cancer-linked DNA hypomethylation and its relationship to hypermethylation. *Curr Top Microbiol Immunol* 310: 251-274, 2006.

16. Estecio MR, Gharibyan V, Shen L, Ibrahim AE, Doshi K, He R, Jelinek J, Yang AS, Yan PS, Huang TH, Tajara EH, and Issa JP. LINE-1 hypomethylation in cancer is highly variable and inversely correlated with microsatellite instability. *PLoS One* 2: e399, 2007.
17. Faulkner GJ, Kimura Y, Daub CO, Wani S, Plessy C, Irvine KM, Schroder K, Cloonan N, Steptoe AL, Lassmann T, Waki K, Hornig N, Arakawa T, Takahashi H, Kawai J, Forrest AR, Suzuki H, Hayashizaki Y, Hume DA, Orlando V, Grimmond SM, and Carninci P. The regulated retrotransposon transcriptome of mammalian cells. *Nat Genet* 41: 563-571, 2009.
18. Figueiredo JC, Grau MV, Wallace K, Levine AJ, Shen L, Hamdan R, Chen X, Bresalier RS, McKeown-Eyssen G, Haile RW, Baron JA, and Issa J-PJ. Global DNA Hypomethylation (LINE-1) in the Normal Colon and Lifestyle Characteristics and Dietary and Genetic Factors. *Cancer Epidemiology Biomarkers & Prevention* 18: 1041-1049, 2009.
19. Flori AR, Lower R, Schmitz-Drager BJ, and Schulz WA. DNA methylation and expression of LINE-1 and HERV_K provirus sequences in urothelial and renal cell carcinomas. *British Journal of Cancer* 80: 1312-1321, 1999.
20. Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, Heine-Suñer D, Cigudosa JC, Urioste M, Benitez J, Boix-Chornet M, Sanchez-Aguilera A, Ling C, Carlsson E, Poulsen P, Vaag A, Stephan Z, Spector TD, Wu Y-Z, Plass C, and Esteller M. Epigenetic differences arise during the lifetime of monozygotic twins. *Proc Natl Acad Sci Sci USA* 102: 10604-10609, 2005.
21. Graham JS, Kaye SB, and Brown R. The promises and pitfalls of epigenetic therapies in solid tumours. *Eur J Cancer* 45: 1129-1136, 2009.
22. Guz J, Foksinski M, Siomek A, Gackowski D, Rozalski R, Dziaman T, Szpila A, and Olinski R. The relationship betwen 8-oxo-7,8-dihydro-2'-deoxyguanosine level and extent of cytosine methylation in leukocytes DNA of healthy subjects and in patients with colon adenomas and carcinomas. *Mutation research* 640: 170-173, 2008.
23. Hoal-van Helden EG, and Van Helden PD. Age-related methylation changes in DNA may reflect the proliferative potential of organs *Mutation research* 219: 263-266, 1989.
24. Hoffmann MJ, and Schulz WA. Causes and consequences of DNA hypomethylation in human cancer. *Biochem Cell Biol* 83: 296-321, 2005.
25. Hsiung DT, Marsit CJ, Houseman A, Eddy K, Furniss CS, McClean MD, and Kelsey KT. Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. *Cancer Epidemiol Biomakers Prev* 16: 108-114, 2007.
26. Huh JW, Kim DS, Kang DW, Ha HS, Ahn K, Noh YN, Min DS, Chang KT, and Kim HS. Transcriptional regulation of GSDML gene by antisense-oriented HERV-H LTR element. *Arch Virol* 153: 1201-1205, 2008.
27. Issa J-P. Aging, DNA methylation and cancer. *Critical Reviews in Oncology/Hematology* 32: 31-43, 1999.
28. Jordan K, Rogozin IB, Glazko GV, and Koonin EV. Origin of a substantial fraction of human regulatory sequences from transposable elements. *Trends in Genetics* 19: 68-72, 2003.

29. Kazazian HH, Jr., and Moran JV. The impact of L1 retrotransposons on the human genome. *Nat Genet* 19: 19-24, 1998.
30. Kim JH, Cho NY, Kim BH, Kim WY, Kim BS, Moon KC, and Kang GH. The Relationship between the Methylenetetrahydrofolate Reductase Genotypes and the Methylation Status of the CpG Island Loci, LINE-1 and Alu in Prostate Adenocarcinoma. *Korean Journal of Pathology* 43: 26-35, 2009.
31. Kim MJ, White-Cross JA, Shen LL, Issa JPJ, and Rashid A. Hypomethylation of long interspersed nuclear element-1 in hepatocellular carcinomas. *Modern Pathology* 22: 442-449, 2009.
32. Lee HS, Kim BH, Cho NY, Yoo EJ, Choi M, Shin SH, Jang JJ, Suh KS, Kim YS, and Kang GH. Prognostic implications of and relationship between CpG island hypermethylation and repetitive DNA hypomethylation in hepatocellular carcinoma. *Clin Cancer Res* 15: 812-820, 2009.
33. Makalowski W. The human genome structure and organization. *Acta Biochim Pol* 48: 587-598, 2001.
34. Mansour A. Epigenetic activation of genomic retrotransposon. *Journal of cell and molecular biology* 6: 99-107, 2007.
35. Mills RE, Bennett EA, Iskow RC, and Devine SE. Which transposable elements are active in human genome? *Trends in Genetic* 123: 183-191, 2007.
36. Moore LE, Pfeiffer RM, Poscablo C, Real FX, Kogevinas M, Silverman D, Garcia-Closas R, Chanock S, Tardon A, Serra C, Carrato A, Dosemeci M, Garcia-Closas M, Esteller M, Fraga M, Rothman N, and Malats N. Genomic DNA hypomethylatio as a marker for bladder cancer susceptibility in the Spanish Bladder Cancer Study:a case-control study. *Lancet Oncol* 9: 359-366 2008.
37. Moss TJ, and Wallrath LL. Connections between epigenetic gene silencing and human disease. *Mutat Res* 618: 163-174, 2007.
38. Mutirangura A. Quantitative PCR analysis for methylation level of genome: clinical implications in cancer. *Asian Biomedicine* 1: 121-128, 2007.
39. Ogino S, Kawasaki T, Nosho K, Ohnishi M, Suemoto Y, Kirkner GJ, and Fuchs CS. LINE-1 hypomethylation is inversely associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer. *International Journal of Cancer* 122: 2767-2773, 2008.
40. Ogino S., Nosho K, Kirkner GJ, Kawasaki T, Chan AT, Schernhammer ES, Giovannucci EL, and Fuchs CS. A Cohort Study of Tumoral LINE-1 Hypomethylation and Prognosis in Colon Cancer. *Journal of the National Cancer Institute* 100: 1734-1738, 2008.
41. Pal A, Srivastava T, Sharma MK, Mehndiratta M, Das P, Sinha S, and Chattopadhyay P. Aberrant methylation and associated transcriptional mobilization of Alu elements contributes to genomic instability in hypoxia. *J Cell Mol Med* 2009.
42. Park SY, Yoo EJ, Cho NY, Kim N, and Kang GH. Comparison of CpG island hypermethylation and repetitive DNA hypomethylation in premalignant stages of gastric cancer, stratified for Helicobacter pylori infection. *J Pathol* 2009.
43. Pattamadilok J, Huapai N, Rattanatanyong P, Vasurattana A, Triratanachat S, Tresukosol D, and Mutirangura A. LINE-1 hypomethylation level as a potential prognostic factor for epithelial ovarian cancer. *Int J Gynecol Cancer* 18: 2008.

44. Perrin D, Ballestar E, Fraga MF, Frappart L, Esteller M, Guerin JF, and Dante R. Specific hypermethylation of LINE-1 elements during abnormal overgrowth and differentiation of human placenta. *Oncogene* 26: 2518-2524, 2007.
45. Pheasant M, and Mattick JS. Raising the estimate of functional human sequences. *Genome Research* 17: 1245-1253, 2007.
46. Phokaew C, Subbalekha K, Kowudtitham S, Shuangshoti S, and Mutirangura A. LINE-1 methylation patterns of differernt loci in normal and cancerous cells. *Nucleic Acids Research* 36: 5704-5712, 2008.
47. Piriyapongsa J, Polavarapu N, Borodovsky M, and McDonald J. Exonization of the LTR transposable elements in human genome. *BMC Genomics* 8: 291, 2007.
48. Romanov GA, and Vanyushin BF. Effects of the tissue type, age, malignancy and hormonal induction. *Biochim Biophys Acta* 653: 204-218, 1981.
49. Schulz WA, Steinhoff C, and Florl AR. Methylation of endogenous human retroelements in health and disease. *Curr Top Microbiol Immunol* 310: 211-250, 2006.
50. Shuangshoti S, Hourpai N, Pulmsuk U, and Mutirangura A. Line-1 hypomethylation in multistage carcinogenesis of the uterine cervix. *Asian Pacific Journal of Cancer Prevention* 8: 307-309, 2007.
51. Tangkijvanich P, Hourpai N, Rattanatanyong P, Wisedopas N, Mahachai V, and Mutirangura A. Serum LINE-1 hypomethylation as a potential prognostic marker for hepatocellular carcinoma. *Clin Chim Acta* 379: 127-133, 2007.
52. Todorovska E. Retrotransposons and their role in plant-genome evolution. *Biotechnol&Biotechnol* 21: 293-305, 2007.
53. Vanyushin BF, Nemirovsky LE, Klimenko VV, Vasiliev VK, and Belozersky AN. The 5-methylcytosine in DNA of rats. Tissue and age specificity and the changes induced by hydrocortisone and other agents. . *Gerontologia* 19: 138-152, 1973.
54. Vanyushin BF, Tkacheva SG, and Belozersky AN. Rare bases in animal DNA. *Rare bases in animal DNA* 225: 948-949, 1970.
55. Wilson AS, Power BE, and Molloy PL. DNA hypomethylation and human diseases *Biochim Biophys Acta* 1775: 138-162, 2007.
56. Wilson VL, and Jones PA. DNA methylation decreases in aging but not in immortal cells. *Science* 220: 1055-1057, 1983.
57. Wilson VL, Smith RA, Ma S, and Cutler RG. Genomic 5-Methyldeoxycytidine decreases with age. *The Journal of Biological Chemistry* 262: 9948-9951, 1987.
58. Xiong Z, and Laird PW. COBRA:a sensitive and quantitative DNA methylation assay. *Nucleic Acids Research* 25: 2532-2534, 1997.
59. Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, and Issa JP. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Res* 32: e38, 2004.
60. Yi J-M, and Kim H-S. Molecular phylogenetic analysis of the human endogenous retrovirus E (HERV-E) family in human tissues and human cancers. *Genes Genet Syst* 82: 89-98, 2007.

61. Youn LJ, Zhe J, and Bin T. Phylogenetic analysis of mRNA polyadenylation sites reveals a role of transposable elements in evolution of the 3'-end of genes. *Nucleic acid research* 36: 5581-5590, 2008.
62. Zvetkova I, Apedaile A, Ramsahoye B, Mermoud JE, Crompton LA, John R, Feil R, and Brockdorff N. Global hypomethylation of the genome in XX embryonic stem cells. *Nat Genet* 37: 1274-1279, 2005.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

Pornrutsami Jintaridth and Apiwat Mutirangura. Distinctive patterns of age-dependent hypomethylation in interspersed repetitive sequences. Physiol Genomics 41:194-200, 2010. (impact factor 3.46)

2. การนำผลงานวิจัยไปใช้ประโยชน์

-เชิงวิชาการ: มีการพัฒนาการเรียนการสอน โดยได้นำผลงานวิจัยไปใช้ประกอบการสอนนักศึกษาในระดับปริญญาเอก คณะเวชศาสตร์เขตร้อน ในหัวข้อ “Epigenetic” และในวิชา “Seminar”

-เชิงวิจัย: นำผลงานวิจัยที่ได้รับไปต่อยอดและใช้อ้างอิงเพื่อไปสู่งานวิจัยอื่นๆในอนาคต

3. การเสนอผลงานในที่ประชุมวิชาการ

3.1 ประชุมวิชาการนานาชาติ 4th Asian Epigenomics Meeting ที่ประเทศสิงคโปร์ จัดโดย Genome Institute of Singapore โดยเสนอผลงานประเภทโปสเตอร์ และได้รับรางวัล Young Scientist Travel Award

3.2 ประชุมวิชาการนานาชาติ Joint International Tropical Medicine Meeting (JITMM 2009) ที่ประเทศไทย จัดโดยคณะเวชศาสตร์เขตร้อน

ภาคผนวก

Physiol Genomics 41: 194–200, 2010.
First published February 9, 2010; doi:10.1152/physiolgenomics.00146.2009.

Distinctive patterns of age-dependent hypomethylation in interspersed repetitive sequences

Pornrutsami Jintaridth (พรรัศม์ จินตฤทธิ์)[1] and Apiwat Mutirangura (อภิวัฒน์ มุทิรางกูร)[2]

[1]*Department of Tropical Nutrition and Food Science, Faculty of Tropical Medicine, Mahidol University; and* [2]*Center for Excellence in Molecular Genetics of Cancer and Human Diseases, Department of Anatomy, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand*

Submitted 28 August 2009; accepted in final form 3 February 2010

Jintaridth P, Mutirangura A. Distinctive patterns of age-dependent hypomethylation in interspersed repetitive sequences. *Physiol Genomics* 41: 194–200, 2010. First published February 9, 2010; doi:10.1152/physiolgenomics.00146.2009.—Interspersed repetitive sequences (IRSs) are a major contributor to genome size and may contribute to cellular functions. IRSs are subdivided according to size and functionally related structures into short interspersed elements, long interspersed elements (LINEs), DNA transposons, and LTR-retrotransposons. Many IRSs may produce RNA and regulate genes by a variety of mechanisms. The majority of DNA methylation occurs in IRSs and is believed to suppress IRS activities. Global hypomethylation, or the loss of genome-wide methylation, is a common epigenetic event not only in senescent cells but also in cancer cells. Loss of LINE-1 methylation has been characterized in many cancers. Here, we evaluated the methylation levels of peripheral blood mononuclear cells of LINE-1, Alu, and human endogenous retrovirus K (HERV-K) in 177 samples obtained from volunteers between 20 and 88 yr of age. Age was negatively associated with methylation levels of Alu ($r = -0.452$, $P < 10^{-3}$) and HERV-K ($r = -0.326$, $P < 10^{-3}$) but not LINE-1 ($r = 0.145$, $P = 0.055$). Loss of methylation of Alu occurred during ages 34–68 yr, and loss of methylation of HERV-K occurred during ages 40–63 yr and again during ages 64–83 yr. Interestingly, methylation of Alu and LINE-1 are directly associated, particularly at ages 49 yr and older ($r = 0.49$, $P < 10^{-3}$). Therefore, only some types of IRSs lose methylation at certain ages. Moreover, Alu and HERV-K become hypomethylated differently. Finally, there may be several mechanisms of global methylation. However, not all of these mechanisms are age-dependent. This finding may lead to a better understanding of not only the biological causes and consequences of genome-wide hypomethylation but also the role of IRSs in the aging process.

global hypomethylation; genome-wide methylation; aging; interspersed repetitive sequences

GLOBAL HYPOMETHYLATION, or the decreasing of genome-wide DNA methylation, is a common epigenetic alteration in senescent cells (23, 56, 57). Overall, total deoxymethylcytosine levels tend to decrease with aging in most vertebrate tissues, including the brain, liver, small intestine mucosa, heart, and spleens of salmon, mice, rats, cows and cattle, and aged human peripheral blood cells (7, 22, 23, 25, 36, 48, 53, 54, 57). Interestingly, although global hypomethylation is related to the replicative senescence associated with aging (56), it is also a common feature of cells with limitless replication potential, including stem cells (62) and cancer cells (8). Age-dependent global hypomethylation has been shown to be associated with many diseases (20, 27, 49, 55). Moreover, in cancer, global hypomethylation is associated with more advanced stages and poor prognosis (30, 31, 36, 39, 40, 43, 50, 51). Therefore, it is important to understand how the same epigenetic characteristic is associated with a variety of cellular phenotypes.

Interspersed repetitive sequences (IRSs) are a major contributor to genome size, accounting for ~45% of human genomic DNA (35, 45). Under normal circumstances, IRSs are methylated to variable degrees depending on their location (46). Therefore, loss of IRS methylation was believed to be the major contributor of global hypomethylation (8). IRSs can be subdivided by size and the presence of transposable elements. Commonly known IRSs are short interspersed elements (SINEs) being <500 bp, long interspersed elements (LINEs) being 6–8 Kb, long terminal repeat (LTR)-retrotransposons, and DNA transposons (14). SINEs, LINEs, and LTR-retrotransposons possess transcriptional activity, and several reports have suggested they are cis regulatory elements (28, 34, 52, 61). Therefore, changes in SINE, LINE, or LTR-retrotransposon methylation should alter cellular functions. Because cellular biology of cancer and aging are different, we hypothesized that the age-dependent pattern of methylation losses in SINEs, LINEs, and LTRs should be different.

Alu is the most abundant SINE (4, 14). Human endogenous retroviruses (HERVs) are a major subset of LTR-retrotransposons (5, 14, 47). LINE-1, or L1, is the most abundant and well-known LINE (14, 29). The methylation status of Alu, LINE-1, and HERV-K, a well-known member of HERV, has been reported for many cancers (5, 8, 16, 19, 50, 51, 55, 60). Recently, Bollati et al. (6) reported an age-related methylation of peripheral blood mononuclear cells (PBMC) of Alu and LINE-1. They found a significant association between age and Alu hypomethylation, but the significance of the association between age and LINE-1 hypomethylation was borderline (6). In the present study, we describe patterns of methylation alternations in Alu, LINE-1, and HERV-K. Our analysis suggests that IRSs lose methylation at a certain age. Moreover, different IRSs lose methylation differently. Finally, there may be several global methylation mechanisms and not all of them are dependent on age.

MATERIALS AND METHODS

Subjects. Our study population consisted of 42 men and 135 women aged 20–90 yr. Healthy volunteer subjects in the urban area of Bangkok were recruited for sample collection according to the following criteria: each individual *1*) agreed to participate in the study and gave informed consent, *2*) had no history of chronic or genetic diseases, *3*) had a body mass index <30 kg/m^2, *4*) had no history of smoking and alcohol drinking 10 yr prior to sample collection,

Address for reprint requests and other correspondence: A. Mutirangura, Center for Excellence in Molecular Genetics of Cancer and Human Diseases, Dept. of Anatomy, Chulalongkorn Univ., Bangkok, Thailand (e-mail: mapiwat@chula.ac.th).

5) engaged in no supplementation with vitamins for 3 mo before study, and *6*) had normal living and eating habits. This study was approved by the ethics committee of the Faculty of Tropical Medicine, Mahidol University.

Blood collection. Heparinized blood was collected, and PBMC were prepared by Ficoll density gradient centrifugation according to published methods (3). Blood hematological parameters and blood chemistries of participants were analyzed. Volunteers with normal hematological parameters and blood chemistries were chosen to participate. DNA methylation in PBMC from healthy subjects of different ages was measured. Hematological data and the amount of PBMC in whole blood were not significantly different between individuals.

DNA extraction and modified combined bisulfite restriction analysis. DNA was extracted from PBMC using QiAmp DNA blood kits (Qiagen, Hilden, Germany). A total of 200 ng DNA (concentration 4 ng/μl) was used for bisulfite treatment. The combined bisulfite restriction analysis (COBRA) consisted of a standard sodium bisulfite PCR treatment followed by restriction digestion and quantitation (58). Bisulfite modification of genomic DNA was performed using previously published methods (8). In brief, 200 ng of DNA was dissolved in 50 μl distilled water and then denatured in 5.5 μl of 2 M NaOH for 10–30 min at 37°C. Next, 30 μl of freshly prepared 10 mM hydroquinone (Sigma) and 520 μl of 3 M bisulfite (pH 5.0) were added and mixed. The samples were incubated at 50°C for 16 h. The bisulfite-treated DNA was isolated using the Wizard DNA Clean-up System. The DNA was eluted with 50 μl of warm water and desulfonated with 5.5 μl of 3 M NaOH for 5 min. The DNA was precipitated (NH_4OAC-EtOH) using glycogen as a carrier and resuspended in 20 μl of water. Bisulfite-treated DNA was stored at −20°C until ready for use.

COBRA-IRS. COBRA is a standard technique for measuring methylation levels of IRSs (2, 8, 9, 25, 32, 38, 41–43, 50, 51, 59). A schematic representation and examples of COBRA of LINE-1, Alu, and HERV-K are shown in Fig. 1. The primer sequences that correspond to the nucleotides in the regulatory region of the LINE-1 sequence (GenBank: M80343) are forward (F), 5′-CGT AAG GGC TTA GGG AGT TTT T-3′ and reverse (R), 5′-(AG)TA AAA CCC TCC (AG)AA CCA AAT ATA AA-3′ (8). The PCR reactions consisted of 35 cycles of 95°C for 1 min, 53°C for 1 min, and 72°C for 1 min. The PCR products were subsequently digested with 2 U of *TaqI* (MBI Fermentas) and 2 U of *TasI* (MBI Fermentas) in TE buffer 3 (Biolab) at 65°C overnight and were then run on an 8% nondenaturing polyacrylamide gel. The gel was stained with SyBr Green, and band intensities were measured by PhosphoImager using Image Quant software (Molecular Dynamics). The Alu primer sequences, which correspond to the nucleotides of the Alu Sx subfamily sequence (4), are F, 5′-GG(T/C) G(C/T)G GTG GTT TA(C/T) GTT TGT AA-3′ and R, 5′-CAC CAT ATT AAC CAA ACT AAT CCC GA-3′. Alu PCR conditions and restriction digestion conditions were similar to those of LINE-1. The HERV-K primer sequences (GenBank accession number AF394944.1), which correspond to the nucleotides in the regulatory region of the HERV-K sequence, are F, 5′-TGG GAA GGG AAA GAT TTG AT-3′ and R, 5′-ACA AAA AAC AAA TAC CTT CCT CTT-3′. The PCR reactions consisted of 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The PCR products were then digested with 2 U of *TaqI* (MBI Fermentas) and in TE buffer 3

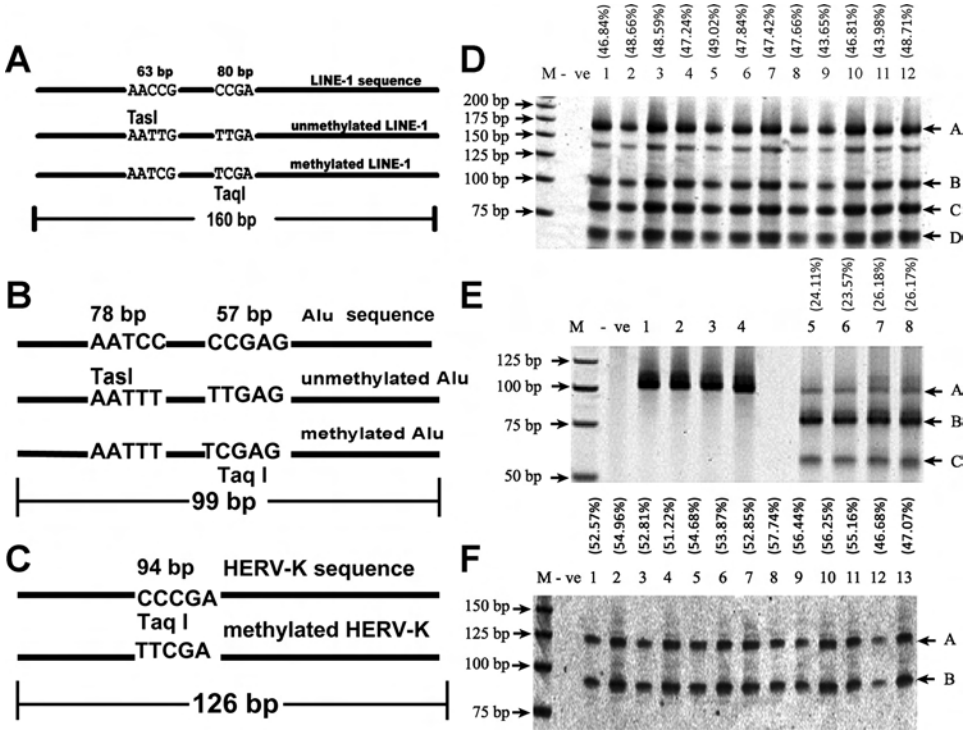


Fig. 1. Combined bisulfite restriction analysis-interspersed repetitive sequence (COBRA-IRS) of sodium bisulfite-treated peripheral blood mononuclear cell (PBMC) DNA of healthy subjects. *A* and *B*: COBRA of long intersperse element (LINE)-1. Unmethylated CpGs within AACCG sequences and methylated CpGs within CCGA sequences create *TasI*-specific AATT(G) and *TaqI*-specific TCGA sites, respectively, after sodium bisulfite treatment. Methylation levels can be assessed by *TasI*-*TaqI* double digestion within the 160 bp amplicon. The presence of an 80 bp fragment was yielded from *TaqI* digestion, whereas *TasI* digestion yielded 63 and 97 bp products. *C* and *D*: COBRA of Alu. *TasI*-specific unmethylated AATTTT and *TaqI*-specific methylated TCGAG sites were created from the original AATCC and CCGAG sequences, respectively, of Alu after sodium bisulfite treatment. A 99 bp amplicon was cut and a 78 bp methylation-specific band was generated in the presence of *TasI*, and a 57 bp band was present with *TaqI* digestion when the CpG of the AATCC was methylated. *E* and *F*: COBRA of human endogenous retrovirus (HERV)-K. A 126 bp amplicon was cut, resulting in 94 and 32 bp fragments when the CCGA sequences of the original bisulfite-treated HERV-K sequences were methylated and converted to TCGA sequences. M, DNA marker; -ve, negative (water and no template) control. Percentage of methylation is listed above each test.

(Biolab) at 65°C overnight and were run on an 8% nondenaturing polyacrylamide gel. The gel was stained with SyBr Green, and band intensities were measured by PhosphoImager using Image Quant software (Molecular Dynamics). COBRA LINE-1, Alu, and HERV-K amplicons were 160, 99, and 126 bp, respectively. After digestion, the LINE-1, Alu, and HERV-K methylated bands were 80, 57, and 94 bp, respectively. Unmethylated bands of LINE-1 were 97 and 63 bp, and the unmethylated band of Alu was 78 bp. Methylation levels were calculated as the intensity of methylated bands divided by the sum of the methylated and unmethylated bands. Each COBRA-IRS was performed two to four times. To ensure that measurements of IRS methylation from band intensities of samples reduced interassay variation, we used Daudi, Jurkat, HeLa, and Molt4 cell lines from the same stocks as a control to validate the interassay variation. IRS methylation levels of the stock cell lines was standardized, and IRS methylation in each experiment was adjusted so that all experiments had the same control IRS methylation levels. The average differences in IRS methylation of the LINE-1, Alu, and HERV-K repetitive elements between adults of different ages were evaluated. IRS methylation of LINE-1, Alu, and HERV-K repetitive elements were also analyzed for associations with age intervals. Associations between IRS methylation of LINE-1, Alu, and HERV-K in each group of age intervals were also determined.

Statistical analysis. Data were analyzed using SPSS statistical software. The Mann-Whitney *U*-test was used to make nonparametric comparisons of the median methylation levels of LINE-1, Alu, and HERV-K between males and females. To analyze different age intervals, ages were divided into 20 yr intervals, and the age interval of each group overlapped adjacent intervals by 15 yr. Some groups were not normally distributed. The averages of methylation levels of LINE-1, Alu, and HERV-K in each group were determined as the median value and the Kruskal-Wallis test was used to make nonparametric comparisons between the groups. To evaluate the correlations between ages and IRS methylation levels in the population and groups of age intervals, Pearson and Spearman correlations were used to determine associated 95% confidence intervals. The associations between LINE-1, Alu, and HERV-K relative methylation levels in the population and groups of age intervals were examined while controlling for confounding variable such as age. Partial correlation was used to determine 95% confidence intervals. All tests were two-tailed analyses.

RESULTS

Association between age and methylation of LINE-1, Alu, and HERV-K. This study consisted of PBMC of 177 study subjects aged between 20 and 90 yr old (mean age 52.92 yr, SE = 1.19). Methylation levels of LINE-1, Alu, and HERV-K were measured by COBRA (2, 8, 9, 25, 32, 38, 41–43, 50, 51, 59). Examples of COBRA-IRS were demonstrated (Fig. 1, *A–F*). Our tests produced limited result deviations (Supporting Fig. S1).[1]

First, we evaluated the relationship between IRS methylation levels and age. LINE-1 methylation was not associated with age ($r = 0.145$, $P = 0.055$) (Fig. 2*A*). However, DNA methylation of both Alu and HERV-K was inversely correlated with age ($r = -0.452$, $P < 10^{-3}$ for Alu; $r = -0.326$, $P < 10^{-3}$ for HERV-K) (Fig. 2, *B* and *C*). Therefore, age-related genomic hypomethylation occurred at only specific types of IRSs, i.e., Alu and HERV-K, but not LINE-1. We then evaluated whether sex influences IRS methylation. Similar to a previous report (8), there were no differences in the methylation of LINE-1, Alu, or HERV-K between males and females (Fig. 3, *A–C*).

Association between LINE-1, Alu, and HERV-K methylation. We evaluated the correlations of methylation levels between IRSs. Because only Alu and HERV-K are demethylated with age, we expected a positive correlation exclusively between Alu and HERV-K. In contrast, we found a significant association between LINE-1 and Alu ($r = 0.290$, $P < 10^{-4}$) or ($r = 0.272$, $P < 10^{-3}$ when excluding outlier) (Fig. 4*A*). There was no association between LINE-1 and HERV-K methylation ($r = -0.058$, $P = 0.45$) (Fig. 4*B*). Unexpectedly, Alu and HERV-K methylation were also not correlated ($r = 0.018$, $P = 0.81$) (Fig. 4*C*).

Changes in LINE-1, Alu, and HERV-K methylation levels in different age intervals. To determine the certain age at which hypomethylation occurs, we grouped samples according to age into 20 yr intervals, with the intervals of group overlapping each adjacent interval by 15 yr (Figs. 5–7). Figure 5, *A*, *B*, and *C*, shows the median, minimum, and maximum of LINE-1, Alu, and HERV-K methylation, respectively, of each interval. Figure 6 shows the r values of the correlation between IRS methylation and each interval of age. No significant LINE-1 losses of methylation were observed in any age, but levels of LINE-1 methylation might increase during ages 40–59 yr ($r = 0.32$, $P = 0.02$) (Fig. 6*A*). An increase of LINE-1 methylation has been seen previously in placentas (44) and in some specific loci in cancer (46). More importantly, we discovered that age-related hypomethylation was found at certain ages. Age-dependent Alu loss of methylation was significant from 34 to

[1] The online version of this article contains supplemental material.

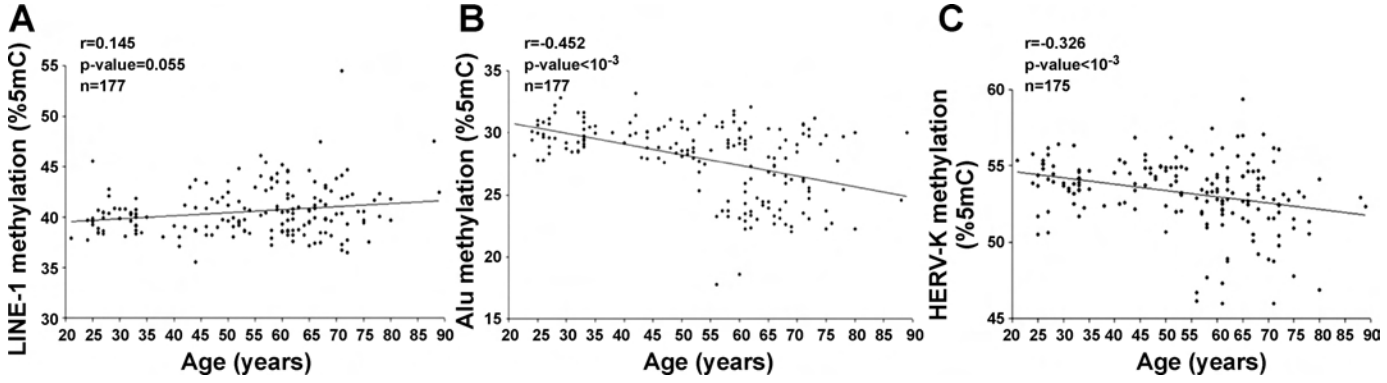


Fig. 2. Association between age and IRS methylation of LINE-1 (*A*), Alu (*B*), and HERV-K (*C*). Significance of correlation coefficients (r) between age and IRS methylation was set at $P < 0.05$. %5 mC, %5-methylcytosine.

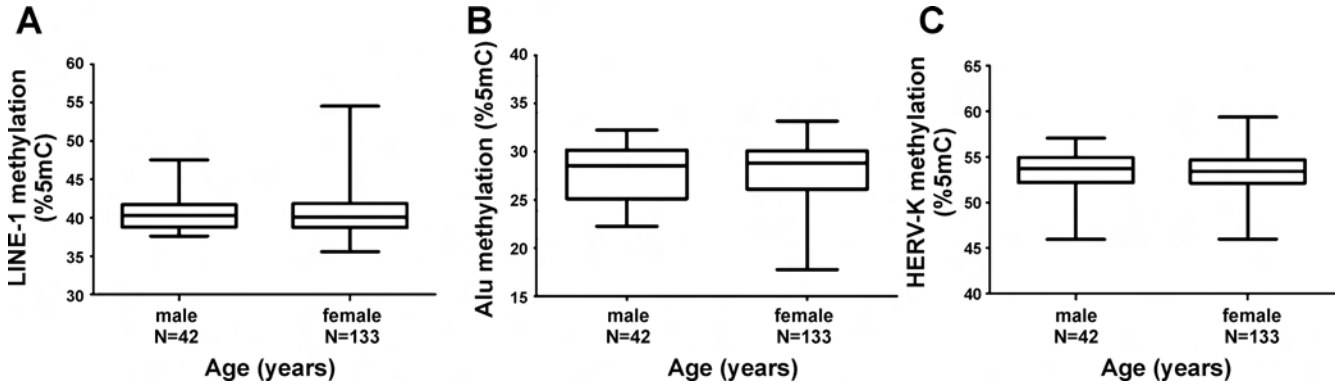


Fig. 3. Methylation differences of LINE-1 (*A*), Alu (*B*), and HERV-K (*C*) in males and females. Comparisons of methylation levels were made between males and females. Error bars represent 95% confidence intervals. %5 mC, %5-methylcytosine.

68 yr of age ($r = -0.477$, $P < 10^{-3}$) (Figs. 5*B*, 6*B*, and Supporting Table S1). The shape of the association between Alu methylation and the age interval 34–68 yr had significant linear and curvilinear trends (Supporting Fig. S2, Supporting Table S2). HERV-K lost methylation twice, during the 40–63 and 64–83 age intervals (Figs. 5*C*, 6*C*, and Supporting Table S1). The shape of association between HERV-K methylation and the age interval 40–83 yr did not fit with both linear and nonlinear models due to errors from curve fit having nonnormal distribution (Supporting Fig. S3, Supporting Table S3). These data support the previous correlation results that the age-related hypomethylations of Alu and HERV-K did not occur via the same process.

Associations between LINE-1, Alu, and HERV-K methylation levels in different age intervals. We analyzed the association between Alu and LINE-1 methylation to study how LINE-1 was not demethylated by age despite its methylation levels being directly correlated with Alu. Alu and LINE-1 methylation were not correlated at younger ages but were significantly correlated at ages 49 yr and older ($r = 0.49$, $P < 10^{-3}$) (Fig. 7*A* and Supporting Table S4). These data support the previous conclusion that Alu methylation may be classified as both age-dependent and -independent. Moreover, the age-independent process may be the same mechanism as that for LINE-1. Alu and HERV-K methylation were directly and significantly correlated during the 40–59 yr age interval (Fig. 7*B* and Supporting Table S4). Interestingly, LINE-1 and HERV-K were inversely correlated during the 44–68 yr age interval (Fig. 7*C* and Supporting Table S4). Therefore, there may be a link between LINE-1 hypermethylation and HERV-K hypomethylation during these ages. Finally, there was direct association between LINE-1 and HERV-K during the 69–88 yr age interval (Fig. 7*C* and Supporting Table S4). This may be similar to the association between Alu and LINE-1 at advanced ages.

DISCUSSION

COBRA-IRS. There is a premise that COBRA-IRS should have been more subject to error. COBRA detects methylation of only one CpG dinucleotide. If there is a mutation, for example C→T, the methylation status will be misinterpreted. COBRA-IRS, however, detects thousands of loci. Consequently, if a mutation does occur, it will not interfere with the interpretation. Moreover, methylation levels of each LINE-1 CpG dinucleotide is in positive linear correlation with the others on the same locus (46). Moreover, pyrosequencing demonstrated frequent positive linear correlations between methylation levels of means and each IRS CpG dinucleotide, including TaqI CpG sites of COBRA-Alu, LINE-1, and HERV-K (Supporting Fig. S4). Therefore, COBRA-IRS is an accurate and reliable technique. Up to now, there are several

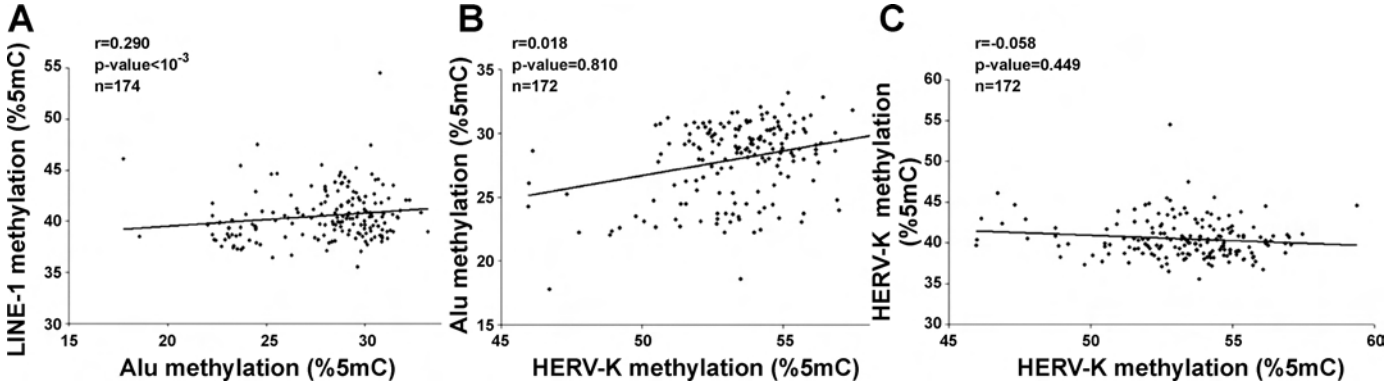


Fig. 4. Association between methylation of repetitive elements. Each plot represents methylation levels of individuals, and error bars represent 95% confidence intervals. Pearson's correlation coefficients (r) with *P* values are indicated. Alu and LINE-1 methylation levels (*A*) are significantly correlated ($P < 0.0001$). In contrast, no correlation was observed either for Alu and HERV-K methylation levels (*B*) or for LINE-1 and HERV-K methylation levels (*C*). %5 mC, %5-methylcytosine.

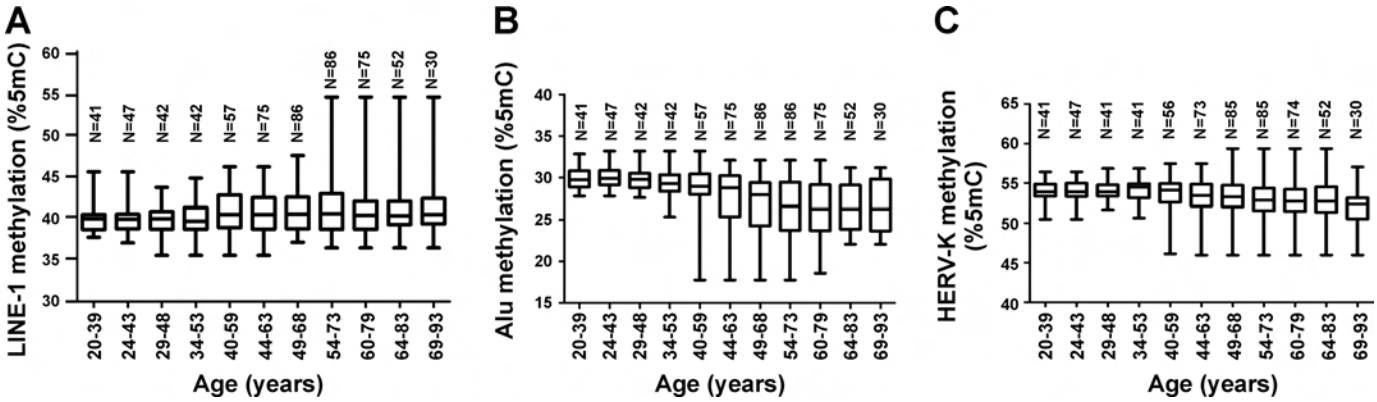


Fig. 5. Changes in LINE-1 (*A*), Alu (*B*), and HERV-K (*C*) methylation levels in different age intervals. Error bars indicate ± SD, which was calculated using the averaged methylation level of all individuals in each group. %5 mC, %5-methylcytosine.

IRS methylation studies. Nonetheless, there has been no conflict result yielded because of different techniques (6, 8, 10, 11, 13, 15, 24, 25, 31, 39, 40, 43, 46, 49–51, 55). It is important to note that IRS methylation studies will not display absolute levels. Therefore, methylation levels between different IRS types or PCR protocols cannot be compared. IRS sequences vary. Therefore, each PCR protocol will target different number of IRS templates. Consequently, IRS methylation levels are relative numbers and the levels between samples can be compared only by the same detection protocol. As a result, we do not compare Alu (Fig. 3*B*) or LINE-1 methylation levels in this study with Bollati et al. (6).

Age- and cancer-related global hypomethylation. In the past, IRSs were wrongly called "junk DNA" because they were thought not to possess any physiological role (33). Consequently, it has been expected that, in general, the level of methylation at each IRS should represent genome-wide levels. Here, our data suggest that different types of IRSs lose methylation via different age-dependent mechanisms. While age-dependent hypomethylation of Alu and HERV-K was observed, hypomethylation of LINE-1 was not. These data do not conflict with previous findings. Although Bollati et al. (6) reported a trend of LINE-1 hypomethylation with aging, they observed only borderline significant levels, unlike Alu. Several other reports did not identify an age-associated hypomethyla-

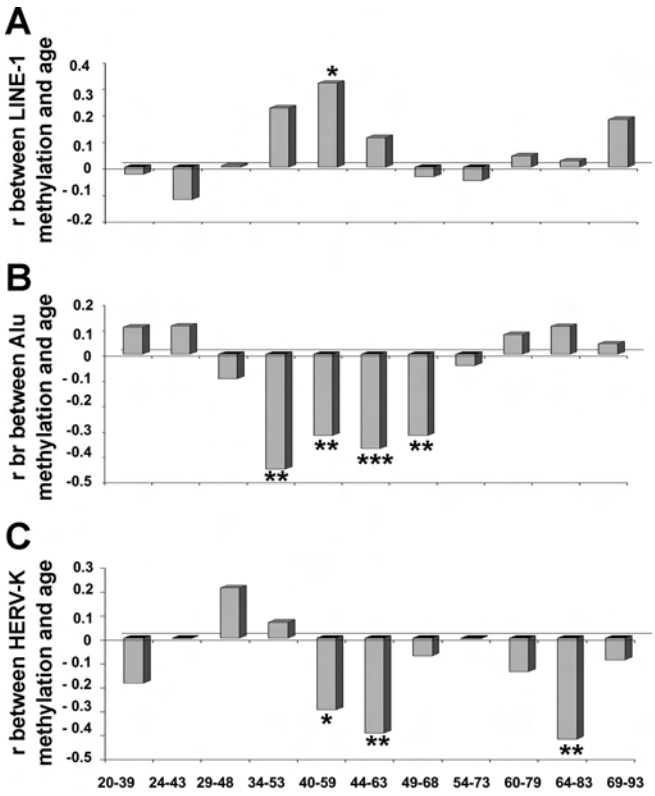


Fig. 6. Association between age and IRS methylation [LINE-1 (*A*), Alu (*B*), and HERV-K (*C*)] in different age intervals. Different levels of significance are denoted by asterisks: *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

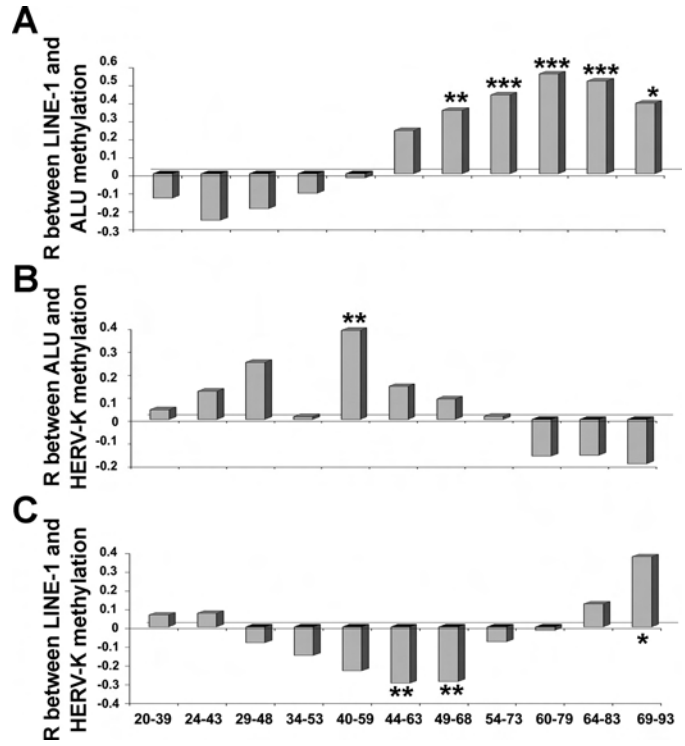


Fig. 7. Pair-wise association of methylation of repetitive elements in different age intervals. LINE-1 and Alu (*A*), Alu and HERV-K (*B*), LINE-1 and HERV-K (*C*). Asterisks are used to denote different levels of significance: $P < 0.05$ (*), 0.01 (**), and 0.001 (***).

tion of LINE-1 (8, 18, 25). Instead of age-associated hypomethylation, LINE-1 hypomethylation is common in cancer cells (8, 10, 11, 13, 15, 19, 24, 25, 31, 39, 40, 43, 46, 49–51, 55). Interestingly, head and neck squamous cell carcinoma risk factors such as smoking could lower levels of LINE-1 methylation in peripheral blood cells (25). Finally, hypomethylation of both Alu or HERV-K and LINE-1 elements often coexists in cancer cells (10, 13, 19). Therefore, age- and cancer-related global hypomethylation are two distinctive processes.

Age-dependent and -independent mechanisms of IRS methylation. Age-dependent and -independent mechanisms of IRS methylation are complex. Because Alu and HERV-K losses of methylation do not occur simultaneously, age-dependent hypomethylation of Alu and HERV-K may be independent events. Moreover, there is IRS methylation that is not age-dependent. While hypomethylation of Alu and HERV-K is age-dependent, hypomethylation of LINE-1 is not. We found that Alu and HERV-K methylation was directly associated with LINE-1 methylation at older ages, which may be after age-related IRS loss of methylation has ended. Therefore, Alu methylation levels may be divided into two classes. The first reduced methylation in relation to age. The other persists and is directly correlated with age-independent LINE-1 methylation. Because LINE-1 hypomethylation is commonly associated with cancer (8, 10, 11, 13, 15, 19, 24, 25, 31, 39, 40, 43, 46, 49–51, 55), it is intriguing to hypothesize whether age-independent mechanisms of IRS methylation may prevent cancer development. It is possible that the same IRS element, such as Alu or HERV-K, may be methylated by both age-dependent and -independent mechanisms. Recently, we reported the methylation statuses of several LINE-1 loci and found some were resistant to cancer hypomethylation processes (46).

IRS methylation as epigenetic marks of aging. Several physiological and anatomical alterations occur during aging. These changes occur and progress at different rates at certain ages. We have demonstrated that Alu methylation decreased during ages 34–68 yr and HERV-K decreased during ages 40–63 yr and again during ages 64–83 yr. Both were also correlated within age intervals. This may be due to the loss of methylation processes of both IRSs occurring commonly at the period. Because genomic methylation was proposed to be related to several diseases (55), it would be interesting to determine whether hypomethylation of SINE and LTR is associated with age-related disabilities. At present, IRSs are no longer considered junk DNA. Instead, several lines of evidence suggest that SINEs, LINEs, and LTRs are gene cis regulatory elements (12, 17, 26, 44, 45, 47). Because SINEs, LINEs, and HERVs produce RNA, DNA methylation should alter their gene regulation activities. In addition, IRS hypomethylation patterns associated with cancer and aging are different. Therefore, global hypomethylation in cancer and aging should lead to different cellular and physiological consequences. Finally, modifying epigenetic marks that contribute to disease development has been proposed as a novel therapeutic approach for disease treatment and/or prevention (1, 21, 37). This study may provide crucial information on future therapeutic targets for the prevention of aging or age-associated disabilities.

ACKNOWLEDGMENTS

We thank all personnel in the Department of Tropical Nutrition and Food Science, Faculty of Tropical Medicine, Mahidol University; Genetic Analysis Professional Center, Faculty of Medicine, Chulalongkorn University; and Center for Excellence in Molecular Genetics of Cancer and Human Diseases, Department of Anatomy, Faculty of Medicine, Chulalongkorn University.

GRANTS

This work was supported by the Thailand Research Fund (TRF) Grant MRG 5180072 and a TRF Senior Research Scholar grant.

DISCLOSURES

All authors have no potential conflict of interest.

REFERENCES

1. **Altucci L, Minucci S.** Epigenetic therapies in haematological malignancies: searching for true targets. *Eur J Cancer* 45: 1137–1145, 2009.
2. **Asada K, Kotake Y, Asada R, Saunders D, Broyles RH, Towner RA, Fukui H, Floyd RA.** LINE-1 hypomethylation in a choline-deficiency-induced liver cancer in rats: dependence on feeding period. *J Biomed Biotechnol* 2006: 17142, 2006.
3. **Baser K, Sunguroglu A, Bokesoy I, Uysal A.** Spontaneous micronuclei in cytokinesis-blocked bone marrow and peripheral blood lymphocytes of CML patients. *Tr J Med Sci* 29: 125–128, 1999.
4. **Batzer MA, Deininger PL.** Alu repeats and human genomic diversity. *Nat Rev* 3: 370–380, 2002.
5. **Belgnaoui SM, Gosden RG, Semmes OJ, Haoudi A.** Human LINE-1 retrotransposon induces DNA damage and apoptosis in cancer cells. *Cancer Cell Intl* 6: 1–10, 2006.
6. **Bollati V, Schwatz J, Wright R, Litonjua A, Taratini L, Suh H, Sparrow D, Vokonas P, Baccarelli A.** Decline in genomic DNA methylation through aging in a cohort of elderly subjects. *Mech Aging Dev* 130: 234–239, 2009.
7. **Castro R, Rivera I, Struys EA, Jansen EEW, Ravasca P, Camilo ME, Blom HJ, Jakobs C, Almeida ITD.** Increased homocysteine and S-adenosylhomocysteine concentrations and DNA hypometylation in vascular disease. *Clin Chem* 49: 1292–1296, 2003.
8. **Chalitchagorn K, Shuangshoti S, Hourpai N, Kongruttanachok N, Tangkijvanich P, Thong-ngam D, Voravud N, Sriuranpong V, Mutirangura A.** Distinctive pattern of LINE-1 methylation level in normal tissues and the association with carcinogenesis. *Oncogene* 23: 8841–8846, 2004.
9. **Cho NY, Kim BH, Choi M, Yoo EJ, Moon KC, Cho YM, Kim D, Kang GH.** Hypermethylation of CpG island loci and hypomethylation of LINE-1 and Alu repeats in prostate adenocarcinoma and their relationship to clinicopathological features. *J Pathol* 211: 269–277, 2007.
10. **Choi IS, Estecio MRH, Nagano Y, Kim DH, White JA, Yao JC, Issa JPJ, Rashid A.** Hypomethylation of LINE-1 and Alu in well-differentiated neuroendocrine tumors (pancreatic endocrine tumors and carcinoid tumors). *Mod Pathol* 20: 802–810, 2007.
11. **Choi SH, Worswick S, Byun HM, Shear T, Soussa JC, Wolff EM, Douer D, Garcia-Manero G, Liang GN, Yang AS.** Changes in DNA methylation of tandem DNA repeats are different from interspersed repeats in cancer. *Intl J Cancer* 125: 723–729, 2009.
12. **Conley AB, Piriyapongsa J, Jordan IK.** Retroviral promoters in the human genome. *Bioinformatics* 24: 1563–1567, 2008.
13. **Daskalos A, Nikolaidis G, Xinarianos G, Savvari P, Cassidy A, Zakopoulou R, Kotsinas A, Gorgoulis V, Field JK, Liloglou T.** Hypomethylation of retrotransposable elements correlates with genomic instability in non-small cell lung cancer. *Intl J Cancer* 124: 81–87, 2009.
14. **Deininger PL, Moran JV, Batzer MA, Kazazian JHH.** Mobile elements and mammalian genome evolution. *Curr Opin Genet Dev* 13: 651–658, 2003.
15. **Ehrlich M.** Cancer-linked DNA hypomethylation and its relationship to hypermethylation. *Curr Top Microbiol Immunol* 310: 251–274, 2006.
16. **Estecio MR, Gharibyan V, Shen L, Ibrahim AE, Doshi K, He R, Jelinek J, Yang AS, Yan PS, Huang TH, Tajara EH, Issa JP.** LINE-1 hypomethylation in cancer is highly variable and inversely correlated with microsatellite instability. *PLoS One* 2: e399, 2007.
17. **Faulkner GJ, Kimura Y, Daub CO, Wani S, Plessy C, Irvine KM, Schroder K, Cloonan N, Steptoe AL, Lassmann T, Waki K, Hornig N, Arakawa T, Takahashi H, Kawai J, Forrest AR, Suzuki H, Hayashizaki Y, Hume DA, Orlando V, Grimmond SM, Carninci P.** The regulated retrotransposon transcriptome of mammalian cells. *Nat Genet* 41: 563–571, 2009.

18. **Figueiredo JC, Grau MV, Wallace K, Levine AJ, Shen L, Hamdan R, Chen X, Bresalier RS, McKeown-Eyssen G, Haile RW, Baron JA, Issa JPJ.** Global DNA hypomethylation (LINE-1) in the normal colon and lifestyle characteristics and dietary and genetic factors. *Cancer Epidem Biomarkers Prev* 18: 1041–1049, 2009.
19. **Flori AR, Lower R, Schmitz-Drager BJ, Schulz WA.** DNA methylation and expression of LINE-1 and HERV_K provirus sequences in urothelial and renal cell carcinomas. *Br J Cancer* 80: 1312–1321, 1999.
20. **Fraga MF, Ballestar E, Paz MF, Ropero S, Setien F, Ballestar ML, Heine-Suñer D, Cigudosa JC, Urioste M, Benitez J, Boix-Chornet M, Sanchez-Aguilera A, Ling C, Carlsson E, Poulsen P, Vaag A, Stephan Z, Spector TD, Wu YZ, Plass C, Esteller M.** Epigenetic differences arise during the lifetime of monozygotic twins. *Proc Natl Acad Sci USA* 102: 10604–10609, 2005.
21. **Graham JS, Kaye SB, Brown R.** The promises and pitfalls of epigenetic therapies in solid tumours. *Eur J Cancer* 45: 1129–1136, 2009.
22. **Guz J, Foksinski M, Siomek A, Gackowski D, Rozalski R, Dziaman T, Szpila A, Olinski R.** The relationship between 8-oxo-7,8-dihydro-2′-deoxyguanosine level and extent of cytosine methylation in leukocytes DNA of healthy subjects and in patients with colon adenomas and carcinomas. *Mutat Res* 640: 170–173, 2008.
23. **Hoal-van Helden EG, Van Helden PD.** Age-related methylation changes in DNA may reflect the proliferative potential of organs. *Mutat Res* 219: 263–266, 1989.
24. **Hoffmann MJ, Schulz WA.** Causes and consequences of DNA hypomethylation in human cancer. *Biochem Cell Biol* 83: 296–321, 2005.
25. **Hsiung DT, Marsit CJ, Houseman A, Eddy K, Furniss CS, McClean MD, Kelsey KT.** Global DNA methylation level in whole blood as a biomarker in head and neck squamous cell carcinoma. *Cancer Epidemiol Biomakers Prev* 16: 108–114, 2007.
26. **Huh JW, Kim DS, Kang DW, Ha HS, Ahn K, Noh YN, Min DS, Chang KT, Kim HS.** Transcriptional regulation of GSDML gene by antisense-oriented HERV-H LTR element. *Arch Virol* 153: 1201–1205, 2008.
27. **Issa JP.** Aging, DNA methylation and cancer. *Crit Rev Oncol/Hematol* 32: 31–43, 1999.
28. **Jordan K, Rogozin IB, Glazko GV, Koonin EV.** Origin of a substantial fraction of human regulatory sequences from transposable elements. *Trends Genet* 19: 68–72, 2003.
29. **Kazazian HH Jr, Moran JV.** The impact of L1 retrotransposons on the human genome. *Nat Genet* 19: 19–24, 1998.
30. **Kim JH, Cho NY, Kim BH, Kim WY, Kim BS, Moon KC, Kang GH.** The relationship between the methylenetetrahydrofolate reductase genotypes and the methylation status of the CpG island Loci, LINE-1 and Alu in prostate adenocarcinoma. *Kor J Pathol* 43: 26–35, 2009.
31. **Kim MJ, White-Cross JA, Shen LL, Issa JPJ, Rashid A.** Hypomethylation of long interspersed nuclear element-1 in hepatocellular carcinomas. *Mod Pathol* 22: 442–449, 2009.
32. **Lee HS, Kim BH, Cho NY, Yoo EJ, Choi M, Shin SH, Jang JJ, Suh KS, Kim YS, Kang GH.** Prognostic implications of and relationship between CpG island hypermethylation and repetitive DNA hypomethylation in hepatocellular carcinoma. *Clin Cancer Res* 15: 812–820, 2009.
33. **Makalowski W.** The human genome structure and organization. *Acta Biochim Pol* 48: 587–598, 2001.
34. **Mansour A.** Epigenetic activation of genomic retrotransposon. *J Cell Mol Biol* 6: 99–107, 2007.
35. **Mills RE, Bennett EA, Iskow RC, Devine SE.** Which transposable elements are active in human genome? *Trends Genet* 123: 183–191, 2007.
36. **Moore LE, Pfeiffer RM, Poscablo C, Real FX, Kogevinas M, Silverman D, Garcia-Closas R, Chanock S, Tardon A, Serra C, Carrato A, Dosemeci M, Garcia-Closas M, Esteller M, Fraga M, Rothman N, Malats N.** Genomic DNA hypomethylation as a marker for bladder cancer susceptibility in the Spanish Bladder Cancer Study: a case-control study. *Lancet Oncol* 9: 359–366, 2008.
37. **Moss TJ, Wallrath LL.** Connections between epigenetic gene silencing and human disease. *Mutat Res* 618: 163–174, 2007.
38. **Mutirangura A.** Quantitative PCR analysis for methylation level of genome: clinical implications in cancer. *Asian Biomed* 1: 121–128, 2007.
39. **Ogino S, Kawasaki T, Nosho K, Ohnishi M, Suemoto Y, Kirkner GJ, Fuchs CS.** LINE-1 hypomethylation is inversely associated with microsatellite instability and CpG island methylator phenotype in colorectal cancer. *Intl J Cancer* 122: 2767–2773, 2008.
40. **Ogino S, Nosho K, Kirkner GJ, Kawasaki T, Chan AT, Schernhammer ES, Giovannucci EL, Fuchs CS.** A cohort study of tumoral LINE-1 hypomethylation and prognosis in colon cancer. *J Natl Cancer Inst* 100: 1734–1738, 2008.
41. **Pal A, Srivastava T, Sharma MK, Mehndiratta M, Das P, Sinha S, Chattopadhyay P.** Aberrant methylation and associated transcriptional mobilization of Alu elements contributes to genomic instability in hypoxia. *J Cell Mol Med* in press.
42. **Park SY, Yoo EJ, Cho NY, Kim N, Kang GH.** Comparison of CpG island hypermethylation and repetitive DNA hypomethylation in premalignant stages of gastric cancer, stratified for Helicobacter pylori infection. *J Pathol* 219: 410–416 2009.
43. **Pattamadilok J, Huapai N, Rattanatanyong P, Vasurattana A, Triratanachat S, Tresukosol D, Mutirangura A.** LINE-1 hypomethylation level as a potential prognostic factor for epithelial ovarian cancer. *Int J Gynecol Cancer* 18: 2008.
44. **Perrin D, Ballestar E, Fraga MF, Frappart L, Esteller M, Guerin JF, Dante R.** Specific hypermethylation of LINE-1 elements during abnormal overgrowth and differentiation of human placenta. *Oncogene* 26: 2518–2524, 2007.
45. **Pheasant M, Mattick JS.** Raising the estimate of functional human sequences. *Genome Res* 17: 1245–1253, 2007.
46. **Phokaew C, Subbalekha K, Kowudtitham S, Shuangshoti S, Mutirangura A.** LINE-1 methylation patterns of different loci in normal and cancerous cells. *Nucl Acids Res* 36: 5704–5712, 2008.
47. **Piriyapongsa J, Polavarapu N, Borodovsky M, McDonald J.** Exonization of the LTR transposable elements in human genome. *BMC Genomics* 8: 291, 2007.
48. **Romanov GA, Vanyushin BF.** Effects of the tissue type, age, malignancy and hormonal induction. *Biochim Biophys Acta* 653: 204–218, 1981.
49. **Schulz WA, Steinhoff C, Florl AR.** Methylation of endogenous human retroelements in health and disease. *Curr Top Microbiol Immunol* 310: 211–250, 2006.
50. **Shuangshoti S, Hourpai N, Pulmsuk U, Mutirangura A.** Line-1 hypomethylation in multistage carcinogenesis of the uterine cervix. *Asian Pacific J Cancer Prev* 8: 307–309, 2007.
51. **Tangkijvanich P, Hourpai N, Rattanatanyong P, Wisedopas N, Mahachai V, Mutirangura A.** Serum LINE-1 hypomethylation as a potential prognostic marker for hepatocellular carcinoma. *Clin Chim Acta* 379: 127–133, 2007.
52. **Todorovska E.** Retrotransposons and their role in plant-genome evolution. *Biotechnol & Biotechnol Eq* 21: 294–305, 2007.
53. **Vanyushin BF, Nemirovsky LE, Klimenko VV, Vasiliev VK, Belozersky AN.** The 5-methylcytosine in DNA of rats. Tissue and age specificity and the changes induced by hydrocortisone and other agents. *Gerontologia* 19: 138–152, 1973.
54. **Vanyushin BF, Tkacheva SG, Belozersky AN.** Rare bases in animal DNA. *Nature* 225: 948–949, 1970.
55. **Wilson AS, Power BE, Molloy PL.** DNA hypomethylation and human diseases. *Biochim Biophys Acta* 1775: 138–162, 2007.
56. **Wilson VL, Jones PA.** DNA methylation decreases in aging but not in immortal cells. *Science* 220: 1055–1057, 1983.
57. **Wilson VL, Smith RA, Ma S, Cutler RG.** Genomic 5-methyldeoxycytidine decreases with age. *J Biol Chem* 262: 9948–9951, 1987.
58. **Xiong Z, Laird PW.** COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res* 25: 2532–2534, 1997.
59. **Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, Issa JP.** A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. *Nucleic Acids Res* 32: e38, 2004.
60. **Yi JM, Kim HS.** Molecular phylogenetic analysis of the human endogenous retrovirus E (HERV-E) family in human tissues and human cancers. *Genes Genet Syst* 82: 89–98, 2007.
61. **Youn LJ, Zhe J, Bin T.** Phylogenetic analysis of mRNA polyadenylation sites reveals a role of transposable elements in evolution of the 3′-end of genes. *Nucleic Acid Res* 36: 5581–5590, 2008.
62. **Zvetkova I, Apedaile A, Ramsahoye B, Mermoud JE, Crompton LA, John R, Feil R, Brockdorff N.** Global hypomethylation of the genome in XX embryonic stem cells. *Nat Genet* 37: 1274–1279, 2005.