CpG methylation changes in Alu repetitive sequences in normal aging due to diastolic hypertension in human dermal fibroblasts from the facial area

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Abstract. Aging fibroblasts, an important factor contributing to skin aging, are affected by numerous mechanisms, including alterations in DNA methylation and age-related diseases. The current study aimed to investigate the role of Alu methylation in aging fibroblasts and hypertension. The Alu methylation levels in dermal fibroblasts obtained from patients of different ages and blood pressure status were analyzed using the combined bisulfite restriction analysis technique. An inverse correlation was observed between Alu methylation in dermal fibroblasts and patient age. Dermal fibroblasts from the high-normal diastolic blood pressure group had higher Alu methylation levels compared with those from the normal group. The findings of the present study suggest that Alu methylation alterations can be observed with chronological aging and hypertension, and are a potential aging marker or therapeutic target.

Introduction

Aging is a biological process attributed to the functional decline of an organism, and it has been the subject of extensive research. It contributes to the pathogenesis of several degenerative diseases, such as cancer, diabetes, atherosclerosis, hypertension and Alzheimer's disease, which affect the quality of life of individuals and lead to functional decline and subsequent death (1). The key to reversing aging lies in understanding its pathophysiology and underlying mechanisms. Several theories have been proposed to describe the phenomenon of aging, including increased reactive oxygen species levels, mitochondrial dysfunction and telomere attrition (2,3). Epigenetic alterations have been identified as a contributing factor and have been proposed as one of the mechanisms underlying aging. These alteration have garnered the attention of researchers in recent years (4).

DNA methylation is a crucial epigenetic mechanism. The role of DNA methylation varies depending on its position in the genome. The loss or gain of methylation at particular positions can result in altered gene expression, the basis for various diseases such as Beckwith-Wiedemann syndrom and various type of cancers (5,6). In addition to the coding region, DNA methylation occurs in interspersed repetitive sequences (IRS) of the genome such as LINE-1 and Alu. Alu is a short IRS that accounts for 13.7% of the human genome (7). The Alu element is a transposable element capable of replicating and inserting itself into different areas of the genome. It contains a high proportion of CpG islands which are sites of DNA methylation. As a result, 25% of the methylation in the genome is located in Alu (7). Alu methylation suppresses transposon activity, while Alu hypomethylation can lead to high transposon activity, resulting in genomic instability and aging (8). The methylation level of Alu is dynamic during normal cell differentiation and tumorigenesis (7,8). With regards to aging, previous studies have illustrated the association between the methylation of the Alu elements and aging, but others have reported conflicting results (8-10). Alu hypomethylation is associated with age-related, noncommunicable diseases such as osteoporosis, diabetes and Alzheimer's disease (11-13).

The skin is the largest organ of the human body. The aging process in the skin can be observed externally through morphological changes, such as wrinkles, pigmentation and abnormal hyperplasia. Owing to its location, the skin is the most susceptible organ to external aging factors, such as ultraviolet

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radiation and pollution. Skin aging can cause delayed wound healing and loss of functional barriers to microbes (14,15).

Dermal fibroblasts, which are responsible for various physiological functions of the skin, play a central role in skin aging. As dermal fibroblasts rarely proliferate, they are more susceptible to age-related damage, thus compromising cell function (14). In skin aging, collagen, the most abundant component of the extracellular matrix (ECM) secreted by dermal fibroblasts, is fragmented and decreasing in collagen production (15-17). This is due to an increase in matrix metalloproteinase activity and impaired growth factor signal transduction, a change that occurs as a result of fibroblast aging (18). Other components of the ECM also change during aging. Elastin, another major component of the ECM produced by fibroblasts, is selectively degraded during the intrinsic skin aging process (19). Such alterations in fibroblast function and ECM remodeling can be observed externally in the aging phenotypes of the skin, such as wrinkles and loss of elasticity (20). Most prominently, these changes can be visualized in the facial area, as fibroblasts in this region are thin and subjected to photoaging and intrinsic chronological aging. Age-related phenotypes of the skin in the periorbital area are accurate predictors of biological aging (21).

In addition to the direct effects of aging, dermal fibroblasts are affected by hypertension, an important age-related disease with a high prevalence rate of 30-45% (22,23). *In vitro* studies have shown that dermal fibroblasts subjected to increased pressure exhibit accelerated aging phenotypes (24,25). This is clinically associated with ulcers in chronic venous hypertension, wherein fibroblasts lose their ability to proliferate resulting in aberrant wound healing (26). The effect of hypertension on dermal fibroblasts is speculated, given the results of a previous study on fibroblasts from other organs (26-28). Hypertension has been linked to the altered activity of cardiac fibroblasts, such as collagenase dysregulation and cellular dedifferentiation (27,28). Although hypertension alters the gene expression profile and cellular phenotypes of dermal fibroblasts, the epigenetic events taking place remain poorly understood (29,30). Understanding epigenetic events will provide useful insights into whether and how hypertension accelerates aging in dermal fibroblasts.

Several studies have investigated the mechanisms and pathophysiology of fibroblast aging, including the role of epigenetic alterations. Changes in the DNA methylation status of human dermal fibroblasts at specific loci have been observed during aging (31,32). It is proposed that rather than occurring randomly during ontogenetic development, these site-specific changes are specifically regulated through unknown mechanisms. Moreover, most of the identified loci were located in the gene and promoter regions, resulting in a lack of extensive research on IRS (32). These specific modifications in the methylation status cannot explain the global hypomethylation phenomenon, which is considered to be the result of passive random loss of methylation, a phenomenon which the methylation status of the IRS is more reflective (9,33,34). The role of DNA methylation in IRS, specifically that of Alu methylation in the process of skin cell aging, whether by the chronological aspect of aging itself or the effect of aging through intermediary age-related diseases such as hypertension, is yet to be investigated. In the current study, the aim was to explore the correlation between the Alu methylation status in human dermal fibroblasts and the chronological age of patients whose specimens were analyzed. The weather age-related diseases, namely hypertension, affect the dermal fibroblast methylation profile was also investigated.

Materials and methods

Study design, sample size and population. The present study is an analytical cross-sectional study. A total of 39 samples were obtained from patients who visited the plastic and reconstructive surgery clinic at King Chulalongkorn Memorial Hospital (Bangkok, Thailand) for surgical procedures taken place between September 2020 and September 2021. All patients were recruited following the inclusion and exclusion criteria. The inclusion criteria included: i) Indication for skin surgery with the excision of normal tissues; ii) ≥18 years old; and iii) ability to make an informed decision. The exclusion criteria included low quality or quantity of DNA from the cultured cells and the presence of active skin disease at the site of operation. The patients were then divided into two groups according to their age at the date of tissue resection: The young age group (<60 years old; n=22) and the old age group (>60 years old; n=17). Patients were also divided into three age groups: Young (<45 years old; n=8), middle (45-60 years old; n=14), and old (>60 years old; n=17). The patients were then categorized into three groups according to their systolic blood pressure (SBP) and diastolic blood pressure (DBP) as: Normal (SBP<130 mmHg; DBP<85 mmHg), high-normal (SBP range, 130-139 mmHg; DBP range, 85-89 mmHg) and hypertensive (SBP≥140 mmHg; DBP≥90 mmHg) (22); based on SBP, there were a total of 14, 14 and 11 patients in every group, respectively, and based on DBP, there were 17, 12 and 10 patients in every group, respectively. Tissue samples were grouped according to the surgical site as eyelid or non-eyelid (Table SI). There was a total of 29 eyelid and 10 non-eyelid samples (three brow samples, four sub-brow samples, one ear sample and two alar samples). The lifestyle factors of the patients were not recorded, therefore, the samples were grouped randomly for these factors.

Ethical statement. The current study was reviewed and approved by the institutional review board of the Faculty of Medicine, Chulalongkorn University (approval no. 353/63). All 39 samples were acquired from patients who underwent surgery at the plastic and reconstructive surgery clinic at King Chulalongkorn Memorial Hospital from September 2020 to September 2021. Written informed consent was obtained from all patients before they participated in the study. The establishment of primary cell lines from patients in the present study was approved by the same institutional review board.

Cells and culture. Dermal tissues were collected from patients during surgical procedures. The participants had not received systemic or topical treatment in the month before the surgery. After excision, specimens were cut into pieces of size range 0.5-1 cm^2 and immersed in Dulbecco's Modified Eagle medium (DMEM) (Sigma-Aldrich; Merck KGaA) for transportation to the laboratory. The dermis was explanted from the surgical specimen and cut into several 5-mm^2 pieces in the laboratory. The pieces were then placed in Roux culture

bottles and cultured in DMEM high glucose (Sigma-Aldrich; Merck KGaA) mixed with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). The bottles were placed in an incubator at 37˚C in humidified air containing 5% CO_2. The cells were subcultured every 7 days.

DNA preparation and measurement of the CpG methylation level of the Alu repetitive sequence. Cells in passages I, III and V were harvested by trypsinization. The suspension was centrifuged at 1,500 x g at 4˚C for 3 min to separate the cells from the solution. The supernatants were discarded, and 500 μl Lysis Buffer II (0.75 M NaCl and 0.024 M EDTA at pH 8.0), 50 μl 10% sodium dodecyl sulfate (Sigma-Aldrich; Merck KGaA) and 50 μl Proteinase K (United States Biological) were then added for DNA extraction. The suspension was incubated overnight at 50˚C until the cells were lysed.

Phenol/chloroform was then added to the cell lysate, and the mixture was centrifuged to separate DNA from the organic compounds. The DNA-containing supernatant was removed and precipitated with 100% isopropanol. The combined bisulfite restriction analysis (COBRA) technique was used to identify methylation sites (35). The DNA was treated with sodium bisulfite using the EZ DNA Methylation-Gold kit (Zymo Research Corp.), according to the manufacturer's protocol.

To determine the methylation level of the CpG site, specifically at the Alu element, polymerase chain reaction (PCR) using the Alu forward primer 5'-GGYGUGGTGGTTTAY GTTTGTAA-3' and the Alu reverse primer 5'-CTAACTTTT TATATTTTTAATAAAAACRAAATTTCACCA-3' with the following conditions was performed: Initial denaturation at 95˚C for 15 min and 35 cycles of denaturation at 95˚C for 45 sec, annealing at 57˚C for 45 sec and extension at 72˚C for 45 sec followed by a final extension at 72˚C for 15 min. The Alu sequence primers were based on the Alu nucleotide sequences from the accession number NM_031483.7 (36), and the aforementioned set of primers was used in our previous study (9,11,35,37,38). The PCR products were subjected to sodium bisulfite treatment (35). The PCR product was digested with TaqI cut enzyme (Thermo Fisher Scientific, Inc.) and incubated at 65˚C for 16 h. The product was analyzed by gel electrophoresis on an 8% non-denaturing polyacrylamide gel. The gels were submerged in SYBR Green (Lonza Group, Ltd.) for 30 min for staining. Band intensity was observed and measured using Strom840 and ImageQuanNT Software (Amersham; Cytiva). DNA from HeLa cells was used as a positive control for agarose gel electrophoresis and band intensity measurement (Fig. S1).

In the COBRA method, the methylation pattern of two CpG loci is reflected in the band length, which can be classified as follows: Alu loci with two unmethylated CpGs ($^{u}C^{u}C$; 133 bp); Alu loci with two methylated CpGs ($^{m}C^{m}C$; 58 and 32 bp, respectively); Alu loci with a 5'-unmethylated CpG and a 3'-methylated CpG ($^{u}C^{m}C$; 75 bp); and Alu loci with a 5'-methylated CpG and a 3'-unmethylated CpG ($^{m}C^{u}C$; 90 bp).

The intensity of each band was calculated by dividing the measured intensity (arbitrary unit according to the ImageQuanNT) by the length of each band as follows: A) 133 bp/133; B) 58 bp/58; C) 75 bp/73; D) 90 bp/90; and E) 43 bp/41. The percentage of Alu methylation was calculated by comparing the number of methylated CpG loci to all the CpG loci located in Alu ([B +E]/[2A + B + C + D + E]) x100.

Statistical analysis. Statistical analysis was performed using SPSS (version 25.0; IBM Corp.). The Alu methylation status was assessed, and compared between the young and old age groups, two categories of surgical sites and two sexes using an unpaired Student's t-test, with the effect of sex regressed out in the first two analyses due to the high skewness between sexes. Owing to the high skewness, the homogeneity of variance and normality of distribution were also investigated, both of which met the assumption of an unpaired t-test (Levene's test, P>0.05; Shapiro-Wilk test, P>0.05). Age and Alu methylation were evaluated using Spearman's rho. Subgroup analysis was performed for each surgical site group to determine the relationship between age and Alu methylation in eyelid and non-eyelid tissues using an unpaired t-test. The comparisons of the Alu methylation overall level and $^{u}C^{u}C$, $^{u}C^{m}C$ and $^{m}C^{u}C$ patterns between the SBP and the DBP groups were performed using one-way analysis of variance (ANOVA), assuming the variances were unequal (Welch's) and homogeneity of variance was equal (Levene's test, P>0.05) followed by Tukey's post hoc test. An unpaired t-test was also performed to compare the Alu methylation status between the SBP and DBP groups. Two-way ANOVA was performed to assess the interaction between age groups, DBP groups and Alu methylation status. Homogeneity of variance and normality of distribution were determined (Levene's test, P>0.05; Shapiro-Wilk test, P>0.05). Sex could not be included in these analyses because of multicollinearity. P<0.05 was considered to indicate a statistically significant difference.

Results

Methylation level and sexes. The differences in Alu methylation levels between male and female patients were not statistically significant (P>0.05; Fig. 1).

Alu methylation pattern and chronological ages. The Alu methylation levels of human dermal fibroblasts obtained from patients of different ages were analyzed. Patients were classified using two criteria as follows, first criteria into two age groups: Young age (≤60 years old) and old age (>60 years old), and second criteria into three age groups: Young age (<45 years old), middle age (45-60 years old) and old age (>60 years old). The additional stratification in the three groups criterion was done to assess subtle changes that occur during the aging process and to enhance the clarity of the observed trends.

In the two-group criteria, there were statistically significant differences in the Alu methylation between young and old age groups (30.90±1.66 and 29.40±1.99%, respectively; Fig. 1A) using two-way ANOVA considering the surgical site, and SBP and DBP groups as co-independent variables (P=0.023, 0.036 and 0.013, respectively). When sex was used as a co-independent variable, a trend with an almost statistically significant difference was observed (P=0.051). Regarding the pattern of Alu methylation, the percentage of $^{u}C^{u}C$ was found to be significantly higher in the old age group using two-way ANOVA with DBP status as covariate (young age

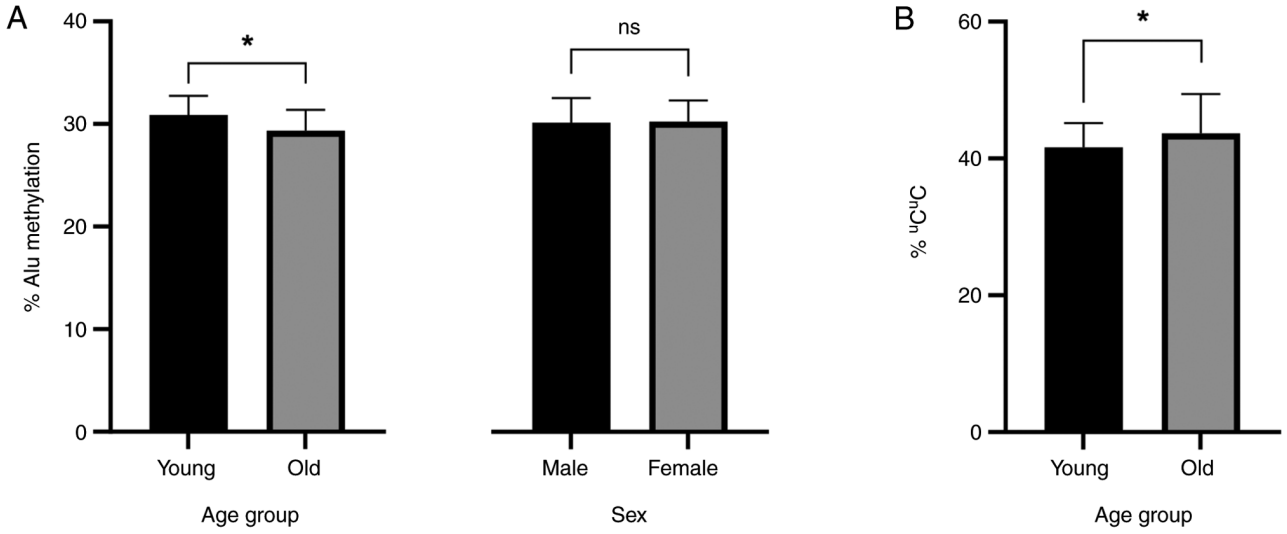

Figure 1. (A) Alu methylation levels in dermal fibroblasts collected from patients of different ages and sexes. The young age group was classified as ≤60 years, while the old age group was defined as >60 years. Regarding mean Alu methylation shown as mean ± SD, that was 30.9±1.66% for 22 samples in the young group and 29.40±1.99% for 17 samples in the old group. The statistical significance of the differences was calculated using two-way ANOVA considering the surgical site, SBP and DBP groups as co-independent variables (P=0.023, 0.036 and 0.013, respectively). There were 34 females and five males with a mean ± SD Alu methylation level of 30.20±2.06 and 30.19±2.38%, respectively. An independent samples t-test was performed (P>0.05). (B) Percentage of the unmethylated allele $^{u}C^{u}C$ in the young and old age groups. The mean ± SD Alu methylation levels of the young and old groups were 41.60±3.56 and 43.67±5.74%, respectively; P=0.016, calculated using two-way ANOVA with DBP status as a covariate. *P<0.05. Ns, not significant; ANOVA, analysis of variance; SBP, systolic blood pressure; DBP, diastolic blood pressure.

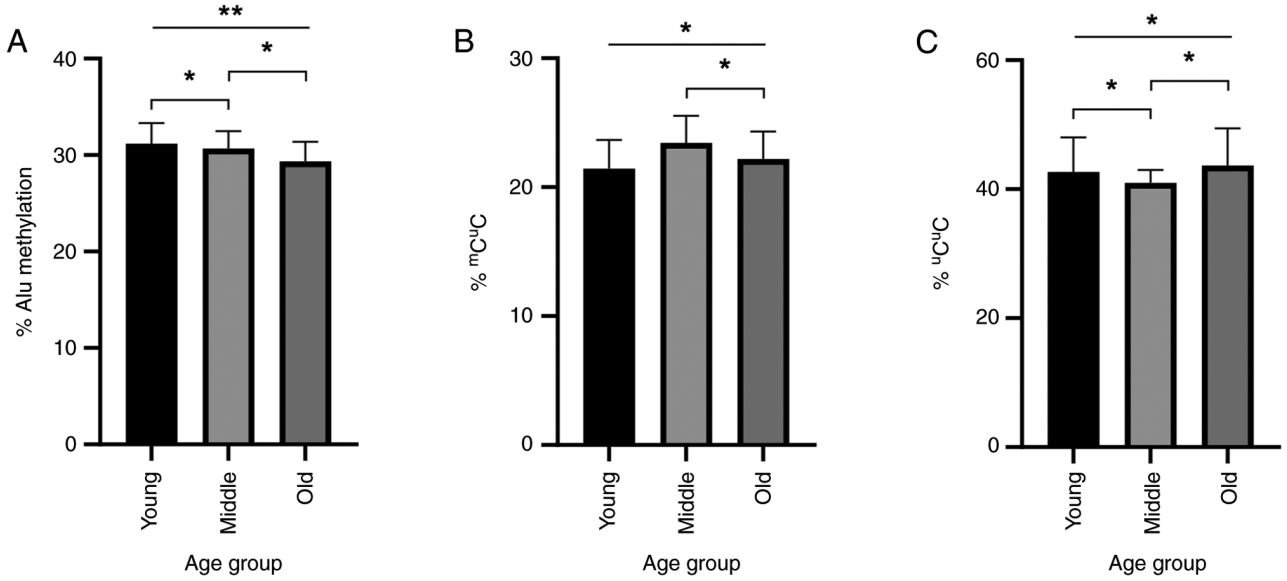

Figure 2. Alu methylation level and percentage of methylation pattern alleles in dermal fibroblasts collected from patients of different ages. In the three-group criteria, patients were classified into the young age group (<45 years old; n=8), middle age group (45-60 years old; n=14) and old age group (>60 years old; n=17). Statistical significance was calculated using two-way analysis of variance with DBP status as a covariate. (A) Alu methylation level in young, middle and old age groups. The mean ± SD Alu methylation levels were 31.20±2.14, 30.70±1.79 and 29.40±2.03%, respectively (P<0.01). Post-hoc analysis revealed P<0.05 in both young-middle and middle-old comparisons. (B) Percentage of the partial methylated allele $^{m}C^{u}C$. The mean ± SD Alu methylation levels were 21.40±2.21, 23.40±2.10 and 22.20±2.13 for the young, middle and old groups, respectively (P<0.05). (C) Percentage of the unmethylated allele $^{u}C^{u}C$. The mean ± SD Alu methylated levels were 42.70±5.34, 41.00±2.00 and 43.70±5.74 for the young, middle and old groups, respectively (P<0.05). DBP, diastolic blood pressure. *P<0.05, **P<0.01.

group, 41.60±3.56% and old age group: 43.70±5.74%; P<0.05; Fig. 1B).

In the three-group criteria, there was a significant difference between young, middle and old age groups using two-way ANOVA with DBP status as a covariate (31.20±2.14, 30.70±1.79 and 29.40±2.03, respectively; P<0.01). Post-hoc analysis showed statistically significant differences between the young and old age, and the middle and old age groups with both the young age and the middle group having significantly higher methylation than the old age group (P<0.05; Fig. 2A).

To avoid the effect of arbitrary grouping, correlation matrix analysis was performed using Spearman's rho since age

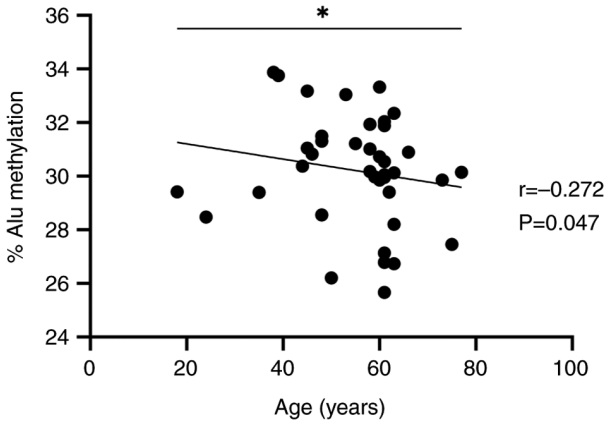

Figure 3. Plot of Alu methylation level with age. All 39 samples were included in the Spearman's rho analysis. A negative correlation was hypothesized, and the correlation was found to be statistically significant (P=0.047 and r=-0.272). *P<0.05.

was considered only in years and not as continuous numbers. Assuming that the age and Alu methylation would be negatively correlated, the correlation was statistically significant (P=0.047; r=-0.272; Fig. 3).

Using two-way ANOVA with DBP status as a covariate statistical test for the methylation pattern analysis, the $^{m}C^{u}C$ methylation was shown to be highest in the middle age group, followed by the old and young age groups (21.40±2.21, 23.40±2.10 and 22.20±2.13% in young, middle and old respectively; P<0.05; Fig. 2B. A similar but inverse trend was observed in the $^{u}C^{u}C$ methylation, where this pattern was lowest in the middle age group, followed by young and old age (42.70±5.34, 41.00±2.00 and 43.70±5.74% in young, middle and old respectively; P<0.05; Fig. 2C). Post-hoc analysis showed a difference in the $^{m}C^{u}C$ methylation percentage between the old and middle age groups, and a difference in the $^{u}C^{u}C$ methylation percentage between the middle and both the young and old age groups; all differences described were statistically significant (P<0.05; Fig. 2B and C).

Surgical site and aging pattern. Tissue samples were also classified into two groups according to the surgical site: Eyelid and non-eyelid. No statistically significant differences were shown in the Alu methylation between the two groups (Fig. 4A). Subgroup analysis revealed that the correlation between Alu methylation and age was stronger in the eyelid group than in the non-eyelid group (P=0.087 and P=0.112, respectively; Fig. 4B). The number of Alu loci with $^{u}C^{u}C$, $^{u}C^{m}C$ and $^{m}C^{u}C$ methylation patterns was not statistically different among age groups in tissues from both eyelid and non-eyelid groups. The methylation of samples from different surgical sites is shown in Fig. 4. A general trend but non statistically significant of lower Alu methylation levels can be observed in the old age group compared with those in the young age group in both eyelid and non-eyelid samples. Notably, some surgical sites, namely the ear and alar, only have samples from the young age group. This was attributed to the difficulty in recruiting subjects as these areas tend to exhibit fewer features of aging and, therefore, have fewer needs for reconstructive surgery.

Methylation level and blood pressure. Patients were classified into three groups based on their blood pressure measured on the day of the surgery: Normal, high-normal and hypertensive, according to the ESC/ESH guidelines on hypertension published in 2018 (21). The Alu methylation levels in the normal, high-normal and hypertensive groups according to SBP were 30.30±2.15, 30.20±2.15 and 30.10±2.06%, respectively (Fig. 5A), whereas the Alu methylation levels in the DBP groups were 29.60±1.85, 31.10±1.78 and 30.20±2.48% for the normal, high-normal and hypertensive groups, respectively (Fig. 5B). The percentage of $^{u}C^{u}C$ methylation was also analysed for the normal, high-normal and hypertensive groups; for SBP, these were 42.80±5.57, 42.70±3.84 and 41.90±4.84%, respectively (Fig. 5C), and for DBP these were 44.20±5.19, 40.40±2.95 and 42.30±4.79%, respectively (Fig. 5D). Analysis of DBP showed a statistically significant increase in the overall Alu methylation in the high-normal group compared with that in the normal group (P<0.05; Fig. 5B). The $^{u}C^{u}C$ Alu methylation pattern was also found at lower proportions in the high-normal DBP group compared with that in the normal group (P<0.05; Fig. 5D). Two-way ANOVA with the three age groups as a covariate also revealed a statistically significant difference in the $^{u}C^{u}C$ methylation percentage between the prehypertensive (diastolic high-normal) and hypertensive groups. No statistically significant differences in SBP were observed among the groups (P>0.05; Fig. 5A and D).

Methylation pattern, age and DBP. Two-way ANOVA of the percentage of $^{u}C^{u}C$ patterns showed an interaction between age and DBP when analyzing both factors as covariates (P=0.01; data not shown). Other interactions between age, DBP and Alu methylation level, the percentages of $^{m}C^{u}C$ and $^{u}C^{m}C$ patterns were not statistically significant (P>0.05).

Discussion

Samples were classified into two groups based on age at the time of sampling. Results showed that the Alu methylation levels in the young and old age groups were statistically different (P≤0.05), with the Alu methylation levels in the old age group being statistically lower than those in the young group.

Several studies have reported an inverse correlation between advanced chronological age and global hypomethylation in various tissues, including fibroblasts from the lung (9,39,40). The methylation level of the Alu element, with ~1.4 million copies interspersed in the human genome, has long been regarded to reflect the global methylation level (10). Cho *et al* (41) showed that Alu hypomethylation is present in cancerous samples from older patients. A similar association between age and Alu hypomethylation was observed in normal cells, such as white blood cells (13). However, the correlation between Alu methylation patterns and age in human dermal fibroblasts is yet to be determined.

COBRA was selected to measure the methylation levels based on the comparability of the results with those of pyrosequencing and high-throughput sequencing methods (9,37). COBRA-IRS can also illustrate methylation patterns that are potential markers in the diagnosis of several diseases, including cancer, autism spectrum disorders and schizophrenia (42-44).

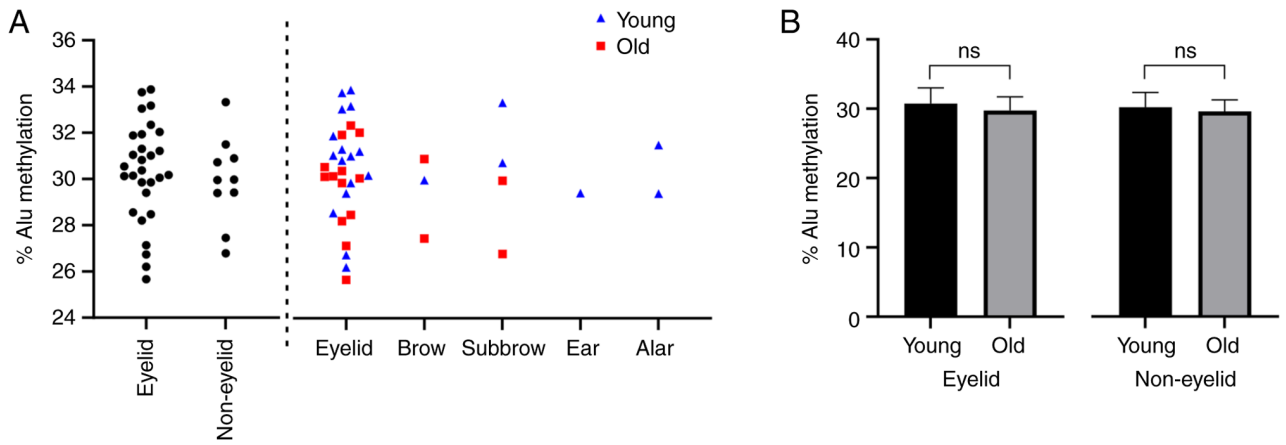

Figure 4. Alu methylation level and surgical sites. (A) There were 28 samples from eyelid sites and 10 from non-eyelid sites (three brow samples, four sub-brow samples, one ear sample and two alar samples). (B) Among the eyelid samples, a total of 15 and 13 were from the young and the old age group, respectively. Among the non-eyelid samples, six and four samples were from the young and the old age group, respectively. Statistical analysis was done in each surgical site group to compare the two age groups using an independent samples t-test (P>0.05). Ns, not significant.

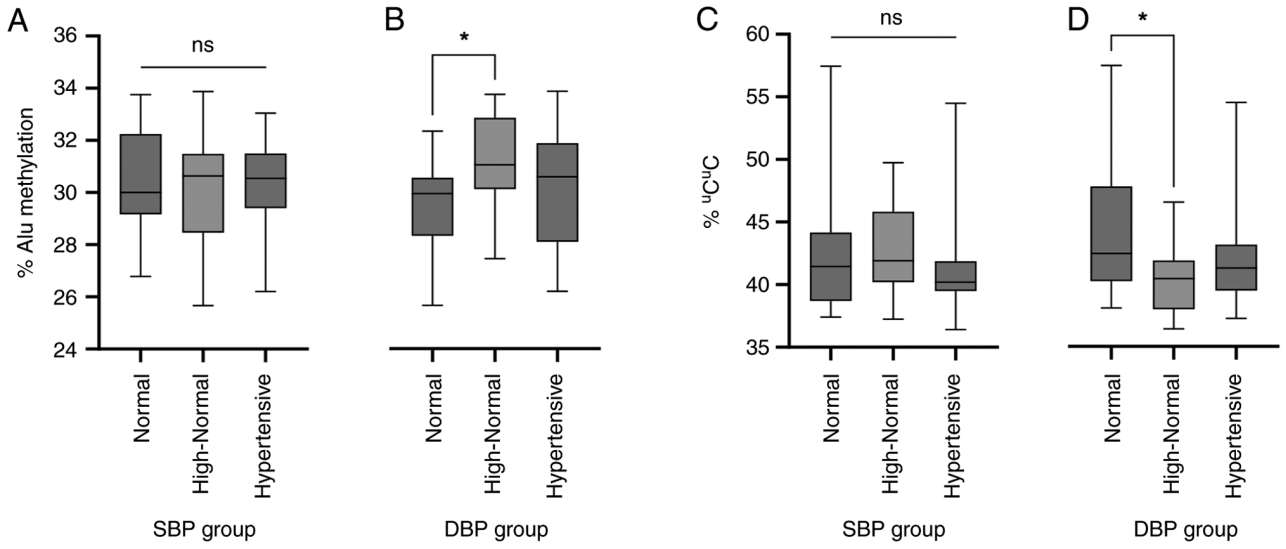

Figure 5. Alu methylation and blood pressure status. SBP and DBP were used to categorize patients into three groups: Normal (SBP<130 mmHg; DBP<85 mmHg), high-normal (SBP range, 130-139 mmHg; DBP range, 85-89 mmHg) and hypertensive (SBP≥140 mmHg; DBP≥90 mmHg). Based on SBP, there were 14, 14 and 11 patients in the normal, high-normal and hypertensive group, respectively. Based on DBP, there were 17, 12 and 10 patients in the normal, high-normal and hypertensive group, respectively. (A) Alu methylation level and SBP status. No statistical difference was found using ANOVA, assuming the variances were unequal (Welch's) and the homogeneity of variance was equal (Levene's test, P>0.05). (B) Alu methylation level and DBP status. A statistical difference was observed between the high-normal and normal groups using an independent samples t-test (P<0.05). (C) Percentage of the unmethylated allele $^{u}C^{u}C$ and SBP status. No statistical difference was found using one-way ANOVA. (D) Percentage of the unmethylated allele $^{u}C^{u}C$ and DBP status. A statistical difference was observed between the high-normal and normal groups using independent samples t-test. *P<0.05. SBP, systolic blood pressure; DBP, diastolic blood pressure; ANOVA, one-way analysis of variance; ns, not significant.

The results of the present study are similar to those of other studies in different tissues; the Alu methylation levels in human dermal fibroblasts decrease with age (10,45). There are two possible explanations for such a correlation: First, in agreement with multiple studies on human diploid fibroblasts, the change in methylation patterns may be the result of age-related chromatin remodeling, which is thought to be the basis of altered epigenetic control of retrotransposable elements in aging (45,46). Second, according to a study by Patchsung *et al* (37), the manipulation of Alu methylation levels could result in the reversal of aging phenotypes and increase cell resistance to DNA damage. These results led to the hypothesis that, rather than being a downstream event of other aging mechanisms, Alu hypomethylation may play an active role in the aging process or both. Alu hypomethylation, an effect of chronological aging, may reactivate retrotransposable elements, causing genomic instability and making cells more susceptible to DNA damage accumulation. Another possible mechanism by which Alu methylation stabilizes DNA is by relieving DNA tension, which makes the DNA less susceptible to pathological endogenous double-strand breaks. This was illustrated in a study by Patchsung *et al* (37), who found that increasing Alu methylation reduced endogenous double-strand breaks and made cells less susceptible

to DNA-damaging agents (37,47). The current methylation pattern analysis also suggested that the age-related methylation change of Alu may be a dynamic process similar to $^{u}C^{u}C$ and $^{m}C^{u}C$ loci percentage, which was found to be at the bottom and peak in the middle-aged group compared with the expected trend of continuous change from young to old age. It was hypothesized that this phenomenon may be explained by the dynamic transfer of methyl groups between loci, resulting in a distinct pattern for each age range while retaining the overarching trend of overall methylation loss. Further studies are required to examine this hypothesis by increasing the sample size and age stratification to study the molecular events underlying the process in depth. It was also shown that no notable changes in the $^{u}C^{m}C$ loci were reported in another study by our team in burned skin (35). Based on this contradiction, the study of methylation patterns points to multiple mechanisms underlying the Alu methylation change. Further studies should confirm these hypotheses and determine whether the proposed mechanisms apply to human dermal fibroblasts.

Subgroup analyses based on sex and surgical site found no differences in the Alu methylation levels. The observation regarding Alu methylation and sex is consistent with a study conducted in another cell type, the peripheral blood mononuclear cell, by Jintaridth and Mutirangura (9). The correlation between Alu methylation and age found in the current study, although statistically significant, was presented with a small margin between the age groups. This is in concordance with other studies, one of which found that the difference in Alu methylation between young and old age groups was only 1% (48). Furthermore, high intragroup variability was observed, which suggests that the human dermal fibroblast Alu methylation levels may depend on several factors other than age, such as DBP. Future studies should include other demographic traits as control variables to avoid confounding effects. Intragroup variability may also occur because the Alu methylation status is more strongly correlated with physiological aging than with chronological aging (45). To uncover this, further studies should consider classifying samples using an aging phenotype based on criteria such as the activity of β-galactosidase, and the proliferation profile and expression of apoptotic proteins. Studies should also collect information on lifestyle factors including occupation, diet, exercise and outdoor activities, a number of which are important in aging and Alu methylation. It is noteworthy that the present study was conducted using skin from the eyelids and other facial areas. Skins in these regions are most prone to aging due to their thinness and greater exposure to sunlight (21). Therefore, the effect of aging may be exacerbated, and intrinsic chronological aging may also be supplemented by photoaging. Although these two modes of aging are similar epigenetically, further research should be carried out involving the use of fibroblasts from other areas of the body to confirm the phenomenon observed in the current study is generally applicable to dermal fibroblasts (49).

The current study classified the patients into three groups according to their blood pressure levels. SBP and DBP were considered separately. The results showed a statistically significant increase in Alu methylation in the high-normal DBP group compared with that in the normal DBP group. No differences were found between groups classified according to SBP. This is in line with most studies showing that changes in DNA methylation, including Alu methylation, usually occur in conjunction with elevated DBP (49,50). SBP and Alu methylation were reported only by one study and are considered to be more susceptible to transient changes such as anxiety, exertion and other disrupting factors; they are therefore not reflective of the baseline patient status (49,51). A debatable aspect of the results of the present study was that it showed a positive correlation between DBP and Alu methylation, a result supported by a study by Alexeeff *et al* (51) but contradicting others (48-50). This contradiction may be due to the different tissue types used in the analysis. All previous studies have used peripheral leukocytes as a model, whereas the present study used dermal fibroblasts. Therefore, the alteration of methylation levels might be regulated by a different process.

Results also confirm the findings of several studies showing that blood pressure can affect dermal fibroblasts. A study by Delva *et al* (52) illustrated the link between hypertension and alterations in collagen synthesis and the proliferative activity of dermal fibroblasts. Another study by Kosugi *et al* (29) reported an increase in the activity of phospholipase C, a target of angiotensin II, vasopressin and thromboxane A2 in dermal fibroblasts obtained from patients with hypertension. Both studies suggested that dermal fibroblasts may play a role in the pathogenesis of hypertension.

Similar to that in the aging study, patterns of Alu methylation were identified using COBRA analysis. Overall, methylation was higher in the high-normal DBP group than that in the normal DBP group. The unmethylated allele, the $^{u}C^{u}C$ methylation pattern, was significantly lower in the high-normal DBP group than in the normal and high DBP groups. Therefore, it was hypothesized that similar to that in aging, these alterations are dynamic processes. Two-way ANOVA analysis of the $^{u}C^{u}C$ methylation pattern revealed a statistically significant interaction between aging and DBP, suggesting that both processes share an underlying epigenetic event or complement each other in the progression of the pathogenetic process. This is a likely possibility, given that hypertension is known to be an age-related disease. Given the connection between dermal fibroblasts and hypertension in a previous study and the pattern of our results, it is also possible that rather than being the victim of damage by hypertension, dermal fibroblasts may play an active role. Further studies could answer this question as this may serve as a basis for a better understanding of the pathogenesis of hypertension. Furthermore, the result of two-way ANOVA, which revealed a statistically significant difference in the $^{u}C^{u}C$ percentage but not overall Alu methylation, indicating a correlation between $^{u}C^{u}C$ percentage and diastolic hypertension. Therefore, further validation is suggested to investigate the potential utility of the $^{u}C^{u}C$ percentage as a more efficient marker than the Alu methylation level.

In conclusion, Alu hypomethylation is correlated with chronological age in human dermal fibroblasts. The Alu methylation levels were significantly lower in the young age group than those in the old age group. Alu methylation was also higher in fibroblasts in patients with high-normal DBP than in those with normal DBP. Dynamic alteration in the methylation of Alu element was also observed in both aging and diastolic hypertension. Taken together, Alu methylation may play an active role in the aging of skin fibroblasts and the

pathogenesis of hypertension. The present study could serve as a foundation for further investigations of skin aging, its role in the pathogenesis of hypertension and the development of therapeutics to reverse this process.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

SJ, NK, AM and JM conceptualized the study. SJ, PH, SK, NB, TS and JM carried out formal analysis. NK, AM and JM acquired funding. SJ, PH, SK, NB, TS, NK, AM and JM carried out the investigation. SJ, PH, SK, NB, TS, NK, AM and JM developed the methodology used. JM completed project administration and provided resources. JM and NK used software. AM and JM supervised the study. NK, AM and JM visualized and validated the data, and wrote the original draft. SJ, PH, SK, NB,TS, NK, AM and JM carried out investigation. SJ, PH, SK, NB, TS, NK, AM and JM reviewed and edited the draft. NK, AM and JM confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

The authors state that they have obtained ethics approval from the institutional review board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB No. 353/63), for the research described in the present study. In addition, written informed consent was obtained from all participants before skin sample collection.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Campisi J, Andersen JK, Kapahi P and Melov S: Cellular senescence: A link between cancer and age-related degenerative disease? Semin Cancer Biol 21: 354-359, 2011.
2. Sesti F, Liu S and Cai SQ: Oxidation of potassium channels by ROS: A general mechanism of aging and neurodegeneration? Trends Cell Biol 20: 45-51, 2010.
3. Levy MZ, Allsopp RC, Futcher AB, Greider CW and Harley CB: Telomere end-replication problem and cell aging. J Mol Biol 225: 951-960, 1992.
4. Calvanese V, Lara E, Kahn A and Fraga MF: The role of epigenetics in aging and age-related diseases. Ageing Res Rev 8: 268-276, 2009.
5. Shames DS, Minna JD and Gazdar AF: DNA methylation in health, disease, and cancer. Curr Mol Med 7: 85-102, 2007.
6. Lim DH and Maher ER: DNA methylation: A form of epigenetic control of gene expression. Obstet Gynaecol 12: 37-42, 2010.
7. Luo Y, Lu X and Xie H: Dynamic Alu methylation during normal development, aging, and tumorigenesis. Biomed Res Int 2014: 784706, 2014.
8. Erichsen L, Beermann A, Arauzo-Bravo MJ, Hassan M, Dkhil MA, Al-Quraishy S, Hafiz TA, Fischer JC and Santourlidis S: Genome-wide hypomethylation of LINE-1 and Alu retroelements in cell-free DNA of blood is an epigenetic biomarker of human aging. Saudi J iol Sci 25: 1220-1226, 2018.
9. Jintaridth P and Mutirangura A: Distinctive patterns of age-dependent hypomethylation in interspersed repetitive sequences. Physiol Genomics 41: 194-200, 2010.
10. Bollati V, Schwartz J, Wright R, Litonjua A, Tarantini L, Suh H, Sparrow D, Vokonas P and Baccarelli A: Decline in genomic DNA methylation through aging in a cohort of elderly subjects. Mech Ageing Dev 130: 234-239, 2009.
11. Jintaridth P, Tungtrongchitr R, Preutthipan S and Mutirangura A: Hypomethylation of Alu elements in post-menopausal women with osteoporosis. PLoS One 8: e70386, 2013.
12. Thongsroy J, Patchsung M and Mutirangura A: The association between Alu hypomethylation and severity of type 2 diabetes mellitus. Clin Epigenetics 9: 93, 2017.
13. Bollati V, Galimberti D, Pergoli L, Dalla Valle E, Barretta F, Cortini F, Scarpini E, Bertazzi PA and Baccarelli A: DNA methylation in repetitive elements and Alzheimer disease. Brain Behav Immun 25: 1078-1083, 2011.
14. Tigges J, Krutmann J, Fritsche E, Haendeler J, Schaal H, Fischer JW, Kalfalah F, Reinke H, Reifenberger G, Stühler K, *et al*: The hallmarks of fibroblast ageing. Mech Ageing Dev 138: 26-44, 2014.
15. Varani J, Dame MK, Rittie L, Fligiel SE, Kang S, Fisher GJ and Voorhees JJ: Decreased collagen production in chronologically aged skin: Roles of age-dependent alteration in fibroblast function and defective mechanical stimulation. Am J Pathol 168: 1861-1868, 2006.
16. Quan T, Qin Z, Xia W, Shao Y, Voorhees JJ and Fisher GJ: Matrix-degrading metalloproteinases in photoaging. J Investig Dermatol Symp Proc 14: 20-24, 2009.
17. Talwar HS, Griffiths CE, Fisher GJ, Hamilton TA and Voorhees JJ: Reduced type I and type III procollagens in photodamaged adult human skin. J Invest Dermatol 105: 285-290, 1995.
18. Shin JW, Kwon SH, Choi JY, Na JI, Huh CH, Choi HR and Park KC: Molecular mechanisms of dermal aging and antiaging approaches. Int J Mol Sci 20: 2126, 2019.
19. Langton AK, Sherratt MJ, Griffiths CE and Watson RE: Differential expression of elastic fibre components in intrinsically aged skin. Biogerontology 13: 37-48, 2012.
20. Boukamp P: Skin aging: A role for telomerase and telomere dynamics? Curr Mol Med 5: 171-177, 2005.
21. Zou Z, Long X, Zhao Q, Zheng Y, Song M, Ma S, Jing Y, Wang S, He Y, Esteban CR, *et al*: A single-cell transcriptomic atlas of human skin aging. Dev Cell 56: 383-397.e8, 2021.
22. Williams B, Mancia G, Spiering W, Agabiti Rosei E, Azizi M, Burnier M, Clement DL, Coca A, de Simone G, Dominiczak A, *et al*: 2018 ESC/ESH Guidelines for the management of arterial hypertension: The Task Force for the management of arterial hypertension of the European Society of Cardiology (ESC) and the European Society of Hypertension (ESH). G Ital Cardiol (Rome) 19 (11 Suppl 1): 3S-73S, 2018.
23. Huang C and Ogawa R: The link between hypertension and pathological scarring: Does hypertension cause or promote keloid and hypertrophic scar pathogenesis? Wound Repair Regen 22: 462-466, 2014.
24. Stanley AC, Fernandez NN, Lounsbury KM, Corrow K, Osler T, Healey C, Forgione P, Shackford SR and Ricci MA: Pressure-induced cellular senescence: A mechanism linking venous hypertension to venous ulcers. J Surg Res 124: 112-117, 2005.
25. Healey C, Forgione P, Lounsbury KM, Corrow K, Osler T, Ricci MA and Stanley A: A new in vitro model of venous hypertension: The effect of pressure on dermal fibroblasts. J Vasc Surg 38: 1099-1105, 2003.
26. Etufugh CN and Phillips TJ: Venous ulcers. Clin Dermatol 25: 121-130, 2007.

27. Díez J: Mechanisms of cardiac fibrosis in hypertension. J Clin Hypertens (Greenwich) 9: 546-550, 2007.
28. Wall SJ, Sampson MJ, Levell N and Murphy G: Elevated matrix metalloproteinase-2 and-3 production from human diabetic dermal fibroblasts. Br J Dermatol 149: 13-16, 2003.
29. Kosugi T, Osanai T, Kamada T, Nakano T and Okumura K: Phospholipase C activity is enhanced in skin fibroblasts obtained from patients with essential hypertension. J Hypertens 21: 583-590, 2003.
30. Ansurudeen I, Sunkari VG, Grünler J, Peters V, Schmitt CP, Catrina SB, Brismar K and Forsberg EA: Carnosine enhances diabetic wound healing in the db/db mouse model of type 2 diabetes. Amino Acids 43: 127-134, 2012.
31. Moulin L, Cenizo V, Antu AN, André V, Pain S, Sommer P and Debret R: Methylation of LOXL1 Promoter by DNMT3A in aged human skin fibroblasts. Rejuvenation Res 20: 103-110, 2017.
32. Koch CM, Suschek CV, Lin Q, Bork S, Goergens M, Joussen S, Pallua N, Ho AD, Zenke M and Wagner W: Specific age-associated DNA methylation changes in human dermal fibroblasts. PLoS One 6: e16679, 2011.
33. Casillas MA Jr, Lopatina N, Andrews LG and Tollefsbol TO: Transcriptional control of the DNA methyltransferases is altered in aging and neoplastically-transformed human fibroblasts. Mol Cell Biochem 252: 33-43, 2003.
34. Tirado-Magallanes R, Rebbani K, Lim R, Pradhan S and Benoukraf T: Whole genome DNA methylation: Beyond genes silencing. Oncotarget 8: 5629-5637, 2017.
35. Meevassana J, Serirodom S, Prabsattru P, Boonsongserm P, Kamolratanakul S, Siritientong T, Mutirangura A and Angspatt A: Alu repetitive sequence CpG methylation changes in burn scars. Burns 48: 1417-1424, 2022.
36. Dagan T, Sorek R, Sharon E, Ast G and Graur D: AluGene: A database of Alu elements incorporated within protein-coding genes. Nucleic Acids Res 32(Database issue): D489-D492, 2004.
37. Patchsung M, Settayanon S, Pongpanich M, Mutirangura D, Jintarith P and Mutirangura A: Alu siRNA to increase Alu element methylation and prevent DNA damage. Epigenomics 10: 175-185, 2018.
38. Thongsroy J and Mutirangura A: The association between Alu hypomethylation and the severity of hypertension. PLoS One 17: e0270004, 2022.
39. Unnikrishnan A, Hadad N, Masser DR, Jackson J, Freeman WM and Richardson A: Revisiting the genomic hypomethylation hypothesis of aging. Ann N Y Acad Sci 1418: 69-79, 2018.
40. Ciccarone F, Tagliatesta S, Caiafa P and Zampieri M: DNA methylation dynamics in aging: how far are we from understanding the mechanisms? Mech Ageing Dev 174: 3-17, 2018.
41. 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: 269-727, 2007.
42. Pobsook T, Subbalekha K, Sannikorn P and Mutirangura A: Improved measurement of LINE-1 sequence methylation for cancer detection. Clin Chim Acta 412: 314-321, 2011.
43. Saeliw T, Tangsuwansri C, Thongkorn S, Chonchaiya W, Suphapeetiporn K, Mutirangura A, Tencomnao T, Hu VW and Sarachana T: Integrated genome-wide Alu methylation and transcriptome profiling analyses reveal novel epigenetic regulatory networks associated with autism spectrum disorder. Mol Autism 9: 27, 2018.
44. Kalayasiri R, Kraijak K, Mutirangura A and Maes M: Paranoid schizophrenia and methamphetamine-induced paranoia are both characterized by a similar LINE-1 partial methylation profile, which is more pronounced in paranoid schizophrenia. Schizophr Res 208: 221-227, 2019.
45. Cardelli M: The epigenetic alterations of endogenous retroelements in aging. Mech Ageing Dev 174: 30-46, 2018.
46. Gentilini D, Mari D, Castaldi D, Remondini D, Ogliari G, Ostan R, Bucci L, Sirchia SM, Tabano S, Cavagnini F, *et al*: Role of epigenetics in human aging and longevity: Genome-wide DNA methylation profile in centenarians and centenarians' offspring. Age (Dordr) 35: 1961-1973, 2013.
47. Mutirangura A: Is global hypomethylation a nidus for molecular pathogenesis of age-related noncommunicable diseases? Epigenomics 11: 577-579, 2019.
48. Xiao FH, Kong QP, Perry B and He YH: Progress on the role of DNA methylation in aging and longevity. Brief Funct Genomics 15: 454-459, 2016.
49. Orioli D and Dellambra E: Epigenetic regulation of skin cells in natural aging and premature aging diseases. Cells 7: 268, 2018.
50. Bellavia A, Urch B, Speck M, Brook RD, Scott JA, Albetti B, Behbod B, North M, Valeri L, Bertazzi PA, *et al*: DNA hypomethylation, ambient particulate matter, and increased blood pressure: Findings from controlled human exposure experiments. J Am Heart Assoc 2: e000212, 2013.
51. Alexeeff SE, Baccarelli AA, Halonen J, Coull BA, Wright RO, Tarantini L, Bollati V, Sparrow D, Vokonas P and Schwartz J: Association between blood pressure and DNA methylation of retrotransposons and pro-inflammatory genes. Int J Epidemiol 42: 270-280, 2013.
52. Delva P, Lechi A, Pastori C, Degan M, Sheiban I, Montesi G, Pea M, Meneguzzi A and Menegazzi M: Collagen I and III mRNA gene expression and cell growth potential of skin fibroblasts in patients with essential hypertension. J Hypertens 20: 1393-1399, 2002.