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Breast Cancer Sera Changes in Alu Element Methylation Predict Metastatic Disease Progression

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Abstract. *Background/Aim: During metastatic disease development, the cancer-immune system crosstalk induces epigenetic modifications to immune cells, impairing their functions. Recently, Alu elements methylation changes were widely studied in terms of early cancer detection. This study aimed to demonstrate in vitro Alu element methylation changes in peripheral immune cells in a metastatic setting and examine their prognostic values in metastatic breast cancer. Materials and Methods: Sera from sixteen metastatic cancer patients and sixteen healthy participants were obtained and used to culture normal peripheral immune cells. After 48 h of incubation, the percentage and pattern of Alu element methylation were examined for clinical relevance. Results: We found that the Alu element hypomethylation was affected by age in the cancer group. Intriguingly, a decrease in Alu element methylation was found in patients with early progressive disease. Moreover, an increase in unmethylated cytosine (mCuC) loci was related to the poorer prognosis group. Accordingly, the decrease in Alu element methylation and the increase in mCuC loci pattern in peripheral immune cells correlated with poorer prognosis and early progression in metastatic breast cancer. Conclusion: Alu element hypomethylation in immune cells and their increased mCuC foci were related to the early progression of breast cancer. These warrant the use of Alu element methylation changes for diagnostic and therapeutic purposes in breast cancer.*

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Breast cancer is the most globally prevalent cancer and the leading cause of death in females (1). Although multimodality treatment has been proven to improve oncologic outcomes, 10%-20% of curative breast cancer patients still develop metastatic diseases (2). To date, the understanding of cancer biology provides specific treatments and precise prognoses (3, 4). However, metastatic recurrences are not prevented. Currently, immune cell surveillance is found to be necessary to control the occurrence of transformed cells, and these durable immune responses could prevent metastatic events (5).

The infiltrating immune cells and circulating immune cells are widely examined in breast cancer research (6, 7). Although the self-immune responses are the self-protective systems which recognize and eradicate cancer development, the cancer-associated epigenetic alteration of immune cells eventually causes host immune dysfunction, which promotes cancer cell survival (8, 9). This plasticity of immune cells results from global methylation changes in breast cancer-associated immune cells (10).

The blood DNA methylation level has been studied as a non-invasive biomarker because blood DNA methylation is affected by cancer-immune cell interactions. Therefore, blood DNA methylation can early detect carcinogenesis (11). Many researchers use the methylation level of transposons as global methylation surrogates. Because the intersperse elements approximate 45% of human genomes, the Alu element methylation is generally demonstrated as the global methylation status (12). Since the CpG sites of Alu elements are usually methylated in normal cells, the hypomethylation

of these elements has been stated as one of the early signs of carcinogenesis (13).

Furthermore, these repetitive transposons are currently known as factors involved in genomic instability (14, 15). In this circumstance, blood cell Alu element methylation is altered after the occurrence of metastasis (16). Furthermore, the aggressiveness of the metastatic disease depends on cancer signatures (17). The presence of dysfunctional immune cells accelerates disease progression. In metastatic disease, alterations in Alu element methylation are affected by several factors, including disease prognostic factors, treatment toxicity, and genomic stability of immune cells. Moreover, alterations in Alu element methylation were found in immunopathologic diseases (18). Therefore, blood cell Alu element methylation and disease biology reciprocally determine disease prognosis.

In this study, we aimed to demonstrate cancer-associated methylation changes of normal immune cells. We designed an experiment to measure Alu element methylation in peripheral blood mononuclear cells (PBMCs) after being exposed to cancer serum from metastatic breast cancer patients. The Combine Bisulfite Restriction Analysis (COBRA) was used to analyze the level and patterns of methylation (19). These changes were compared with those of normal and bovine serum. We examined whether these changes could provide prognostic values in metastatic breast cancer.

Materials and Methods

The recruitment of 16 metastatic breast cancer participants and 16 healthy participants with normal mammographic results was performed at Queen Sirikit Centre for Breast Cancer from December 2020-May 2021. The follow-up data were updated until the progressive disease was noted radiographically or the 90-day follow-up duration was reached. Time to progression was calculated from the sample retrieval date to the disease progression date. The exclusion criteria included other chronic medical diseases, other malignancies, and a history of blood transfusion within 3 months. A written informed consent was obtained from each participant. The demographic data and medical records were retrieved from King Chulalongkorn Memorial Hospital database. The study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University (IRB no. 789/63).

Blood and sera samples. A total of 16 ETDA tubes were collected from 16 healthy participants. The fresh blood was diluted by Roswell Park memorial institute (RPMI) 1640 medium. The blood samples were gently layered on Lymphoprep™ (Stemcell™, Waterbeach, Cambridge, UK) and centrifuged at 500 × *g* for 30 min with a brake-off. The interface PBMC layer was softly collected and maintained in a 6-well plate of complete RPMI medium. Trypan blue was used to determine cell number.

Blood was also collected in clot blood tubes for all study participants. The fluid part of the samples was collected and placed in Eppendorf tubes. The samples were centrifuged, and the serum was collected.

Culture of PBMCs in sera. PBMCs were cultured in bovine, normal, or cancer serum. The experiment was performed in triplicate. The RPMI 1640 medium was supplemented with 20% of allocated serum in a 6-well plate. PBMCs were seeded at 2.5×10^5 cells/ml per well. The culture well plates were maintained at 37˚C in a humidified atmosphere containing 5% CO_2 for 48 h. Then, PBMCs were collected and centrifuged. The cell pellets were used for DNA extraction.

Quantitative combine bisulfite restriction analysis for Alu elements (COBRA Alu). The cell pellets were lysed with cell lysis buffer II (0.75 M NaCl, 0.024 M EDTA at pH 8), 10% sodium dodecyl sulfate (Sigma-Aldrich, St Louis, MO, USA), and 20 mg/ml proteinase K (Usbio, Salem, MA, USA). The phenol-chloroform extraction method was used to extract genomic DNA. Five hundred ng of genomic DNA was used for quantification of the methylation level. The samples were treated with sodium bisulfite using an EZ DNA methylation-GoldTM kit (Zymo Research, Irvine, CA, USA) based on the manufacturer's instructions. Subsequently, a total of 1 µl of bisulfited DNA was subjected to 45 cycles of PCR using the following Alu elements primers.

Forward (5'-GGYGYGGTGGTTTAYGTTTGTAA-3')

Reverse (5'-CTAACTTTTTATATTTTTAATAAAAACRAAATTTCACCA-3').

The PCR was performed to generate 133 bp amplicons. The amplified amplicons were digested using 2 units of Taq1 (Thermo Scientific, Waltham, MA, USA) endonuclease in NE buffer III at 65˚C overnight. The PCR products were analyzed by 8% polyacrylamide gel electrophoresis and stained with SYBR green (SYBR® Green, Sigma-Aldrich). After incubation in SYBR solution, the gels were captured with epiblue light, and band intensity analysis was performed by using a phosphor imager and Image Quant Software (Molecular Dynamics, GE Healthcare®, Slough, UK).

Methylation analysis. In the COBRA technique, two sites of PCR products were used to determine the status of CpG methylation. An unmethylated site was modified by sodium bisulfite to avoid the restriction enzyme digestion. There were four bands that determine their Alu methylation patterns including 133 bp (uCuC), 90 bp (mCuC), 75 bp (uCmC), and 32 bp (mCmC) as shown in Figure 1. The intensity of each band was normalized by its paired length to calculate the amount of their amplicons as follows: 133 bp/133 (A), 58 bp/58 (B), 75 bp/73 (C), 90 bp/90 (D) and 43 bp/41 (E). The methylation percentage of each sample was calculated using the following formula:

The percentage of Alu element methylation=[(E + B)/(E +B + 2A + C +D)]×100

All gels were normalized by bisulfite-converted 100% methylated human DNA (Zymo Research).

Statistical analysis. Data are reported as mean and percentages. The paired and unpaired *t*-tests were used to compare quantitative data. The Pearson correlation was used to determine correlations with age. The unpaired *t*-test and ANOVA were used in clinical data analysis. Statistical significance was set at p-value <0.05. The analysis was performed using IBM SPSS software (version 26.0, Chicago, IL, USA).

Results

Participant characteristics. The study included 16 healthy participants with a mean age of 53.06 years, range=37-62

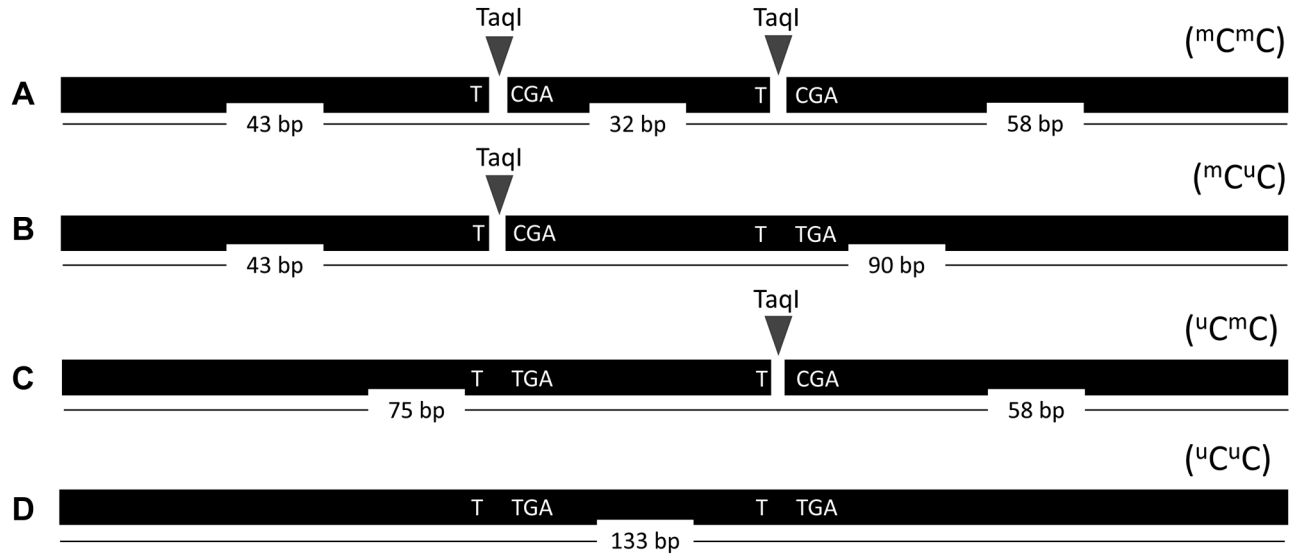

Figure 1. *The restriction sites of methylated CpGs were cut by Taq-I enzyme. The figure shows (A) 2 unmethylated sites, (B) and (C) one unmethylated site, and (D) 2 methylated sites.*

years. In the breast cancer group, there were 16 sera from metastatic breast cancer patients. Their mean age was 50.5 years, range=33-62 years. All participants in the cancer group received systemic treatment, including chemotherapy and anti-hormonal treatment for their disseminated diseases. The median follow-up time was 140.5 days in the breast cancer group. The clinicopathological characteristics are presented in Table I. Disease progression was determined by radiographic evidence. Time to progression was calculated from the sample retrieval date to the disease progression date.

Alu element methylation changes in PBMCs after culture with cancer or normal serum. After 48 h of culture, the mean percentages of Alu element methylation were 67.61, 66.17, and 66.87 in the bovine, normal, and cancer serum group, respectively (Figure 2). The paired *t*-test revealed no statistical significance (p=0.54 in cancer *vs*. normal serum, and p=0.41 in cancer *vs*. bovine serum).

Alu element methylation changes related to cancer prognosis. Percentage changes in Alu element methylation after 48-h incubation with cancer serum were calculated as the differences from bovine serum and healthy serum groups. These differences were examined for clinical relevance. The breast cancer prognostic factors were used to examine whether the differences in Alu methylation changes determined good and poor prognostic factors, as demonstrated in Table I. Moreover, time to progression was used as a prognostic factor in this study. Cancer patients were divided according to time to progression (TTP) into two groups; in the TTP≤90 group where patients progressed in less than 90 days and the TTP>90 group in which patients progressed in more than 90 days. There were 7 samples in the TTP≤90 and 9 samples in the TTP>90 group. Their mean ages were 53 years in the TTP≤90 group and 48 years in the TTP>90 group (p=0.31), and the age ranges were comparable between groups (39-62 years in the TTP≤90 group and 33-61 years in the TTP>90 group). Interestingly, the mean difference in Alu elements methylation was significantly lower in the TTP≤90 group, which was -3.48%, whereas in the TTP>90 group was +1.37% (p=0.008) (Figure 3). However, the means were not significantly different between cancer serum and normal serum. These findings suggest that the progression of breast cancer was related to hypomethylated Alu elements of circulating immune cells.

Changes in Alu element methylation pattern determined early progression in metastatic breast cancer. In the COBRA technique, the pattern of Alu element methylation was divided into four patterns, including double methylated cytosine loci (mCmC), unmethylated cytosine followed by methylated cytosine loci (uCmC), methylated cytosine followed by unmethylated cytosine loci (mCuC), and double unmethylated cytosine loci (uCuC). In this study, hypomethylation in circulating immune cells was found in the early cancer progression group, which showed significant patterns of the hypomethylation including increased mCuC loci and decreased mCmC loci. In detail, the percentages of mCuC were 17.24 and 21.34 in TTP>90 and TTP≤90 groups, respectively (p=0.016). Moreover, the percentages of mCmC were 46.27 and 39.80 in TTP>90 and TTP≤90 groups, respectively (p=0.022) (Figure 4).

Table I. *The mean of differences in Alu elements methylation according to clinicopathological characteristics of breast cancer.*

Factors	N	Cancer serum - Bovine serum		Cancer serum – Normal serum	
		Mean of differences	*p*-Value	Mean of differences	*p*-Value
Grade					
1-2	6	–1.010	0.793	–0.870	>0.999
3	10	–0.120		2.340	
Estrogen receptor					
Positive	10	0.960	0.220	2.925	0.118
Negative	6	–2.755		–2.535	
Progesterone receptor					
Positive	6	1.600	0.635	2.675	0.492
Negative	10	–1.205		–0.870	
HER2					
Positive	3	0.950	0.704	4.600	0.146
Negative	13	–1.190		0.670	
Treatment					
Chemotherapy	9	–1.190	0.837	–2.410	0.758
Others	7	–0.160		2.580	
Time to progression					
≤90 days	7	–4.66	0.0079	2.58	0.252
>90 days	9	1.56		–2.66	

Discussion

The methylation of Alu elements in blood cells has been used for cancer diagnosis and prognosis for many years (12, 20, 21). For instance, alterations of Alu element methylation were demonstrated in cancer patients, including breast, colon, and pancreatic cancer (22-25). As we know, the methylation status is dynamic. External factors, such as viral infection, burn, and tobacco, could alter the methylation status (26-29). Furthermore, several studies have shown early changes of Alu element methylation in cancer (28, 30). Moreover, this alteration in methylation has also been found preclinically (22). In this study, methylation changes in immune cells were found after 48 h incubation with cancer sera, and the hypomethylation in immune cells was related to early disease progression.

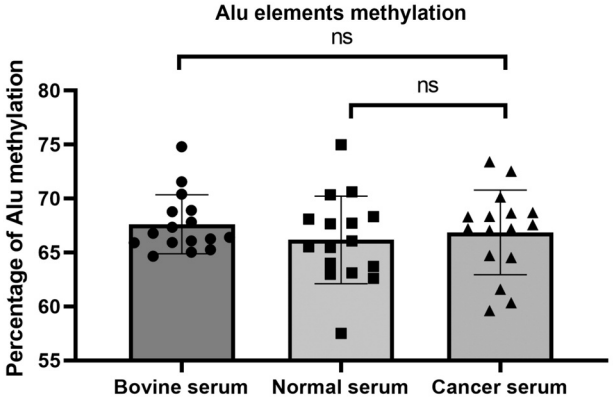

Figure 2. *Means of Alu elements methylation in each group. The figure shows scatter plots and bars of the percentage of Alu elements methylation in bovine serum, normal serum, and cancer serum groups.*

In this study, changes in Alu element methylation were related to the early progression of metastatic breast cancer. These findings are in accordance with a previous study showing Alu element hypomethylation in immune cells in relation to carcinogenesis (12). Moreover, other studies showed a higher odd ratio of hypomethylated Alu elements with advanced staging and lymph node involvement (16, 31). In this study, all cancer sera were retrieved from metastatic breast cancer patients. Although we did not find an association between Alu element hypomethylation and tumor characteristics, this change in immune cells tended to predict the progression of metastatic breast cancer, whereas the sensitivity of current tumor markers and circulating tumor cells was not as effective (32, 33). Therefore, the changes in Alu element methylation in circulating immune cells are challenging to study in metastatic breast cancer.

Apart from global genome-wide methylation status, Alu element methylation has also been related to aging and organ function impairment (34). Furthermore, another study found Alu element hypomethylation in the elderly (35). As in chronic diseases, such as diabetes and osteoporosis, Alu element hypomethylation was found in those diseases with high severity (36, 37). It is known that aging is the consequence of DNA damages and metabolic disturbances. The accumulation

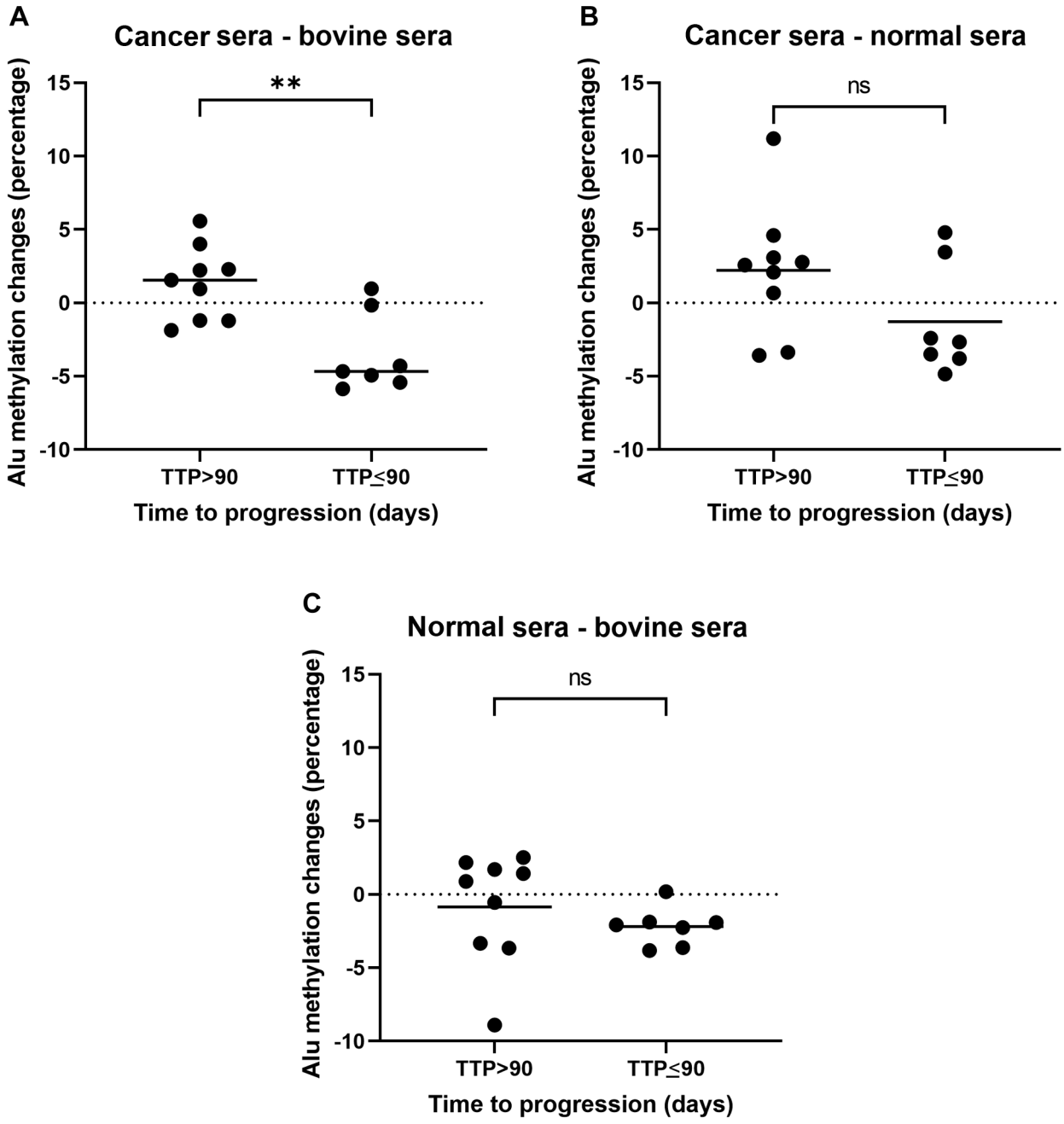

Figure 3. *The Alu elements methylation changes after a 48-h cancer serum incubation in the TTP>90 and TTP≤90 groups. These figures show the differences in Alu elements methylation between cancer serum and bovine serum group (A), the differences in Alu elements methylation between cancer serum and normal serum groups (B), and the differences in Alu elements methylation between normal serum and bovine serum groups (C).*

of these events creates genomic instability (38). Recently, siRNA of Alu elements have been used to increase methylation of Alu elements, and lower levels of DNA damage markers were found after exposure to DNA damage damaging agents in the siRNA treated group (15). These findings suggested the association of Alu element methylation and cellular aging.

According to the COBRA technique, there were 2 restriction sites of methylated CpG for evaluation. In our study, hypomethylation in immune cells was found to be partial, and the mCuC loci were prominent. This pattern of hypomethylation in immune cells was found to be associated with shorter survival of patients with head and neck cancer (16, 26). On the

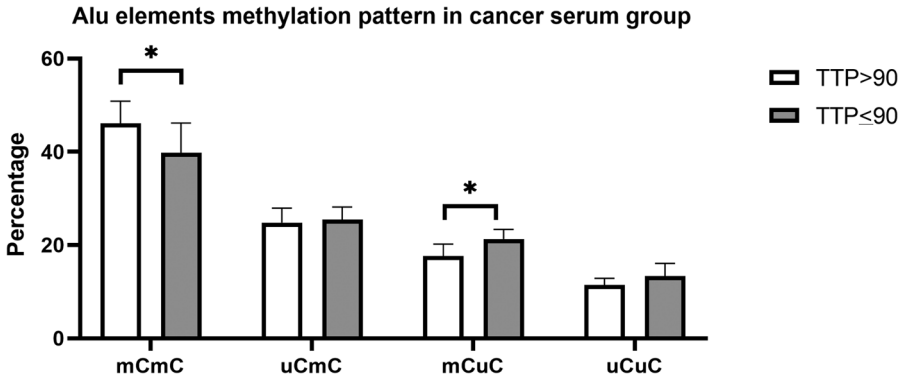

Figure 4. *The patterns of Alu elements methylation in TTP>90 and TTP≤90 groups.*

contrary, the percentage of mCuC loci was decreased in autoimmune diseases (39). Here, the increase in mCuC loci percentage represented Alu element hypomethylation was associated with a worse prognosis.

In conclusion, this study demonstrated breast cancer-induced epigenetic modifications in immune cells. Although the pattern of Alu element methylation changes was not consistent, hypomethylation in immune cells was related to the early progression of breast cancer. Moreover, the most prominent pattern of Alu element hypomethylation was mCuC loci. These findings warrant the use of Alu element methylation changes in leukocytes for diagnostic and therapeutic purposes in breast cancer.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Research design: Muthirangura A., Puttipanyalears C, and Denariyakoon S; experimental work: Denariyakoon S; review and revision; Muthirangura A., Puttipanyalears C, and Chatamra K.

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