Relationship between Long Interspersed Nuclear Element-1 DNA Methylation in Leukocytes and Dyslipidemia in the Japanese General Population

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Aim: Aberrant global DNA methylation is involved in the development of several diseases, including cardiovascular disease (CVD). We investigated whether the methylation of long interspersed nuclear element-1 (LINE-1) in leukocytes is associated with dyslipidemia, a major risk factor for CVD, in the Japanese general population.

Methods: We conducted a cross-sectional study consisting of 420 Japanese subjects (187 men and 233 women) without a clinical history of cancer, stroke, or ischemic heart disease. LINE-1 DNA methylation levels in leukocytes were measured using a pyrosequencing method.

Results: Significantly higher odds ratios (ORs) for hypermethylation were observed in the high LDL cholesterol and high LDL/HDL ratio groups than the corresponding normal group (high LDLC group: OR, 1.88; 95% confidence interval [CI], 1.20–2.96, high LDL/HDL ratio group: OR, 1.90; 95% CI, 1.20–3.01). Subjects with 2 or more lipid abnormalities had significantly higher ORs for hypermethylation than those with no lipid abnormality (OR, 2.31; 95% CI, 1.11–4.82).

Conclusion: LINE-1 DNA hypermethylation in leukocytes was associated with CVD risk profiles: high LDLC, high LDL/HDL ratio, and the degree of abnormal lipid metabolism.

Introduction

DNA methylation is the addition of a methyl group to DNA bases and is an epigenetic mechanism that regulates gene expression without alternating the DNA sequence. DNA methylation occurs predominantly on cytosine bases of the dinucleotide sequence CpG[1]. Methylation of a CpG island within a cluster

of CpG sites in the promoter region of a gene represses the expression of that gene[2]. Methylation directly hinders the binding of transcription factors to the promoter region[3], is reversible, and affects basic biological functions such as aging and cell development[4]. Global hypomethylation and gene-specific promoter hypermethylation are common events not only in tumor tissues but also in blood leukocytes[5, 6].

Long interspersed nuclear element-1 (LINE-1) belongs to the family of non-long terminal repeat retrotransposons. The gene constitutes about 17% of the human genome and is spread ubiquitously throughout the genome[7]. Methylation levels of LINE-1 are believed to be surrogate markers of global DNA methylation[8], and they are commonly heavily methylated in normal tissues[9]. Several epidemiological studies have shown that global DNA methylation levels in leukocytes are associated with diseases such as cardiovascular disease (CVD) and diabetes[10, 11]. Previous studies have suggested that DNA methylation may play an important role in chronic diseases, especially circulatory diseases[12, 13]. It has been reported that LINE-1 DNA hypomethylation in leukocytes is significantly associated with ischemic heart disease, stroke[12], and myocardial infarction[13]. Therefore, we expect that knowledge of LINE-1 DNA methylation levels will allow prediction of the development of lifestyle diseases.

Dyslipidemia is recognized as a potential risk for CVD and is caused by unhealthy lifestyle factors such as poor diet and physical inactivity. Several studies have reported an association between DNA methylation and dyslipidemia; however, no consistent results have been obtained[14, 15]. Although there is evidence that ethnic groups differ in their patterns of DNA methylation[16, 17], to the best of our knowledge there are no reported studies of the association of DNA methylation with dyslipidemia in the Japanese population.

Aim

In the present cross-sectional study, we investigated whether aberrant LINE-1 DNA methylation in leukocytes was associated with dyslipidemia, a risk factor for CVD, in the Japanese general population.

Methods

The present cross-sectional study is part of the ongoing Yakumo Study in Hokkaido, Japan. A total of 525 Japanese people attended a health examination in August 2015. We excluded 105 subjects who had a clinical history of cancer, stroke, or ischemic heart dis-

ease, did not provide informed consent for the present study, or for whom adequate samples could not be obtained to measure LINE-1 DNA methylation levels. The remaining 420 eligible subjects (187 men and 233 women) were included in the analysis. The protocol of this study was approved by the Ethics Committee of Fujita Health University (approval No. 164).

A trained public health nurse obtained health information, including smoking habits (never, former, or current), alcohol consumption (never, former, or current), antihyperlipidemic drug use (user/non-user), and a history of major illnesses (yes/no) using self-administered questionnaires during the health examination. Height, weight, waist circumference, and blood pressure were measured during the health examination. Body mass index (BMI) was calculated by dividing weight (kg) by height squared (m^2).

Fasting blood samples were taken during the health examination. Plasma glucose, whole blood hemoglobin A1c, and serum lipids such as triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDLC), and low-density lipoprotein cholesterol (LDLC) were determined using autoanalyzers, and serum C-reactive protein (CRP) concentrations were measured by a latex-immunoassay (LT-auto Wako CRP, Wako Pure Chemical Industries, Ltd., Osaka, Japan) at the laboratory of Yakumo General Hospital on the day of the health examination.

Blood samples were drawn into tubes containing ethylenediaminetetraacetic acid (EDTA), then centrifuged at 1500 g for 10 min. The buffy coat was collected and treated with a lysis solution (pH 7.4) containing 150 mmol/L NH$_4$Cl, 14 mmol/L NaHCO$_3$, and 0.11 mmol/L EDTA-2Na in distilled water to remove erythrocytes. Treated buffy coat samples were centrifuged at 100 g for 5 min at 4℃ and the supernatant was discarded. DNA was extracted from peripheral leukocytes using a NucleoSpin® Tissue kit (Takara, Japan) and stored at −80℃ until use.

Extracted DNA (500 ng, 50 ng/μL) was treated with sodium bisulfite using an EpiTect Fast DNA bisulfite kit (QIAGEN, Germany) according to the manufacturer's protocol to convert only non-methylated cytosine to uracil. LINE-1 elements were amplified by polymerase chain reaction (PCR) using Takara EpiTaq™ HS (for bisulfite-treated DNA) (Takara). PCR was performed using a 20 μL reaction sample containing 1 ng/μL bisulfite converted DNA, 0.05 U/μL Takara EpiTaq HS, 1x EpiTaq PCR buffer, 2.5 mmol/L MgCl$_2$, 0.3 mmol/L dNTP mixture, 0.2 μmol/L each of forward and reverse primer (forward primer 5'-TTTTGAGTTAGGTGTGGGATATA-3' and reverse biotinylated primer 5'-AAAAT-CAAAAAATTCCCTTTC-3'), and RNase-free water.

The PCR protocol consisted of an initial denaturing step at 98℃ for 20 s, followed by 45 cycles of 98℃ for 10 s, 55℃ for 30 s, and 72℃ for 30 s. We measured methylation by the pyrosequencing method (analysis device: PyroMark Q24 Advanced, QIAGEN) using the sequence primer 5'-AGTTAGGTGT-GGGATATAGT-3' and PyroMark® Q24 Advanced CpG reagents (QIAGEN). We quantified LINE-1 DNA methylation at three CpG sites[18, 19]. The levels of LINE-1 DNA methylation were expressed as the percentage of methylated cytosine and the mean LINE-1 DNA methylation levels of three CpG sites were calculated.

Dyslipidemia was defined according to the definition released in 2012 by the Japan Atherosclerosis Society[20]. Dyslipidemia subjects meet at least one of the following conditions: serum TG ≥ 150 mg/dL, serum LDLC ≥ 140 mg/dL, or serum HDLC < 40 mg/dL. The number of lipid abnormalities was defined as the sum of these three factors (0–3). Subjects were classified into three groups according to the number of lipid abnormalities (0, 1, ≥ 2) because there were only three subjects with three lipid abnormalities. The LDL/HDL ratio was calculated as the serum LDLC level divided by the serum HDLC level. We defined a high LDL/HDL ratio as being more than 2.5[21] and high TC as ≥ 200 mg/dL[22], and these scores raise the risk for CVD.

Statistical analyses were performed using JMP version 12.0 (SAS Institute, Cary, NC). We used log-transformed levels of plasma glucose, serum TG, and serum CRP for the analyses because these data provided log-normal distributions. Plasma glucose, serum TG, and serum CRP were presented as geometric mean and interquartile range, respectively. Other continuous parameters were represented as mean ± standard deviation (SD). We analyzed the differences of continuous parameters by the number of lipid abnormalities using analysis of variance (ANOVA) and Tukey–Kramer HSD test and compared categorized parameters using the chi-square test. Pearson's correlation coefficients were calculated to measure the linear relationship between LINE-1 DNA methylation and serum levels of various lipids. We defined the hypermethylation group as the third tertile of the LINE-1 DNA methylation levels (>57.65%). Multivariate adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for hypermethylation were calculated by logistic regression analysis according to the dyslipidemia. We used sex, age, smoking habits, alcohol consumption, BMI, CRP, and antihyperlipidemic drug use (user/non-user) as confounding factors. Probability values less than 0.05 were considered to be statistically significant.

Results

LINE-1 DNA methylation in leukocytes has an approximately normal distribution (mean ± SD = 56.98 ± 2.86%). There is no sex difference in LINE-1 DNA methylation (mean ± SD = 57.19 ± 2.89% in men; mean ± SD = 56.82 ± 2.82% in women; p = 0.182). Age, BMI, waist circumference, diastolic blood pressure, HbA1c, and CRP were significantly higher in the 2 or more lipid abnormalities than in those with 0 and 1 lipid abnormality (**Table 1**).

Correlation coefficients between LINE-1 DNA methylation levels and serum levels of various lipids are shown in **Table 2**. There was a significant positive association between LINE-1 DNA methylation and LDL/HDL ratio (r = 0.118, p = 0.016). HDLC was weakly and negatively associated with LINE-1 DNA methylation levels (r = − 0.094, p = 0.055).

The high LDLC and high LDL/HDL ratio groups had higher LINE-1 DNA methylation levels than the normal group (high LDLC group: mean ± SD = 57.49 ± 3.11%, normal LDLC group: 56.75 ± 2.71%, p = 0.009; high LDL/HDL ratio group: 57.51 ± 3.14%, normal LDL/HDL ratio group: 56.71 ± 2.66%, p = 0.004) (**Table 3**). However, there were no significant differences in LINE-1 DNA methylation between the TG, TC, and HDLC abnormal (i.e., high TG, high TC, and low HDLC levels) and normal groups.

We also calculated the confounding factor-adjusted ORs and 95% CIs for hypermethylation (**Table 4**). Significantly higher ORs for hypermethylation were observed in the high LDLC and the high LDL/HDL ratio groups than in the normal group (high LDL group: OR, 1.88; 95% CI, 1.20–2.96; high LDL/HDL ratio group: OR, 1.90; 95% CI, 1.20–3.01).

Table 5 shows the difference in LINE-1 DNA methylation in leukocytes by the number of lipid abnormalities and the confounding factor-adjusted ORs and 95% CIs for hypermethylation. Compared with the no lipid abnormality group, the group of 2 or more lipid abnormalities had significantly higher LINE-1 DNA methylation levels (56.70 ± 2.78% vs 57.95 ± 3.20%) and a higher prevalence of hypermethylation. Moreover, subjects with 2 or more lipid abnormalities had significantly higher ORs for hypermethylation than those with no lipid abnormality (OR, 2.31; 95% CI, 1.11–4.82, p for trend = 0.024).

Moreover, we divided the subjects by the non-user and user of antihyperlipidemic drugs and analyzed the association between LINE-1 DNA methylation and serum lipids (**Tables 3–5**). In the non-user group, we obtained results similar to those analyzed in

Table 1. Characteristics of the study subjects

	The number of lipid abnormalities			
	0	1	2 or more	*p* values
N	244	135	41	
Age[a] (y)	64.0±9.84	62.2±9.26	58.2±8.92ef	0.001[c]
Triglyceride[b] (mg/dL)	75.0 (59.0-103.8)	113.0 (78.0-151.0)[e]	179.0 (157.0-203.5)[ef]	<0.001[c]
Total cholesterol[a] (mg/dL)	196.6±23.2	233.3±32.8[e]	247.4±38.3[ef]	<0.001[c]
LDL cholesterol[a] (mg/dL)	110.8±20.0	145.4±30.1[e]	159.9±31.3[ef]	<0.001[c]
HDL cholesterol[a] (mg/dL)	62.1±12.8	57.2±13.5[e]	48.0±11.7[ef]	<0.001[c]
LDL/HDL ratio[a]	1.87±0.52	2.63±0.65[e]	3.42±0.70[ef]	<0.001[c]
Smoking habits				
Never *n* (%)	128 (52.5)	63 (46.7)	12 (29.3)	0.002[d]
Former *n* (%)	83 (34.0)	49 (36.3)	13 (31.7)	
Current *n* (%)	33 (13.5)	23 (17.0)	16 (39.0)	
Alcohol consumption				
Never *n* (%)	114 (46.7)	71 (52.6)	20 (48.8)	0.413[d]
Former *n* (%)	5 (2.1)	1 (0.7)	2 (4.9)	
Current *n* (%)	125 (51.2)	63 (46.7)	19 (46.3)	
Body mass index[a] (kg/m^2)	23.1±3.1	23.9±3.47	25.5±2.75[ef]	<0.001[c]
Waist circumference[a] (cm)	80.7±9.5	82.7±10.5	87.0±7.4[ef]	<0.001[c]
Systolic blood pressure[a] (mmHg)	130.4±20.4	129.7±19.9	136.9±21.6	0.130[c]
Diastolic blood pressure[a] (mmHg)	74.6±12.7	76.0±14.0	85.1±14.4[ef]	<0.001[c]
Glucose[b] (mg/dL)	86.0 (81.0-92.0)	86.0 (79.0-93.0)	89.0 (84.0-97.5)	0.086[c]
Hemoglobin A1c[a] (%)	5.71±0.51	5.72±0.47	5.96±0.66[ef]	0.014[c]
Antihyperlipidemic drug user *n* (%)	51 (20.9)	20 (14.8)	6 (14.6)	0.277[d]
C-reactive protein[b] (mg/dL)	0.030 (0.015-0.072)	0.041 (0.023-0.082)	0.072 (0.042-0.214)[ef]	<0.001[c]

[a] Mean ± SD.
[b] Median (interquartile range).
[c] one-way-ANOVA.
[d] chi-squared test.
[e] *p* < 0.05, Tukey-Kramer HSD test (vs. Low).
[f] *p* < 0.05, Tukey-Kramer HSD test (vs. Middle).

Table 2. Correlation between LINE-1 DNA methylation levels and serum levels of various lipids

	r[a]	*p* values
Ln Triglyceride[b]	0.053	0.278
Total cholesterol	0.021	0.673
LDL cholesterol	0.078	0.110
HDL cholesterol	−0.094	0.055
LDL/HDL ratio	0.118	0.016

[a] pearson's correlation coefficients   [b] log-transformed levels

all subjects. On the contrary, the significant associations between LINE-1 DNA methylation and serum lipids were not observed in the user group.

Discussion

In this study, we found that the levels of LINE-1 DNA methylation in leukocytes were significantly and positively associated with the prevalence of dyslipidemia, and were particularly associated with high LDLC and a high LDL/HDL ratio. Moreover, the degree of abnormal lipid metabolism, expressed as the number of lipid abnormalities, was positively associated with LINE-1 DNA methylation in leukocytes. There was no significant linear relationship between LINE-1 DNA methylation and LDLC. A previous study[14] also observed no significant linear relationship between LINE-1 DNA methylation levels and LDLC. We suggested that the global hypermethylation levels in leukocytes, assessed by LINE-1 methylation analysis, were associated with abnormal lipid metabolism. Epidemiological studies[23] have reported that combined dyslipidemia (the higher LDLC and the lower HDLC) increased the risk for CVD. LINE-1 DNA methylation levels may be associated with the risk for CVD because patients with combined lipid abnormalities had high LINE-1 DNA methylation levels.

Several epidemiological studies have reported the association of LINE-1 DNA methylation and dyslipidemia, but those results remain controversial. The Newcastle Thousand Families Study (NTFS) reported positive associations of LINE-1 DNA methylation with serum levels of TG, TC, and LDLC, and negative associations of LINE-1 DNA methylation with

Table 3. Differences in LINE-1 DNA methylation levels in leukocytes according to the serum levels of various lipids

		Total			Non-user of antihyperlipidemic drugs		
		n (%)	LINE-1 DNA methylation levels (%)[a]	p values[b]	n (%)	LINE-1 DNA methylation levels (%)[a]	p values[b]
Triglyceride	<150 mg/dL	348 (82.3)	56.89 ± 2.83	0.182	284 (82.8)	56.93 ± 2.89	0.174
	≥ 150 mg/dL	72 (17.1)	57.46 ± 2.94		59 (17.2)	57.51 ± 3.21	
Total cholesterol	<200 mg/dL	140 (33.3)	57.15 ± 2.86	0.366	103 (30.0)	57.32 ± 3.02	0.229
	≥ 200 mg/dL	280 (66.7)	56.89 ± 2.85		240 (70.0)	56.90 ± 2.92	
LDL cholesterol	<140 mg/dL	291 (69.3)	56.75 ± 2.71	0.009	227 (66.2)	56.79 ± 2.83	0.038
	≥ 140 mg/dL	129 (30.7)	57.49 ± 3.11		116 (33.8)	57.49 ± 3.14	
HDL cholesterol	≥ 40 mg/dL	401 (95.5)	56.95 ± 2.89	0.414	330 (96.2)	57.30 ± 2.25	0.735
	<40 mg/dL	19 (4.5)	57.53 ± 2.05		13 (3.8)	57.02 ± 2.98	
LDL/HDL ratio	<2.5	277 (66.0)	56.71 ± 2.66	0.004	217 (63.3)	56.76 ± 2.78	0.026
	≥ 2.5	143 (34.0)	57.51 ± 3.14		126 (36.7)	57.50 ± 3.19	

		User of antihyperlipidemic drugs		
		n (%)	LINE-1 DNA methylation levels (%)[a]	p values[b]
Triglyceride	<150 mg/dL	64 (83.1)	56.68 ± 2.55	0.439
	≥ 150 mg/dL	13 (16.9)	57.24 ± 1.21	
Total cholesterol	<200 mg/dL	37 (48.1)	56.68 ± 2.36	0.753
	≥ 200 mg/dL	40 (51.9)	56.85 ± 2.42	
LDL cholesterol	<140 mg/dL	64 (83.1)	56.62 ± 2.23	0.226
	≥ 140 mg/dL	13 (16.9)	57.51 ± 2.98	
HDL cholesterol	≥ 40 mg/dL	71 (92.2)	56.67 ± 2.41	0.187
	<40 mg/dL	6 (7.8)	58.01 ± 1.62	
LDL/HDL ratio	<2.5	60 (77.9)	56.52 ± 2.21	0.084
	≥ 2.5	17 (22.1)	57.65 ± 2.80	

[a] Mean ± SD.
[b] Student's t-test.

Table 4. Multivariate adjusted ORs and 95% CIs for LINE-1 DNA hypermethylation according to the serum levels of various lipids

		Total LINE-1 DNA hypermethylation		Non-user of antihyperlipidemic drugs LINE-1 DNA hypermethylation		User of antihyperlipidemic drugs LINE-1 DNA hypermethylation	
		n (%)	OR (95%CI)[a]	n (%)	OR (95%CI)[b]	n (%)	OR (95%CI)[b]
Triglyceride	<150 mg/dL	112/348 (32.2)	1.00	91/284 (32.0)	1.00	23/64 (35.9)	1.00
	≥ 150 mg/dL	26/72 (36.1)	1.07 (0.60-1.87)	21/59 (35.6)	1.11 (0.59-2.05)	5/13 (38.5)	0.84 (0.20-3.26)
Total cholesterol	<200 mg/dL	44/140 (31.4)	1.00	32/103 (31.1)	1.00	13/37 (35.1)	1.00
	≥ 200 mg/dL	94/280 (33.6)	1.01 (0.71-1.73)	80/240 (33.3)	1.10 (0.67-1.83)	15/40 (37.5)	0.93 (0.33-2.55)
LDL cholesterol	<140 mg/dL	83/291 (28.5)	1.00	62/227 (27.3)	1.00	22/64 (34.4)	1.00
	≥ 140 mg/dL	55/129 (42.6)	1.88 (1.20-2.96)	50/116 (43.1)	2.08 (1.28-3.39)	6/13 (46.2)	1.25 (0.30-4.85)
HDL cholesterol	≥ 40 mg/dL	130/401 (32.4)	1.00	107/330 (32.4)	1.00	25/71 (35.2)	1.00
	<40 mg/dL	8/19 (42.1)	1.32 (0.47-3.57)	5/13 (38.5)	1.25 (0.35-4.15)	3/6 (50.0)	1.55 (0.21-11.35)
LDL/HDL ratio	<2.5	78/277 (28.2)	1.00	61/217 (28.1)	1.00	18/60 (30.0)	1.00
	≥ 2.5	60/143 (42.0)	1.90 (1.20-3.01)	51/126 (40.5)	1.83 (1.11-3.02)	10/17 (58.8)	2.67 (0.79-9.36)

[a] Adjusted for sex, age, smoking habits, alcohol consumption, BMI, CRP, and antilipidemic drug use.
[b] Adjusted for sex, age, smoking habits, alcohol consumption, BMI, and CRP.

Table 5. Multivariate adjusted ORs and 95% CIs for LINE-1 DNA hypermethylation in leukocytes according to the number of lipid abnormalities

		The number of lipid abnormalities			
		0	1	2 or more	*p* values
Total					
LINE-1 DNA methylation levels[a]	%	56.70 ± 2.78	57.20 ± 2.82	57.95 ± 3.20[g]	0.019[d]
LINE-1 DNA hypermethylation	*n* (%)	70/244 (28.7)	48/135 (35.6)	20/41 (48.8)	0.029[e]
	OR (95%CI)[b]	1.00	1.37 (0.86-2.17)	2.31 (1.11-4.82)	0.024[f]
Non-user of antihyperlipidemic drugs					
LINE-1 DNA methylation levels[a]	%	56.79 ± 2.88	57.10 ± 2.88	58.10 ± 3.39[h]	0.051[b]
LINE-1 DNA hypermethylation	*n* (%)	55/193 (28.5)	39/115 (33.9)	18/35 (51.4)	0.027[c]
	OR (95%CI)[c]	1.00	1.31 (0.79-2.18)	2.95 (1.32-6.67)	0.013[f]
User of antihyperlipidemic drugs					
LINE-1 DNA methylation levels[a]	%	56.35 ± 2.34	57.78 ± 2.40	57.02 ± 1.61	0.070[b]
LINE-1 DNA hypermethylation	*n* (%)	16/51 (31.4)	10/20 (50.0)	2/6 (33.3)	0.336[c]
	OR (95%CI)[c]	1.00	1.64 (0.51-5.24)	0.72 (0.07-5.07)	0.967[f]

[a] Mean ± SD.
[b] Adjusted for sex, age, smoking habits, alcohol consumption, BMI, CRP, and antilipidemic drug use.
[c] Adjusted for sex, age, smoking habits, alcohol consumption, BMI,and CRP.
[d] one-way-ANOVA.
[e] chi-squared test.
[f] trend *p*.
[g] *p* = 0.026, Tukey-Kramer HSD test (vs. the 0 lipid abnormality group).
[h] *p* = 0.041, Tukey-Kramer HSD test (vs. the 0 lipid abnormality group).

HDLC and the HDLC/LDLC ratio for 228 subjects aged 49–51 years[15]. On the contrary, Cash *et al.*[14] observed that LINE-1 DNA hypermethylation was associated with lower LDLC and higher HDLC levels in 355 subjects of the Samoan Family Study of Overweight and Diabetes. Our results showed that LINE-1 DNA hypermethylation was associated with dyslipidemia in the Japanese population.

We cannot offer a clear explanation for these conflicting findings, but differences in the characteristics of the study subjects may be involved. A previous study showed that global DNA methylation differed by race[16]. There was a difference in LINE-1 DNA methylation levels between the Samoan study and our study. The mean ± SD of LINE-1 DNA methylation in Samoans was 83.2 ± 1.8%. The median and interquartile range (IQR) of LINE-1 DNA methylation of the NTFS study subjects was similar to our results (median: 52.76%; IQR: 51.51–54.92). We quantified LINE-1 DNA methylation at three CpG sites, as did the NTFS study, but the Samoan study examined the methylation status at four CpG sites in the LINE-1 region. We assumed that the observed difference of methylation levels was due to differences in the quantified LINE-1 DNA methylation sites. Moreover, the subjects in the Samoan study had a substantially higher BMI than our study subjects and the NTFS

study subjects. Recent reports indicate that alterations in DNA methylation are closely associated with obesity[24, 25]. The Multi-Ethnic Study of Atherosclerosis (MESA) reported that individuals with high BMI (≥ 40 kg/m²) had higher LINE-1 DNA methylation levels in leukocytes than those with normal BMI (< 25 kg/m²) in a cohort of 987 individuals aged 45–84 years[26]. Severe obesity may be involved in altering DNA methylation. In our study, obesity had little effect on LINE-1 DNA methylation, since none of our study subjects had BMI ≥ 40 kg/m². Furthermore, the subjects in the Samoan and NTFS studies were younger than our subjects. The age range of the NTFS study subjects was 49–51 years and the mean ages of the Samoan study subjects were 29.2–39.2 years. Age is reported to be negatively correlated with LINE-1 DNA methylation levels[27]. Antihyperlipidemic drugs may be associated with LINE-1 DNA methylation, since statin, a cholesterol-lowering drug, is reported to reduce DNMT activity[28]. Although the number of subjects may be insufficient to conclude from these analyzes , similar results were obtained in non-antihyperlipidemic drug users but not in antihyperlipidemic drug users. The rate of severe obesity, age, and the use of an antihyperlipidemic drug may affect the association between DNA methylation and dyslipidemia, potentially resulting in conflicting findings between

studies. However, we adjusted these factors in our study analysis.

Lipoproteins are reported to influence DNA methylation. LDLC causes vascular dysfunction by changing DNA methylation. LDLC inhibits the transcription of Krüppel-like factor 2 (KLF2), a protein that plays an important role in maintaining endothelial function[29] in human umbilical vein endothelial cells through its activation of DNMT 1 and by inducing transcription repressing proteins such as methyl-CpG-binding protein-2[30]. Consequently, global DNA methylation may be affected by LDLC. ApoA1, a major component of HDLC, is related to the risk of coronary artery disease[31], and its expression has also been reported to be directly regulated by the DNA methylation pattern on the promoter region. Hypermethylation on the ApoA1 promoter region causes a decrease in the expression of ApoA1[32]. No statistically significant relationship between HDLC and LINE-1 DNA methylation was obtained in this study, although the small number of subjects with low HDLC levels (n [%] = 19 [4.5]) may have had an impact on the results.

Inflammation, a risk factor for atherosclerosis, is associated with DNA methylation in leukocytes. Positive correlations between inflammation markers such as CRP and interleukin-6 (IL-6) and global DNA methylation in leukocytes were observed in 155 patients with renal disease[33]. IL-6 may have a function in epigenetic changes by regulating the methyltransferase gene[34]. Pro-inflammatory gene (encoding IL-1β and IL-6) promoter hypermethylation and down-regulation of expression were observed in THP-1 cells, a model for studying the role of inflammatory cells in atherosclerosis, following treatment with atherogenic lipoproteins, including high levels of LDLC and very low-density lipoprotein cholesterol[35]. Global DNA hypermethylation may fluctuate due to obesity and inflammation. We did not observe large alterations in our results for the relationship between LINE-1 DNA methylation and dyslipidemia, although our analyses were adjusted for BMI and serum CRP levels.

There have been several recent studies on associations between atherosclerosis-related diseases and DNA methylation in leukocytes. Stenvinkel et al. found that global DNA hypermethylation was significantly associated with high cardiovascular disease mortality in 98 patients with severe chronic kidney disease[33]. In contrast, a previous prospective study observed that elderly men (mean age = 72.3 years) with LINE-1 DNA hypomethylation were at a high risk for ischemic heart disease and stroke[12]. A nested case–control study also showed that LINE-1 DNA hypo-methylation was associated with myocardial infarction in a European prospective cohort study[13]. An experimental study using ApoE knock-out mice reported a significant reduction in DNA methylation in atherosclerotic lesions[36] and in aorta tissues prior to the appearance of atherosclerotic lesions[37]. Although the association of DNA methylation in leukocytes with methylation levels in vascular tissues is not clear, the levels of global DNA methylation in leukocytes may also be altered during cardiovascular pathogenic processes, such as those in atherosclerotic lesions or aorta tissues.

This is the first report to examine the relationship between LINE-1 DNA methylation levels in leukocytes and dyslipidemia in the Japanese population. Our study has several limitations. First, we could not prove causal relationships between LINE-1 DNA methylation and the serum level of each lipid because of the cross-sectional study design, which warrants a longitudinal study in future. Second, we did not consider the type of white blood cells when we measured the methylation levels of leukocyte DNA. It has been reported that the proportion of white blood cell subsets affects LINE-1 DNA methylation levels[38]. Although we calculated ORs adjusted for the percentage of neutrophils, this did not appear to alter our results (data not shown).

Conclusion

The main finding of our study is that high LINE-1 DNA methylation levels in leukocytes were associated with high CVD risk profiles: high LDLC, high LDL/HDL ratio, and the degree of abnormal lipid metabolism in the Japanese population. Our results suggest that LINE-1 DNA hypermethylation in leukocytes may be associated with an association between dyslipidemia and onset of CVD. Further studies are needed to elucidate the mechanism of the association between LINE-1 DNA methylation in leukocytes and dyslipidemia.

Conflict of Interest

The authors declare that no competing financial interests exist.

References

1) Jaenisch R, Bird A: Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet, 2003; 33: 245-254

2) Kulis M, Esteller M: DNA methylation and cancer. Adv Genet, 2010; 70: 27-56

3) Bayarsaihan D: Epigenetic mechanisms in inflammation. J Dent Res, 2011; 90: 9-17

4) Feinberg AP: Phenotypic plasticity and the epigenetics of human disease. Nature, 2007; 447: 433-440

5) Brennan K, Flanagan JM: Is there a link between genome-wide hypomethylation in blood and cancer risk? Cancer Prev Res (Phila), 2012; 5: 1345-1357

6) Joyce BT, Gao T, Zheng Y, Liu L, Zhang W, Dai Q, Shrubsole MJ, Hibler EA, Cristofanilli M, Zhang H, Yang H, Vokonas P, Cantone L, Schwartz J, Baccarelli A, Hou L: Prospective changes in global DNA methylation and cancer incidence and mortality. Br J Cancer, 2016; 115: 465-472

7) Lander ES, Linton LM, Birren B, Nusbaum C, Zody MC, Baldwin J, Devon K, Dewar K, Doyle M, FitzHugh W, Funke R, Gage D, Harris K, Heaford A, Howland J, Kann L, Lehoczky J, LeVine R, McEwan P, McKernan K, Meldrim J, Mesirov JP, Miranda C, Morris W, Naylor J, Raymond C, Rosetti M, Santos R, Sheridan A, Sougnez C, Stange-Thomann Y, Stojanovic N, Subramanian A, Wyman D, Rogers J, Sulston J, Ainscough R, Beck S, Bentley D, Burton J, Clee C, Carter N, Coulson A, Deadman R, Deloukas P, Dunham A, Dunham I, Durbin R, French L, Grafham D, Gregory S, Hubbard T, Humphray S, Hunt A, Jones M, Lloyd C, McMurray A, Matthews L, Mercer S, Milne S, Mullikin JC, Mungall A, Plumb R, Ross M, Shownkeen R, Sims S, Waterston RH, Wilson RK, Hillier LW, McPherson JD, Marra MA, Mardis ER, Fulton LA, Chinwalla AT, Pepin KH, Gish WR, Chissoe SL, Wendl MC, Delehaunty KD, Miner TL, Delehaunty A, Kramer JB, Cook LL, Fulton RS, Johnson DL, Minx PJ, Clifton SW, Hawkins T, Branscomb E, Predki P, Richardson P, Wenning S, Slezak T, Doggett N, Cheng JF, Olsen A, Lucas S, Elkin C, Uberbacher E, Frazier M, Gibbs RA, Muzny DM, Scherer SE, Bouck JB, Sodergren EJ, Worley KC, Rives CM, Gorrell JH, Metzker ML, Naylor SL, Kucherlapati RS, Nelson DL, Weinstock GM, Sakaki Y, Fujiyama A, Hattori M, Yada T, Toyoda A, Itoh T, Kawagoe C, Watanabe H, Totoki Y, Taylor T, Weissenbach J, Heilig R, Saurin W, Artiguenave F, Brottier P, Bruls T, Pelletier E, Robert C, Wincker P, Smith DR, Doucette-Stamm L, Rubenfield M, Weinstock K, Lee HM, Dubois J, Rosenthal A, Platzer M, Nyakatura G, Taudien S, Rump A, Yang H, Yu J, Wang J, Huang G, Gu J, Hood L, Rowen L, Madan A, Qin S, Davis RW, Federspiel NA, Abola AP, Proctor MJ, Myers RM, Schmutz J, Dickson M, Grimwood J, Cox DR, Olson MV, Kaul R, Raymond C, Shimizu N, Kawasaki K,

Minoshima S, Evans GA, Athanasiou M, Schultz R, Roe BA, Chen F, Pan H, Ramser J, Lehrach H, Reinhardt R, McCombie WR, de la Bastide M, Dedhia N, Blocker H, Hornischer K, Nordsiek G, Agarwala R, Aravind L, Bailey JA, Bateman A, Batzoglou S, Birney E, Bork P, Brown DG, Burge CB, Cerutti L, Chen HC, Church D, Clamp M, Copley RR, Doerks T, Eddy SR, Eichler EE, Furey TS, Galagan J, Gilbert JG, Harmon C, Hayashizaki Y, Haussler D, Hermjakob H, Hokamp K, Jang W, Johnson LS, Jones TA, Kasif S, Kaspryzk A, Kennedy S, Kent WJ, Kitts P, Koonin EV, Korf I, Kulp D, Lancet D, Lowe TM, McLysaght A, Mikkelsen T, Moran JV, Mulder N, Pollara VJ, Ponting CP, Schuler G, Schultz J, Slater G, Smit AF, Stupka E, Szustakowki J, Thierry-Mieg D, Thierry-Mieg J, Wagner L, Wallis J, Wheeler R, Williams A, Wolf YI, Wolfe KH, Yang SP, Yeh RF, Collins F, Guyer MS, Peterson J, Felsenfeld A, Wetterstrand KA, Patrinos A, Morgan MJ, de Jong P, Catanese JJ, Osoegawa K, Shizuya H, Choi S, Chen YJ, Szustakowki J: Initial sequencing and analysis of the human genome. Nature, 2001; 409: 860-921

8) Weisenberger DJ, Campan M, Long TI, Kim M, Woods C, Fiala E, Ehrlich M, Laird PW: Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res, 2005; 33: 6823-6836

9) 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, 2004; 32: e38

10) Muka T, Nano J, Voortman T, Braun KVE, Ligthart S, Stranges S, Bramer WM, Troup J, Chowdhury R, Dehghan A, Franco OH: The role of global and regional DNA methylation and histone modifications in glycemic traits and type 2 diabetes: A systematic review. Nutr Metab Cardiovasc Dis, 2016; 26: 553-566

11) Zhong J, Agha G, Baccarelli AA: The Role of DNA Methylation in Cardiovascular Risk and Disease: Methodological Aspects, Study Design, and Data Analysis for Epidemiological Studies. Circ Res, 2016; 118: 119-131

12) Baccarelli A, Wright R, Bollati V, Litonjua A, Zanobetti A, Tarantini L, Sparrow D, Vokonas P, Schwartz J: Ischemic heart disease and stroke in relation to blood DNA methylation. Epidemiology, 2010; 21: 819-828

13) Guarrera S, Fiorito G, Onland-Moret NC, Russo A, Agnoli C, Allione A, Di Gaetano C, Mattiello A, Ricceri F, Chiodini P, Polidoro S, Frasca G, Verschuren MW, Boer JM, Iacoviello L, van der Schouw YT, Tumino R, Vineis P, Krogh V, Panico S, Sacerdote C, Matullo G: Gene-specific DNA methylation profiles and LINE-1 hypomethylation are associated with myocardial infarction risk. Clin Epigenetics, 2015; 7: 133

14) Cash HL, McGarvey ST, Houseman EA, Marsit CJ, Hawley NL, Lambert-Messerlian GM, Viali S, Tuitele J, Kelsey KT: Cardiovascular disease risk factors and DNA methylation at the LINE-1 repeat region in peripheral blood from Samoan Islanders. Epigenetics, 2011; 6: 1257-1264

15) Pearce MS, McConnell JC, Potter C, Barrett LM, Parker L, Mathers JC, Relton CL: Global LINE-1 DNA methylation is associated with blood glycaemic and lipid profiles. Int J Epidemiol, 2012; 41: 210-217

16) Zhang FF, Cardarelli R, Carroll J, Fulda KG, Kaur M, Gonzalez K, Vishwanatha JK, Santella RM, Morabia A: Significant differences in global genomic DNA methylation by gender and race/ethnicity in peripheral blood. Epigenetics, 2014; 6: 623-629

17) Heyn H, Moran S, Hernando-Herraez I, Sayols S, Gomez A, Sandoval J, Monk D, Hata K, Marques-Bonet T, Wang L, Esteller M: DNA methylation contributes to natural human variation. Genome Res, 2013; 23: 1363-1372

18) Alexeeff SE, Baccarelli AA, Halonen J, Coull BA, Wright RO, Tarantini L, Bollati V, Sparrow D, Vokonas P, Schwartz J: Association between blood pressure and DNA methylation of retrotransposons and pro-inflammatory genes. Int J Epidemiol, 2013; 42: 270-280

19) Piyathilake CJ, Badiga S, Alvarez RD, Partridge EE, Johanning GL: A lower degree of PBMC L1 methylation is associated with excess body weight and higher HOMA-IR in the presence of lower concentrations of plasma folate. PLoS One, 2013; 8: e54544

20) Teramoto T, Sasaki J, Ishibashi S, Birou S, Daida H, Dohi S, Egusa G, Hiro T, Hirobe K, Iida M, Kihara S, Kinoshita M, Maruyama C, Ohta T, Okamura T, Yamashita S, Yokode M, Yokote K: Executive summary of the Japan Atherosclerosis Society (JAS) guidelines for the diagnosis and prevention of atherosclerotic cardiovascular diseases in Japan -2012 version. J Atheroscler Thromb, 2013; 20: 517-523

21) Chen QJ, Lai HM, Chen BD, Li XM, Zhai H, He CH, Pan S, Luo JY, Gao J, Liu F, Ma YT, Yang YN: Appropriate LDL-C-to-HDL-C Ratio Cutoffs for Categorization of Cardiovascular Disease Risk Factors among Uygur Adults in Xinjiang, China. Int J Environ Res Public Health, 2016; 13: 235

22) Kodama K, Sasaki H, Shimizu Y: Trend of coronary heart disease and its relationship to risk factors in a Japanese population: a 26-year follow-up, Hiroshima/Nagasaki study. Jpn Circ J, 1990; 54: 414-421

23) Castelli WP: Cholesterol and lipids in the risk of coronary artery disease--the Framingham Heart Study. Can J Cardiol, 1988; 4 Suppl A: 5a-10a

24) Carraro JC, Mansego ML, Milagro FI, Chaves LO, Vidigal FC, Bressan J, Martinez JA: LINE-1 and inflammatory gene methylation levels are early biomarkers of metabolic changes: association with adiposity. Biomarkers, 2016; 21: 625-632

25) Wang X, Zhu H, Snieder H, Su S, Munn D, Harshfield G, Maria BL, Dong Y, Treiber F, Gutin B, Shi H: Obesity related methylation changes in DNA of peripheral blood leukocytes. BMC Med, 2010; 8: 87

26) Perng W, Villamor E, Shroff MR, Nettleton JA, Pilsner JR, Liu Y, Diez-Roux AV: Dietary intake, plasma homocysteine, and repetitive element DNA methylation in the Multi-Ethnic Study of Atherosclerosis (MESA). Nutr Metab Cardiovasc Dis, 2014; 24: 614-622

27) Bollati V, Schwartz J, Wright R, Litonjua A, Tarantini L, Suh H, Sparrow D, Vokonas P, Baccarelli A: Decline in genomic DNA methylation through aging in a cohort of elderly subjects. Mech Ageing Dev, 2009; 130: 234-239

28) Houde AA, Legare C, Biron S, Lescelleur O, Biertho L, Marceau S, Tchernof A, Vohl MC, Hivert MF, Bouchard L: Leptin and adiponectin DNA methylation levels in adipose tissues and blood cells are associated with BMI, waist girth and LDL-cholesterol levels in severely obese men and women. BMC Med Genet, 2015; 16: 29

29) Atkins GB, Jain MK: Role of Kruppel-like transcription factors in endothelial biology. Circ Res, 2007; 100: 1686-1695

30) Kumar A, Kumar S, Vikram A, Hoffman TA, Naqvi A, Lewarchik CM, Kim YR, Irani K: Histone and DNA methylation-mediated epigenetic downregulation of endothelial Kruppel-like factor 2 by low-density lipoprotein cholesterol. Arterioscler Thromb Vasc Biol, 2013; 33: 1936-1942

31) Maciejko JJ, Holmes DR, Kottke BA, Zinsmeister AR, Dinh DM, Mao SJ: Apolipoprotein A-I as a marker of angiographically assessed coronary-artery disease. N Engl J Med, 1983; 309: 385-389

32) Guardiola M, Oliva I, Guillaumet A, Martin-Trujillo A, Rosales R, Vallve JC, Sabench F, Del Castillo D, Zaina S, Monk D, Ribalta J: Tissue-specific DNA methylation profiles regulate liver-specific expression of the APOA1/C3/A4/A5 cluster and can be manipulated with demethylating agents on intestinal cells. Atherosclerosis, 2014; 237: 528-535

33) Stenvinkel P, Karimi M, Johansson S, Axelsson J, Suliman M, Lindholm B, Heimburger O, Barany P, Alvestrand A, Nordfors L, Qureshi AR, Ekstrom TJ, Schalling M: Impact of inflammation on epigenetic DNA methylation - a novel risk factor for cardiovascular disease? J Intern Med, 2007; 261: 488-499

34) Hodge DR, Xiao W, Clausen PA, Heidecker G, Szyf M, Farrar WL: Interleukin-6 regulation of the human DNA methyltransferase (HDNMT) gene in human erythroleukemia cells. J Biol Chem, 2001; 276: 39508-39511

35) Rangel-Salazar R, Wickstrom-Lindholm M, Aguilar-Salinas CA, Alvarado-Caudillo Y, Dossing KB, Esteller M, Labourier E, Lund G, Nielsen FC, Rodriguez-Rios D, Solis-Martinez MO, Wrobel K, Wrobel K, Zaina S: Human native lipoprotein-induced de novo DNA methylation is associated with repression of inflammatory genes in THP-1 macrophages. BMC Genomics, 2011; 12: 582

36) Hiltunen MO, Turunen MP, Hakkinen TP, Rutanen J, Hedman M, Makinen K, Turunen AM, Aalto-Setala K, Yla-Herttuala S: DNA hypomethylation and methyltransferase expression in atherosclerotic lesions. Vasc Med, 2002; 7: 5-11

37) Lund G, Andersson L, Lauria M, Lindholm M, Fraga MF, Villar-Garea A, Ballestar E, Esteller M, Zaina S: DNA methylation polymorphisms precede any histological sign of atherosclerosis in mice lacking apolipoprotein E. J Biol Chem, 2004; 279: 29147-29154

38) Zhu ZZ, Hou L, Bollati V, Tarantini L, Marinelli B, Cantone L, Yang AS, Vokonas P, Lissowska J, Fustinoni S, Pesatori AC, Bonzini M, Apostoli P, Costa G, Bertazzi PA, Chow WH, Schwartz J, Baccarelli A: Predictors of global methylation levels in blood DNA of healthy subjects: a combined analys. Int J Epidemiol, 2012; 41: 126-139