DNA methylation is associated with muscle loss in community-dwelling older men -the Yakumo study- : a preliminary experimental study

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ABSTRACT

Frailty is a state of reduced muscle strength and activity in older people. DNA methylation is associated with osteoporosis and muscle loss in murine and other animal studies, but there are no epidemiological studies in humans. This study aimed to assess the association of osteoporosis and muscle loss with DNA methylation in community-dwelling older people. This cross-sectional study was performed in a rural part of Japan. We analyzed 204 subjects (98 men and 106 women). In univariate analysis, the two groups were compared according to the presence or absence of osteoporosis and of muscle loss. Logistic regression analysis was performed to determine predictors of frailty in the muscle loss group. We used age, sex, body mass index, smoking history, drinking history, serum albumin and C-reactive protein levels, diabetes, hypertension, hyperlipidemia, heart disease history, and LINE-1 DNA methylation as the factors. Probability values < 0.05 were considered to be statistically significant. The levels of LINE-1 DNA methylation in leukocytes were associated with muscle loss in men over the age of 60. LINE-1 DNA methylation levels were not associated with bone mineral density in either the men or women over the age of 60. LINE-1 DNA methylation levels in leukocytes correlated significantly with the risk of frailty in men over the age of 60. Promoting an understanding of DNA methylation may lead to a better understanding of the pathophysiology of muscle loss.

Keywords: cross-sectional study, frailty, LINE-1 DNA methylation, muscle loss, osteoporosis

Abbreviations:
BMI: body mass index
CI: confidence interval
CRP: C-reactive protein
OR: odds ratio
SMI: skeletal muscle mass index

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INTRODUCTION

Frailty is a state described by the Japanese Society of Geriatrics in which “elderly people have reduced muscle strength and activity (weakness)”.1-3 Frail older people are more likely to have health problems such as dysfunction in daily living4,5 and are more likely to be admitted to a facility,6 at risk of falling,7 and hospitalized.8 In addition, their mortality rate is high.9 Therefore, it is important to evaluate their vulnerability and apply appropriate interventions. Frailty is strongly associated with physical factors, of which muscle loss and osteoporosis are considered the significant causes.10

DNA methylation is a chemical reaction in which a methyl group is added to a DNA base at a cytosine base CpG island of a dinucleotide sequence CpG in DNA.11 Methylation of CpG islands within a cluster of CpG sites in the promoter region of a gene represses that gene.12 Methylation has the effect of directly interfering with the binding of transcription factors to the promoter region.13 Its action is reversible and is thought to affect important biological functions such as aging and cell development.14

LINE-1 makes up about 17% of the human genome, is ubiquitous throughout the genome, and belongs to the family of non-long terminal repeat retrotransposons.15 Therefore, LINE-1 methylation levels are considered to be surrogate markers for global DNA methylation.16 It is highly methylated in normal tissue.17 Several epidemiological studies have shown an association between global leukocyte DNA methylation levels and diseases such as cardiovascular disease and diabetes.18,19

DNA methylation is associated muscle loss in animal studies. DNA methylation has been shown to be essential for muscle differentiation and regeneration, and muscle regeneration was inhibited in DNA methylase knockout mice.20 There are few epidemiological studies in humans. Gale et al showed in a cross-sectional study that there are no widespread differences in methylation patterns between physically frail and non-frail people, suggesting that blood DNA methylation is not a good biomarker of physical frailty.21 The association between blood DNA methylation and physical frailty is still controversial. In this study, we focused on sarcopenia and osteoporosis as physical findings. This study aimed to assess the association of osteoporosis and muscle loss with DNA methylation in community-dwelling older people.

METHODS

This study was approved by the institutional review board of our institution’s Ethics Committee (NUEC; approval number, 2014-0207), and the study protocol was approved by our institutional review board. All subjects gave their written informed consent. All authors approved the publication of this study.

Subjects

This research is part of the Yakumo study we are conducting based on the health examination conducted in Yakumo-cho, Hokkaido. In 2015, a total of 525 Japanese people participated in the health examination. The study excluded those who did not participate in the musculoskeletal test, did not have enough samples to measure LINE-1 DNA methylation levels, and were under the age of 60. Persons with a history of cancer, stroke, or ischemic heart disease and who did not provide informed consent were also excluded (Table 1). Eventually, 204 eligible persons (98 men and 106 women) remained in this analysis.
Table 1  Demographic, blood test results and lifestyle habits of the participants

| Variable                  | >60 years | Men  | Women | P value |
|---------------------------|-----------|------|-------|---------|
| Age (years)               | 68.7±5.86 | 68.9±5.65 | 68.5±6.08 | 0.627a |
| SMI (kg/m²)               | 6.93±0.98 | 7.62±0.66 | 6.28±0.75 | <0.001a |
| Muscle loss, n            | 41 (20.1%) | 16 (16.3%) | 25 (23.6%) | 0.223b |
| Back strength (kg)        | 75.3±29.7 | 96.3±25.5 | 55.6±17.2 | <0.001a |
| BMD %Y AM                 | 77.6±14.2 | 82.8±15.7 | 72.9±10.7 | <0.001a |
| Knee OA, n                | 72 (35.3%) | 29 (29.6%) | 43 (40.6%) | 0.109b |
| BMI (kg/m²)               | 23.4±3.13 | 23.9±2.44 | 22.8±3.59 | <0.05a |
| Serum albumin (mg/dl)     | 4.28±0.26 | 4.28±0.29 | 4.28±0.24 | 0.962a |
| C-reactive protein (mg/dl)| 0.12±0.33 | 0.15±0.42 | 0.09±0.20 | 0.162a |
| DM, n                     | 43 (21.1%) | 23 (23.5%) | 20 (18.9%) | 0.493b |
| Hypertension, n           | 80 (39.2%) | 44 (44.9%) | 36 (34.0%) | 0.453b |
| Hyperlipidemia n          | 65 (31.9%) | 27 (27.6%) | 38 (35.8%) | 0.102b |
| Heart disease, n          | 7 (3.4%)  | 6 (6.1%)  | 1 (0.9%)  | 0.0933b |
| Smoking habit             | +, n      | 34 (16.7%) | 17 (17.3%) | 7 (6.6%) |
|                          | −, n      | 170 (83.3%) | 81 (82.7%) | 99 (93.7%) |
| Alcohol consumption       | <0.001b   | Current, n  | 98 (48.0%) | 72 (73.5%) | 26 (24.5%) |
|                          | Former, n | 7 (3.4%)  | 5 (5.1%)  | 2 (1.9%)  |
|                          | Never, n  | 99 (48.5%) | 21 (21.4%) | 78 (73.6%) |

SMI: skeletal muscle mass index
BMD: bone mineral density
YAM: young adult mean
BMI: body mass index
DM: diabetes mellitus

Data collection

We used a self-administered questionnaire during the health examination and obtained health information such as smoking habit (never, previously, or now), alcohol consumption (never, previously, or now), and history of significant illness (yes/no). We also obtained basic physical data such as height, weight, waist circumference, and blood pressure. Body mass index (BMI) was calculated by dividing weight (kg) by height squared (m²).

Muscle mass evaluation

Skeletal muscle mass index (SMI) was measured by the bioelectrical impedance analysis method using a medical body composition meter (InBody 720, Biospace). Based on the sarcopenia diagnostic criteria of the Sarcopenia Asia Working Group, SMI <7.0 kg/m² for men and SMI <5.4 kg/m² for women were defined as muscle loss groups. The bioelectrical impedance analysis method measures the electrical resistance (impedance) of tissues by passing weak alternating current electricity through the living body and uses the fact that the electrical resistance differs...
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depending on muscles, bones, adipose tissue, and other tissue. A previous report described this method.\textsuperscript{22} The subject stands on a table and holds electrodes in both hands. The measurement time is about 90 seconds, and the result can be explained to the subject immediately. This is a non-invasive and easy way to determine SMI.

Blood tests

Blood samples taken during the medical examination were mixed in tubes with ethylenediaminetetraacetic acid (EDTA) and then centrifuged at 1500 × g for 10 minutes. Erythrocytes were removed from the buffy coat sample. The buffy coat was collected and treated with a lysis solution (pH 7.4) containing 150 mmol/L NH\textsubscript{4}Cl, 14 mmol/L NaHCO\textsubscript{3}, and 0.11 mmol/L EDTA-2Na in distilled water to remove erythrocytes. Extraction of DNA from peripheral leukocytes was done using a NucleoSpin\textsuperscript{®} Tissue kit (Takara, Japan). The extracted DNA (500 ng, 50 ng/µL) was then converted from unmethylated cytosine to uracil. It was treated with sodium bisulfite according to the manufacturer’s protocol using an EpiTect Fast DNA bisulfite kit (QIAGEN, Germany). To amplify the LINE-1 element, a polymerase chain reaction was performed using EpiTaq\textsuperscript{™} HS (for bisulfite-treated DNA) (Takara). Methylation was measured by the pyrosequencing method using the PyroMark Q24 Advanced system (QIAGEN) with sequence primers 5’-AGTTAGGTGT-GGGATATAGT-3’ and PyroMark\textsuperscript{®} Q24 Advanced CpG reagent (QIAGEN). The level of LINE-1 DNA methylation was expressed as a percentage of methylated cytosine. LINE-1 DNA methylation at the three CpG sites was quantified to calculate mean LINE-1 DNA methylation levels.

Serum albumin, whole blood hemoglobin A\textsubscript{1c}, and serum lipids were measured by an automated analyzer, 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 Yakumo General Hospital.

Statistical analysis

The continuous parameters are presented as mean ± standard deviation. In the univariate analysis, the two groups were compared according to the presence or absence of osteoporosis and of muscle loss. Multivariate-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) for hypermethylation were calculated by logistic regression analysis in the muscle loss groups. We used age, sex, BMI, smoking history, drinking history, serum albumin level, CRP level, diabetes, hypertension, hyperlipidemia, heart disease history, and LINE-1 DNA methylation as the factors. Probability values less than 0.05 were considered to be statistically significant. Statistical analyses were performed using EZR (Jichi Medical School, Tochigi, Japan).

RESULTS

The participants’ demographic factors, blood test results, and physical performance data are listed in Table 1. BMI (p<0.01), back muscle strength (p<0.001), bone mineral density (% young adult mean) (p<0.001), smoking habit (p<0.001), and alcohol consumption (p<0.001) were significantly different between the men and women; however, no significant difference was noted in age or the rate of muscle loss.

The correlation between LINE-1 DNA methylation levels and the physical factors (osteoporosis and muscle loss) are shown in Table 2. In men over 60 years old, LINE-1 DNA methylation levels of the muscle loss group were lower than those in the non-muscle loss group (muscle loss group: 55.71 ± 0.77%, non-muscle loss group: 57.01 ± 2.58%, p<0.001) (Figure 1). In
contrast, there were no significant differences in LINE-1 DNA methylation between the muscle loss group and the non-muscle loss group in the women over 60 years (muscle loss group: 56.80 ± 2.18%, non-muscle loss group: 57.23 ± 3.10%, p=0.521). Further, the LINE-1 DNA methylation level did not differ between the osteoporosis group and the non-osteoporosis group (men over 60 years, osteoporosis group: 57.37 ± 3.33%, non-osteoporosis group: 56.81 ± 2.42%, p=0.387; women over 60 years, osteoporosis group: 57.03 ± 2.27%, non-muscle loss group: 57.19 ± 3.30%, p=0.653) (Figure 1).

Table 2  Correlation between LINE-1 DNA methylation levels and physical factors of osteoporosis and muscle loss  

|                          | r    | P value |
|--------------------------|------|---------|
| Men >60 years (n=98)     |      |         |
| Osteoporosis (BMD: YAM <70%) | 0.0136 | 0.897   |
| Muscle loss (SMI <7.0 kg/m²) | 0.0321 | 0.756   |
| Women >60 years (n=104)  |      |         |
| Osteoporosis (BMD: YAM <70%) | 0.0195 | 0.845   |
| Muscle loss (SMI <5.4 kg/m²) | −0.00573 | 0.954   |

BMD: bone mineral density  
YAM: young adult mean  
SMI: skeletal muscle mass index  
Pearson’s correlation coefficient.

Fig. 1  Differences in LINE-1 DNA methylation levels in leukocytes according to the physical factors of osteoporosis and muscle loss  

Fig. 1A: In men over 60 years old, LINE-1 DNA methylation levels of the muscle loss group were lower than those in the non-muscle loss group. In contrast, there were no significant differences in LINE-1 DNA methylation between the muscle loss group and the non-muscle loss group in women over 60 years old.

Fig. 1B: The LINE-1 DNA methylation levels did not differ between the osteoporosis group and the non-osteoporosis group for either sex. The statistical differences shown on each graph were analyzed by Student t-test.

*p<0.001. porosis osteoporosis.
Table 3 shows multivariate adjusted ORs and 95% CIs for LINE-1 DNA methylation according to muscle loss. The men over 60 years with muscle loss had significantly lower ORs for hypermethylation than those with non-muscle loss (OR, 0.49; 95% CI, 0.27–0.86, p=0.012). In this logistic regression analysis, age, sex, smoking history, drinking history, serum albumin level, CRP level, diabetes, hypertension, hyperlipidemia, and heart disease history were not significantly correlated with muscle loss, whereas BMI was correlated (OR, 0.50; 95% CI, 0.33–0.75, p<0.001).

Table 3  Multivariate adjusted ORs and 95% CIs for LINE-1 DNA methylation according to muscle loss

| Muscle loss in men >60 years old (n=98) | SMI ≥7.0 kg/m² | SMI <7.0 kg/m² |
|--------------------------------------|---------------|---------------|
| LINE-1 DNA methylation               |               |               |
| Levels (%)                           | 57.01±2.58    | 55.71±0.77    |
| n (%)                                | 82 (83.7)     | 16 (16.3)     |
| OR (95% CI)                          | 1.00          | 0.49 (0.27–0.86) |

OR: odds ratio
CI: confidence interval
SMI: skeletal muscle mass index

The ROC curve of the predicted value of LINE-1 DNA methylation level for the presence or absence of the risk of frailty in men over 60 years of age showed an area under the curve of 0.685 (Figure 2).
DISCUSSION

Few studies have investigated the relationship between the risk of frailty in the general population and LINE-1 DNA methylation in leukocytes. We showed that the levels of LINE-1 DNA methylation in leukocytes were associated with muscle loss in men over 60 years old. However, LINE-1 DNA methylation levels were not associated with bone mineral density in either men or women over the age of 60.

The levels of LINE-1 DNA methylation in leukocytes were associated with muscle loss in the men aged 60 and older. A previous study also showed that LINE-1 mRNA expression and the DNA copy count increased with age in mouse skeletal muscle. Another study showed that skeletal muscle-specific Dnmt3a KO impairs skeletal muscle regeneration and causes muscle loss. At that time, the level of DNA methylation was reduced.

Further, the association between muscle loss and the expression of LINE-1 methylation was present only in the men. A study reported that sex hormone-binding globulin and testosterone were associated with genome-wide DNA methylation. The decrease in secreted sex hormones is significantly affected by aging, especially in men. Age-related muscle loss begins around the age of 40, and over the 35 years from 40–44 years to 75–79 years, about 11% of males and 6% of females lose limb muscle mass. The higher rate of decrease in men versus women is thought to be related to age-related changes in the endocrine system, such as in the amount of testosterone secreted, which has anabolic effects.

The LINE-1 DNA methylation levels were not associated with bone mineral density in either the men or women over the age of 60. The relationship between osteoporosis and LINE-1 methylation has been debated. An animal study showed the association between osteoporosis and LINE-1 methylation by DNA methylation regulation of vitamin D biosynthesis. In contrast, there is a report that osteoporosis and methylation are related in Alu elements but not in LINE-1, and thus, further investigation is considered necessary.

It is possible to prevent the progression of frailty if early screening can be performed. Several studies showed that LINE-1 methylation could be used as a biomarker in cardiovascular disease, dyslipidemia, and chronic liver diseases. We investigated LINE-1 DNA methylation in men over 60 years as a biomarker associated with frailty, but its AUC value was rather low at 0.683. Further consideration is thus needed regarding its usefulness as a biomarker. In recent years, other reports have shown that other epigenetic factors such as microRNA are useful as biomarkers in liver disease, chronic kidney disease, and osteoporosis. Thus, other factors such as microRNA need to be considered for the early diagnosis of frailty.

This study has some limitations. First, because it is a cross-sectional study, an association was shown but not a causal relationship. Second, the number of participants is relatively small. Third, the study targeted rural residents, who may have different living and working environments than urban residents, and this may have influenced the determination of risk levels of frailty.

In conclusion, LINE-1 DNA methylation levels in leukocytes correlated significantly with the risk of frailty in men over 60 years of age. Promoting an understanding of DNA methylation may lead to a better understanding of the pathophysiology of muscle loss.

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CONFLICTS OF INTEREST

None

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