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Repetitive element hypomethylation in blood leukocyte DNA and cancer incidence, prevalence and mortality in elderly individuals: the Normative Aging Study

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Abstract

Background—Global genomic hypomethylation is a common epigenetic event in cancer that mostly results from hypomethylation of repetitive DNA elements. Case-control studies have associated blood leukocyte DNA hypomethylation with several cancers. Because samples in case-control studies are collected after disease development, whether DNA hypomethylation is causal or just associated with cancer development is still unclear.

Methods—In 722 elderly subjects from the Normative Aging Study cohort, we examined whether DNA methylation in repetitive elements (Alu, LINE-1) was associated with cancer incidence (30 new cases, median follow-up: 89 months), prevalence (205 baseline cases), and mortality (28 deaths, median follow-up: 85 months). DNA methylation was measured by bisulfite pyrosequencing.

Results—Individuals with low LINE-1 methylation (<median) had a 3.0-fold (95%CI 1.3-6.9) increased incidence of all cancers combined. LINE-1 and Alu methylation were not significantly associated with cancer prevalence at baseline (all cancers combined). However, individuals with low LINE-1 methylation (<median) had a 3.2-fold (95% CI 1.4-7.5) higher prevalence of lung cancer. Individuals with low LINE-1 or Alu methylation (<median) had increased cancer mortality (HR=3.2, 95%CI 1.3-7.9 for LINE-1; HR=2.5, 95%CI 1.1-5.8 for Alu).

Conclusion—These findings suggest that individuals with lower repetitive element methylation are at high risk of developing and dying from cancer.

Keywords

Repetitive elements; DNA methylation; Epigenetics; Blood; Cancer risk

Introduction

DNA methylation is a reversible mechanism of epigenetic regulation, which plays a key role in a wide variety of fundamental biological processes including gene expression and maintenance of genomic stability [1]. In mammals, methylation involves addition of methyl groups to cytosine to form 5-methyl cytosine (5mC). Genome-wide DNA methylation derives from the overall level of 5mC in the genome. About 55% of the human genome consists of repetitive elements [2], among which LINE-1 and Alu are the most plentiful families representing approximately 30% of the human genome [3]. Because of their high representation throughout the genome, LINE-1 and Alu have been used as global surrogate markers for estimating the genomic DNA methylation level [4]. DNA methylation patterns are largely established in utero or during early life, and stably maintained during later development, but can be changed in response to endogenous and exogenous exposure factors [5, 6].

Global DNA hypomethylation is regarded as an important and, in many cases, essential component of cancer development [7]. In a broad panel of cancers, lower global DNA methylation was observed in tumor tissues compared with their normal tissue counterparts [3]. Global hypomethylation is primarily due to demethylation of repetitive DNA elements. Based on case-control design, global hypomethylation measured in blood DNA has been associated with colorectum, bladder, breast, and head and neck cancers [8-11]. Given that DNA methylation in target tissues or blood DNA in previous case-control studies was

measured in subjects after cancer diagnosis, the global blood hypomethylation observed in patients with cancer may represent a consequence of cancer development and/or treatment [12, 13], rather than a determinant of cancer risk. Animal models have shown that rodents with experimentally induced hypomethylation develop cancers [14-16], suggesting that global DNA hypomethylation may be causally involved in carcinogenesis. However, whether subjects with global DNA hypomethylation have an increased risk of developing cancers has never been evaluated in human prospective investigations. On the other hand, hypomethylation of LINE-1 elements in tumor tissues or serum has been associated with poorer survival of patient with cancer [17, 18]. Whether hypomethylation in blood leukocyte DNA of LINE-1 or other repetitive elements is associated with cancer mortality has not been investigated, either.

In the present cohort study of elderly individuals in the Boston area, we determined whether LINE-1 and Alu methylation levels were associated with: i) risk of incident cancers among subjects without baseline cancers; ii) cancer prevalence at baseline; and iii) increased risk of death from cancers in the entire study population.

Materials and methods

Study subjects

Our study population included 722 elderly individuals, who, as of March 1st 1999, were active participants in the Normative Aging Study (NAS), a longitudinal investigation of aging established in 1963 by the U.S. Veterans Administration [19]. The NAS participants are all male subjects who are recalled for comprehensive clinical examinations every 3-5 years, and at each visit, all study participants are asked to donate a 7-ml blood sample. Between March 1999 and October 2007, 723 (89.8%) of the 805 active participants agreed to donate blood for DNA methylation analysis. Age at the time of blood drawing was between 55 and 100 years (mean=72 years; SD=6.8). DNA methylation analysis was unsuccessful on 11 subjects for LINE-1 elements and 1 subject for Alu elements, leaving a final number of 722 study participants. This study was approved by the Institutional Review Boards of all participating Institutions, and all participants gave written informed consent.

Baseline diagnoses of cancers

All subjects were questioned by a physician about existing cancer diagnoses (baseline) on the date of collection of the blood sample used for methylation analysis. In total, 205 (28.4%) participants were identified with cancers at baseline, including 85 prostate cancers, 30 lung cancers, 23 colorectal cancers, and 67 other cancers, whereas 517 (71.6%) individuals were cancer-free.

Incidence follow-up

Of the 517 participants free of baseline cancers, 390 (75.4%) had follow-up visits before December 31, 2008 and 30 (7.7%) new occurrences of cancers were ascertained, including 11 prostate cancers, 4 lung cancers, 3 colorectal cancers, 2 leukemias, 2 skin melanomas, and 8 other cancers. Thirty-six (28.3%) of the 127 participants who did not have follow-up examinations had died before the date of the next scheduled visit. Median incidence follow-up was 89 months (min=11, max=118), for 34,727 months (2,894 person-years) of total analysis time at risk. Ascertainment of incident cancer cases was based on self-report by the patients. Cancer diagnoses were confirmed on clinical records. Nearly all incident cases of cancer were confirmed by histological reports.

Mortality follow-up

Most deaths occurring in this cohort were notified through next of kin or postal authorities. Birthday cards and supplemental questionnaires mailed to participants provided additional opportunities to ascertain the vital records as well as the records of the VA and the Social Security Administration Death Master File to pick up possible unreported deaths. For participants who died, death certificates were obtained from the appropriate state health department. These were reviewed to ensure accurate classification of primary causes of death. Mortality follow-up was available for 714 (98.9%) of the 722 study participants. In total, 28 (3.9%) subjects died from cancers during the follow-up, among which 5 subjects died from prostate cancer, 4 from lung cancer, 2 from colorectal cancer, 2 from leukemia, 2 from lymphoma, 2 from soft tissue sarcoma, 2 from skin melanoma, and 9 from other cancers. Median mortality follow-up was 85 months (min=2, max=118), for 60,954 months (5,080 person-years) of total analysis time at risk.

DNA methylation analysis of LINE-1 and Alu elements

We performed DNA methylation analyses of LINE-1 and Alu elements on bisulfite-treated DNA using highly quantitative analysis based on PCR pyrosequencing, as described in detail elsewhere [20]. In brief, 7 ml of whole blood was collected, and buffy coat was extracted and stored in cell lysis solution until DNA extraction. All samples were coded and frozen at −20°C. Buffy coat DNA was extracted using the QiAmp DNA blood kits (QIAGEN, Hilden, Germany) and bisulfite treated using EZ-DNA Methylation-Gold™ Kits (Zymo Research, Orange, CA, USA) according to manufacturer's protocols. Pyrosequencing was performed using the PSQ Q96 MD Pyrosequencing System (Pyrosequencing, Inc). The degree of methylation was expressed for both LINE-1 and Alu as percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines (%5mC). For all assays we used built-in controls to verify bisulfite conversion. In the LINE-1 and Alu assays, we measured the %5mC at each of three CpG dinucleotide positions that are repeated over the human genome with the sequence of interest, as described in detail elsewhere by Tarantini et al.[21]. Each marker was tested in two replicates and their average was used in final analysis. The within-sample coefficients of variation were 0.7% for LINE-1 and 1.6% for Alu.

Statistical analysis

Differences between subjects with and without baseline cancers in age, body mass index (BMI), race, education, smoking history, and alcohol drinking were evaluated using the Student's t or Fisher's exact tests. The associations between LINE-1 or Alu methylation and the prevalence of cancers were estimated using multivariate logistic regression models. LINE-1 or Alu methylation related to the risk of new cancer event or mortality from cancers was analyzed using Cox proportional hazard regression models. Mortality analysis, which included subjects with and without baseline cancers, used Cox regression with baseline cancer as a stratification variable, allowing a different hazard estimate in each stratum.

For all models, we showed results adjusted for age (continuous variable), BMI (continuous variable), race (white/others), education (≤12, 13-15, ≥16 years), smoking (ever/never), pack-years (continuous variable), and alcohol drinking (two drinks/day or more: yes/no). As a sensitivity analysis, we further adjusted for variables that have been previously associated with LINE-1/Alu or global methylation, including dietary folate intake [22], diabetes [23], and cardiovascular diseases [24]. This sensitivity analysis did not yield meaningful differences (data not shown). All tests were two-sided and a *P* value of less than 0.05 was considered significant. All these statistical analyses were conducted using Stata 10.1 (Stata Corporation, College Station, TX).

Results

Baseline characteristics of the study participants

Table 1 shows the distribution of subjects by cancer diagnosis at baseline. Overall, subjects with baseline cancer diagnosis had lower BMI (P=0.017) and higher cumulative pack-years of smoking (P=0.005) than those without. When evaluated by cancer sites, individuals with prostate cancer were older (P=0.01), those with lung cancer had higher education (P=0.030) and cumulative pack-years of smoking (P=0.038), those with colorectal cancer were less educated (P=0.002), and those with other cancers had lower BMI (P=0.027) and higher cumulative pack-years of smoking (P=0.015).

Longitudinal risk of cancers

Risks of developing cancers were inversely associated with the level of LINE-1 methylation among subjects without cancers at baseline (n=517) (Table 2). Relative to subjects in the fourth quartile of LINE-1 methylation, hazard ratios (HRs) for cancer incidence were 1.7 (95% CI 0.4-7.4) for the subjects with methylation in the third quartile, 3.3 (95% CI 0.9-12.4) for the second quartile, and 5.1 (95% CI 1.4-18.3) for the first quartile ($P_{\text{-trend}}$=0.004). Using the alternative cut-point based on the median, individuals with lower (<median) LINE-1 methylation had a 3.0-fold (95% CI 1.3-6.9) increased risk of developing cancers compared with subjects with higher (>median) LINE-1 methylation. The excess risk associated with LINE-1 hypomethylation appeared to be distributed across all cancer types evaluated, although precision of risk estimates was limited due to the small number of events. No association between Alu methylation and longitudinal risk of cancers was observed (Table 2).

Also, we stratified our incidence analysis according to the time interval between the baseline blood DNA sampling and cancer diagnosis (Supplementary Table S1). The results showed no major differences in the risk estimate of LINE-1 or Alu methylation (Supplementary Table S1). However, individuals with lower (<median) LINE-1 tended to have a larger risk of incident cancer within 2.7 years (median diagnosis time) from the baseline (HR=4.3, 95% CI 1.2-15.5), and a less pronounced risk afterward (HR=2.4, 95% CI 0.8-7.2).

Association of LINE-1 and Alu methylation with cancer prevalence at baseline

We did not observe significant associations between LINE-1 or Alu methylation and the overall cancer prevalence at baseline (Table 3). However, the lung cancer prevalence increased with decreasing levels of LINE-1 methylation. Compared with subjects in the fourth (highest) quartile of LINE-1 methylation, individuals with methylation in the third, second, and first (lowest) quartile had 1.4-fold (95% CI 0.3-6.2), 3.5-fold (95% CI 0.9-13.1), and 4.4-fold (95% CI 1.2-16.4) increased relative odds of baseline lung cancer prevalence, respectively ($P_{\text{-trend}}$=0.007). When using the alternative cut-point based on median LINE-1, individuals with LINE-1 methylation below the median had a 3.2-fold (95% CI 1.4-7.5) increased relative odds of lung cancer prevalence at baseline, relative to subjects with higher (>median) LINE-1 methylation. DNA methylation in both LINE-1 and Alu elements was not associated with prostate cancer, colorectal cancer or other cancers.

As those subjects who were cancer-free at baseline but developed cancer during follow-up had lower LINE-1 methylation compared with those who remained cancer-free (Table 2), we further analyzed the baseline associations using only the controls who remained cancer-free during the follow-up. In this analysis (Supplementary Table S2), we found no major differences from the results reported previously, with no changes in statistical significance.

Cancer mortality

Mortality from cancers increased with decreasing methylation levels of either LINE-1 or Alu elements (Table 4). When compared with subjects in the fourth quartile of LINE-1 methylation, HRs for cancer mortality were 1.0 (95% CI 0.2-4.8) for the subjects with methylation in the third quartile, 3.3 (95% CI 0.9-11.9) for the second quartile, and 2.9 (95% CI 0.8-10.7) for the first quartile (P_{trend}=0.031). Using the alternative cut-point based on median, subjects with lower (<median) LINE-1 methylation had a 3.2-fold (95% CI 1.3-7.9) and individuals with lower (<median) Alu methylation had a 2.5-fold (95% CI 1.1-5.8) increased risk of cancer mortality, compared with subjects with higher (>median) methylation. The excess mortality associated with Alu or LINE-1 hypomethylation appeared to be distributed across all the cancer types evaluated, although precision of risk estimates was limited due to the small number of events (Table 4).

As LINE-1 methylation has been found to be significantly associated with increased prevalence of baseline lung cancer in our cross-sectional analysis, we further examined whether the association between LINE-1 methylation and cancer mortality depended on cases who had lung cancer at baseline. Adding baseline lung cancer into Cox proportional hazard regression resulted in no major difference in the risk estimate of LINE-1 methylation on cancer mortality (HR=3.3, 95% CI 1.3-8.2).

Additional models testing for the interaction between LINE-1 or Alu methylation and baseline cancers showed that the associations between hypomethylation and cancer mortality were not significantly different in subjects with or without baseline cancers (P≥0.432).

Discussion

Our results from a cohort study of elderly subjects demonstrated for the first time that individuals with leukocyte DNA hypomethylation of repetitive elements had increased incidence and mortality from cancer. In particular, we observed that individuals with LINE-1 hypomethylation had an increased risk of developing cancers and those with lower LINE-1 or Alu methylation had increased cancer mortality. In addition, we showed in our cross-sectional analyses that individuals with lower LINE-1 methylation at baseline had higher prevalence of lung cancer.

Growing evidence suggests that global DNA hypomethylation is causally involved in carcinogenesis. Rodents with experimentally induced hypomethylation – by decreasing methyltransferase activity [14], feeding a low-methyl diet [15], or methyl-inhibitor treatment [16] – have been shown to develop cancers at multiple sites. Additionally, it has been reported that patients with colorectal adenoma, the precursor of colorectal cancer, had lower global methylation levels in leukocyte DNA than controls [10, 25]. Our study yielded new information on risk of developing cancers among individuals with LINE-1 hypomethylation in leukocyte DNA. The results provide evidence that repetitive DNA element hypomethylation can precede cancer occurrence and can be detected in an easily obtainable DNA source such as blood leukocytes that may help identify individuals at risk of developing cancers. Repetitive element methylation levels in leukocyte DNA have been inversely associated with older age [26] and other risk factors for carcinogenesis, such as environmental exposures of tobacco smoke, benzene, and persistent organic pollutants [20, 27, 28], suggesting that blood DNA hypomethylation may reflect cumulative effects from aging and carcinogenic exposures. Results showing that the offspring's global DNA methylation levels estimated in CCGG sequences are associated with those in paternal DNA [29], and that DNA methylation maintenance demonstrates familial clustering [30] suggest that global methylation change may be partly under genetic control. Thus, it is also possible

that DNA hypomethylation may be part of the processes that determine transgenerational risks of common human diseases, including cancers.

Global DNA hypomethylation in cancer tissues has been proposed to participate in determining chromosomal instability, loss of imprinting, and activation of oncogenes, thereby causing formation of abnormal chromosomal structures, increased mutation rates, and aberrant activation of a wide spectrum of genes conveying various growth advantages [14, 31]. In addition, global DNA hypomethylation is expected to lead to the direct reactivation and subsequent expression of LINE-1 transcripts [32, 33], which may participate in carcinogenesis through insertion into functional sequences, homologous recombination, and deregulation of protein expression [14, 31]. However, further studies are warranted to clarify whether these biological processes operate in blood leukocytes, and if so, how they might affect the formation of a tumor at a different site.

Global or repetitive element hypomethylation in genomic DNA derived from tumor tissues has been associated with advanced tumor stage, late tumor grade, large tumor size, lymph node metastasis, and poorer survival [17, 34-38]. It has also been reported that LINE-1 hypomethylation in DNA derived from sera of hepatocellular carcinoma patients is correlated with large tumor size, advanced tumor stage and poorer patient survival [18]. However, the association between Alu and LINE-1 methylation in DNA derived from blood leukocytes and disease survival of patients with cancers has not previously been investigated. Our data showed for the first time that individuals with LINE-1 or Alu hypomethylation in blood leukocyte DNA had an increased risk of death from cancers. Further analysis showed that this association between repetitive element hypomethylation and cancer mortality did not differ in subjects with or without baseline cancers.

In the cross-sectional analyses of the present study, LINE-1 element hypomethylation tended to be associated with lung cancer prevalence, but not with prostate, colorectal or other cancers. Global or repetitive element hypomethylation in leukocyte DNA has been reported to be associated with cancers of several specific sites in some [8-11] but not all [39] case-control studies. Taken together, these findings suggest that there exists an association between leukocyte global or repetitive element hypomethylation and some cancer subtypes. However, it should be noted that leukocyte DNA hypomethylation observed in cancer patients may be the consequence of the cancer-bearing state, rather than on the causal pathway. Proliferating cancer cells usually take up substantial methyl nutrients to support high rate biosynthesis of nucleic acids and other macromolecules [12]. On the other hand, patients with cancer frequently have reduced nutritional intake due to poor appetite, malabsorption, and metabolic disruption [13]. Thus, patients with cancer are more likely to be in a state of methyl-donor deficiency, which has been shown to induce decreased global DNA methylation content in animal models [15]. Therefore, cross-sectional analyses are not informative as to whether global hypomethylation is the cause or the consequence of cancer development.

The longitudinal nature of this study has enabled us to minimize the potential for biases that are often encountered in cross-sectional or case-control studies and to establish more clearly the temporal nature of the association between DNA hypomethylation and cancer. We used quantitative analysis by pyrosequencing methodology, which is suitable for measuring subtle changes in DNA methylation [4]. One limitation of our study is the limited number of new cancer occurrence and mortality events during follow-up, and thus, significant findings by chance cannot be excluded. Larger studies are needed to validate our findings. Similarly, the relatively small sample size of our study, along with the DNA origin for methylation analysis from buffy coats instead of peripheral blood lymphocytes, may contribute to the lack of association of LINE-1 or Alu methylation with the overall cancer prevalence. Buffy

coats are made up of a mixed cell population that contains all leukocyte subtypes in addition to non-nucleated platelets. The methylation measures of DNA extracted from peripheral leukocytes might have reflected shifts in leukocyte subtypes [40]. Also, because cancer diagnosis were self-reported, we cannot exclude misclassification of cancer outcomes. However, diagnoses were confirmed on clinical records and, for nearly all cases, on pathology reports. Also, lack of differences between cancer-free subjects and subjects with various cancers with regard to subject characteristics (e.g., age, BMI) (Table 1) may be partly due to the relatively small sample size. In addition, the NAS cohort investigated in the present study is made up of older men who are almost all white. Since age, sex, and ethnicity have been shown to be associated with global DNA methylation levels [26, 41, 42], future studies should address the role of global blood DNA hypomethylation in carcinogenesis among women, as well as in different age and ethnic groups. Finally, although DNA methylation analyses of LINE-1 and Alu elements have been widely used as surrogates for global DNA methylation content, methylation levels of both repetitive elements are not equivalent to, albeit vastly represents, global DNA methylation content [4]. Although studies have shown that the LINE-1 and Alu methylation in tumor tissue DNA were correlated with each other [43], no significant correlation between LINE-1 and Alu methylation levels has been found in surrounding normal tissues [43], or in non-target tissues such as blood and buccal cells [20, 28, 44, 45]. It has been reported that the associations of LINE-1 and Alu methylation with gastric cancer risk were modified differently by certain grastric cancer risk factors and genetic polymorphisms [45]. As expected, we observed that the associations of LINE-1 and Alu methylation in blood leukocyte DNA with cancer were different, and that LINE-1 and Alu methylation levels were not significantly correlated (Pearson's coefficient = 0.069, $P = 0.129$). In this respect, the differences might have been expected based on the differences between LINE-1 and Alu in their methylation regulation mechanisms [46], responses to cellular stressors and environmental exposures [20, 28, 44], and baseline methylation levels [4, 28]. Although LINE-1 and Alu have been long considered to be part of ‘junk’ DNA, i.e. stretches of DNA with structural properties and little if any functional activity [47], growing evidence has shown that each of them has a distinct functional role that may participate in cancer development and progression [46-48]. In this context, our results provide further indirect evidence of the independent and distinct functional roles of LINE-1 and Alu, which may be related to the cancer risks we observed in our study population.

In summary, although limited by lower statistical power, the results suggest that repetitive element hypomethylation in blood leukocyte DNA is associated with increased risk of developing cancers, as well as increased cancer mortality. Because of the limited statistical power of the present study, as well as of the moderate consistency of the prevalence, incidence and mortality analyses, future studies will be needed to verify our findings. The identification in easily obtainable biospecimens such as leukocyte DNA of hypomethylation that anticipates the onset of cancers may help identify individuals at risk. Because hypomethylation is potentially reversible, our findings indicate a new direction to develop lifestyle or pharmacological interventions aimed at removing or modifying deleterious epigenetic features.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Baseline characteristics of the Normative Aging Study participants according to existing cancer diagnosis at baseline

Variable	Cancer-free	Cancer									
		All		Prostate cancer		Lung cancer		Colorectal cancer		Other cancers	
	n=517	*n*=205	*P* value[a]	*n*=85	*P* value[a]	*n*=30	*P* value[a]	*n*=23	*P* value[a]	*n*=67	*P* value[a]
Age (years), mean (SD)	72.2 (6.9)	73.1 (6.8)	0.099	74.2 (6.4)	0.010	71.0 (7.0)	0.387	74.3 (6.2)	0.133	72.1 (7.1)	0.985
Body Mass Index (kg/m^2), mean (SD)	28.2 (4.2)	27.4 (3.9)	0.017	27.8 (3.8)	0.379	27.2 (3.9)	0.174	27.5 (4.2)	0.411	27.0 (3.8)	0.027
Race											
White	494 (97.1)	201 (98.5)		84 (98.8)		29 (96.7)		23 (100.0)		65 (98.5)	
Others	15 (2.9)	3 (1.5)	0.304	1 (1.2)	0.714	1 (3.3)	0.605	0 (0.0)	(-)	1 (1.5)	0.472
Education (years), *n* (%)											
≤12	182 (35.2)	62 (30.2)		24 (28.2)		8 (26.7)		16 (69.6)		14 (20.9)	
13-15	146 (28.2)	57 (27.8)		27 (31.8)		4 (13.3)		5 (21.7)		21 (31.3)	
≥16	189 (36.6)	86 (42.0)	0.333	34 (40.0)	0.453	18 (60.0)	0.030	2 (8.7)	0.002	32 (47.8)	0.055
Ever smoked, *n* (%)											
Never	146 (28.2)	65 (31.7)		31 (36.5)		10 (33.3)		8 (34.8)		16 (23.9)	
Ever	371 (71.8)	140 (68.3)	0.356	54 (63.5)	0.123	20 (66.7)	0.548	15 (65.2)	0.497	51 (76.1)	0.453
Cumulative smoking (pack-years), mean (SD)	28.7 (23.1)	37.9 (41.8)	0.005	37.1 (46.4)	0.062	42.1 (33.3)	0.038	29.9 (24.8)	0.873	39.2 (43.9)	0.015
Alcohol drinkers (≥2 drinks/day), *n* (%)											
No	427 (83.1)	163 (79.9)		65 (77.4)		21 (70.0)		20 (87.0)		57 (85.1)	
Yes	87 (16.9)	41 (20.1)	0.317	19 (22.6)	0.205	9 (30.0)	0.068	3 (13.0)	0.626	10 (14.9)	0.680

[a] Student's t or Fisher's exact tests evaluating differences relative to cancer-free subjects

Table 2

Association of LINE-1 and Alu methylation with longitudinal risk of cancers[a]

Methylation	Person-years	All cancers		Prostate cancer		Lung cancer		Colorectal cancer		Other cancers	
		No.	HR (95% CI)[b]	No.	HR (95% CI)[b]	No.	HR (95% CI)[b]	No.	HR (95% CI)[b]	No.	HR (95% CI)[b]
LINE-1 (%5mC)											
Quartile[c]											
Q4 (86.2-78.5)	787	3	Ref.	1	Ref.	0	Ref.	0	Ref.	2	Ref.
Q3 (78.4-77.2)	766	5	1.7 (0.4-7.4)	3	3.0 (0.3-29.4)	1	N/A	0	N/A	1	0.7 (0.1-8.0)
Q2 (77.1-75.9)	713	9	3.3 (0.9-12.4)	3	3.5 (0.4-33.7)	1	N/A	1	N/A	4	2.4 (0.4-13.3)
Q1 (75.8-68.1)	594	12	5.1 (1.4-18.3)	4	6.3 (0.7-57.4)	2	N/A	2	N/A	4	2.3 (0.4-13.6)
			P-trend=0.004		*P*-trend=0.086		*P*-trend=0.116		*P*-trend=0.097		*P*-trend=0.208
Median[c]											
High (86.2-77.2)	1,553	8	Ref.	4	Ref.	1	Ref.	0	Ref.	3	Ref.
Low (77.1-68.1)	1,307	21	3.0 (1.3-6.9)	7	2.3 (0.7-8.0)	3	4.8 (0.5-48.3)	3	N/A	8	2.7 (0.7-10.4)
Alu (%5mC)											
Quartile[c]											
Q4 (32.4-26.8)	735	8	Ref.	2	Ref.	1	Ref.	1	Ref.	4	Ref.
Q3 (26.7-26.1)	678	7	1.0 (0.3-2.7)	4	2.3 (0.4-13.1)	3	4.1 (0.4-44.5)	0	N/A	0	N/A
Q2 (26.0-25.6)	717	9	1.2 (0.5-3.1)	3	1.6 (0.3-10.2)	0	N/A	0	N/A	6	1.6 (0.4-5.6)
Q1 (25.5-23.0)	756	6	0.7 (0.2-2.1)	2	1.0 (0.1-7.5)	0	N/A	2	2.1 (0.2-25.6)	2	0.5 (0.1-2.6)
			P-trend=0.695		*P*-trend=0.880		*P*-trend=0.241		*P*-trend=0.436		*P*-trend=0.895
Median[c]											
High (32.4-26.1)	1,413	15	Ref.	6	Ref.	4	Ref.	1	Ref.	4	Ref.
Low (26.0-23.0)	1,473	15	1.0 (0.5-2.0)	5	0.8 (0.2-2.7)	0	N/A	2	2.2 (0.2-24.6)	8	1.9 (0.5-6.3)

%5mC percentage of 5-methyl cytosine, *CI* confidence interval, *HR* hazard ratio, *N/A* not available

[a] Analysis restricted to individuals (n=517) in the Normative Aging Study cohort without existing diagnosis of cancer at the time of blood collection for DNA methylation analysis (baseline)

[b] Adjusted by age, BMI, race, education, smoking, pack-years, and alcohol drinking

[c] The quartiles and the medians of LINE-1 or Alu measures were based on values among subjects free of baseline cancers

Table 3

Association of LINE-1 and Alu methylation with prevalence of baseline cancers

Methylation	Cancer-free	Cancer									
		All		Prostate cancer		Lung cancer		Colorectal cancer		Other cancers	
	n (%)	*n* (%)	OR (95% CI)[a]	*n* (%)	OR (95% CI)[a]	*n* (%)	OR (95% CI)[a]	*n* (%)	OR (95% CI)[a]	*n* (%)	OR (95% CI)[a]
LINE-1 (%5mC)											
Quartile[b]											
Q4 (86.2-78.5)	128 (25.2)	56 (27.4)	Ref.	27 (31.8)	Ref.	3 (10.0)	Ref.	6 (26.1)	Ref.	20 (30.3)	Ref.
Q3 (78.4-77.2)	127 (25.1)	43 (21.1)	0.7 (0.4-1.2)	18 (21.2)	0.6 (0.3-1.2)	5 (16.7)	1.4 (0.3-6.2)	5 (21.7)	0.9 (0.2-3.0)	15 (22.7)	0.7 (0.3-1.5)
Q2 (77.1-75.9)	127 (25.1)	60 (29.4)	1.0 (0.7-1.6)	21 (24.7)	0.7 (0.4-1.4)	10 (33.3)	3.5 (0.9-13.1)	9 (39.1)	1.5 (0.5-4.5)	20 (30.3)	1.0 (0.5-2.1)
Q1 (75.8-68.1)	125 (24.6)	45 (22.1)	0.8 (0.5-1.2)	19 (22.3)	0.6 (0.3-1.2)	12 (40.0)	4.4 (1.2-16.4)	3 (13.1)	0.5 (0.1-2.0)	11 (16.7)	0.5 (0.2-1.2)
			P-trend=0.569		*P*-trend=0.253		*P*-trend=0.007		*P*-trend=0.588		*P*-trend=0.289
Median[b]											
High (86.2-77.2)	255 (50.3)	99 (48.5)	Ref.	45 (52.9)	Ref.	8 (26.7)	Ref.	11 (47.8)	Ref.	35 (53.0)	Ref.
Low (77.1-68.1)	252 (49.7)	105 (51.5)	1.1 (0.8-1.5)	40 (47.1)	0.9 (0.5-1.4)	22 (73.3)	3.2 (1.4-7.5)	12 (52.2)	1.1 (0.4-2.5)	31 (47.0)	0.9 (0.5-1.6)
Alu (%5mC)											
Quartile[b]											
Q4 (32.4-26.8)	128 (24.8)	57 (27.8)	Ref.	23 (27.1)	Ref.	10 (33.3)	Ref.	5 (21.7)	Ref.	19 (28.3)	Ref.
Q3 (26.7-26.1)	130 (25.2)	39 (19.0)	0.7 (0.4-1.2)	17 (20.0)	0.8 (0.4-1.5)	6 (20.0)	0.6 (0.2-1.8)	3 (13.1)	0.7 (0.2-2.9)	13 (19.4)	0.7 (0.3-1.5)
Q2 (26.0-25.6)	127 (24.6)	51 (24.9)	0.9 (0.6-1.5)	19 (22.3)	0.8 (0.4-1.6)	8 (26.7)	0.8 (0.3-2.2)	7 (30.4)	1.3 (0.4-4.4)	17 (25.4)	0.9 (0.4-1.8)
Q1 (25.5-23.0)	131 (25.4)	58 (28.3)	1.0 (0.7-1.6)	26 (30.6)	1.0 (0.6-2.0)	6 (20.0)	0.6 (0.2-1.8)	8 (34.8)	1.5 (0.5-4.7)	18 (26.9)	1.1 (0.5-2.2)
			P-trend=0.683		*P*-trend=0.800		*P*-trend=0.449		*P*-trend=0.369		*P*-trend=0.786
Median[b]											
High (32.4-26.1)	258 (50.0)	96 (46.8)	Ref.	40 (47.1)	Ref.	16 (53.3)	Ref.	8 (34.8)	Ref.	32 (47.8)	Ref.
Low (26.0-23.0)	258 (50.0)	109 (53.2)	1.1 (0.8-1.6)	45 (52.9)	1.1 (0.7-1.7)	14 (46.7)	0.9 (0.4-1.9)	15 (65.2)	1.6 (0.7-4.1)	35 (52.2)	1.1 (0.7-1.9)

%5mC percentage of 5-methyl cytosine, *CI* confidence interval, *OR* odds ratio

[a] Adjusted by age, BMI, race, education, smoking, pack-years, and alcohol drinking

[b] The quartiles and the medians of LINE-1 or Alu measures were based on values among subjects free of baseline cancers

Table 4

Association of LINE-1 and Alu methylation with cancer mortality

Methylation	Person-years	All cancers		Prostate cancer		Lung cancer		Colorectal cancer		Other cancers	
		No.	HR (95% CI)[a]	No.	HR (95% CI)[a]	No.	HR (95% CI)[a]	No.	HR (95% CI)[a]	No.	HR (95% CI)[a]
LINE-1 (%5mC)											
Quartile[b]											
Q4 (86.2-78.5)	1,287	3	Ref.	0	Ref.	0	Ref.	0	Ref.	3	Ref.
Q3 (78.4-77.2)	1,207	3	1.0 (0.2-4.8)	1	N/A	1	N/A	0	N/A	1	0.3 (0.03-3.3)
Q2 (77.1-75.9)	1,298	11	3.3 (0.9-11.9)	1	N/A	3	N/A	1	N/A	6	1.9 (0.5-7.7)
Q1 (75.8-68.1)	1,230	10	2.9 (0.8-10.7)	3	N/A	0	N/A	1	N/A	6	1.6 (0.4-6.6)
			P-trend=0.031		*P*-trend=0.093		*P*-trend=0.598		*P*-trend=0.250		*P*-trend=0.232
Median[b]											
High (86.2-77.2)	2,494	6	Ref.	1	Ref.	1	Ref.	0	Ref.	4	Ref.
Low (77.1-68.1)	2,528	21	3.2 (1.3-7.9)	4	4.5 (0.5-41.5)	3	4.0 (0.3-47.6)	2	N/A	12	2.6 (0.8-8.1)
Alu (%5mC)											
Quartile[b]											
Q4 (32.4-26.8)	1,355	4	Ref.	1	Ref.	0	Ref.	0	Ref.	3	Ref.
Q3 (26.7-26.1)	1,149	4	1.2 (0.3-4.9)	0	N/A	2	N/A	0	N/A	2	0.8 (0.1-4.8)
Q2 (26.0-25.6)	1,245	12	3.4 (1.1-10.9)	2	2.0 (0.2-23.3)	2	N/A	1	N/A	7	2.5 (0.6-10.0)
Q1 (25.5-23.0)	1,323	8	2.1 (0.6-7.2)	2	1.4 (0.1-16.7)	0	N/A	1	N/A	5	1.6 (0.4-7.0)
			P-trend=0.103		*P*-trend=0.504		*P*-trend=0.671		*P*-trend=0.226		*P*-trend=0.285
Median[b]											
High (32.4-26.1)	2,504	8	Ref.	1	Ref.	2	Ref.	0	Ref.	5	Ref.
Low (26.0-23.0)	2,568	20	2.5 (1.1-5.8)	4	3.3 (0.4-30.5)	2	1.5 (0.2-12.1)	2	N/A	12	2.3 (0.8-6.6)

%5mC percentage of 5-methyl cytosine, *CI* confidence interval, *HR* hazard ratio, *N/A* not available

[a]Adjusted by age, BMI, race, education, smoking, pack-years, and alcohol drinking

[b]The quartiles and the medians of LINE-1 or Alu measures were based on values among subjects free of baseline cancers