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Prospective study of DNA methylation at *LINE-1* and *Alu* in peripheral blood and the risk of prostate cancer

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Abstract

BACKGROUND—Evidence suggests that global blood DNA methylation levels may be associated with the risk of various cancers, but no studies have evaluated this relationship for prostate cancer.

METHODS—We used pyrosequencing to quantify DNA methylation levels at the long interspersed nuclear element 1 (*LINE-1*) and *Alu* repetitive elements in pre-diagnostic blood samples from 694 prostate cancer cases and 703 controls from the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial. We evaluated prostate cancer risk associated with the mean methylation level for each element using logistic regression, adjusting for potential confounders.

RESULTS—We did not observe a significant association with prostate cancer for *LINE-1* [Odds Ratio (OR) for the highest compared to the lowest quartile=1.01, 95% Confidence Interval (CI): 0.73-1.39, p_{trend}=0.99] or *Alu* (OR=0.94, 95% CI: 0.68-1.29, p_{trend}=0.69) methylation levels overall. However, for *Alu*, we observed that higher DNA methylation levels were associated with a significant increased risk for those diagnosed 4 or more years after blood draw (OR=2.26, 95% CI: 1.27-4.00, $p_{trend}=4.4\times10^{-3}$). In contrast, there was no association for those diagnosed 2 (OR=1.13, 95% CI: 0.67-1.90, p_{trend}=0.64) or 3 years after draw (OR=1.22, 95% CI: 0.71-2.07, p_{trend}=0.32), and a decreased risk for those diagnosed less than 2 years after draw (OR=0.40, 95% CI: 0.25-0.65, $p_{trend}=3.8\times10^{-5}$; $p_{heterogeneity}=5.3\times10^{-6}$).

CONCLUSIONS—While *LINE-1* DNA methylation levels were not associated with prostate cancer, we observed an association for *Alu* that varied by time from blood draw to diagnosis. Our study suggests that elevated *Alu* blood DNA methylation levels several years before diagnosis may be associated with an increased prostate cancer risk.

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Keywords

global DNA methylation; pre-diagnostic; prostate cancer; repetitive element

INTRODUCTION

Global DNA hypomethylation has been observed in tumor relative to histologically normal tissue for a number of cancers [1], including prostate cancer [2]. Although usually thought to occur early in the development of cancer, some data have suggested that this epigenetic alteration may occur late in prostate cancer progression [3]. Some studies have also suggested that global DNA methylation in peripheral blood may be associated with the risk of various cancers, including bladder, breast, renal, gastric, hepatocellular, and head and neck cancer [4-11]; however, the evidence is mixed and not all studies have observed an association [12]. Few of the studies have been prospective and, to date, no previous study has evaluated global DNA methylation levels in peripheral blood in relation to the risk of prostate cancer.

Global DNA methylation can be measured in a variety of different ways [13], which may contribute to the mixed findings with cancer risk in the literature [12]. Long interspersed nuclear element 1 (*LINE-1*) and *Alu* elements are abundant transposable DNA elements located within repetitive elements in the genome. As more than one-third of DNA methylation throughout the genome is estimated to occur at repetitive elements, DNA methylation at CpG sites in these elements has been used as a proxy of global cytosine methylation [14]. In this prospective study, we aimed to evaluate the relationship between CpG site DNA methylation at *LINE-1* and *Alu* in peripheral blood DNA and prostate cancer risk.

MATERIALS AND METHODS

Study population

We conducted a nested case-control study of prostate cancer in the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial using pre-diagnostic peripheral blood samples. The PLCO Cancer Screening Trial is a randomized trial of more than 150,000 men and women ages 55 to 74 who were enrolled from 10 centers across the United States between 1993 and 2001 to evaluate the impact of specific cancer screening regimens on the risk of mortality from prostate, lung, colorectal, and ovarian cancers [15]. As described previously [15,16], men randomized to the screening arm were offered prostate-specific antigen (PSA) testing at baseline and annually for 5 years and digital rectal exam (DRE) at baseline and annually for 3 years. Men with a PSA test result >4 ng/ml or a DRE exam suspicious for prostate cancer were referred to their personal physician for follow-up. Medical and pathologic records were obtained for participants suspected to have prostate cancer based on screening examination results or self-report on annual follow-up questionnaires. The study was approved by the institutional review boards at the ten centers and the National Cancer Institute.

For the present study, only men randomized to the screening arm of the trial who provided a blood specimen, consented to participate in etiologic studies of cancer, completed a baseline questionnaire, and had no history of cancer prior to randomization were eligible. Cases were preferentially selected from among the non-Hispanic Caucasian men that participated in the Cancer Genetic Markers of Susceptibility (CGEMS) initiative [17], where men with aggressive disease were oversampled. Only pathologically confirmed cases were included, and all cases selected were diagnosed at least one year after providing the blood sample. Controls were free of prostate cancer prior to the censor date for case diagnosis, December 31, 2007. Controls were frequency-matched to cases on age at randomization (five year intervals), year of randomization, year of blood draw, and study year of diagnosis/selection. A total of 707 cases and 707 controls were selected for the study. Excluding participants who had insufficient pre-diagnostic DNA resulted in a final sample size of 694 cases and 703 controls.

Pyrosequencing assays

DNA samples were extracted using Qiagen QIAamp DNA Blood Midi/Maxi kits and shipped to EpigenDx, Inc. (Hopkinton, MA) for analysis. The DNA was bisulfite-converted using a Zymo Research EZ DNA Methylation kit and DNA methylation levels were quantified at 4 CpG sites in *LINE-1* and 4 CpG sites in *Alu*, respectively, using pyrosequencing with assays in triplicate as described elsewhere [7]. Four artificial control samples were included on each plate [one negative control sample (no DNA added), as well as three positive control samples with known global DNA methylation levels: low (0%), partial (50%), and highly methylated (100%)]. In addition, we included 58 blind replicate samples for four of the study subjects (about 15 replicates per subject) interspersed within and between plates for quality control. Sequencing was performed using the Pyrosequencing PSQ96 HS System (Pyrosequencing Qiagen). The methylation status at each CpG site was analyzed as an artificial C/T SNP using QCpG software (Pyrosequencing Qiagen), and the percent of methylation was calculated for each CpG site as methylated cytosine divided by the sum of methylated and unmethylated cytosines. We calculated mean DNA methylation levels across the 4 CpG sites for *LINE-1* and the 4 CpG sites for *Alu*, respectively, to estimate the overall percent methylated cytosine within each element. We excluded results that failed bisulfite control or that had poor bisulfite conversion (based on percent unconverted bisulfite>7%), as well as results that had no signal or a low signal. In addition, we excluded individual triplicate results that had a coefficient of variation (CV) greater than 10 for *LINE-1* measures or a CV greater than 15 for *Alu* measures. The average CV and Intraclass Correlation Coefficient were 4.0 and 0.18 for the triplicate *LINE-1* measures and 5.9 and 0.47 for the *Alu* measures, respectively. After QC exclusions, mean *LINE-1* results were available for 691 cases and 700 controls and mean *Alu* results for 690 cases and 699 controls. The average CVs for QC replicate samples within and between plates were 2.1 and 3.5 for mean *LINE-1* and 3.9 and 6.2 for mean *Alu*, respectively.

Statistical analysis

Using the controls to create cutpoints, we categorized the individual and mean *LINE-1* and *Alu* DNA methylation measures into quartiles and used unconditional logistic regression models to evaluate prostate cancer risk associated with each CpG site individually and the

mean across the sites, adjusting for age at blood draw (continuous), year of blood draw, family history of prostate cancer (yes/possible, no), smoking status (never, former, current, pipe/cigar), total folate intake (quartiles defined among controls), and body mass index (BMI) (<25, 25-29, 30 or higher). We selected covariates for adjustment based on an observed association with prostate cancer in the literature or in the present study. We computed p-values for trend using the median value among controls for each quartile entered as a continuous variable in the model.

We also evaluated the relationships between the DNA methylation measures and prostate cancer risk separately for aggressive (defined as Stage III/IV or Gleason score>8) and non-aggressive (Stage I/II and Gleason score<8) cases using polytomous regression. We also used polytomous regression to evaluate these relationships by time from blood draw to diagnosis for the cases (< 2 years, 2 to < 3 years, 3 to < 4 years, and 4 or more years). We computed p-values for heterogeneity across strata using Wald Chi-square tests. In addition, we evaluated potential effect modification of the associations for the mean DNA methylation measures with prostate cancer risk by age at diagnosis/selection (< 65 or ≥ 65), family history of prostate cancer, smoking status, total folate intake, and BMI, using likelihood ratio tests comparing nested models with and without the interaction terms with the DNA methylation measures.

RESULTS

In our study population, about 25% of the prostate cancer cases were aggressive. Cases were more likely to have a family history of prostate cancer than controls and tended to consume higher folate than controls and were less likely than controls to have smoked (Table I). We did not observe differences between cases and controls with respect to age at blood draw or year of blood draw (study matching factors) or BMI (Table I).

Mean *LINE-1* and *Alu* methylation levels, which were moderately correlated with each other (among controls, Spearman rho=0.26, p-value=1.7×10^{-12}), were similar between cases and controls. The median value for mean *LINE-1* was 74.1% for both cases and controls (overall range: 63.3%-82.9%), and the median for mean *Alu* was 25.7% for both groups (overall range: 20.5%-29.1%). Overall, we did not observe a significant association with prostate cancer risk for mean *LINE-1* and *Alu* (Table II) or any of the individual CpG sites (Supplementary Table SI). For mean *LINE-1*, the Odds Ratio (OR) for the highest compared to the lowest quartile was 1.01, 95% Confidence Interval (CI): 0.73-1.39, p_{trend}=0.99, and for mean *Alu*, the OR was 0.94, 95% CI: 0.68-1.29, p_{trend}=0.69 (Table II).

There was no difference by disease aggressiveness. For mean *LINE-1*, the OR for the highest compared to the lowest quartile was 0.95 (95% CI: 0.56-1.60, p_{trend}=0.95) for aggressive disease and 1.04 (95% CI: 0.73-1.49, p_{trend}=0.85) for non-aggressive disease. For mean *Alu*, the OR was 0.99 (95% CI: 0.60-1.66, p_{trend}=0.89) for aggressive disease and 0.93 (95% CI: 0.66-1.32, p_{trend}=0.75) for non-aggressive disease.

When we evaluated differences by time from blood draw to diagnosis, we observed that higher mean *Alu* DNA methylation levels were associated with an increased risk of prostate

cancer for those diagnosed 4 or more years after blood draw (OR for the highest compared to the lowest quartile=2.26, 95% CI: 1.27-4.00, $p_{trend}=4.4\times10^{-3}$) (Table III). This association became stronger for those diagnosed 5 or more years after draw (case n=103), with an OR for the highest compared to the lowest quartile of 4.91 (95% CI: 2.25-10.69, $p_{trend}=5.1\times10^{-5}$). In contrast, no association was observed for those diagnosed 2 (OR=1.13, 95% CI: 0.67-1.90, $p_{trend}=0.64$) or 3 years after draw (OR=1.22, 95% CI: 0.71-2.07, $p_{trend}=0.32$), and a decreased risk was observed for those diagnosed less than 2 years after draw (OR=0.40, 95% CI: 0.25-0.65, $p_{trend}=3.8\times10^{-5}$; $p_{heterogeneity}=5.3\times10^{-6}$) (Table III). No association was observed with mean *LINE-1* regardless of the time from blood draw to diagnosis (Table III). Results for the individual *LINE-1* and *Alu* positions by time from blood draw to diagnosis (Supplementary Tables SII and SIII) were similar to the results observed for mean *LINE-1* and *Alu*.

Following up on our findings for *Alu*, we further evaluated the role of timing of DNA methylation levels relative to diagnosis by conducting a case-only analysis. Among the prostate cancer cases in our study, we observed a significant positive relationship between time from blood draw to diagnosis and mean *Alu* DNA methylation levels (Spearman rho=0.23, p-value=9.6×10^{-10}), corresponding to higher DNA methylation levels for measurements taken further in time before diagnosis (or, equivalently, lower DNA methylation levels approaching the time of diagnosis). To investigate whether age could have contributed to our findings for *Alu* by time from blood draw to diagnosis, we also computed the correlation between mean *Alu* DNA methylation levels and age at blood draw. We did not observe a significant association among either cases or controls (Spearman rho= −0.03 and −0.05, p=0.47 and 0.21, respectively).

We did not observe significant differences (p-interaction<0.05) in the association between mean *LINE-1* or *Alu* DNA methylation levels and prostate cancer risk by age at diagnosis/ selection, family history of prostate cancer, smoking status, folate intake, or BMI (data not shown).

DISCUSSION

This is the first study to evaluate the relationship between DNA methylation at *LINE-1* and *Alu* in peripheral blood and prostate cancer risk. We did not observe a significant association with prostate cancer risk for either element overall. However, for *Alu*, we observed an increased risk of prostate cancer associated with increasing methylation levels (i.e. hypermethylation among cases) for those diagnosed 4 or more years after draw. Notably, the association for *Alu* tended to further increase with increasing time between blood draw and diagnosis, although estimates were less precise due to smaller case numbers. The lack of association to protective association (i.e. hypomethylation among cases) for *Alu* methylation levels with blood draws closer to diagnosis and our finding of an association between time from blood draw to diagnosis and *Alu* methylation levels among the cases suggest that DNA methylation patterns may change over time as the cancer begins to develop.

It is widely held that global DNA hypomethylation may be associated with an increased cancer risk (and thus global hypermethylation associated with a decreased risk) [1] and we

recognize that our findings for *Alu* for those diagnosed 4 or more years after draw are contrary to this hypothesis. However, previous studies that have observed increased cancer risk associated with global hypomethylation in blood (i.e. decreased risk associated with global hypermethylation) have largely used post-diagnostic samples [12] as opposed to pre-diagnostic samples as in our study. The few prospective studies of global DNA methylation that have been conducted to date (i.e. for bladder and renal cancers), although *Alu* was not evaluated specifically, have tended to observe an increased risk of cancer associated with global hypermethylation [18,19]. The mechanism by which global DNA hypermethylation may increase cancer risk is unclear; however, it has been hypothesized that cells with higher methylation levels may be longer-lived and thus, combined with carcinogen exposure, this methylation pattern may favor clonal expansion of damaged cells [4].

Interestingly, similar contrasting associations to those we observed in the present study by time from blood draw to diagnosis have been reported for pre-diagnostic and post-diagnostic blood samples in studies of global DNA methylation and bladder cancer [4,10,18,20]. In these studies, hypermethylation was observed among cases with pre-diagnostic samples (relative to controls) as opposed to hypomethylation among cases with post-diagnostic samples. Although we excluded prostate cancer cases diagnosed within one year of blood draw, it is likely that those diagnosed less than 2 years after blood draw in our study may have already had prostate cancer at the time of blood draw. Thus, the observed association for *Alu* for this group in our study may reflect changes in DNA methylation due to the disease process. Previous studies have shown that DNA methylation may also change with age [21], which could potentially contribute to findings by time from draw to diagnosis; however, *Alu* DNA methylation levels were not associated with age in our study and therefore we do not expect that our findings were driven by an age effect.

While some previous studies evaluating cancer risk in relation to DNA methylation at repetitive elements in peripheral blood have reported similar results for both *LINE-1* and *Alu* [22,24,25], others have found different associations for the two elements [5,23]. Zhu et al reported an association with the risk of all cancers combined for *LINE-1* DNA methylation levels, but not for *Alu* [23]. Similar to our study, a prospective study of gastric cancer by Gao et al observed an association for *Alu* DNA methylation, but not for *LINE-1* DNA methylation [5]. Although both measures have been used as proxies for global DNA methylation levels in the literature, the two measures were not highly correlated in our study, as has also been reported previously in blood [22]. Previous research has also observed different transcription patterns for *LINE-1* and *Alu* in response to cellular stressors [26], suggesting that these elements may have different functional roles.

As we limited our study population to non-Hispanic Caucasians, our study may not be generalizable to other populations. We also acknowledge that DNA methylation levels in blood may not reflect levels in tissue; however, there is growing evidence that blood DNA methylation levels may serve as independent predictors of cancer risk for a variety of solid tumors [12]. We had limited numbers for our stratified analyses by disease aggressiveness and time from blood draw to diagnosis, necessitating replication in future studies. Our ability to evaluate time from blood draw to diagnosis was also limited by the diagnosis of most cases in our study within several years of blood draw (75^{th} percentile=3.7 years),

although there was a large range (1-13.9 years). In addition, while it is possible that our findings for *Alu* may have been driven by changes in DNA methylation levels among the cases over time, we did not evaluate within-individual temporal variability using serial blood samples to directly address this question.

Strengths of our study included the use of pyrosequencing to measure DNA methylation levels as this is a high-throughput, quantitative method that is considered highly sensitive to detect differences in DNA methylation between individuals. In addition, the large, prospective design of the study and our ability to separately evaluate the association between blood DNA methylation measures and prostate cancer risk among those diagnosed several years after providing the blood sample was a further strength.

CONCLUSIONS

While *LINE-1* DNA methylation levels in peripheral blood were not associated with prostate cancer risk in our study, we observed an association for *Alu* DNA methylation levels that varied by time from blood draw to diagnosis. Although replication is needed, our study suggests that elevated blood DNA methylation levels at *Alu* several years prior to diagnosis may be associated with an increased risk of prostate cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SYNOPSIS

There is growing evidence for a number of cancers that global DNA methylation levels in peripheral blood may be associated with risk. However, few studies have been prospective and, to date, no previous study has evaluated global DNA methylation levels in peripheral blood in relation to the risk of prostate cancer. Using a large, prospective design, we conducted the first study to evaluate blood DNA methylation levels at the *LINE-1* and *Alu* repetitive elements, which are commonly used as proxies of global DNA methylation, in relation to the risk of prostate cancer. While *LINE-1* DNA methylation levels were not associated with prostate cancer risk, we observed an association for *Alu* DNA methylation levels that varied by time from blood draw to diagnosis. Although requiring replication, our findings suggest that elevated *Alu* DNA methylation levels in peripheral blood DNA several years before diagnosis may be associated with an increased risk of prostate cancer.

Table I

Study population characteristics for the prostate cancer cases and controls

Characteristic [mean±sd or n (%)]	Cases (n=694)	Controls (n=703)
Age at diagnosis/selection[a] (years)	68.6 ± 5.6	68.2 ± 5.6
Age at blood draw[a]	65.5 ± 5.3	65.4 ± 5.2
Year of blood draw[a]	1997.6 ± 2.2	1997.7 ± 2.4
Family history of prostate cancer		
Yes/possible	93 (13.4%)	43 (6.1%)
No	595 (85.7%)	654 (93.0%)
Missing	6 (0.9%)	6 (0.9%)
Smoking		
Never	247 (35.6%)	171 (24.3%)
Former	333 (48.0%)	371 (52.8%)
Current	52 (7.5%)	90 (12.8%)
Pipe/cigar	62 (8.9%)	71 (10.1%)
Folate		
Q1	124 (17.9%)	168 (23.9%)
Q2	192 (27.7%)	167 (23.8%)
Q3	186 (26.8%)	167 (23.8%)
Q4	164 (23.6%)	167 (23.8%)
Missing	28 (4.0%)	34 (4.8%)
BMI		
<25	177 (25.5%)	175 (24.9%)
25-29	367 (52.9%)	361 (51.4%)
≥30	139 (20.0%)	161 (22.9%)
Missing	11 (1.6%)	6 (0.9%)
Aggressive prostate cancer[b]		
Yes	172 (24.8%)	---
No	516 (74.4%)	
Missing	6 (0.9%)	

[a] Study matching factor or combination of study matching factors

[b] Aggressive defined as Stage III/IV or Gleason score≥8; non-aggressive defined as Stage I/II and Gleason score<8

Table II

Mean *LINE-1* and *Alu* DNA methylation levels and prostate cancer risk

	DNA methylation marker quartiles[a]	*LINE-1*[b]		DNA methylation marker quartiles[a]	*Alu*[b]	
Quartile	**Range**	**Case/Control**	**OR (95% CI)[c]**	**Range**	**Case/Control**	**OR (95% CI)[c]**
Q1	63.3-73.0	171/182	REF	20.5-24.6	178/175	REF
Q2	>73.0-74.1	188/178	1.16 (0.85-1.57)	>24.6-25.7	192/199	1.01 (0.74-1.37)
Q3	>74.1-75.3	185/177	1.10 (0.81-1.50)	>25.7-26.3	169/162	0.96 (0.70-1.32)
Q4	>75.3-82.9	147/163	1.01 (0.73-1.39)	>26.3-29.1	151/163	0.94 (0.68-1.29)
	ptrend[d]		0.99	ptrend[d]		0.69

[a] Defined among controls

[b] Average across positions 1-4

[c] Adjusted for age at blood draw, year of blood draw, family history of prostate cancer (yes/possible, no), smoking status (never, former, current, pipe/cigar), total folate intake (quartiles), and BMI (<25, 25-29, 30 or higher)

[d] Based on a variable assigned the median value (among controls) for each quartile entered as a continuous variable in the model

Table III

Mean *LINE-1* and *Alu* DNA methylation levels and prostate cancer risk by time from blood draw to diagnosis for the cases

		Controls[a]	Cases								
	DNA methylation marker quartiles[b]		< 2 years[c]		2 to < 3 years		3 to <4 years		≥ 4 years		$P_{heterogeneity}$[d,e]
	LINE-1[f]										
Quartile	Range	n	n	OR (95% CI)[e]	n	OR (95% CI)[e]	n	OR (95% CI)[e]	n	OR (95% CI)[e]	
Q1	63.3-73.0	182	53	REF	41	REF	38	REF	39	REF	
Q2	>73.0-74.1	178	39	0.75 (0.46-1.20)	50	1.22 (0.75-1.98)	60	**1.83 (1.13-2.98)**	39	1.07 (0.64-1.79)	
Q3	>74.1-75.3	177	72	1.30 (0.84-2.00)	41	0.98 (0.59-1.63)	37	1.06 (0.62-1.80)	35	1.00 (0.59-1.68)	
Q4	>75.3-82.9	163	54	1.04 (0.66-1.64)	32	0.91 (0.53-1.55)	26	0.88 (0.49-1.57)	35	1.18 (0.70-1.99)	0.83
	ptrend[g]			0.43		0.60		0.40		0.62	
	Alu[f]										
Q1	20.5-24.6	175	82	REF	40	REF	35	REF	21	REF	
Q2	>24.6-25.7	199	65	0.71 (0.47-1.06)	46	1.13 (0.69-1.86)	42	1.15 (0.68-1.94)	39	**1.80 (1.01-3.21)**	
Q3	>25.7-26.3	162	41	**0.50 (0.32-0.78)**	39	1.08 (0.65-1.81)	48	1.39 (0.83-2.35)	41	**1.90 (1.06-3.41)**	
Q4	>26.3-29.1	163	31	**0.40 (0.25-0.65)**	39	1.13 (0.67-1.90)	37	1.22 (0.71-2.07)	44	**2.26 (1.27-4.00)**	$\mathbf{5.3\times10^{-6}}$
	ptrend[g]			$\mathbf{3.8\times10^{-5}}$		0.64		0.32		$\mathbf{4.4\times10^{-3}}$	

[a] Comparison group

[b] Defined among controls

[c] Prostate cancer cases diagnosed less than one year after blood draw were excluded in the study design

[d] P-value for heterogeneity from Wald Chi-square test comparing effects of Q4 (vs. Q1) on the four case groups relative to controls

[e] Adjusted for age at blood draw, year of blood draw, family history of prostate cancer (yes/possible, no), smoking status (never, former, current, pipe/cigar), total folate intake (quartiles), and BMI (<25, 25-29, 30 or higher)

[f] Average across positions 1-4

[g] Based on a variable assigned the median value (among controls) for each quartile entered as a continuous variable in the model