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Research Article

Correlation of *LINE-1* Methylation Levels in Patient-Matched Buffy Coat, Serum, Buccal Cell, and Bladder Tumor Tissue DNA Samples

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Abstract

Background: Evidence suggests that global methylation levels in blood cell DNA may be a biomarker for cancer risk. To date, most studies have used genomic DNA isolated from blood or urine as a surrogate marker of global DNA methylation levels in bladder tumor tissue.

Methods: A subset of 50 bladder cancer cases was selected from the New England Bladder Cancer Case–Control Study. Genomic DNA was isolated from buffy coat, buccal cells, serum, and formalin-fixed, paraffin-embedded tissue for each participant. DNA methylation at four CpG sites within the long interspersed nucleotide element (*LINE-1*) repetitive element was quantified using pyrosequencing and expressed as a mean methylation level across sites.

Results: Overall, the mean percent (%) *LINE-1* 5-methylcytosine (%5MeC) level was highest in serum (80.47% ± 1.44%) and lowest in bladder tumor DNA (61.36% ± 12.74%) and levels varied significantly across tissue types ($P = 0.001$). An inverse association between *LINE-1* mean %5MeC and tumor stage ($P = 0.001$) and grade ($P = 0.002$) was observed. A moderate correlation between patient-matched serum and buffy coat DNA *LINE-1* %5MeC levels was found ($r = 0.32$, $P = 0.03$) but levels were uncorrelated among other matched genomic DNA samples.

Conclusions: The mean promoter *LINE-1* %5MeC measurements were correlated between buffy coat and serum DNA samples. No correlation was observed between genomic DNA sources and tumor tissues; however a significant inverse association between tumor percent *LINE-1* methylation and tumor stage/grade was found.

Impact: *LINE-1* methylation measured in case blood DNA did not reflect that observed in bladder tumor tissue but may represent other factors associated with carcinogenesis. *Cancer Epidemiol Biomarkers Prev; 21(7); 1143–8. ©2012 AACR.*

Introduction

Global hypomethylation is thought to contribute to carcinogenesis by increasing genome instability whereas gene-specific hypermethylation is associated with gene silencing (1, 2). Bladder cancer studies have shown that chromosomal instability is widespread, occurs early, and is associated with chromosome 9 aberrations, the first and most frequently altered chromosome in bladder cancer. One of the earliest epigenetic changes associated with genomic instability is believed to be global hypomethylation. Methylation at CpG sites within long interspersed nucleotide elements (*LINE-1*) in serum, buffy coat, buccal cell, and urine DNA have been analyzed as biomarkers of total global methylation status in cancer studies (3–8), however, the usefulness of genomic DNA *LINE-1* methylation as a biomarker remains uncertain and is the subject of continued investigation (9, 10).

High-performance capillary electrophoresis of global methylation and PCR-based analysis of *LINE-1* repeat sequences in blood DNA have been used as biomarkers of bladder cancer risk. In 3 case–control studies, inverse associations were observed using postdiagnostic blood samples (4, 5, 11). It is unclear whether differences in

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global methylation among cases and controls in genomic DNA represent an epigenetic biomarker of recent exposures, a general marker of cancer susceptibility (such as chromosomal alterations), or differences related to carcinogenesis. It is also unknown how methylation levels in tumor DNA correlate with those in surrogate tissues and within the same individuals.

To understand associations between *LINE-1* methylation levels across tissue types and in bladder tumor tissue, we compared levels from matched DNA specimens to determine whether % 5-methylcytosine (%5MeC) levels in the *LINE-1* promoter region correlated in DNA isolated from buccal cells, buffy coat, serum, and formalin-fixed, paraffin-embedded (FFPE) bladder tumor tissue from the same individuals. A secondary aim was to determine which peripheral DNA source best reflected *LINE-1* methylation levels in tumor tissue.

Materials and Methods

Study population

The New England Bladder Cancer Study (NEB) is a population-based case–control study conducted in Maine, Vermont, and New Hampshire (12). Cases, aged 30 to 79 years with histologically confirmed urothelial carcinoma were included (12). Rapid case ascertainment was conducted through pathology departments and hospital/state registries. We interviewed 1,213 bladder cancer cases. Participants were interviewed at home by a trained interviewer using a detailed computer-assisted personal interview (CAPI). Information collected included general demographics, occupational and residential histories, tobacco and hair coloring use, family history of cancer, and diet. Biologic samples were collected, including blood and buccal cells. FFPE bladder tumor tissue was obtained from hospital pathologists. Fifty cases were selected from Maine and Vermont from whom buffy coat, serum, buccal cell, and FFPE tumor tissue was available.

Laboratory methods

Genomic DNA was extracted from samples using standard phenol-chloroform methods. FFPE tissue blocks, considered representative of the patient's primary tumor, and hematoxylin and eosin (H&E)-stained diagnostic slides, were obtained from hospitals where cases were treated. Serial, 5-μm tissue sections were placed onto charged slides. One slide was stained with H&E and scanned on an Aperio ScanScope XT at 20× (Aperio Technologies). Digital images were transferred into an Aperio Spectrum database and harvest regions identified by a study pathologist via Aperio ImageScope annotation software. Tumor tissue was macrodissected and placed into 1.5 mL centrifuge tubes. One hundred and seventy-five microliters of microwave retrieval solution (0.1% guanidium thiocyanate and 0.1 mol/L NaOH; ref. 13) was added, heated to 100°C for 10 minutes followed by 10 minutes at 100°C using the microwave module of a Milestone RH-1 tissue processor (Hacker Instruments and Industries, Inc.). Samples were cooled for 5 minutes, placed into a Thermomixer R (Eppendorf) for 10 minutes at 65°C at 1,400 rpm. One microliter of RNAse (100 mg/mL) was added, samples were incubated for 30 minutes at 55°C and 5 minutes at 65°C without shaking. Samples were centrifuged (5 min/14,000 × g/4°C). DNA was isolated from supernatant using the phenol-based AutoGenprep 245T DNA extraction kit according to instructions. DNA yield and purity were determined by NanoDrop 1000 spectrophotometer (NanoDrop technologies). Bisulfite modification of DNA (0.25–0.5 μg/μL) was conducted by the EZ DNA Methylation Kit (Zymo Research), according to the manufacturer's instructions, and stored at −20°C. An unmethylated cytosine site was included in the PCR amplification product as a positive, internal bisulfite conversion control.

LINE-1 methylation levels were quantified using pyrosequencing (EpigenDx; refs. 14–16), to examine the methylation status at 4 CpG sites in the *LINE-1* promoter (−492 to −419 bp from ATG). Bisulfite-treated DNA was eluted to 20 μL. Each 50 μL PCR contained the bisulfite-treated DNA, 10× PCR buffer, 3.0 mmol/L $MgCl_2$, 200 μmol/L dNTPs, 0.2 μmol/L primers, 1.25 U DNA polymerase (HotStar, Qiagen Inc.). A biotinylated primer was used to capture one single-stranded DNA template for pyrosequencing, (17, 18). PCR products (10 μL) were sequenced using the Pyrosequencing PSQ96 HS System (Biotage). Loci were analyzed as a T/C SNP using QCpG software (Biotage). Each plate contained unmethylated, partially methylated, and heavily methylated DNAs (SssI-treated DNA) for quality control.

Statistical analysis

STATA (version 10.0) was used for data analysis. The study was designed to have 90% power to detect a ≥1.27% difference in mean methylation levels with an SD of ±3.0 and 80% power to observe a correlation of $r = 0.4$ between mean methylation (N = 50). The quality and quantity of extracted DNA from FFPE tumor tissue limited our bladder tumor sample size to N = 34. Within that sample size (N = 34), we had 80% power to detect a ≥1.27% difference in mean methylation levels with an SD ±3.0 and 80% power to observe a correlation of $r = 0.5$ between mean methylation levels.

Triplicate %5MeC measurements were averaged across 4 CpG dinucleotides in the *LINE-1* promoter and expressed as a methylation index (%5MeC). Initially, samples with a coefficient of variation (CV) >10% ($n = 3$) or a percent unconverted internal cytosine control >15% ($n = 19$) were excluded. Sensitivity analyses were conducted excluding samples with a CV >5% ($n = 5$) and those with unconverted cytosine >15% (internal cytosine control; $n = 19$). The difference in calculated mean values was <1% for both criterion. Therefore, we included all samples regardless of the percent unconverted cytosine and excluded only samples with CVs >10% from the analysis. Data were distributed nonnormally. Both nonparametric (Kruskall–Wallis) and parametric tests (Student

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t tests/ordered logistic regression) were conducted on log-transformed *LINE-1* methylation levels. Correlation coefficients were evaluated using Pearson's test on transformed continuous data. Correlations using other cutoff points (medians, tertiles, and quartiles) were also examined.

Results

A sufficient quality and quantity of genomic DNA was extracted from all buccal, serum, and buffy coat samples; and 34 FFPE tumor tissue blocks (68%). Pyrosequencing data were obtained on 184 samples. Three samples were excluded (buffy coat, $n = 1$; serum, $n = 2$) with CVs >10%. Table 1 summarizes case characteristics and the mean percent *LINE-1* methylation by genomic DNA source. Case demographics were similar to NEB cases overall (12). More subjects were white (94%), male (80%), ≥55 years old (70%), and smokers (70%).

Initially, we analyzed the mean *LINE-1* methylation CVs by DNA source. The lowest variance was observed for buccal cell (CV, 4.24 ± 0.89; range, 1.39–5.82), and buffy coat DNA (CV, 4.54 ± 0.65; range, 3.20–6.14; data not shown). Variation was highest for tumor tissue (CV, 4.26 ± 1.59; range, 2.17–8.71) and serum DNA (CV, 5.06 ± 4.51;

Table 1. Mean *LINE-1* methylation levels (%) in matched tissue DNAs by patient/tumor characteristics

Characteristics of cases	Buccal		Buffy coat		Serum		Bladder tumor tissue			P^c (without tissue)
	Cases	Mean ± SD	Cases	Mean ± SD	Cases	Mean ± SD	Cases	Mean ± SD	P^c	
	50	77.09 ± 2.09	49	79.36 ± 1.34	48	80.47 ± 1.44	34	61.36 ± 12.74	0.001	0.0001
Sex										
Female	10	76.15 ± 2.21	9	78.51 ± 1.24	9	79.72 ± 1.30	6	58.86 ± 15.33	0.002	0.002
Male	40	77.3 ± 2.07	40	79.56 ± 1.30	39	80.64 ± 1.43	28	61.9 ± 12.37	0.001	0.0001
P^b		0.10		0.03		0.08		0.56		
Age, y										
<55	19	77.14 ± 1.83	18	79.23 ± 1.47	18	80.05 ± 1.43	15	63.51 ± 13.78	0.0001	0.0001
55–64	16	77.41 ± 2.21	16	79.72 ± 0.78	16	80.82 ± 1.57	8	64.5 ± 7.15	0.0001	0.0001
65–74	10	76.88 ± 2.10	10	79.02 ± 1.85	10	80.76 ± 1.49	7	52.65 ± 16.5	0.001	0.006
75+	5	76.27 ± 3.01	5	79.44 ± 1.28	4	80.21 ± 0.18	4	62.27 ± 2.91	0.03	0.06
P_{trend}^b		0.44		0.95		0.38		0.38		
Race										
White	47	77.07 ± 2.13	46	79.45 ± 1.33	45	80.55 ± 1.43	31	60.58 ± 13.01	0.001	0.001
Non-white	3	77.34 ± 1.71	3	78.08 ± 0.87	3	79.19 ± 1.23	3	69.42 ± 5.68	0.18	0.18
P^b		0.82		0.09		0.11		0.29		
Smoking										
Never	14	76.95 ± 1.69	13	79.06 ± 1.17	13	80.68 ± 1.38	10	59.22 ± 9.40	0.001	0.0001
Former[a]	16	77.57 ± 1.71	16	79.52 ± 1.51	15	80.30 ± 1.37	9	59.85 ± 17.66	0.001	0.0002
Current	20	76.79 ± 2.60	20	79.44 ± 1.34	20	80.45 ± 1.59	15	63.70 ± 11.70	0.001	0.0001
P_{trend}^b		*0.72*		*0.47*		*0.70*		*0.48*		
Ever[a]	36	77.13 ± 2.25	36	79.48 ± 1.40	35	80.39 ± 1.48	24	63.26 ± 13.98	0.0001	0.0001
P^b		0.80		0.35		0.53		0.74		
Tumor grade										
1	29	76.77 ± 2.42	29	79.35 ± 1.33	28	80.61 ± 1.31	20	66.31 ± 9.03	0.0001	0.0001
2	8	77.47 ± 0.24	8	78.63 ± 1.67	8	80.89 ± 2.10	5	63.71 ± 2.91	0.005	0.01
3	11	77.97 ± 1.47	11	79.82 ± 0.94	11	79.74 ± 1.25	8	50.66 ± 15.65	0.0003	0.005
Unknown	1	74.97	1	80.75	1	81.02	1	36.35	N/A	N/A
Missing	1		—		—		—			
P_{trend}^b		0.09		0.52		0.14		0.002		
Tumor stage										
Ta	39	76.98 ± 2.19	39	79.25 ± 1.38	38	80.68 ± 1.45	26	64.98 ± 8.62	0.0001	0.0001
T1	6	78.43 ± 1.14	6	79.48 ± 10.70	6	79.29 ± 0.92	4	53.33 ± 18.57	0.02	0.15
T2a-T3a	4	76.43 ± 1.94	4	80.27 ± 1.31	4	80.21 ± 1.41	4	45.86 ± 17.32	0.15	0.14
Missing	1	75.68	—		—					
P_{trend}^b		0.78		0.17		0.14		0.001		

[a]Includes one occasional smoker.
[b]*P* value for Student *t* test for dichotomous variables, *P* value for ordered logistic regression analysis of categorical variables.
[c]*P* value for Kruskall–Wallis test.

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range, 2.65–12.06). The mean CVs of *LINE-1* methylation levels across DNA sources per individual were similar, with (CV, 4.41 ± 0.63; range, 3.52–7.04) and without the inclusion of tumor DNAs (CV, 4.39 ± 0.53; range, 3.35–5.63).

Mean *LINE-1* methylation levels were highest in serum (80.47% ± 1.44%) followed by buffy coat (79.36% ± 1.34%) and buccal cell DNA (77.09% ± 2.09%; Table 1). Bladder tumor DNA had the lowest *LINE-1%* 5MeC and the most variation across samples (61.36% ± 12.74%). Mean *LINE-1* methylation levels differed significantly across DNA source both with ($P = 0.001$) and without ($P = 0.0001$) inclusion of tumor levels. When stratified by case characteristics, buffy coat *LINE-1* methylation levels differed by gender, with males having higher methylation than females ($P = 0.03$). We did not observe significant differences by the other DNA sources analyzed; however tumor DNA *LINE-1* methylation levels were inversely associated with stage ($P = 0.001$) and grade ($P = 0.002$).

In Table 2, correlations between *LINE-1* methylation levels are presented between DNA pairs extracted from different sources and by tumor stage/grade. By DNA source, a moderate correlation was observed between patient-matched serum and buffy coat DNAs ($r = 0.32$, $P = 0.03$). *LINE-1* methylation levels were uncorrelated among other matched genomic DNA samples or with tumor tissue. Grouping methylation levels by medians, tertiles, or quartiles, did not improve correlations between DNA sources (data not shown). Tumor tissue %5MeC levels showed a strong inverse association with tumor stage ($r = -0.56$, $P = 0.0005$) and grade ($r = -0.52$, $P = 0.002$).

Discussion

CpG methylation in repetitive regions of the genome is becoming widely used to estimate global methylation (reviewed in ref. 10). Yet, few studies have compared %5MeC within repetitive genomic regions in DNA from matched sources. The NEB study provided an opportunity to evaluate these comparisons. In this study of 50 patients, associations were not observed between *LINE-1* mean %5MeC measured across DNA types. No correlation was observed between genomic DNA sources and tumor tissues. *LINE-1* %5MeC in buffy coat DNA was higher among males than females; and a significant inverse association between tumor %5MeC levels and tumor stage and grade was found. This difference between males and females is consistent with several recent studies of *LINE-1* in blood DNA (4–6, 19–21), but not all (22). Similar to published studies, associations between smoking or age and tissue methylation were not observed (4–6, 11, 19, 23, 24).

Our analysis also supports reports that levels of *LINE-1* methylation in tumor tissue decreases with stage and grade. One study by Choi and colleagues (25) examined different interspersed repeats (*LINE-1* and *ALU*) and tandem repeats (Sat-α, NBL-2, and D4Z4) in blood among 10 individually matched tissue DNAs in 4 cancer-free individuals undergoing autopsy. In contrast to work presented here, the investigators reported *LINE-1* levels were consistent between tissues ($n = 10$) and individuals ($n = 4$). An inverse association between *LINE-1* %5MeC and matched adjacent normal urothelium and bladder tumor samples from cases was also observed; however correlations were not provided. The study by Choi and colleagues was one of the first studies showing that tumor tissue methylation levels were lower than normal urothelial tissue and blood cell DNA. This study was one of the first to report substantial variability and lack of comparability in methylation outcome using different DNA sequences to estimate global methylation.

In the current study, the strongest correlation in methylation levels was observed between buffy coat and serum, both originating from blood. A recent study compared different blood cell types among 48 matched DNAs and noted similar moderate correlations within *LINE-1* methylation of blood cell subtypes (26). It has been suggested that a proportion of serum DNA is actually blood cell DNA although the exact proportion remains uncertain in patients with bladder cancer. Other literature suggests that serum DNA originates from lysed, malignant, haemopoietic apoptotic, and/or necrotic circulating cells in patients with cancer (27).

Table 2. Pearson's correlation coefficients (*P* values) of mean *LINE-1* methylation levels in case-matched DNAs and tumor stage and grade

DNA source	Buccal cell	Buffy coat	Serum	Bladder tumor tissue	Stage
Buffy coat	0.04 (0.76)				
Serum	0.04 (0.78)	0.32 (0.03)			
Bladder tumor tissue	−0.08 (0.67)	−0.13 (0.46)	0.08 (0.64)		
Tumor characteristic					
Stage	0.04 (0.78)	0.20 (0.17)	−0.22 (0.14)	−0.56 (0.0005)	
Grade	0.24 (0.09)	0.09 (0.52)	−0.22 (0.14)	−0.52 (0.002)	0.69[a] (<0.00001)

[a]Tumor stage and grade classifications are predicted to be highly correlated.

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The potential for *LINE-1* methylation as a predictive biomarker of bladder cancer risk is an active area of research. Three case–control studies report associations between blood cell methylation and bladder cancer risk using postdiagnostic samples; but it is unclear whether a similar relationship is observed before diagnosis (8, 28). Such prediagnostic studies will elucidate whether hypomethylation in *LINE-1* is an independent risk factor for bladder cancer, or a question of reverse causality. Environmental exposures such as smoking may impact *LINE-1* methylation and its use as a biomarker. Smoking modified the association between *LINE-1* levels and bladder cancer risk in 2 studies (4, 11), but not in a third study (5). These data suggest the need for researchers to continue modeling environmental exposures (when possible) during development of disease specific biomarkers.

It has been hypothesized that *LINE-1* hypomethylation in case blood DNA may reflect levels in bladder tumor tissue. Our study suggests that they are not directly related. Rather, it appears that hypomethylation of *LINE-1* in genomic DNA reflects other factors associated with bladder carcinogenesis such as retropositioning and enhancement of gene expression. Hypomethylation of a specific *LINE-1* promoter has been shown to activate an alternate transcript of the *MET* oncogene in bladder tumors and normal bladder urothelium (29, 30). It is unclear whether the *LINE-1* methylation levels in genomic DNA actually change expression of specific functional *LINE-1* elements and other cancer genes; however these findings provide a mechanism through which *LINE-1* methylation could modify cancer susceptibility.

Limitations of this study include lack of inclusion of healthy controls, limiting the analysis of bladder cancer risk factors such as smoking exclusively to cases. Healthy tissues or exfoliated cells were collected in this cohort, therefore we were unable to compare *LINE-1* methylation levels across the 4 types of DNA isolated from healthy controls. This study was sufficiently powered to evaluate the study aims of examining modest correlations between paired DNA samples, however a larger sample of cases would have allowed for additional subgroup analyses. For example, the low proportion of nonsmoking cases limited power to estimate differences by smoking status. Strengths of this study include the study design; 50 patients with matched genomic, and tumor tissue DNAs for comparison. Isolation of DNA from FFPE tissue did limit our analysis to 34 tissue case samples. Yet even with the smaller sample size, we had sufficient power to compare %5MeC measured in tissue to %5MeC measured in buffy coat, buccal, and serum. Another strength of the study was the use of pyrosequencing; an accurate and reproducible method used to measure %5MeC. The role of incomplete bisulfite conversion and high CVs across replicates was evaluated through sensitivity analyses, strengthening the overall interpretation of our results.

To our knowledge, this is the first study to systematically compare *LINE-1* methylation across these specific types of matched DNAs. The mean promoter *LINE-1* %5MeC measurements were correlated between buffy coat and serum DNA samples but not with tumor or buccal cell DNA. Although genomic *LINE-1* levels were not directly related to those observed in bladder tumor tissue, current case–control studies appear to support an association between global hypomethylation in blood and cancer risk; however the possibility of reverse causality cannot be eliminated. Future studies should include case–control and prospective studies of prediagnostic blood samples, to elucidate temporal associations between *LINE-1* methylation and bladder cancer risk.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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