Epigenetics 9:3, 404–415; March 2014; © 2014 Landes Bioscience

LINE1 methylation levels associated with increased bladder cancer risk in pre-diagnostic blood DNA among US (PLCO) and European (ATBC) cohort study participants

Gabriella Andreotti[1,†], Sara Karami[1,†], Ruth M Pfeiffer[1], Lauren Hurwitz[1], Linda M Liao[1], Stephanie J Weinstein[1], Demetrius Albanes[1], Jarmo Virtamo[2], Debra T Silverman[1], Nathaniel Rothman[1], and Lee E Moore[1,*]

[1]Division of Cancer Epidemiology and Genetics (DCEG); U.S. National Cancer Institute (NCI); National Institutes of Health (NIH); Department of Health and Human Services (DHHS); Bethesda, MD USA; [2]Department of Chronic Disease Prevention; National Institute for Health and Welfare; Helsinki, Finland

[†]These authors contributed equally to this work.

Keywords: global methylation, bladder cancer, nested case-control, pre-diagnostic blood DNA, PLCO, ATBC

Global methylation in blood DNA has been associated with bladder cancer risk in case-control studies, but has not been examined prospectively. We examined the association between *LINE1* total percent 5-methylcytosine and bladder cancer risk using pre-diagnostic blood DNA from the United States-based, Prostate, Lung, Colorectal, Ovarian Cancer Screening Trial (PLCO) (299 cases/676 controls), and the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) cohort of Finnish male smokers (391 cases/778 controls). Logistic regression adjusted for age at blood draw, study center, pack-years of smoking, and sex was used to estimate odd ratios (ORs) and 95% confidence intervals (CIs) using study- and sex-specific methylation quartiles. In PLCO, higher, although non-significant, bladder cancer risks were observed for participants in the highest three quartiles (Q2–Q4) compared with the lowest quartile (Q1) (OR = 1.36, 95% CI: 0.96 -1.92). The association was stronger in males (Q2–Q4 vs. Q1 OR = 1.48, 95% CI: 1.00–2.20) and statistically significant among male smokers (Q2–Q4 vs. Q1 OR = 1.83, 95% CI: 1.14–2.95). No association was found among females or female smokers. Findings for male smokers were validated in ATBC (Q2–Q4 vs. Q1: OR = 2.31, 95% CI: 1.62–3.30) and a highly significant trend was observed ($P = 8.7 \times 10^{-7}$). After determining that study data could be combined, pooled analysis of PLCO and ATBC male smokers (580 cases/1119 controls), ORs were significantly higher in Q2-Q4 compared with Q1 (OR = 2.03, 95% CI: 1.52–2.72), and a trend across quartiles was observed ($P = 0.0001$). These findings suggest that higher global methylation levels prior to diagnosis may increase bladder cancer risk, particularly among male smokers.

Introduction

The incidence of urothelial carcinoma varies worldwide with the highest rates occurring among males residing in industrialized countries.[1] Primarily a smoking-associated disease, bladder cancer incidence is expected to rise in developing areas of the world due to increases in smoking prevalence and the aging world population.[2] Although most bladder cancers present as superficial tumors, many recur and require regular follow-up screening and intervention.[3]

Previously, four case-control studies using post-diagnostically collected blood DNA found that genomic hypomethylation may be involved in bladder carcinogenesis.[4-7] Using a radioactive bioincorporation assay in a Spanish case-control study, Moore et. al. reported that lower methylation levels in leukocyte DNA were associated with an increased bladder cancer risk.[4] This finding was replicated in two subsequent population-based case-control studies conducted in New Hampshire[5] and China[6] using a high-throughput pyrosequencing assay measuring methylation throughout long interspersed nuclear element sequences (*LINE1*) to estimate total percent 5-methylcytosine (%5mC) in blood DNA.[8,9] A fourth hospital-based bladder cancer case-control study conducted in Thailand estimated overall and regional *LINE1* methylation levels by COBRA PCR.[7] This study found that bladder cancer patients had lower methylation levels in blood and exfoliated bladder cell DNA compared with healthy controls, with the lowest methylation levels measured in case tumor DNA.[7] These retrospective findings support an association between DNA hypomethylation and bladder carcinogenesis; however, since global methylation at CpG loci throughout the genome may change in response to environmental exposures, immune response, and the carcinogenic process itself, findings

*Correspondence to: Lee E Moore; Email: moorele@mail.nih.gov
Submitted: 09/01/2013; Revised: 11/15/2013; Accepted: 12/02/2013; Published Online: 12/06/2013
http://dx.doi.org/10.4161/epi.27386

observed retrospectively could be due to epigenetic changes that occur subsequent to cancer diagnosis.[10-12] Additionally, questionnaire data collected post-diagnosis could be subject to recall bias. We therefore designed a nested case-control study within the Prostate, Lung, Colorectal, Ovarian Cancer Screening Trial (PLCO), a prospective cohort conducted in the United States, selecting male and female bladder cancer cases who were cancer free and provided blood samples prior to diagnosis. For replication, a second nested case-control study was conducted within the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) cohort, a prospective cohort of Finnish male smokers

Results

For PLCO, DNA samples from 419 bladder cancer cases and 843 controls were sent to the lab. Of these, results were obtained from 378 (90.2%) cases and 786 (92.7%) controls. Of these, suitable coefficient of variations (CVs < 10%) from triplicate runs were obtained from 306 (86.5%) cases and 676 (90%) controls. Seven cases were dropped because we could not confirm that their blood sample was taken prior to cancer diagnosis within that year. Cases and controls included from the PLCO study did not differ significantly from those excluded in this study (data not shown). For ATBC, data were available from 391 of 395 cases (99%) and 778 of 790 (99%) of controls, and all CVs were <10%.

Analysis of control samples analyzed per plate did not reveal systematic batch effects. Control samples that were 0, 50, and 100% methylated were 2.7, 55.7, and 81.2% methylated in PLCO, respectively, and 4.9, 48.1, and 88.5% methylated in ATBC, respectively. Averaged across both studies, the percent methylation among control samples was 3.8, 51.9, and 84.9%, respectively.

No significant differences between cases and controls were observed for matching characteristics (age at randomization, sex in PLCO), nor for age at blood draw, study center, and years between blood draw and case diagnosis/control selection (**Table 1**). In PLCO, approximately 25% of cases never smoked, while 45% of controls never smoked. In both studies, cases were significantly more likely than controls to have more pack-years of smoking. ATBC subjects, in addition to being Finnish male smokers, were younger (range: 50–70 vs. 55–74 y in PLCO) and some had a longer time between blood draw and diagnosis date/control selection (range: ≤1–16) compared with PLCO subjects (range: ≤1–13 y).

Mean *LINE1%*5mC levels adjusted for age at blood draw and sex (in PLCO only) among controls in each study are shown in **Table 2**. In PLCO, male controls had significantly higher mean *LINE1%*5mC compared with females (84.2% vs. 83.5%; $P = 0.0004$). In ATBC, mean *LINE1%*5mC levels were lower than PLCO overall (male ever smokers = 79.1%) and in selected subgroups. Age at blood draw was inversely associated with *LINE1%*5mC in PLCO, but positively associated in ATBC. *LINE1%*5mC decreased with increasing years between blood draw and case diagnosis in PLCO, but did not show a monotonic pattern in ATBC. *LINE1%*5mC did not vary significantly by PLCO study centers, but did vary somewhat between ATBC centers. In PLCO, never smokers (84.0%) had significantly higher *LINE1%*5mC compared with ever smokers (83.6%). However, no significant trend was observed for pack-years of smoking in either study or for either gender in PLCO. To note, we found no significant correlations between pack-years of smoking and age among the controls (data not shown). Also, mean *LINE1%*5mC did not vary by dietary factors among controls in either study.

Final risk estimates for bladder cancer and *LINE1%*5mC were adjusted for pack-years of smoking, age at blood draw, sex (PLCO only), and intervention arm (ATBC only) (**Table 3**). In PLCO, higher although non-significant bladder cancer risks were observed for participants in the highest three methylation quartiles (Q2–Q4 combined) compared with the lowest quartile (Q1) (odd ratio [OR] = 1.36, 95% confidence interval [CI]: 0.96–1.92, $P = 0.08$), with no evidence of a monotonic trend by methylation quartiles (P-trend = 0.20). When stratified by sex, the association was stronger among males (Q2–Q4 vs. Q1: OR = 1.48, 95% CI: 1.00–2.20; $P = 0.05$) than females (Q2–Q4 vs. Q1: OR = 1.11, 95% CI: 0.51–2.42, $P = 0.79$), but the test for interaction was not statistically significant (P-interaction = 0.33). After stratification by ever/never smoking, the significant association was restricted to ever smokers (OR = 1.82, 95% CI: 1.18–2.27, $P = 0.01$) and was null among never smokers (OR = 0.82, 95% CI: 0.47–1.45, $P = 0.50$), but the test for interaction was not statistically significant (P-interaction = 0.08). In ATBC, a study comprised of male smokers, significantly higher bladder cancer risk was observed for participants in Q2–Q4 compared with Q1 (OR = 2.31; 95% CI: 1.62–3.30, $P = 1.8 \times 10^{-6}$), with a significant positive trend across quartiles (P-trend = 8.7×10^{-7}). We did not find evidence of risk modification or confounding by age at randomization, age at blood draw, age at diagnosis/control selection, years between blood draw and case diagnosis/control selection, or by the nutrients examined, specifically those involved in methylation reactions.

Because of the differences in associations by gender and smoking status, we conducted additional stratified analyses among PLCO subjects (**Table 4**). A significant increase in bladder cancer risk was observed among male smokers (Q2–Q4 vs. Q1: OR = 1.83, 95% CI: 1.14–2.95, $P = 0.01$), but not never smokers (OR = 0.88, 95% CI: 0.43–1.78, $P = 0.71$), although the interaction was not significant (P-interaction = 0.16). A similar relationship was observed among females, with higher risks among ever smokers (Q2–Q4 vs. Q1: OR = 1.63, 95% CI 0.54–4.93, $P = 0.39$) compared with never smokers (OR = 0.74, 95% CI: 0.27–2.03, $P = 0.55$), but the associations were not statistically significant.

Since the interaction between study and methylation on bladder cancer risk was not statistically significant (P-interaction = 0.26), we pooled male ever smokers from PLCO and ATBC. Pooled ORs were significantly elevated across quartiles compared with Q1 as a referent: Q2 (OR = 1.66; 95% CI: 1.17–2.34, $P = 0.004$), Q3 (OR = 2.29; 95% CI: 1.64–3.19, $P = 4.2 \times 10^{-6}$), and Q4 (OR = 2.18; 95% CI: 1.55–3.06, $P = 0.0001$), (P-trend = 0.0001), adjusted for study, pack-years of smoking, and age

Table 1. Selected characteristics

PLCO[1]						ATBC[2]					
Characteristic	Cases		Controls		P^3	Characteristic	Cases		Controls		P^3
	N	(%)	N	(%)			N	(%)	N	(%)	
Total	299	(100.0)	676	(100.0)	NA	**Total**	391	(100.0)	778	(100.0)	NA
Gender											
Male	242	(80.9)	560	(82.8)		Male	391	(100.0)	778	(100.0)	
Female	57	(19.1)	116	(17.2)	0.47	-					
Age at randomization (years)											
-						50–55	113	(28.9)	239	(30.7)	
55–59	70	(23.4)	147	(21.7)		55–59	130	(33.2)	250	(32.1)	
60–64	91	(30.4)	216	(32.0)		60–64	99	(25.3)	197	(25.3)	
65–69	87	(29.1)	191	(28.3)		65–70	49	(12.5)	92	(11.8)	0.92
70–74	51	(17.1)	122	(18.0)	0.90	-					
Age at blood draw (years)											
-						54	5	(1.3)	23	(3.0)	
55–59	22	7.4	84	12.4		55–59	100	(25.6)	194	(24.9)	
60–64	84	28.1	169	25.0		60–64	137	(35.0)	275	(35.4)	
65–69	95	31.8	213	31.5		65–69	96	(24.6)	184	(23.7)	
70–79	98	32.8	210	31.1	0.12	70–76	53	(13.6)	102	(13.1)	0.52
Center											
Colorado	24	(8.0)	55	(8.1)		Kouvola	17	(4.3)	35	(4.5)	
Georgetown	18	(6.0)	40	(5.9)		Kotka	22	(5.6)	35	(4.5)	
Henry Ford	28	(9.4)	57	(8.4)		Helsinki	109	(27.9)	220	(28.3)	
Minnesota	98	(32.8)	184	(27.2)		Jyvaskyla	29	(7.4)	65	(8.4)	
Washington	34	(11.4)	60	(8.9)		Hameenlinna	24	(6.1)	43	(5.5)	
Pittsburgh	28	(9.4)	102	(15.1)		Lahti	25	(6.4)	36	(4.6)	
Utah	25	(8.4)	70	(10.4)		Meltola	6	(1.5)	13	(1.7)	
Marshfield	36	(12.0)	96	(14.2)		Pori	30	(7.7)	44	(5.7)	
Alabama	8	(2.7)	12	(1.8)	0.19	Tampere	35	(9.0)	77	(9.9)	
						Salo	9	(2.3)	17	(2.2)	
						Seinajoki	31	(7.9)	68	(8.7)	
						Tutku	40	(10.2)	108	(13.9)	
						Rauma	14	(3.6)	17	(2.2)	0.66
Years between age at blood draw and case dx and matching for controls											
≤1	69	(23.1)	149	(22.0)		≤1	43	(11.0)	95	(12.2)	
2–3	83	(27.8)	190	(28.1)		2–3	53	(13.6)	92	(11.8)	
4–6	86	(28.8)	182	(26.9)		4–6	68	(17.4)	133	(17.1)	
7–13	61	(20.4)	155	(22.9)	0.81	7–13	191	(48.9)	383	(49.2)	
						14–16	36	(9.2)	75	(9.6)	0.91

N, number; NA, not applicable; dx, diagnosis; PLCO, Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; ATBC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Cohort. [1]PLCO included 7 cases also selected as controls prior to diagnosis, and 10 controls randomly selected as controls twice. [2]ATBC did not include cases also selected as controls and 110 controls were randomly matched to more than 1 case. [3]χ^2 *P* value.

Table 1. Selected characteristics (continued)

PLCO[1]						ATBC[2]					
Characteristic	Cases		Controls		P^3	Characteristic	Cases		Controls		P^3
	N	(%)	N	(%)			N	(%)	N	(%)	
Pack-Years of Smoking											
0	75	(25.5)	304	(45.4)		-					
1–20	51	(17.3)	120	(17.9)		1–20	56	(14.3)	187	(24.0)	
21–40	57	(19.4)	116	(17.3)		21–40	166	(42.5)	339	(43.6)	
41–60	52	(17.7)	77	(11.5)		41–60	125	(32.0)	197	(25.3)	
>60	59	(20.1)	53	(7.9)	**<0.0001**	>60	44	(11.3)	55	(7.1)	**<0.001**

N, number; NA, not applicable; dx, diagnosis; PLCO, Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; ATBC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Cohort. [1]PLCO included 7 cases also selected as controls prior to diagnosis, and 10 controls randomly selected as controls twice. [2]ATBC did not include cases also selected as controls and 110 controls were randomly matched to more than 1 case. [3]χ^2 *P* value.

at blood draw (quartiles among pooled controls). Further adjustment for study centers and ATBC randomization arm did not change estimates.

Discussion

We examined *LINE1%*5mC levels and bladder cancer risk using prospectively collected blood DNA from nested bladder cancer case-control studies conducted in a US and European population. In PLCO, we did not observe evidence of a linear trend when data were analyzed by quartile; however, a significant trend emerged when analyses were restricted first to males, and subsequently to male smokers. Replication in the ATBC study corroborated these findings among male smokers. Pooling data from PLCO and ATBC male smokers showed that this positive association between *LINE1%*5mC level and bladder cancer risk was highly significant.

Reasons for the different findings between this prospective and previous retrospective case-control studies of bladder cancer, which found associations for hypomethylation[4-7] are unclear, but suggest that *LINE1%*5mC examined in post-diagnostically collected blood may reflect changes associated with immune response and/or the carcinogenic process.[10-13] For example, if a particular blood cell subtype were associated with both *LINE1%*5mC level and bladder cancer risk, then the varying proportions could either confound or modify the association, resulting in different pre- and post-bladder cancer-methylation associations. In bladder cancer, alterations have been observed primarily in the number of neutrophils, and in some cases, leukocytosis (increased leukocyte/white blood cell count) has been associated with poor prognosis, but not in the earliest stages of bladder cancer.[14] It has also been suggested that variation in methylation levels could reflect differences in the proportion of blood cell subtypes.[15-17] Differences in cell proportions (assuming the cell subtypes have different means/distribution of methylation) would have to be very large to significantly affect associations. In addition, if cell proportions were non-differential with methylation, associations observed would be driven toward the null because the added variability would increase the likelihood of false-negative results.

Previous studies of global methylation and other cancer sites have shown varying results. One meta-analysis of 12 studies reported an overall significant 1.5-fold cancer risk with hypomethylation, with significant heterogeneity between studies, but not between retrospective or prospective study designs.[13] A second meta-analysis did not, with the exception of studies using a total 5mC assay.[12] Included in this meta-analysis were two retrospective studies, one of breast[18] and one of renal cancer,[19] that showed increased cancer risk among those with higher methylation levels using the luminometric methylation assay (LUMA) and *LINE1* assays, respectively. Although the Xu et al.,[18] study did not find associations with breast cancer risk using the *LINE1* assay, the inconsistency was considered to be reflecting the different genomic areas examined by each assay: the *LINE1* assay measures CpG sites throughout *LINE1* sequences spanning the genome; the LUMA assay measures 5mC levels in the C^{m}CGG motif, a sequence over-represented in gene promoter regions. These findings together with ours suggest that global DNA methylation varies by cancer site, the method used to measure methylation, and the region of DNA examined.

Although our findings were unexpected, hypermethylation of CpG islands in the promoter region of tumor suppressor genes has been reported in transitional cell carcinomas of the bladder, and have been strongly associated with tumor development and progression, in tumor tissue.[20] However, it is unknown how promoter methylation levels in bladder tumor tissue correlate with those in blood DNA. Future epigenome-wide association studies (EWAS) that are currently being designed will be able to examine an array of specific gene loci using blood DNA.

In PLCO the association between hypermethylation and bladder cancer became significant among males, particularly ever smoker males. This finding is consistent with Liao et al.,[19] where the association for hypermethylation and renal cancer was stronger among current smokers, compared with former or never smokers. Interestingly, Moore et al.[4] and Cash et al.[6] found stronger associations for hypomethylation and bladder cancer risk among never smokers compared with ever smokers. The effect of tobacco use on methylation levels and bladder cancer risk is unclear. In relation to our findings, the higher cancer risk among smokers could suggest that prior to cancer development,

Table 2. Mean *LINE-1*%5mC among controls

PLCO					ATBC				
Characteristic	**N**	**Mean *LINE*-1%5mC[1]**	**(SE[1])**	***P*[1,2]**	**Characteristic**	**N Controls**	**Mean *LINE*-1%5mC[1]**	**(SE[1])**	***P*[1,2]**
Gender									
Male	560	84.2	(0.09)		Male	778	79.08	(0.05)	
Female	116	83.5	(0.19)	**0.0004**	Female	0	-	-	
Age at blood draw (years)									
-					50–55	239	78.57	(0.09)	
55–59	84	84.5	(0.23)		55–59	250	78.99	(0.09)	
60–64	169	83.9	(0.17)		60–64	197	79.59	(0.10)	
65–69	213	83.6	(0.16)		65–70	92	79.53	(0.14)	**<0.001**
70–79	210	83.8	(0.16)	**0.01**	-				
Years between age at blood draw and case diagnosis/control selection									
≤1	149	84.1	(0.18)		≤1	95	79.11	(0.14)	
2–3	190	83.9	(0.16)		2–3	92	78.96	(0.14)	
4–6	182	83.8	(0.17)		4–6	133	78.88	(0.12)	
7–13	155	83.6	(0.18)	**0.02**	7–13	383	78.94	(0.07)	
-					14–16	75	80.22	(0.15)	**0.01**
Center									
Colorado	55	84.4	(0.29)		Kouvola	35	79.77	(0.23)	
Georgetown	40	84.4	(0.33)		Kotka	35	79.06	(0.23)	
Henry Ford	57	84.1	(0.28)		Helsinki	220	78.95	(0.09)	
Minnesota	184	83.5	(0.17)		Jyvaskyla	65	79.47	(0.17)	
Washington	60	84.0	(0.27)		Hameenlinna	43	78.84	(0.21)	
Pittsburgh	102	83.8	(0.21)		Lahti	36	79.44	(0.23)	
Utah	70	83.6	(0.25)		Meltola	13	78.90	(0.39)	
Marshfield	96	83.9	(0.22)		Pori	44	79.43	(0.21)	
Alabama	12	83.8	(0.60)	0.13	Tampere	77	79.16	(0.16)	
					Salo	17	79.19	(0.33)	
					Seinajoki	68	78.78	(0.17)	
					Tutku	108	78.83	(0.13)	
					Rauma	17	79.15	(0.33)	**0.003**
Ever/never smoking									
Never	304	84.0	(0.13)		Never	-	-	-	
Ever	372	83.6	(0.14)	**0.008**	Ever	778	79.08	(0.05)	NA
Pack-years of smoking					**See males**				
1–20	120	83.6	(0.25)						
21–40	116	83.3	(0.27)						
41–60	77	83.3	(0.30)						
>60	53	83.2	(0.35)	0.20					

N, number; NA, not applicable; SE, standard error; mg, micrograms; g, grams; dx, diagnosis; PLCO, Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; ATBC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Cohort. [1]Adjusted for age at blood draw and sex (PLCO only). [2]F-test for categorical variables and *P*-trend for ordinal variables.

Table 2. Mean *LINE-1*%5mC among controls (continued)

PLCO					ATBC				
Characteristic	N	Mean *LINE*-1%5mC[1]	(SE[1])	*P*[1,2]	Characteristic	N Controls	Mean *LINE*-1%5mC[1]	(SE[1])	*P*[1,2]
Pack-years of smoking-males									
1–20	106	84.2	(0.20)		1–20	187	79.00	(0.10)	
21–40	107	84.1	(0.20)		21–40	339	79.10	(0.08)	
41–60	72	84.2	(0.25)		41–60	197	79.13	(0.10)	
>60	50	83.8	(0.30)	**0.05**	>60	55	79.02	(0.19)	0.60
Pack-years of smoking-females					NA				
1–20	14	83.6	(0.68)						
21–40	9	82.3	(0.86)						
>40	8	82.1	(0.91)	0.16					
Alcohol drinking (drinks per day)					**(g per day)**				
0	119	83.8	(0.19)		0	67	79.01	(0.17)	
0–0.5	283	83.9	(0.14)		>0–12.1	332	79.08	(0.07)	
>0.5	274	83.8	(0.15)	0.97	>12.1	379	79.01	(0.07)	0.74
Vegetable intake (servings per day)									
≤5	335	83.8	(0.13)		<749.5	415	79.06	(0.06)	
>5	341	83.9	(0.13)	0.83	≥749.5	363	79.10	(0.08)	0.71
Fruit intake (servings per day)									
≤3	342	83.7	(0.13)		<233.1	459	79.09	(0.06)	
>3	334	84.0	(0.13)	0.09	≥233.1	319	79.05	(0.08)	0.71
Folate (diet and supplement, mg per day)									
≤539.2	338	83.8	(0.13)		<346.1	401	79.1	(0.07)	
>539.2	338	83.9	(0.13)	0.66	≥346.1	377	79.06	(0.08)	0.65
Vitamin B6 (diet and supplement, mg per day)									
≤3.5	339	83.8	(0.13)		<2.5	400	79.06	(0.07)	
>3.5	337	83.9	(0.13)	0.56	≥2.5	378	79.1	(0.08)	0.67
Vitamin B12 (diet and supplement, mg per day)									
≤9.1	341	83.8	(0.13)		<11.0	421	79.15	(0.06)	
>9.1	335	83.9	(0.13)	0.54	≥11.0	357	78.97	(0.08)	0.08
Dietary methionine (mg per day)									
≤1.8	337	83.8	(0.12)		<2.1	409	79.12	(0.06)	
>1.8	339	83.9	(0.14)	0.60	≥2.1	369	79.01	(0.08)	0.27
Dietary protein (g per day)									
≤83.5	338	83.9	(0.12)		<94.8	410	79.1	(0.06)	
>83.5	338	83.8	(0.14)	0.91	≥94.8	368	79.05	(0.08)	0.62

N, number; NA, not applicable; SE, standard error; mg, micrograms; g, grams; dx, diagnosis; PLCO, Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; ATBC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Cohort. [1]Adjusted for age at blood draw and sex (PLCO only). [2]F-test for categorical variables and *P*-trend for ordinal variables.

cells from individuals with higher methylation levels that are also exposed to important bladder carcinogens (such as those found in tobacco) may possess a survival advantage over those from individuals with both low methylation levels and the same carcinogenic exposure. Having both low DNA methylation in the presence of tobacco exposure may direct damaged cells toward programmed cell death decreasing the likelihood of survival and clonal expansion.[4]

In this study, *LINE1*%5mC levels were higher among participants in PLCO compared with ATBC, although similar

comparative associations were observed in both studies and the levels observed are within the range of those observed in other studies.[4-7,12,13] One reason for the different levels in each study may be the ethnic variation in methylation levels between the study populations (US vs. Finnish). Also, PLCO includes both smokers and non-smokers of both sexes, whereas ATBC is comprised of male ever smokers (although the methylation difference was still apparent among male smokers). Another explanation may be the DNA source: buffy coat was used in PLCO, whereas whole blood was used in ATBC. Also, a higher ICC was observed in ATBC compared with the PLCO study. Although ICCs are rarely reported, in comparison to Liao et al.[19] and a previous study of breast cancer,[21] the ICCs we report are comparatively higher.

Strengths of this study include the high quality questionnaire data collected prior to cancer diagnosis, thus reducing the risk of recall bias. Our study design allowed random selection of other cancer cases as controls and comparison to the PLCO population as a whole to ensure that our controls were not healthier than all controls. Each case was histologically confirmed with bladder cancer, eliminating case misclassification. Laboratory error could have increased the likelihood of false-negative results; however, we attempted to reduce intra-and inter-individual variation by excluding individual PCR runs in which low bisulfite conversion levels were observed and subjects for whom a high CV was observed across triplicate runs.

A limitation of this study is that the ATBC cohort only included male smokers and did not allow replication of findings among women. Also, in PLCO, the number of female cases, particularly female smokers, was small and limited the statistical power to detect modest risk estimates. While we were able to examine temporality of the associations using the time between blood draw and date of diagnosis/control selection, changes in methylation levels over time would ideally be conducted using repeat measurements from the same individuals at time points prior to and post-bladder cancer diagnosis, which was beyond the scope of the current study but could be conducted in the future.

In conclusion, to our knowledge, this is the first prospective study of *LINE1*%5mC and bladder cancer risk conducted in two unique populations, but with similar study design. Unlike previous case-control studies, we found higher bladder cancer risks with higher methylation levels. Additional longitudinal studies using samples from the same individuals collected at different time points prior to and post-diagnosis will clarify temporal associations between *LINE1* methylation levels and bladder cancer risk. Studies of genome wide CpG site alterations at specific loci will add to the sensitivity and specificity of using CpG site methylation levels as biomarkers of future cancer risk in healthy populations.

Methods

PLCO screening trial

PLCO study design, sample collection and processing have been previously described.[22,23] In brief, PLCO is a multi-center intervention trial for which participants ages 55 to 74 y were recruited from 1993 to 2001 and randomized into a screening or control arm. Participants provided demographic and lifestyle information at baseline via questionnaire. Participants in the screening arm provided non-fasting blood samples at six annual examinations. Informed consent was obtained from all subjects who provided blood samples. Biospecimen collection was approved by the US. National Cancer Institute (NCI) Special Studies Institutional Review Board (IRB) (OH-C-N041), the US National Institutes of Health, and the IRB at each screening site.

For the current study, approval to use biological specimens and questionnaire data was granted through a peer review process administered by the PLCO Etiologic and Early Marker Studies (EEMS) program (https://www.plcostars.gov). Urinary bladder cancer cases were selected (International Classification of Diseases [ICD] 3 codes C670-C679) from the PLCO screening arm who were self-identified as white, had a complete questionnaire, and were cancer-free at baseline (n = 437). For each case, two controls that were self-identified as white with a completed questionnaire, were frequency matched on sex and 5-y categories for age at randomization to the screening arm (n = 847). Genomic DNA (at least 500 ng) was extracted from pre-diagnostically collected buffy coat samples using standard phenol-chloroform methods, and was available for 419 cases and 843 controls. Seven controls were diagnosed with a cancer other than bladder cancer during follow-up. Because controls were sampled with replacement, ten controls were sampled twice. When more than one blood sample was available per subject, the sample most proximal to the diagnosis date was analyzed.

ATBC study

ATBC is a randomized, double-blind, placebo-controlled, primary prevention trial of Finnish male smokers, designed to evaluate whether daily supplementation with α-tocopherol, β-carotene reduced cancer incidence compared with those without supplementation.[24] Finnish men, 50 to 69 y old who smoked at least five cigarettes per day were recruited between April 1985 and June 1988. Each participant was randomly assigned to one of four intervention groups. Subjects were asked about their smoking status at enrollment via questionnaire. Cohort follow-up continued through the Finnish Cancer Registry. Both cancer incidence and mortality data were collected. Written informed consent was obtained from each participant. ATBC was approved by the IRB of the US NCI and the National Public Health Institute of Finland. The trial was registered as Clinical Trials.gov number NCT00342992 (ClinicalTrials.gov).

For this study, cases were defined as histologically confirmed primary carcinoma of the urinary bladder including carcinoma in situ (ICD9 codes 188.1–188.9). All cases were white, from Finland, had complete questionnaire and nutritional intake data, and were cancer-free at baseline (n = 395). For each case, two controls were selected with complete questionnaire and nutritional intake data, and were frequency matched on 5-y categories for age at randomization (n = 790). Because controls were sampled with replacement, 110 controls were randomly selected twice. Genomic DNA (500 ng) was extracted from pre-diagnostically collected whole blood samples using standard phenol-chloroform methods (http://atbcstudy.cancer.gov).

Table 3. Odds ratios and 95% confidence intervals for bladder cancer risk and *LINE-1*%5mC

PLCO							ATBC					
Characteristics All Subjects		**N Case**	**N Control**	**OR[2]**	**95% CI[2]**	***P*[2]**	**Characteristic All Male Smokers**		**N Case**	**N Control**	**OR[2]**	**95% CI[2]**	***P*[2]**
Q1[1]		68	178	REF	-	-	Q1[1]		49	195	REF	-	-
Q2		69	151	1.29	0.85–1.97	0.23	Q2		86	194	1.74	1.15–2.63	**0.01**
Q3		96	180	1.53	1.03–2.28	0.04	Q3		123	195	2.61	1.75–3.90	**1.1×10^{-5}**
Q4		66	167	1.23	0.80–1.89	0.34	Q4		133	194	2.78	1.85–4.19	**3.8×10^{-6}**
P-trend						0.20	*P*-trend						**8.7×10^{-7}**
Q1		68	178	REF	-	-	Q1		49	195	REF	-	-
Q2–4		231	498	1.36	0.96–1.92	0.08	Q2–4		342	583	2.31	1.62–3.30	**1.8×10^{-6}**
Gender													
Male	Q1	49	141	REF	-	-	NA						
Q2–Q4		193	419	1.48	1.00–2.20	**0.05**							
Female	Q1	19	37	REF	-	-							
Q2–Q4		38	79	1.11	0.51–2.42	0.79							
P-interaction						0.33							
Age at blood draw-years													
55–64	Q1	21	58	REF			50–65	Q1	40	159	REF	-	-
Q2–Q4		85	195	1.38	0.75–2.56	0.30	Q2–Q4		202	333	2.35	1.57–3.53	**<0.001**
65–79	Q1	47	120	REF			65–70	Q1	9	36	REF	-	-
Q2–Q4		146	303	1.42	0.92–2.18	0.11	Q2–Q4		140	250	2.34	1.06–5.15	**0.04**
P-interaction						0.96	*P*-interaction						0.75
Years between blood draw and case diagnosis/control selection													
≤4	Q1	69	94	REF	-	-	≤4	Q1	10	52	REF	-	-
Q2–Q4		37	311	1.57	0.96–2.57	0.07	Q2–Q4		107	174	3.16	1.49–6.67	**0.003**
5–13	Q1	116	84	REF	-	-	5–16	Q1	39	143	REF	-	-
Q2–Q4		77	187	1.19	0.71–2.01	0.51	Q2–Q4		235	409	2.15	1.42–3.24	**<0.001**
P-interaction						0.42	*P*-interaction						0.33
Ever/never smoking							**Ever/never smoking**						
Never	Q1	28	71	REF	-	-	NA						
Q2–Q4		78	233	0.82	0.47–1.45	0.50							
Ever	Q1	47	107	REF	-	-							
Q2-Q4		146	265	1.82	1.18–2.79	**0.01**							
P-interaction						0.08							
Pack-years of smoking							**Pack-years of smoking**						
1–40	Q1	24	67	REF	-	-	1–40	Q1	30	143	REF	-	-
Q2–Q4		84	169	1.71	0.94–3.08	0.08	Q2–Q4		192	383	2.39	1.53–3.74	**<0.001**
>41	Q1	22	39	REF	-	-	>41	Q1	19	52	REF	-	-
Q2–Q4		89	91	1.99	1.03–3.83	**0.04**	Q2–Q4		150	200	2.45	1.3–4.50	**0.004**
P-interaction						0.58	*P*-interaction						0.76

N, number; OR, odds ratio; CI, confidence interval; NA, not applicable; mg, milligrams; g, grams; dx, diagnosis; PLCO, Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; ATBC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Cohort. [1]Quartile cutpoints were based on sex and study-specific control levels (see methods section). [2]Regression models adjusted for age at blood draw, study center, packyears smoking, sex (PLCO), and treatment arm (ATBC).

Table 3. Odds ratios and 95% confidence intervals for bladder cancer risk and *LINE-1*%5mC (continued)

PLCO							ATBC						
Characteristics All Subjects		N Case	N Control	OR[2]	95% CI[2]	*P*[2]	Characteristic All Male Smokers		N Case	N Control	OR[2]	95% CI[2]	*P*[2]
Alcohol drinking (drinks per day)							**(g per day)**						
≤0.5	Q1	36	107	REF	-	-	≤12.1	Q1	31	94	REF	-	-
Q2–Q4		130	295	1.43	0.89–2.30	0.14	Q2–Q4		180	305	1.71	1.07–2.76	**0.03**
>0.5	Q1	32	71	REF	-	-	>12.1	Q1	18	101	REF	-	-
Q2–Q4		101	203	1.25	0.75–2.07	0.39	Q2–Q4		162	278	3.79	2.13–6.76	**<0.001**
P-interaction						0.82	*P*-interaction						0.08
Vegetable intake (servings per day)							**(g per day)**						
≤5	Q1	40	89	REF	-	-	<749.5	Q1	27	98	REF	-	-
Q2–Q4		115	246	1.14	0.71–1.84	0.58	Q2–Q4		204	317	2.51	1.54–4.11	**<0.001**
>5	Q1	28	89	REF	-	-	≥749.5	Q1	22	97	REF	-	-
Q2–Q4		116	252	1.69	1.00–2.85	**0.05**	Q2–Q4		138	266	2.14	1.25–3.66	**0.005**
P-interaction						0.23	*P*-interaction						0.73
Fruit intake (servings per day)													
≤3	Q1	42	97	REF	-	-	<233.1	Q1	28	106	REF	-	-
Q2–Q4		130	245	1.32	0.83–2.09	0.24	Q2–Q4		211	353	2.53	1.56–4.11	**<0.001**
>3	Q1	26	81	REF	-	-	≥233.1	Q1	21	89	REF	-	-
Q2–Q4		101	253	1.48	0.85–2.59	0.16	Q2–Q4		131	230	2.37	1.36–4.15	**0.002**
P-interaction						0.68	*P*-interaction						0.92
Folate (diet and supplement, mg per day)													
≤539.2	Q1	32	86	REF	-	-	< 346.1	Q1	24	97	REF	-	-
Q2–Q4		100	252	1.24	0.75–2.05	0.40	Q2–Q4		187	304	2.61	1.56–4.36	**<0.001**
>539.2	Q1	36	92	REF	-	-	≥346.1	Q1	25	98	REF	-	-
Q2–Q4		131	246	1.53	0.93–2.50	0.09	Q2–Q4		155	279	1.99	1.20–3.31	**0.01**
P-interaction						0.47	*P*-interaction						0.49
Vitamin B6 (diet and supplement, mg per day)													
≤3.5	Q1	36	88	REF	-	-	<2.5	Q1	28	106	REF	-	-
Q2–Q4		105	251	1.19	0.74–1.93	0.48	Q2–Q4		188	294	2.49	1.54–4.05	**<0.001**
>3.5	Q1	32	90	REF	-	-	≥2.5	Q1	21	89	REF	-	-
Q2–Q4		126	247	1.62	0.96–2.72	0.07	Q2–Q4		154	289	2.16	1.24–3.75	**0.01**
P-interaction						0.25	*P*-interaction						0.67
Vitamin B12 (diet and supplement, mg per day)													
≤9.1	Q1	36	90	REF	-	-	<11.0	Q1	20	105	REF	-	-
Q2–Q4		96	251	1.12	0.68–1.82	0.66	Q2–Q4		186	316	3.62	2.11–6.20	**<0.001**
>9.1	Q1	32	88	REF	-	-	≥11.0	Q1	29	90	REF	-	-
Q2–Q4		145	247	1.77	1.06–2.94	**0.03**	Q2–Q4		156	267	1.63	0.98–2.71	0.06
P-interaction						0.17	*P*-interaction						0.07

N, number; OR, odds ratio; CI, confidence interval; NA, not applicable; mg, milligrams; g, grams; dx, diagnosis; PLCO, Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; ATBC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Cohort. [1]Quartile cutpoints were based on sex and study-specific control levels (see methods section). [2]Regression models adjusted for age at blood draw, study center, packyears smoking, sex (PLCO), and treatment arm (ATBC).

Table 3. Odds ratios and 95% confidence intervals for bladder cancer risk and *LINE-1*%5mC (continued)

PLCO							ATBC						
Characteristics All Subjects		N Case	N Control	OR[2]	95% CI[2]	*P*[2]	Characteristic All Male Smokers		N Case	N Control	OR[2]	95% CI[2]	*P*[2]
Dietary protein (g per day)													
≤83.5	Q1	49	94	REF	-	-	<94.8	Q1	22	105	REF	-	-
Q2–Q4		120	244	1.01	0.66–1.56	0.95	Q2–Q4		176	305	2.63	1.56–4.45	**<0.001**
>83.5	Q1	19	84	REF	-	-	≥94.8	Q1	27	90	REF	-	-
Q2–Q4		111	254	2.32	1.25–4.34	**0.01**	Q2–Q4		166	278	1.85	1.12–3.05	**0.02**
P-interaction						**0.02**	*P*-interaction						0.30
Dietary methionine (mg per day)													
≤1.8	Q1	44	95	REF	-	-	<2.1	Q1	25	101	REF	-	-
Q2–Q4		122	242	1.20	0.77–1.86	0.43	Q2–Q4		184	308	2.43	1.46–4.03	**0.001**
>1.8	Q1	24	83	REF	-	-	≥2.1	Q1	24	94	REF	-	-
Q2–Q4		109	256	1.65	0.93–2.93	0.09	Q2–Q4		158	275	2.06	1.23–3.45	**0.01**
P-interaction						0.29	*P*-interaction						0.69

N, number; OR, odds ratio; CI, confidence interval; NA, not applicable; mg, milligrams; g, grams; dx, diagnosis; PLCO, Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial; ATBC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention Cohort. [1]Quartile cutpoints were based on sex and study-specific control levels (see methods section). [2]Regression models adjusted for age at blood draw, study center, packyears smoking, sex (PLCO), and treatment arm (ATBC).

LINE1 methylation quantification

Methylation was quantified using *LINE1,* the most highly expressed transposable element in the genome. With more than 500 000 copies, it comprises approximately 17% of the human genome.[25] Although the assay used in most recent studies to quantify *LINE1* methylation (bisulfite treatment and pyrosequencing) does not measure total CpG content (as does the HPCE analysis), the accuracy, high-throughput and quantitative nature of this surrogate biomarker makes it attractive for use in large epidemiologic studies.[25,26] Quantification of *LINE1* for both PLCO and ATBC was conducted at the same laboratory using the same methods. Bisulfite DNA modification (0.25–0.5 μg/μl) was conducted using the EZ DNA Methylation Kit (Zymo Research) according to the manufacturer's instructions. One T/C SNP was used to evaluate the completeness of the bisulfite treatment by the proportion of Cs altered and unaltered with bisulfite treatment. When the bisulfite treatment is complete, the unmethylated cytosine will be modified. In addition to the T/C SNP, there were four additional CpG sites evaluated and averaged to determine mean CpG site methylation of the *LINE1* promoter region. *LINE1*%5mC levels were quantified using pyrosequencing (EpigenDx).[27] Methylation levels were examined at four CpG sites in the *LINE1* promoter (−492 to −419 bp from ATG). Each 50 μl PCR contained the bisulfite-treated DNA, 10× PCR buffer, 3.0 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM primers, 1.25 U DNA polymerase (HotStar, Qiagen Inc.). A biotinylated primer was used to capture one single-stranded DNA template for pyrosequencing[27,28] using the Pyrosequencing PSQ96 HS System (Biotage). One T/C SNP per locus was evaluated using QCpG software (Biotage).

For quality control, each plate contained blank wells and controls containing unmethylated (0%), partially methylated (50%), and heavily methylated (~100%) DNA samples (SssI treated).[29] Triplicate measurements were averaged across each of the four CpG dinucleotides. Individual runs with >7.5% bisulfite-unconverted cytosine loci were eliminated from calculations. Subjects with a CV >10% for triplicate runs were also excluded. Two plates were excluded from the PLCO analysis due to instrumentation problems during aliquotting that resulted in insufficient DNA quantities per well. However, because all samples were randomized across plates, the excluded samples did not differ significantly from those included in the current study (data not shown). After exclusions, the PLCO analysis totaled 299 cases and 676 controls, and the ATBC analysis included 391 cases and 778 controls. The ICC was calculated using the triplicate PCR runs from each individual using the GLM procedure in SAS: 0.34 and 0.47 for PLCO and ATBC studies, respectively.

Statistical analyses

We first analyzed the data from PLCO and ATBC separately using the same statistical methods, unless otherwise noted. Selected characteristics were compared between cases and controls using chi-square tests. The distribution of methylation levels among controls was nearly normal (Kolmogorov-Smirnov test $P = 0.05$), therefore methylation levels were not transformed. Among the controls, we calculated mean methylation levels adjusting for age at blood draw and sex (PLCO only) by selected characteristics, and tested for differences in mean methylation values using the F-test for categorical variables and the p-trend for ordinal variables.

ORs and 95% CIs for associations between quartiles of *LINE1*%5mC and bladder cancer risk were computed using logistic regression models, which considered the random repeat sampling of some participants in the variance computation using a generalized estimating equation approach. Because of the systematically lower methylation levels in PLCO than in

Table 4. Odds ratios and 95% confidence intervals for bladder cancer risk and *LINE-1*%5mC by gender and smoking status in PLCO

	Never smoker					Ever smoker				
Gender	**N Case**	**N Control**	**OR[2]**	**95% CI[2]**	***P*[2]**	**N Case**	**N Control**	**OR[2]**	**95% CI[2]**	***P*[2]**
Male										
Q1[1]	13	49	REF	-	-	36	92	FER	-	-
Q2	12	49	0.91	0.36–2.28	0.84	50	82	1.77	1.02–3.10	0.04
Q3	15	63	0.92	0.40–2.13	0.84	62	88	2.03	1.18–3.50	0.01
Q4	13	58	0.79	0.33–1.91	0.60	41	79	1.64	0.91–2.94	0.10
P-trend					0.63					0.07
P-interaction										0.56
Q1	13	49	REF	-	-	36	92	REF	-	-
Q2–Q4	40	170	0.88	0.43–1.78	0.71	153	249	1.83	1.14–2.95	0.01
P-interaction										0.16
Female										
Q1	7	22	1.00	-	-	12	15			
Q2	2	18	0.35	0.06–1.91	0.23	5	2	1.43	0.21–9.98	0.72
Q3	7	23	0.95	0.29–3.14	0.93	12	6	2.55	0.57–11.46	0.22
Q4	6	22	0.84	0.24–2.91	0.78	6	8	1.06	0.25–4.47	0.94
P-trend					0.99					0.61
P-interaction										0.35
Q1	7	22	1.00	-	-	12	15	1.00	-	-
Q2–Q4	15	63	0.74	0.27–2.03	0.55	23	16	1.63	0.54–4.93	0.39
P-interaction										0.27

N, number; OR, odds ratio; CI, confidence interval; PLCO, Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial. [1]Quartile cutpoints were based on sex-specific control distributions: PLCO males, 78.3: <82.9, <84.3, <85.7, ≤89.7%; females, 78.4: <82.7, <83.4, <84.9, ≤89.5%. [2]Regression models adjusted for age at blood draw, packyears smoking, study center.

ATBC, as well as lower levels among female vs. male controls in PLCO, study and sex-specific quartile cut-points (Q1–Q4) were calculated among controls. Male quartile cut-points in PLCO were: 82.9, 84.3, 85.7, and 89.7%; and female quartile cut-points in PLCO were: 82.7, 83.4, 84.9, and 89.5%. Quartile cut-points among ATBC controls (male smokers) were: 78.1, 78.9, 80.1, and 84.9%.

We evaluated the following potential confounders: nutrients related to one-carbon metabolism, study center, age at blood draw, years between blood draw and case diagnosis/control selection, pack-years of smoking, and intervention arm (for ATBC). We also evaluated the correlation between pack-years of smoking and age (examined as age at randomization, age at blood draw, and age at control selection) among all controls, as well ever smokers and male smokers, using the Pearson correlation coefficient.

Statistical interactions between methylation Q1 (referent), Q2–Q4 and sex (in PLCO only), age at blood draw, years between blood draw and diagnosis/control selection date, ever/never smoking status (in PLCO only), cigarette pack-years, alcohol drinks per day, and dietary factors hypothesized to modify DNA methylation levels and/or bladder cancer risk were examined using an interaction term in logistic regression models. We also calculated risk estimates for bladder cancer and methylation stratified by the above-mentioned factors. Some dietary intake measurement units differed between studies; therefore, study-specific median values were used (reported in **Table 3**).

ATBC data were pooled with PLCO data for male smokers. ORs and 95% CIs were estimated using logistic regression with study-specific quartiles of methylation and quartiles of age at blood draw among the pooled controls. We also evaluated the interaction between the two studies using an interaction term in the logistic regression models. Analyses were conducted using SAS version 9.1 (SAS Institute) and STATA version 10.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Drs Liying Yan and Matthew Poulin, EpigenDx.

Author Contributions

All authors participated in the critical review of this report for its intellectual content. In particular Andreotti G, Karami S, Pfeiffer RM, Hurwitz L, Liao LM, Weinstein SJ, Albanes D, Virtamo J, and Moore LE participated in the design, data

collection, data interpretation, and writing of this report. Andreotti G, Karami S, Pfeiffer RM, LN, Liao LM, and Moore LE participated in data analysis. Albanes D and Virtamo designed/conducted the original cohort studies. Weinstein SJ, Albanes D, and Virtamo J collected data, provided risk factor, dietary and exposure data, and biological samples for laboratory analyses. Silverman DT, Rothman N, and Moore LE developed the study concept, design, data interpretation, revising and finalizing this report for publication.

Funding

This research was supported in part by the Intramural Research Program of the National Cancer Institute, US National Institutes of Health, and Department of Health and Human Services. Additionally, this research was supported by US. Public Health Service contracts N01-CN-45165, N01-RC-45035, N01-RC-37004 and HHSN261201000006C from the National Cancer Institute, National Institutes of Health, and Department of Health and Human Services.

References

1. Ploeg M, Aben KK, Kiemeney LA. The present and future burden of urinary bladder cancer in the world. World J Urol 2009; 27:289-93; PMID:19219610; http://dx.doi.org/10.1007/s00345-009-0383-3
2. Silverman DT, Devesa SS, Moore LE, Rothman N. Bladder cancer. In: Schottenfeld D, Fraumeni Jr. JF, editors. Cancer epidemiology and prevention. 3rd ed. Oxford; Oxford University Press;2006.
3. Amling CL. Diagnosis and management of superficial bladder cancer. Curr Probl Cancer 2001; 25:219-78; PMID:11514784; http://dx.doi.org/10.1067/mcn.2001.117539
4. Moore LE, Pfeiffer RM, Poscablo C, Real FX, Kogevinas M, Silverman D, García-Closas R, Chanock S, Tardón A, Serra C, et al. Genomic DNA hypomethylation as a biomarker for bladder cancer susceptibility in the Spanish Bladder Cancer Study: a case-control study. Lancet Oncol 2008; 9:359-66; PMID:18339581; http://dx.doi.org/10.1016/S1470-2045(08)70038-X
5. Wilhelm CS, Kelsey KT, Butler R, Plaza S, Gagne L, Zens MS, Andrew AS, Morris S, Nelson HH, Schned AR, et al. Implications of LINE1 methylation for bladder cancer risk in women. Clin Cancer Res 2010; 16:1682-9; PMID:20179218; http://dx.doi.org/10.1158/1078-0432.CCR-09-2983
6. Cash HL, Tao L, Yuan JM, Marsit CJ, Houseman EA, Xiang YB, Gao YT, Nelson HH, Kelsey KT. LINE-1 hypomethylation is associated with bladder cancer risk among nonsmoking Chinese. Int J Cancer 2012; 130:1151-9; PMID:21445976; http://dx.doi.org/10.1002/ijc.26098
7. Patchsung M, Boonla C, Amnattrakul P, Dissayabutra T, Mutirangura A, Tosukhowong P. Long interspersed nuclear element-1 hypomethylation and oxidative stress: correlation and bladder cancer diagnostic potential. PLoS One 2012; 7:e37009; PMID:22615872; http://dx.doi.org/10.1371/journal.pone.0037009
8. Yang AS, Estécio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res 2004; 32:e38; PMID:14973332; http://dx.doi.org/10.1093/nar/gnh032
9. Weisenberger DJ, Campan M, Long TI, Kim M, Woods C, Fiala E, Ehrlich M, Laird PW. Analysis of repetitive element DNA methylation by MethyLight. Nucleic Acids Res 2005; 33:6823-36; PMID:16326863; http://dx.doi.org/10.1093/nar/gki987
10. Nelson HH, Marsit CJ, Kelsey KT. Global methylation in exposure biology and translational medical science. Environ Health Perspect 2011; 119:1528-33; PMID:21669556; http://dx.doi.org/10.1289/ehp.1103423
11. Schulz WA. Do DNA-methylation changes also occur in blood? Lancet Oncol 2008; 9:312-3; PMID:18374285; http://dx.doi.org/10.1016/S1470-2045(08)70083-4
12. Brennan K, Flanagan JM. Is there a link between genome-wide hypomethylation in blood and cancer risk? Cancer Prev Res (Phila) 2012; 5:1345-57; PMID:23135621; http://dx.doi.org/10.1158/1940-6207.CAPR-12-0316
13. Woo HD, Kim J. Global DNA hypomethylation in peripheral blood leukocytes as a biomarker for cancer risk: a meta-analysis. PLoS One 2012; 7:e34615; PMID:22509334; http://dx.doi.org/10.1371/journal.pone.0034615
14. Dukes JW, Tierney LM Jr. Paraneoplastic leukemoid reaction as marker for transitional cell carcinoma recurrence. Urology 2009; 73:e17-9; PMID:18718647; http://dx.doi.org/10.1016/j.urology.2008.05.023
15. Wu HC, Delgado-Cruzata L, Flom JD, Kappil M, Ferris JS, Liao Y, Santella RM, Terry MB. Global methylation profiles in DNA from different blood cell types. Epigenetics 2011; 6:76-85; PMID:20890131; http://dx.doi.org/10.4161/epi.6.1.13391
16. Reinius LE, Acevedo N, Joerink M, Pershagen G, Dahlén SE, Greco D, Söderhäll C, Scheynius A, Kere J. Differential DNA methylation in purified human blood cells: implications for cell lineage and studies on disease susceptibility. PLoS One 2012; 7:e41361; PMID:22848472; http://dx.doi.org/10.1371/journal.pone.0041361
17. Adalsteinsson BT, Gudnason H, Aspelund T, Harris TB, Launer LJ, Eiriksdottir G, Smith AV, Gudnason V. Heterogeneity in white blood cells has potential to confound DNA methylation measurements. PLoS One 2012; 7:e46705; PMID:23071618; http://dx.doi.org/10.1371/journal.pone.0046705
18. Xu X, Gammon MD, Hernandez-Vargas H, Herceg Z, Wetmur JG, Teitelbaum SL, Bradshaw PT, Neugut AI, Santella RM, Chen J. DNA methylation in peripheral blood measured by LUMA is associated with breast cancer in a population-based study. FASEB J 2012; 26:2657-66; PMID:22371529; http://dx.doi.org/10.1096/fj.11-197251
19. Liao LM, Brennan P, van Bemmel DM, Zaridze D, Matveev V, Janout V, Kollarova H, Bencko V, Navratilova M, Szeszenia-Dabrowska N, et al. LINE-1 methylation levels in leukocyte DNA and risk of renal cell cancer. PLoS One 2011; 6:e27361; PMID:22076155; http://dx.doi.org/10.1371/journal.pone.0027361
20. Sánchez-Carbayo M. Hypermethylation in bladder cancer: biological pathways and translational applications. Tumour Biol 2012; 33:347-61; PMID:22274923; http://dx.doi.org/10.1007/s13277-011-0310-2
21. Brennan K, Garcia-Closas M, Orr N, Fletcher O, Jones M, Ashworth A, Swerdlow A, Thorne H, Riboli E, Vineis P, et al.; KConFab Investigators. Intragenic ATM methylation in peripheral blood DNA as a biomarker of breast cancer risk. Cancer Res 2012; 72:2304-13; PMID:22374981; http://dx.doi.org/10.1158/0008-5472.CAN-11-3157
22. Gohagan JK, Prorok PC, Hayes RB, Kramer BS; Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial Project Team. The Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial of the National Cancer Institute: history, organization, and status. Control Clin Trials 2000; 21(Suppl):251S-72S; PMID:11189683; http://dx.doi.org/10.1016/S0197-2456(00)00097-0
23. Hayes RB, Sigurdson A, Moore L, Peters U, Huang WY, Pinsky P, Reding D, Gelmann EP, Rothman N, Pfeiffer RM, et al.; PLCO Trial Team. Methods for etiologic and early marker investigations in the PLCO trial. Mutat Res 2005; 592:147-54; PMID:16054167; http://dx.doi.org/10.1016/j.mrfmmm.2005.06.013
24. Albanes D, Heinonen OP, Taylor PR, Virtamo J, Edwards BK, Rautalahti M, Hartman AM, Palmgren J, Freedman LS, Haapakoski J, et al. Alpha-Tocopherol and beta-carotene supplements and lung cancer incidence in the alpha-tocopherol, beta-carotene cancer prevention study: effects of base-line characteristics and study compliance. J Natl Cancer Inst 1996; 88:1560-70; PMID:8901854; http://dx.doi.org/10.1093/jnci/88.21.1560
25. Nelson HH, Marsit CJ, Kelsey KT. Global methylation in exposure biology and translational medical science. Environ Health Perspect 2011; 119:1528-33; PMID:21669556; http://dx.doi.org/10.1289/ehp.1103423
26. Brennan K, Flanagan JM. Is there a link between genome-wide hypomethylation in blood and cancer risk? Cancer Prev Res (Phila) 2012; 5:1345-57; PMID:23135621; http://dx.doi.org/10.1158/1940-6207.CAPR-12-0316
27. Royo JL, Hidalgo M, Ruiz A. Pyrosequencing protocol using a universal biotinylated primer for mutation detection and SNP genotyping. Nat Protoc 2007; 2:1734-9; PMID:17641638; http://dx.doi.org/10.1038/nprot.2007.244
28. Brakensiek K, Wingen LU, Länger F, Kreipe H, Lehmann U. Quantitative high-resolution CpG island mapping with Pyrosequencing reveals disease-specific methylation patterns of the CDKN2B gene in myelodysplastic syndrome and myeloid leukemia. Clin Chem 2007; 53:17-23; PMID:17095538; http://dx.doi.org/10.1373/clinchem.2007.072629
29. England R, Pettersson M. Pyro Q-CpG™: Quantitative analysis of methylation in multiple CpG sites by pyrosequencing. Nat Methods 2005; 2:1-2; http://dx.doi.org/10.1038/nmeth800