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Genomic DNA Hypomethylation is a Biomarker for Bladder Cancer Susceptibility in the Spanish Bladder Cancer Case-Control Study

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Abstract

Background—DNA hypomethylation has been suggested to cause genomic instability and increase cancer risk. In this study we aimed to test the hypothesis that DNA hypomethylation was associated with bladder cancer risk.

Methods—We measured cytosine methylation (%5-mC) content in genomic DNA from blood cells in subjects enrolled in a large case-control study in Spain. The %5-mC content was measured in leukocyte DNA from 775 cases and 397 controls using a combination of high performance capillary electrophoresis (HPCE), HPA II digestion, and densitometry. In addition to personal characteristics such as age and sex, data on 34 polymorphisms in 9 folate metabolism genes and nutritional intake

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Contributors All authors participated in the critical review of this manuscript for its intellectual content. In particular, FR, MK, DS, RC, AT, CS, AC, MD, SC, MGC, NR, NM participated in the design and conduct of the original case-control study, data collection, provided risk factor, dietary and exposure data, and biological samples for laboratory analysis. LM, DS, MK, MGC, NM, FR, MD, ME, MF, NR developed the concept, design, analysis and interpretation of this epigenetic analysis. All authors participated in the conduct, data analysis, interpretation and participated in drafting, revising and finalizing this manuscript. All authors participated in developing the study concept, conducted laboratory analysis, data interpretation, and prepared the manuscript for publication.

of five B vitamins, folate, alcohol, and smoking were assessed as potential confounders. Informed consent was obtained from all participants in accordance with the National Cancer Institute and local IRB Boards.

Findings—The median %5-mC DNA content was significantly lower among cases than controls (3.03% Interquartile Range (IQR)=2.17–3.56)) and (3.19% (IQR=2.46–3.68, respectively (p=0.0002). Subjects were subsequently categorized into quartiles by %5-mC levels among controls. When the highest quartile of %5-mC content was considered as a reference category (Q4), the following adjusted odds ratios (OR) and 95% confidence intervals (95% CI) were observed for decreasing methylation quartiles $OR_{(Q3)}$=2.05 (95% CI:1.37–3.06); $OR_{(Q2)}$=1.62 (95% CI: 1.07–2.44) and $OR_{(Q1)}$=2.67 (1.77–4.03), p-trend<0.0001. The lowest cancer risk was observed among never smokers in the highest methylation quartile (NS-Q4). In comparison to never smokers in the highest quartile, current smokers in the lowest methylation quartile (Q1: OR=25.51; 95% CI:9.61–67.76) presented the highest risk of bladder cancer (p-interaction=0.06). In analyses stratified by smoking, hypomethylation was a strong risk factor among never smokers (OR=6.39; 95% CI:2.37–17.22). When stratified by methylation quartile, the association between smoking and bladder cancer was attenuated among subjects in Q1 compared to the rest of the study population in Q2-4. Methylation levels among controls were not associated with subject characteristics, micronutrients, or selected genotypes in folate metabolism pathways.

Interpretation—For the first time, to our knowledge, we have shown that leukocyte DNA hypomethylation was strongly associated with increased bladder cancer risk in a large case-control study, independently of smoking and other risk factors examined. Global methylation levels in genomic DNA could provide a useful biomarker of susceptibility to certain cancer types and additional studies are warranted.

Keywords

bladder cancer; DNA methylation; folate metabolism; tobacco

Introduction

In tumor tissue, changes in DNA methylation patterns such as promoter CpG-island hypermethylation and global (genome-wide) hypomethylation are frequently observed. Global hypomethylation of DNA is thought to contribute to carcinogenesis by inducing genome instability and gene-specific hypomethylation (1,2). Genomic instability is a common occurrence in many cancer types and both chromosomal instability and to a lesser extent microsatellite instability has been observed in urothelial carcinomas of the bladder (3–8). Additional studies have also shown that in bladder cancer, chromosomal instability is wide-spread, occurs early in the carcinogenic process, and is associated with alterations on chromosome 9, the first and most frequently altered chromosome in bladder cancer development (9–12). One study also suggested that LOH of chromosome 9 was associated with genome-wide hypomethylation (12).

Measures of global cytosine methylation in blood cell and normal tissue DNA has been used recently as a phenotypic marker of genomic instability and potential cancer risk. One study of head and neck cancer patients found that global cytosine methylation levels were lower in blood DNA from cancer cases compared to controls (13). Smoking and the methylene tetrahydrofolate reductase (*MTHFR*) variant genotype were associated with lower methylation levels whereas exposure to human papilloma virus 16 was associated with higher global methylation levels (13). Another study of cancer cell lines and tumor tissues revealed that the majority of long interspersed nuclear element-1 (LINE-1) sequences were hypomethylated compared to that observed in lymphocytes and normal colon mucosa (14). Only one smaller investigation studied global methylation as a susceptibility factor for bladder cancer (15), a

tobacco associated tumor for which genomic instability increases with disease progression (16–18). The investigation did not investigate other co-variates that may also influence methylation levels in genomic DNA, such as diet and genes.

To determine whether DNA methylation levels were associated with bladder cancer risk, cytosine methylation (%5-mC) content in leukocyte DNA was measured among bladder cancer cases and controls enrolled in a large hospital-based study conducted in Spain. Information on variation in 34 single nucleotide polymorphisms (SNPs) in 9 folate metabolism pathway genes and on vitamins B_1,B_2, B_3, B_6, B_{12}, folate, total protein, and alcohol intake was also assessed to identify factors contributing to genomic methylation levels in controls and to identify potential confounding factors and risk modifiers for bladder cancer (19–21).

Materials and Methods

Study Population

The study population has been previously described (22,23). Briefly, the Spanish Bladder Cancer Study is a hospital-based case-control study conducted between 1998–2001 in 5 regions of Spain (Asturias, Barcelona, Vallés Occidental/Bages, Alicante, and Tenerife). Cases were male and female patients with newly diagnosed and histologically confirmed urothelial carcinoma of the bladder. Controls were selected among patients admitted to the same hospital for diseases/conditions considered unrelated to smoking and other known bladder cancer risk factors. They were individually matched to cases on age (within 5 years), sex, race, and hospital referral area. Overall, 1,219/1453 eligible cases (84%) and 1,271/1442 eligible controls (88%) were identified who agreed to participate in the study using a computer-based personal interview to collect information on demographic and risk factors. Ninety-four percent (1150/1219) of cases and 1149/1271 (90%) provided suitable genomic DNA for molecular analyses of genotypes and global methylation levels. Methylation analyses were conducted on 775/1150 (67%) of cases and 397/1149 (35%) of controls with available leukocyte DNA for analysis. Nutritional data was available for 588/775 (76%) of cases and 288/397 (73%) of control subjects. Tobacco exposure data was available for 742/775 (96%) of cases and 359/397 (90%) of controls that had global methylation levels quantified. Informed consent was obtained from all participants in accordance with the National Cancer Institute and local Institutional Review Boards.

Quantification of Global Methylation Levels in Leukocyte DNA

Leukocyte genomic DNA was extracted using standard methods. The percentage of methylated cytosine (5-mC) in genomic DNA was estimated using a combination of high performance capillary electrophoresis (HPCE) and Hpa II digestion of DNA to quantify the % of 5-mC and unmethylated cytosine in DNA samples. Global 5mC content was quantified by HPCE as previously described (24). Briefly, genomic DNA samples were boiled, treated with nuclease P1 (Sigma-Aldrich Química, Spain) for 16 h at 37°C, and with alkaline phosphatase (Sigma-Aldrich Química, Spain) for an additional 2 h at 37°C. After hydrolysis, total cytosine and 5-mC content was measured by capillary electrophoresis using a P/ACE MDQ system (Beckman-Coulter,, Spain). Relative 5mC content was expressed as a percentage (%5-mC) with respect to the total cytosine content (the sum of methylated and non-methylated cytosines).

The Hpa II restriction digest was performed as previously described (24). This enzyme selectively cleaves unmethylated sequences. In brief, 1 ug of DNA was digested with the restriction enzyme Hpa II (Fermentas, Inc., Maryland, USA) according to the manufacturer's instructions (2 to 3 U of enzyme/pg of DNA, digestion time, overnight). Subsequently, the degree of digestion was determined by densitometry analysis on 0.001% ethidium bromide stained gels. The percentage of 5-mC was obtained using standard curves constructed from a

panel of DNA samples that were previously quantitated using both densitometry and HPCE as described previously (25–27). The standard sample set included DNA methylated *in vitro*, DNA extracted from normal human lymphocytes, the human cancer cell line HCT116, cells lacking DNA methyltransferase-1 *(DNMT)*activity, from cells lacking *DNMT3b*activity, from cells lacking both *DNMT1*and *DNMT3b*methyltransferase activities and from cells treated with the demethylating drug 5-aza-2-deoxycytidine. Genomic methylation levels in study samples estimated from the standard curve are expressed as a %5-mC. Seventy-two samples were analyzed in duplicate and the coefficient of variation was 0.33%.

Dietary Assessment

Food intake prior to diagnosis for cases and before interview for controls was estimated using a semi-quantitative 127-item food frequency questionnaire (FFQ), previously validated in Spain (19,20). A subset of 917/1219 (75%) cases interviewed and 875/1271 (69%) controls completed the FFQ. Micronutrients and macronutrients that were included in these analyses were suggested to be important co-factors and methyl donors in one-carbon (folate) metabolism in the literature. These included B-vitamins, folate, protein, and alcohol (inversely related to folate levels). Macronutrients measured in food group categories were also assessed for associations with DNA methylation levels. The food groups analyzed included total vegetables, fruits, meats (red and white) and fish consumption. Nutrient density variables were calculated by dividing the total number of micrograms of food consumed daily, by the total daily energy intake in kilocalories (ug/kcal/day). Nutrient data were analyzed as continuous variables and also in quartiles and deciles based on the distribution among controls.

Genotyping

Genotype assays were conducted at the Core Genotyping Facility (CGF), of the National Cancer Institute. DNA for genotype assays was extracted from either leukocytes or mouthwash samples as described previously (20). Single nucleotide polymorphisms (SNPs) in exons (Ex) and intervening sequences (IVS) within nine genes including: cystathione beta-synthase (*CBS; Ex9+33C>T; Ex13+41C>T; IVS15-134G>A; Ex18–391 A>G*), cystathionase (*CTH; -340A>G, IVS3-66A>C, IVS7-799A>G, IVS7-583G>T, IVS10-430C>T, IVS10-303A>G*), 5,10 methylene tetrahydrofolate reductase (*MTHFR; Ex2-120C>T, Ex5+79C>T, IVS7-76T>G, Ex8-62A>G*), 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (*MTR; Ex26-20A>G, IVS26+157T>G, IVS26+43G>A*), 5-methyltetrahydrofolate-homocysteine methyltransferase reductase (*MTRR; Ex2-64A>G, Ex9-85C>T, Ex9+9G>A, Ex14-42G>A, Ex14+14C>T, Ex15-526 G>A, Ex15-526G>A, Ex15-405A>T*), serine hydroxymethyltransferase *1(SHMT1; Ex12+138C>T, Ex12+217G>T, Ex12+236T>C),* solute carrier family 19(folate transporter member *1-SLC19A1; Ex4-114G>A, Ex7-233G>T, Ex7-198C>T),* and thymidylate synthase *(TYMS; IVS7-68T>C, Ex8+157C>T, Ex8 +227A>G),* were selected because of their role in folate metabolism. The glutathione S-transferase mu deletion (*GSTM1*) was selected for analysis because of it's association with bladder cancer risk in several independent and meta-analyses studies (19,20). Haplotype frequencies for *MTHFR*, *CTH*, and *SHMT1*were also were estimated using SAS Genetics (SAS 9.1). Initially we selected SNPs in six genes with expected minor allele frequency (MAF) ≥0.05 in Caucasians, with TaqMan® based assays available at the CGF. SNP selection favored those leading to non-synonymous amino acid changes, those previously evaluated in relation to bladder cancer risk, or those with evidence of functional significance. Later, additional SNPs were included from a large scale evaluation of candidate genes using the Illumina® Golden Gate Assay (28). This panel included 1,433 SNPs in selected candidate genes with assays previously sequenced and genotyped in the SNP500 Cancer project (28). Methods for genotype assays can be found at http://snp500cancer.nci.nih.gov(29). All genotypes included in this study were in Hardy-Weinberg Equilibrium among the controls (p>0.05).

Statistical Analysis

Global methylation levels were analyzed in quartiles and deciles using cut-off points determined among controls and as log transformed continuous values in regression models. When used as a categorical variable, the association between %5- mC content and case-control status and other factors was assessed using χ^2statistics. Linear regression models and median tests of differences were applied to analyses using continuous values and odds ratios are presented as they approximate the relative risk when derived from case-control studies of rare diseases such as bladder cancer (30). To assess association of methylation levels and case-control status, unconditional logistic regression models were used, adjusted for age, sex, region (which were matching variables in the original study), and smoking. To control for smoking we created a variable "combined smoking information" with 9 levels that were used as dummies: 0 (non-smokers, used as referent category), 1–4 (former smokers, pack-years in Q1 through Q4), 5–8 (current smokers, pack-years in quartiles 1 through 4). This coding was used to allow for different effects of pack-years for former and current smokers. Data were analyzed using SAS statistical software (SAS 9.1). For the main analysis we interpret $p<0.05$ as statistically significant. For the analyses that were used to screen the large number of potential confounders (Supplemental Tables), we accounted for the number of tests in the interpretation of the results. This study conforms to STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines for case-control subjects.

Role of the Funding Source

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Results

Characteristics of cases and controls with and without methylation data were compared (Supplemental Table 1). Controls with methylation data were younger than those in the main study, and the proportion included from Asturias was greater, and the proportion from Valles/Bages and Tenerife less among those without methylation data. There were more current smokers in the controls with methylation data however separately, cases and controls did not differ significantly by pack-years of exposure to those in the main case-control study. In the case-control comparison, among those included in this analysis (those with methylation data, Table 1) only region and age remained significantly different, therefore these factors were adjusted for in all analyses. As observed for the study as a whole (23), a higher proportion of cases currently smoked and had a higher number of pack-years of smoking compared to controls ($p<0.0001$). When nutrient intake levels were compared in cases and controls in this study, vitamin $B_{12}$and total protein intake were slightly lower among cases than controls ($p=0.06$ and $p=0.07$, respectively) but folate intake levels were similar as observed among the cases and controls that were compared in the Spanish Bladder Cancer Study nutritional analysis (Table 1)(19,20).

Global methylation in leukocyte DNA in cases and controls

DNA was evaluated from a subset of 775 cases and 397 controls with leukocyte DNA for analysis. The distribution of %5-mC levels among cases and controls was skewed to the right. Overall, the median percent methylated cytosine (%5-mC) content was significantly lower in DNA from cases compared to controls (3.03% (Inter quartile range-(IQR)=2.17–3.56)) and (3.19% (IQR=2.46–3.68)) respectively ($p=0.0002$). The odds ratio (OR), a close approximation to the relative risk (30), for the top compared to the bottom 50% percentile was significantly

elevated (OR=1.38; 95% CI:1.05–1.08, p=0.02). After grouping subjects by methylation percentile, subjects in the lowest three quartiles (Q1-3) had significantly higher bladder cancer risk compared to subjects in the highest quartile (Q4) (p-trend=<0.0001). In analyses stratified by sex, risk was higher among women than men but the risk estimates for women were imprecise due to the small number of subjects (Table 2). No relationship was observed between global methylation levels among diagnosis groups of controls or with tumor stage and grade among cases (data not shown).

To identify potential confounding factors that might explain the association between methylation and bladder cancer risk, the association of demographic, genetic and nutritional risk factors with methylation levels was examined (Supplemental Table 2). Among controls, 16/45 (35.6%) of females were in the highest methylation quartile compared to 84/352 (23.9%) of males (p=0.12). Methylation levels were not associated with age across the age range of cases or controls in this study (whether considered in quartiles (Table 2) or continuously (data not shown)) or smoking status when considered as never, former, current (Supplemental Table 2) or as pack-years (data not shown). To evaluate modification of methylation levels by nutrients potentially associated with bladder cancer and folate metabolism, associations with selected nutrients and food groups were assessed. Nutrients included vitamin B_1, B_2, B_3, B_6, B_{12}, folate, total protein, and alcohol. In controls, methylation was weakly associated with protein intake (p=0.02) however after consideration of multiple comparisons we attributed this result to a chance finding. Food groups included total meat, vegetables, and fruits. None of the variables examined identified new associations that could account for the lower methylation levels observed among cases (Supplemental Table 2).

To evaluate the contribution of genetic variation in folate metabolism genes and DNA methylation, 34 SNPs in 9 genes (*CBS, CTH, GSTM1, MTHFR, MTR, MTRR, SHMT1, SLC19A1,* and *TYMS*) were examined among cases and controls independently. Co-dominant (additive) models were used. Dominant models were only employed when the minor allele frequency was less than 10% in the controls (Supplemental Table 3). None of the genotypes examined, whether considered independently or as haplotypes, explained the lower DNA methylation levels observed among bladder cancer cases compared to controls, including two functional SNPs in the *MTHFR* gene (A222V and E429A). We attribute the association of methylation quartiles with two SNPs in *CTH* and *SHMT1* genes among controls (p-values =0.05 and 0.01 respectively) to chance due to the large number of statistical tests. Haplotypes of SNPs genotyped in *CTH, SHMT1* and *MTHFR* were not associated with methylation levels among cases or controls (Supplemental Table 2)

Because smoking is considered an important risk factor for bladder cancer in Spain (23), we examined the association between bladder cancer risk and smoking status jointly, and after stratification by methylation quartiles (Table 3). Never smokers in the highest methylation quartile (Q4) had the lowest bladder cancer risk. When this group (never-smokers in Q4) was used as a common referent, bladder cancer risk was highest among current smokers in the lowest methylation quartile (OR=25.51; 95% CI: 9.61–67.76, p-interaction=0.06). The interaction was significant when the analysis was restricted to males (p-interaction=0.02). Risk associated with low methylation (Q1) was strongest for never smokers (OR=6.39; 95% CI: 2.37–17.22, p-trend =0.0007), than for former or current smokers (OR (approximately) =13.3/7.8=1.7 and OR (approximately)=25.5/10.3=2.5, respectively). In analyses stratified by methylation quartile using never smokers in each quartile as referent group, bladder cancer risk associated with former and current smoking was significantly less among subjects in Q1 compared to Quartiles 2–4 (Figure 1). Specifically, the risk associated with ever smoking (former and current combined) among subjects in Q2–4 was greater than two-times that observed in Q1 for the group as a whole ($OR_{(Q2–4)}$=5.78; 95% CI: 3.28-10.28 and $OR_{(Q1)}$=2.14; 95% CI:1.01–4.53, p-interaction=0.12, data not shown) and over four-times

greater when the analysis was restricted to males ($OR_{(Q2–4)}$=8.57; 95% CI:4.37–16.78 and $OR_{(Q1)}$=2.01; 95% CI:0.90–4.46, p-interaction=0.009) (data not shown). A similar relationship was observed when smoking status was analyzed in pack-years. Whether considered jointly or individually within strata, smoking and hypomethylation clearly appeared to independently contribute to bladder cancer risk.

Discussion

In this study, the median global methylation level measured as the %5-mC in leukocyte DNA, was significantly lower among bladder cancer cases than controls and was independently associated with cancer risk. Bladder cancer risk was also inversely associated with DNA methylation level in a dose-dependent manner and the relationship was not modified by nutritional or polymorphic variants in genes known to be involved in DNA methylation or folate metabolism that were investigated in this study. Although smoking was not associated with DNA methylation level, it did modify the relationship between DNA methylation and bladder cancer. After stratification by smoking status, risk was lowest among never smokers in the high methylation quartile and highest among current smokers in the lowest methylation group and the p-value for interaction was significant when the analysis was restricted to males. Hypomethylation was a stronger bladder cancer risk factor among never smokers, than former and current smokers when compared to well methylated individuals (Q4) in each strata. In other words, the comparatively lower bladder cancer risk provided by having “highly-methylated” DNA (i.e. Q4) was greater for never smokers, than former or current smokers. After stratification by methylation quartile, risk of bladder cancer associated with smoking was attenuated among subjects in the low methylation quartile (Q1) compared to the rest of the population (Q2–Q4). Again, this relationship was strengthened when the analysis was restricted to males.

Our findings are in agreement with a recent study of head and neck cancer that reported an inverse association between global DNA methylation levels and cancer risk, concluding that global methylation in blood DNA is an independent risk factor for this type of cancer (13). In contrast, this study reported that cigarette smoking and the T variant of the *MTHFR* A222V polymorphism modified methylation levels among controls (13). Our study is in agreement with a smaller study of global hypomethylation and a subset of similar genetic polymorphsims in 1-C metabolism genes which did not observe that *CBS* (Exon 8, 844INS68), *MTHFR* (V677A, C to T), and *MS* (D913G, 2756 A to G) polymorphisms increased susceptibility to bladder cancer or influenced the extent of global DNA hypomethylation (15). Our study is also in agreement with an investigation that observed LINE-1 hypomethylation in DNA from leukocytes was independent of age and gender (31).

It is currently unclear whether DNA methylation in leukocyte DNA is an independent risk factor, or a phenotypic marker of risk associated with other factors such as genetic instability, altered epigenetic regulation, or other characteristics not yet identified. Other phenotypic markers of cancer susceptibility that have been measured in blood cells include chromosomal aberrations (32–34) and telomere length (35–37). Lymphocyte measurements of both markers have been associated with increased cancer risk in prospective studies. Most cytogenetic studies of chromosomal aberrations did not observe that the association between chromosomal aberrations and cancer risk was modified by occupational exposure history or cigarette smoking, although bladder cancer risk was not presented independently in these reports. By contrast, three smaller studies of telomere length have provided evidence that bladder cancer risk was inversely associated with length, and that risks were highest among smokers with the shortest telomeres, although power was limited to detect interactions (35–37). Similar to the study by Wu et al. of several cancer types combined (35), we also observed that bladder cancer risk associated with smoking was attenuated among those in the lowest methylation quartile

(Q1), compared to the remaining subjects. Although hypomethylation overall was associated with higher bladder cancer risk, in the presence of tobacco carcinogens, cells with low %5-mC levels may be less genetically stable than cells with "well-methylated" DNA, and more likely to undergo apoptosis. A survival disadvantage of genetically altered cells may be beneficial to the host when exposed to DNA damaging agents. Additional studies are needed to understand the relationship between global methylation levels in blood DNA, genomic stability at the tissue level and modification by exposures. Bladder cancer provides an excellent model in which to study the relationship between global methylation in leukocyte DNA and in urothelial tissues in vivo because the majority of bladder tumors are histologically similar (TCCs), exfoliated urothelial cells can be non-invasively collected in urine, and bladder cancer risk factors are well-understood. One recent study examined DNA methylation levels in exfoliated urothelial cells using a specific monoclonal antibody for 5-methylcytosine, and detected significantly lower methylation levels in cancer patient cells compared to those from healthy controls or patients with benign disease (38). As stated earlier, genomic instability has been associated with alterations on chromosome 9, the first and most frequently altered chromosome in bladder cancer (3,16,18). Although we did not observe a relationship between bladder cancer stage and grade, genomic instability has been reported to increase with bladder cancer progression (3–8,11). However, it appears that chromosome 9 instability (measured as LOH, or loss) is an early carcinogenic event, and may be related specifically to DNA hypomethylation levels at pericentromeric satellite regions of this chromosome, potentially prior to cancer development (9,11,12).

One limitation of our investigation is that the Spanish Bladder Cancer Study was a hospital-based study, and did not include healthy control subjects and DNA from cases was collected prior to treatment yet post-diagnosis. To test that control diseases were not associated with methylation, we compared levels between controls with disease groups and differences were not observed. We also did not observe differences in methylation levels by tumor stage and grade among cases suggesting that disease progression would not have influenced these results. To extrapolate the importance of global methylation levels and bladder cancer susceptibility in the general population, studies using prospectively collected samples are desirable as well as studies sufficiently powered to study female subjects. Lastly, to apply global methylation as a biomarker in the clinic, it is likely that sensitive high-throughput methods would be applied. The development of new sensitive and quantitative techniques for the analysis of global DNA methylation content, such as pyrosequencing of the long interspersed nuclear element-1 (LINE-1) would be would be extremely useful in the systematic implementation and expansion of these studies in larger clinical settings (39).

In summary, an inverse association was observed between global DNA methylation levels and bladder cancer risk in to our knowledge, the largest study of global DNA methylation and bladder cancer risk conducted to date that included high-quality information on lifestyle factors, nutrition and variation in genes involved in folate metabolism. Both cigarette smoking and global hypomethylation were independently associated with bladder cancer risk. Never smokers with high global methylation had the lowest bladder cancer risks and current smokers in the lowest methylation quartile had the greatest cancer risk. Although smoking is considered the most important bladder cancer risk factor in Spain (23), hypomethylation was strongly associated with bladder cancer risk among never smokers and appeared to increase cancer risk among both current former and current smokers as well. Unexpectedly, risk associated with smoking was attenuated among subjects in the lowest methylation quartile, compared to the rest of the population however these results appear to be similar to those observed for other phenotypic markers of cancer risk such as chromosomal aberrations and telomere length. These findings add to the growing body of evidence that global methylation levels in genomic DNA from blood cells may provide an additional phenotypic marker for cancer risk and merit further investigation in studies of other cancer types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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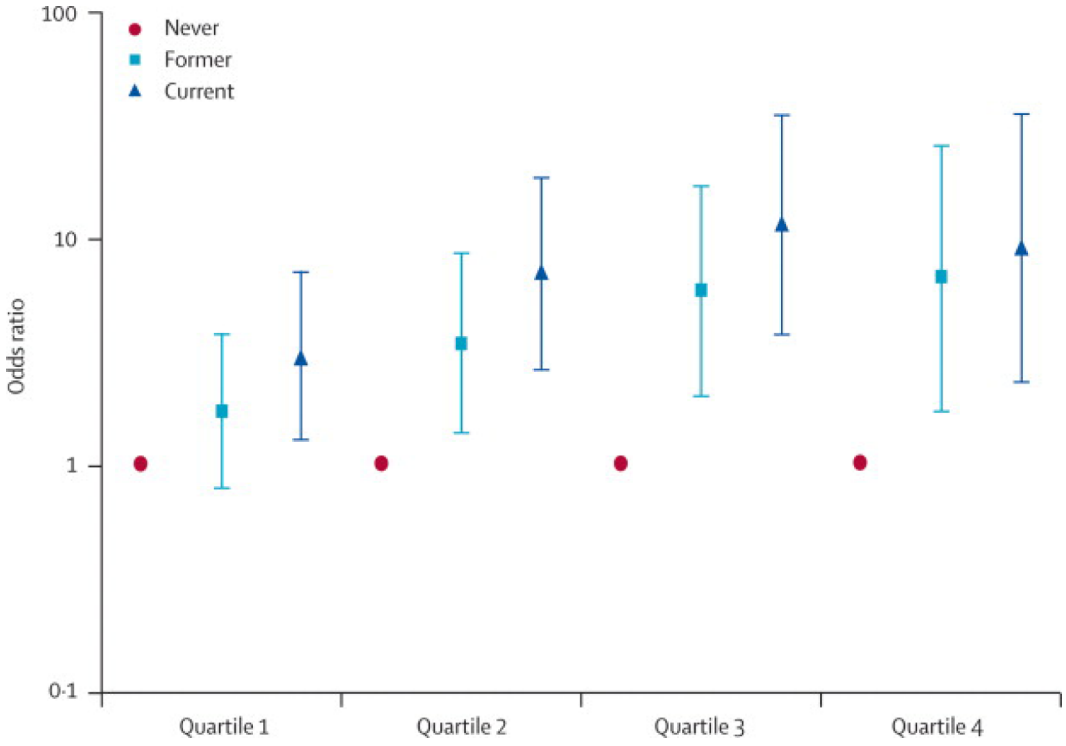

Figure 1.
Risk of bladder cancer for former and current smokers compared to never smokers by methylation quartile.

Table 1

Comparison of controls and cases with Methylation data

Variable	Cases (N=775)	Controls (N=397)	p-value
Age (IQR)	68 yrs (61–74)	65 yrs (56–71)	***<0.0001***
	N (%)	**N (%)**	
Sex			
Male	675 (87.1%)	352 (88.7%)	
Female	100 (12.9%)	45 (11.3%)	***0.44***
Region			
Barcelona	132 (17.0%)	81 (20.4%)	
Vallès/Bages	122 (15.7%)	50 (12.6%)	
Elche	49 (6.3%)	33 (8.3%)	
Tenerife	139 (17.9%)	45 (11.3%)	
Asturias	333 (43.0%)	188 (47.4%)	***0.01***
Education			
Less than primary	354 (46.3%)	168 (43.3%)	
Incomplete high school	304 (39.8%)	161 (41.5%)	
High school or higher	106 (13.9%)	59 (15.2%)	***0.60***
Cigarette Smoking			
Never	108 (14.6%)	89 (24.8%)	
Former	295 (39.8%)	142 (39.6%)	
Current	339 (45.7%)	128 (35.7%)	***<0.0001***
Pack Years[1]			
Q1	55 (8.9%)	64 (24.6%)	
Q2	132 (21.4%)	66 (25.4%)	
Q3	194 (31.4%)	65 (25.0%)	
Q4	237 (38.4%)	65 (25.0%)	***<0.0001***
Vitamin B1 (quartile)[2,3]			
Q1	178 (30.3%)	72 (25.0%)	
Q2	139 (23.6%)	72 (25.0%)	
Q3	111 (18.9%)	72 (25.0%)	
Q4	160 (27.2%)	72 (25.0%)	***0.12***
Vitamin B2 (quartile)[2,4]			
Q1	168 (28.6%)	72 (25.0%)	
Q2	141 (24.0%)	72 (25.0%)	
Q3	139 (23.6%)	72 (25.0%)	
Q4	140 (23.8%)	72 (25.0%)	***0.74***
Vitamin B3 (quartile)[2,5]			
Q1	149 (25.3%)	72 (25.0%)	
Q2	166 (28.2%)	72 (25.0%)	
Q3	142 (24.2%)	72 (25.00%)	
Q4	131 (22.3%)	72 (25.00%)	***0.70***
Vitamin B6 (quartile)[2,6]			
Q1	168 (28.6%)	72 (25.0%)	
Q2	135 (22.9%)	72 (25.0%)	
Q3	157 (26.7%)	72 (25.0%)	
Q4	128 (21.8%)	72 (25.0%)	***0.50***
Vitamin B12 (quartile)[2,7]			
Q1	194 (33.0%)	72 (25.0%)	
Q2	147 (25.0%)	72 (25.0%)	
Q3	133 (22.6%)	72 (25.0%)	
Q4	114 (19.4%)	72 (25.0%)	***0.06***
Folate (quartile)[2,8]			
Q1	166 (28.2%)	72 (25.0%)	
Q2	133 (22.6%)	72 (25.0%)	
Q3	144 (24.5%)	72 (25.0%)	
Q4	145 (24.7%)	72 (25.0%)	***0.74***
Protein (quartile)[2,9]			
Q1	144 (24.5%)	72 (25.0%)	
Q2	182 (31.0%)	72 (25.0%)	
Q3	156 (26.5%)	72 (25.0%)	
Q4	106 (18.0%)	72 (25.0%)	***0.07***
Alcohol (quartile)[2,10]			
Q1	163 (27.7%)	72 (25.0%)	
Q2	125 (21.3%)	72 (25.0%)	
Q3	156 (26.5%)	72 (25.0%)	
Q4	144 (24.5%)	72 (25.0%)	***0.59***

[1] Quartile cutoffs: all controls (18.5, 36.0, 57.0), all cases (30.0, 45.0, 62.0), controls w/ methylation data (18.0, 34.5, 52.9)

[2] Nutrient density; micrograms/kcal/day

[3] Quartile cutoffs: all controls (0.56, 0.66, 0.77), all cases (0.54, 0.64, 0.76), controls w/ methylation data (0.56, 0.65, 0.74)

[4] Quartile cutoffs: all controls (0.77, 0.91, 1.10), all cases (0.74, 0.89, 1.07), controls w/ methylation data (0.76, 0.91, 1.09)

[5] Quartile cutoffs: all controls (8.27, 10.02, 11.99), all cases (8.11, 9.55,11.34), controls w/ methylation data (8.10, 9.78, 11.58)

[6] Quartile cutoffs: all controls (0.85, 1.01, 1.19), all cases (0.81, 0.96, 1.13), controls w/ methylation data (0.83, 0.97, 1.14)

[7] Quartile cutoffs: all controls (2.77, 3.86, 5.71), all cases (2.61, 3.62, 5.32), controls w/ methylation data (2.87, 3.95, 5.85)

[8] Quartile cutoffs: all controls (138.73, 167.37, 209.41), all cases (129.70, 162.61, 201.62), controls w/ methylation data (135.68, 164.22, 202.05)

[9] Quartile cutoffs: all controls (40.31, 46.33, 53.81), all cases (39.10, 45.19, 51.36), controls w/ methylation data (38.57, 46.04, 53.26)

[10] Quartile cutoffs: all controls (0.33, 4.07, 11.10), all cases (0.26, 4.21, 12.82), controls w/ methylation data (0.57, 4.46, 12.97)

Table 1

Association of Global DNA Hypomethylation and case status in the Spanish Bladder Cancer Study

	Total Population					
	775 cases, 397 controls					
Methylation Levels		Crude		Adjusted		
(quartiles in controls)	Cases / Controls	OR	95% CI	OR[1]	95% CI	
Q4 (≥3.68)	122/100	1.00		1.00		
Q3(3.19-<3.68)	222/99	1.84	(1.29-2.62)	2.05	(1.37-3.06)	
Q2 (2.46–<3.19)	185/99	1.53	(1.07-2.20)	1.62	(1.07-2.44)	
Q1 (<2.46)	246 / 99	2.04	(1.43-2.90)	2.67	(1.77-4.03)	
		p=0.001		*p<0.0001*		
	Males					
	675 cases, 352 control					
Methylation Levels		Crude		Adjusted		
(quartiles in controls)	Cases / Controls	OR	95% CI	OR[2]	95% CI	
Q4 (≥3.68)	109/84	1.00		1.00		
Q3(3.19-<3.68)	192/92	1.61	(1.10-2.35)	1.79	(1.17-2.75)	
Q2 (2.46-<3.19)	156/85	1.41	(0.96-2.09)	1.48	(0.95-2.31)	
Q1 (<2.46)	218/91	1.84	(1.27-2.69)	2.50	(1.61-3.88)	
		p=0.007		*p=0.0004*		
	Females					
	100 cases, 45 controls					
Methylation Levels		Crude		Adjusted		
(quartiles in controls)	Cases / Controls	OR	95% CI	OR[2]	95% CI	
Q4 (≥3.68)	13/6	1.00		1.00		
Q3(3.19-<3.68)	30/7	5.27	(1.75-15.86)	5.14	(1.49-17.66)	
Q2 (2.46-<3.19)	29/14	2.55	(0.97-6.83)	3.52	(1.07-11.57)	
Q1 (<2.46)	28/8	4.31	(1.47-12.61)	4.23	(1.19-15.13)	
		p=0.03		*p=0.06*		*p-int=0.57*[3]

[1] Model adjusted for age, gender, region, smoking status (never/former/current), pack years, and smoking status-pack years interaction

[2] Model adjusted for age, region, smoking status (never//former/current), pack years, and smoking status-pack years interaction

[3] P-value for gender interaction calculated using quartile cutoffs among all controls

Table 3

Joint Effect of Hypomethylation and Smoking Status on Bladder Cancer Risk

	SMOKING STATUS (742 cases, 359 controls)								
	Never Smoker			Former Smoker			Current Smoker		
Methylation Levels (quartiles in controls)	**Cases / Controls**	**Adjusted OR[2]**	**95% CI**	**Cases/Controls**	**Adjusted OR[2]**	**95% CI**	**Cases/Controls**	**Adjusted OR[2]**	**95% CI**
Q4 (≥3.68)	11/25	1.00	reference	49/33	7.82	(2.93-20.88)	57/35	10.31	(3.89-27.30)
Q3(3.19-<3.68)	31/21	3.51	(1.33-9.25)	81/37	10.52	(4.05-27.36)	101/33	22.00	(8.35-60.00)
Q2 (2.46-<3.19)	28/25	2.79	(.07-7.30)	69/36	9.74	(3.74-25.38)	81/30	17.75	(6.71-46.98)
Q1 (<2.46)	38/18	6.39	(2.37-17.22)	96/36	13.31	(5.13-34.55)	100/30	25.51	(9.61-67.76)
									p-int=0.06 [2]

[1] Model adjusted for age, gender, and region

[2] Interaction p-value calculated from stratified model of smoking status (ever/never)