

Genomic DNA hypomethylation as a biomarker for bladder cancer susceptibility in the Spanish Bladder Cancer Study: a case–control study



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Summary

Background DNA hypomethylation has been suggested to cause genomic instability and increase cancer risk. We aimed to test the hypothesis that DNA hypomethylation is associated with increased risk of bladder cancer.

Methods We measured cytosine methylation (5-mC) content in genomic DNA from blood cells from patients with bladder cancer enrolled in a large case–control study in Spain between Jan 1, 1998, and Dec 31, 2001. Cases were men and women with newly diagnosed and histologically confirmed urothelial carcinoma of the bladder. Controls were selected from patients admitted to the same hospital for diseases or conditions unrelated to smoking or other known risk factors for bladder cancer. Controls were individually matched to cases on age (within 5 years), sex, race, and area of hospital referral. 5-mC content was measured in leucocyte DNA by use of a combination of high-performance capillary electrophoresis, *Hpa* II digestion, and densitometry. Data on demographics, 34 polymorphisms in nine folate metabolism genes, and nutritional intake of six B vitamins (including folate), alcohol, and smoking were assessed as potential confounders. Relative 5-mC content was expressed as a percentage (%5-mC) with respect to the total cytosine content (the sum of methylated and non-methylated cytosines). The primary endpoint was median %5-mC DNA content.

Findings %5-mC was measured in leucocyte DNA from 775 cases and 397 controls. Median %5-mC DNA was significantly lower in cases (3·03% [IQR 2·17–3·56]) than in controls (3·19% [2·46–3·68], $p=0\cdot0002$). All participants were subsequently categorised into quartiles by %5-mC content in controls. When the highest quartile of %5-mC content was used as the reference category (Q4), the following adjusted odds ratios (OR) and 95% CI were recorded for decreasing methylation quartiles: OR_(Q3) 2·05 (95% CI 1·37–3·06); OR_(Q2) 1·62 (1·07–2·44); and OR_(Q1) 2·67 (1·77–4·03), p for trend $<0\cdot0001$. The lowest cancer risk was noted in never smokers in the highest methylation quartile (never smokers in Q4). By comparison with never smokers in the highest quartile, current smokers in the lowest methylation quartile had the highest risk of bladder cancer (Q1: OR 25·51 [9·61–67·76], p for interaction 0·06). In analyses stratified by smoking, hypomethylation was a strong risk factor in never smokers (OR 6·39 [2·37–17·22]). Amount of methylation in controls were not associated with baseline characteristics, micronutrients, or selected genotypes in folate metabolism pathways.

Interpretation For the first time, to our knowledge, we have shown in a large case–control study that leucocyte DNA hypomethylation is associated with increased risk of developing bladder cancer, and this association is independent of smoking and the other assessed risk factors. Amount of global methylation in genomic DNA could provide a useful biomarker of susceptibility to certain cancer types and further research is warranted.

Funding Intramural Research Program of the National Institutes of Health, National Cancer Institute, Division of Cancer Epidemiology and Genetics, Bethesda, MD, USA, and Fondo de Investigacion Sanitaria, Spain (G03/174).

Introduction

In tumour tissue, changes in patterns of DNA methylation, such as promoter CpG-island hypermethylation and global (genome-wide) hypomethylation, frequently occur. Global hypomethylation of DNA is thought to contribute to carcinogenesis by the induction of genome instability and gene-specific hypomethylation.^{1,2} Genomic instability is a common occurrence in many cancers, and chromosomal instability and, to a lesser extent, microsatellite instability, has been noted in urothelial carcinomas of the bladder.^{3–8} Studies have also shown that in bladder cancer,

chromosomal instability is widespread, occurs early in the carcinogenic process, and is associated with alterations on chromosome 9, the first and most frequently altered chromosome in the development of bladder cancer.^{9–12} One study suggested that loss of heterozygosity (LOH) of chromosome 9 was associated with genome-wide hypomethylation.¹²

Measures of global cytosine methylation in DNA of blood cells and of healthy tissue have been used as a phenotypic marker of genomic instability and potential cancer risk. One study¹³ of patients with head and neck

Published Online
March 12, 2008
DOI:10.1016/S1470-2045(08)70038-X

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cancer noted that amounts of global cytosine methylation were lower in the DNA in blood from patients with cancer compared with controls. Smoking and the methylene tetrahydrofolate reductase (*MTHFR*) variant genotype were associated with less methylation compared with controls, whereas exposure to human papilloma virus 16 was associated with more global methylation compared with controls.¹³ Another study¹⁴ of cancer-cell lines and tumour tissues showed that most long interspersed nuclear element-1 (LINE-1) sequences were hypomethylated compared with those in lymphocytes and healthy colon mucosa. Only one smaller study¹⁵ assessed global methylation as a susceptibility factor for bladder cancer, a tobacco-associated tumour for which genomic instability increases with disease progression.^{16–18} That study did not assess other covariates that might also affect the amount of methylation in genomic DNA, such as diet and genetic variation.

To ascertain whether the amount of DNA methylation was associated with risk of bladder cancer, we aimed to measure cytosine methylation (5-mC) content in leucocyte DNA in patients with bladder cancer (cases) and controls who were enrolled in a large hospital-based study (the Spanish Bladder Cancer Study). Information on variation in 34 single nucleotide polymorphisms (SNPs) in nine folate-metabolism-pathway genes, on vitamins B₁, B₂, B₃, B₆, B₁₂, and folate, total protein, and alcohol intake was assessed to identify factors that contribute to the amount of genomic methylation in controls and to identify potential confounding factors and risk modifiers for bladder cancer.^{19–21}

Methods

Patients

The study population has been previously described.^{22,23} This study was a hospital-based case-control study undertaken between Jan 1, 1998, and Dec 31, 2001, in five regions of Spain (Asturias, Barcelona, Vallés Occidental-Bages, Alicante, and Tenerife). Cases were men and women with newly diagnosed and histologically confirmed urothelial carcinoma of the bladder. Controls were selected from patients admitted to the same hospital for diseases or conditions unrelated to smoking and other known risk factors for bladder cancer. Controls were individually matched to cases on age (within 5 years), sex, race, and area of hospital referral. Eligible cases and controls who agreed to participate in the study provided demographic data and information on risk factors by use of a computer-based personal interview, and provided information on food intake frequency by use of a food-frequency questionnaire. The identified cases and controls provided information on nutrition and tobacco exposure. Blood was collected in Spain, and DNA was extracted at the National Cancer Institute (NCI), Bethesda MD USA. Scientists at the NCI collected suitable genomic DNA

from leucocytes by use of the Puregene DNA Isolation Kit (Gentra systems, Minneapolis, MN, USA) for molecular analyses of genotypes and amount of global methylation in cases and controls. Written informed consent was obtained from all participants in accordance with the NCI and local Institutional Review Boards (IRB). Ethics approval was not needed because this work was already approved under the original IRB approval for the case-control study.

Quantification of global methylation

Leucocyte genomic DNA was extracted by use of standard methods. Percentages of 5-mC and unmethylated cytosine in genomic DNA were estimated by use of a combination of high-performance capillary electrophoresis (HPCE) and *Hpa* II digestion of DNA. Global 5-mC content was quantified by HPCE as previously described.²⁴ Genomic-DNA samples were boiled, treated with nuclease P1 (Sigma-Aldrich Química, Madrid, Spain) for 16 h at 37 °C and with alkaline phosphatase (Sigma-Aldrich Química, Madrid, Spain) for an additional 2 h at 37 °C. After hydrolysis, total cytosine and 5-mC content was measured by capillary electrophoresis by use of a programmable automated capillary electrophoresis (P/ACE) MDQ system (Beckman-Coulter, Madrid, Spain). Relative 5-mC 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 done as previously described.²⁴ This enzyme selectively cleaves unmethylated sequences. 1 µg of DNA was digested with the restriction enzyme *Hpa* II (Fermentas Inc, Glen Burnie, MD, USA) according to the manufacturer's instructions (2–3 U of enzyme/pg of DNA, digestion time: overnight). Subsequently, the extent of digestion was measured by densitometric analysis on 0.001% ethidium-bromide-stained gels. %5-mC was obtained by use of standard curves constructed from a panel of DNA samples that were previously quantified by densitometry and HPCE as described previously.^{25–27} The standard sample set included DNA methylated in vitro, DNA extracted from healthy human lymphocytes, the human cancer-cell line HCT116, cells without DNA methyltransferase-1 (DNMT1) activity, cells with no DNMT3b activity, cells with neither DNMT1 nor DNMT3b methyltransferase activities, and cells treated with the demethylating drug 5-aza-2-deoxycytidine. Amounts of genomic methylation in study samples estimated from the standard curve were expressed as %5-mC. 72 samples were analysed (MF and ME) in duplicate and the coefficient of variation was 0.33%.

Nutritional assessment

Food intake before diagnosis in cases and before interview in controls was estimated by use of a semi-quantitative 127-item food-frequency questionnaire (FFQ) as part of

	Cases (n=775)	Controls (n=397)	p
Median age, years (IQR)	68 (61–74)	65 (56–71)	<0.0001
Sex			
Men	675 (87)	352 (89)	0.44
Women	100 (13)	45 (11)	..
Region			
Barcelona	132 (17)	81 (20)	0.01
Valles-Bages	122 (16)	50 (13)	..
Eiche	49 (6)	33 (8)	..
Tenerife	139 (18)	45 (11)	..
Asturias	333 (43)	188 (47)	..
Education			
Less than primary school	354 (46)	168 (43)	0.60
Incomplete high school	304 (40)	161 (42)	..
High school or higher	106 (14)	59 (15)	..
Missing data	n=11	n=9	..
Smoking status			
Never	108 (15)	89 (25)	<0.0001
Former	295 (40)	142 (40)	..
Current	339 (46)	128 (36)	..
Missing data	n=33	n=38	..
Pack-years*			
Q1	55 (9)	64 (25)	<0.0001
Q2	132 (21)	66 (25)	..
Q3	194 (31)	65 (25)	..
Q4	237 (38)	65 (25)	..
Missing data	n=157	n=137	..
Vitamin B ₆ (quartile), µg/kcal per day†			
Q1	178 (30)	72 (25)	0.12
Q2	139 (24)	72 (25)	..
Q3	111 (19)	72 (25)	..
Q4	160 (27)	72 (25)	..
Missing data	n=187	n=109	..
Vitamin B ₁₂ (quartile), µg/kcal per day‡			
Q1	168 (29)	72 (25)	0.74
Q2	141 (24)	72 (25)	..
Q3	139 (24)	72 (25)	..
Q4	140 (24)	72 (25)	..
Missing data	n=187	n=109	..
Vitamin B ₉ (quartile), µg/kcal per day§			
Q1	149 (25)	72 (25)	0.70
Q2	166 (28)	72 (25)	..
Q3	142 (24)	72 (25)	..
Q4	131 (22)	72 (25)	..
Missing data	n=187	n=109	..

(Continues in next column)

	Cases (n=775)	Controls (n=397)	p
(Continued from previous column)			
Vitamin B ₆ (quartile), µg/kcal per day¶			
Q1	168 (29)	72 (25)	0.50
Q2	135 (23)	72 (25)	..
Q3	157 (27)	72 (25)	..
Q4	128 (22)	72 (25)	..
Missing data	n=187	n=109	..
Vitamin B ₁₂ (quartile), µg/kcal per day			
Q1	194 (33)	72 (25)	0.06
Q2	147 (25)	72 (25)	..
Q3	133 (23)	72 (25)	..
Q4	114 (19)	72 (25)	..
Missing data	n=187	n=109	..
Folate (quartile), µg/kcal per day**			
Q1	166 (28)	72 (25)	0.74
Q2	133 (23)	72 (25)	..
Q3	144 (25)	72 (25)	..
Q4	145 (25)	72 (25)	..
Missing data	n=187	n=109	..
Protein (quartile), µg/kcal per day††			
Q1	144 (25)	72 (25)	0.07
Q2	182 (31)	72 (25)	..
Q3	156 (27)	72 (25)	..
Q4	106 (18)	72 (25)	..
Missing data	n=187	n=109	..
Alcohol (quartile), µg/kcal per day‡‡			
Q1	163 (28)	72 (25)	0.59
Q2	125 (21)	72 (25)	..
Q3	156 (27)	72 (25)	..
Q4	144 (25)	72 (25)	..
Missing data	n=187	n=109	..

Data are n (%) except for age. Percentages are of actual total for each category and might not add to 100% due to rounding. Quartile cut-offs based on controls (n=397) with methylation data. *Quartile cut-offs: 18.0, 34.5, 52.9. †Quartile cut-offs: 0.56, 0.65, 0.74. ‡Quartile cut-offs: 0.76, 0.91, 1.09. §Quartile cut-offs: 8.10, 9.78, 11.58. ¶Quartile cut-offs: 0.83, 0.97, 1.14. ||Quartile cut-offs: 2.87, 3.95, 5.85. **Quartile cut-offs: 135.68, 164.22, 53.26. ††Quartile cut-offs: 38.57, 46.04, 53.26. ‡‡Quartile cut-offs: 0.57, 4.46, 12.97.

Table 1: Comparison of cases and controls with methylation data

the main interview; this questionnaire had been previously validated in Spain.^{19,20} Micronutrients and macronutrients that were included in these previous analyses were suggested to be important co-factors and methyl donors in one-carbon (folate) metabolism—eg, B-vitamins including folate, protein, and alcohol (inversely related to folate concentrations).^{19–21} Macronutrients

measured in food groups were also assessed for associations with amount of DNA methylation. The food groups analysed included total vegetables, fruits, meats (red and white), and fish. Nutrient density variables were calculated by dividing the total number of micrograms of food consumed daily by the total daily energy intake in kilocalories (µg/kcal per day). Nutrient data were analysed as continuous variables and in quartiles and deciles based on the distribution in controls.

Genotyping

Genotype assays were undertaken at the Core Genotyping Facility (CGF), of the NCI, Bethesda, MD, USA. DNA for

genotype assays was extracted from leucocytes or mouthwash samples as described previously.²⁰ Single nucleotide polymorphisms (SNPs) in exons (Ex) and intervening sequences (IVS) within nine genes were selected because of their role in folate metabolism and included: cystathione beta-synthase (*CBS*; *Ex8+33C>T*; *Ex12+41C>T*; *IVS14-134G>A*; *Ex17G>A*), 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*, *Ex15-526G>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*; *IVS6-68C>T*; *rs1059394*). Glutathione S-transferase mu deletion (*GSTM1*) was selected for analysis because of its association with risk of bladder cancer in meta-analyses.^{19,20} Haplotype frequencies for *MTHFR*, *CTH*, and *SHMT1* were estimated by use of SAS Genetics, version 9.1. Initially, we used TaqMan-based assays available at the CGF to select SNPs in six genes that had expected minor allele frequency (MAF) of at least 0.05 in

whites. This method of selection favoured SNPs that led to non-synonymous amino acid changes, those previously assessed in relation to risk of bladder cancer, or those with evidence of functional significance. Later, we used the Illumina Golden Gate Assay²⁸ to include additional SNPs from a large-scale assessment of candidate genes. This panel included 1433 SNPs in selected candidate genes with assays previously sequenced and genotyped in the SNP500 Cancer project.²⁸ For the methods used for genotype assays see [www.http://snp500cancer.nci.nih.gov](http://snp500cancer.nci.nih.gov).²⁹ All genotypes included in this study were in Hardy-Weinberg Equilibrium in the controls ($p > 0.05$).

Statistical analysis

Amounts of global methylation were analysed in quartiles (Q) and deciles (data not shown) by use of cut-off points established in 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 by use of χ^2 statistics. Linear regression models and median tests of differences were applied to analyses by use of continuous values, and odds ratios (OR) are presented because they approximate the relative risk (RR) when derived from case-control studies of rare diseases such as bladder cancer.³⁰ To assess the association of the amount of methylation and case-control status, unconditional logistic regression models were used and these were adjusted for age, sex, demographic region (which were matching variables in the original study), and smoking status. To control for smoking status, we used a variable "combined-smoking information" with nine categories used as dummies: 0 (non-smokers, used as referent category), 1-4 (former smokers, pack-years in Q1 to Q4), and 5-8 (current smokers, pack-years in Q1 to Q4). This coding was used to allow for different effects of pack-years for former and current smokers. Data were analysed by use of SAS statistical software, version 9.1. For the main analysis, we interpreted p less than 0.05 as statistically significant. For the analyses that were used to screen the large number of potential confounders, we accounted for the number of tests in the interpretation of the findings. This study conforms to STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines for case-control studies.

Role of the funding source

This study was funded by the Intramural Research Program of the National Institutes of Health, NCI, Division of Cancer Epidemiology and Genetics (Bethesda, MD, USA). The study sponsors had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

	Cases, n	Controls, n	Crude odds ratio (95% CI)	Adjusted odds ratio (95% CI)
All patients (775 cases, 397 controls)*				
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	< 0.0001
Men (675 cases, 352 controls)†				
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	0.0004
Women (100 cases, 45 controls)‡				
Q4 (≥ 3.68)	13	16	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	0.06; p interaction=0.57‡

*Model adjusted for age, sex, region, smoking status (never/former/current), pack-years, and smoking status-pack-years interaction. †Model adjusted for age, region, smoking status (never/former/current), pack-years, and smoking status-pack-years interaction. ‡p value for sex interaction calculated by use of quartile cut-offs in all controls.

Table 2: Association of global DNA hypomethylation status and risk of bladder cancer in cases and controls

Results

1219 of 1453 (84%) eligible cases and 1271 of 1442 (88%) eligible controls agreed to participate in the study. 1150 of 1219 (94%) cases and 1149 of 1271 (90%) controls provided suitable genomic DNA for molecular analyses of genotypes and amount of global methylation. Methylation analyses were done on DNA from 775 of 1150 (67%) cases and 397 of 1149 (35%) controls who had available leucocyte DNA for analysis. Nutritional data were available for 588 of 775 (76%) cases and 288 of 397 (73%) controls. Data on tobacco exposure was available for 742 of 775 (96%) cases and 359 of 397 (90%) controls who had quantified amounts of global methylation. A subset of 917 of 1219 (75%) and 875 of 1271 (69%) controls that were interviewed completed the FFQ.

Characteristics of cases and controls with and without methylation data were compared (webtable 1). Median age of controls with methylation data was younger than the median age of controls without methylation data. Some differences by demographic region and current smoking status were noted between the controls with and without methylation data. In a comparison of participants included in this analysis (ie, cases and controls with methylation data; table 1), only demographic region and age remained significantly different between the two groups; therefore, these factors were adjusted for in all analyses. As noted for the study as a whole,²³ more cases were current smokers and had a higher number of pack-years than were controls ($p < 0.0001$ for both). When nutrient intake was compared in cases and controls in this study, vitamin B₁₂ ($p = 0.06$) and total protein intake ($p = 0.07$) were slightly lower in cases than controls, but folate intake was comparable between cases and controls, similar to that of a nutritional analysis in our previous study (table 1).^{19,20}

Distribution of 5-mC in cases and controls was skewed to the right. Overall, median %5-mC was significantly lower in DNA from cases (3.03% [IQR 2.17–3.56]) than in controls (3.19% [2.46–3.68], $p = 0.0002$). OR, a close approximation to RR,³⁰ for the top 50% percentile compared with the bottom 50% percentile was significantly higher (OR 1.38 [95% CI 1.05–1.08], $p = 0.02$). After grouping participants by methylation percentile, those in the lowest three quartiles (Q1–Q3) had significantly higher risk of bladder cancer than participants in the highest quartile (Q4; p for trend ≤ 0.0001). In analyses stratified by sex, risk was higher in women than men, but the risk estimates for women were imprecise due to the small number of participants (table 2). No association was noted between amounts of global methylation in groups of controls with different diagnoses, or in cases with different tumour stages or grades (data not shown).

To identify potential confounding factors that might explain the association between methylation and increased risk of bladder cancer, association of demographic, genetic, and nutritional risk factors with amounts of global methylation was assessed (webtables 2 and 3). In controls,

	Cases, n	Controls, n	Adjusted odds ratio (95% CI)
Never smokers	n=108	n=89	
Q4 (≥ 3.68)	11	25	1.00
Q3 (3.19–<3.68)	31	21	3.51 (1.33–9.25)
Q2 (2.46–<3.19)	28	25	2.79 (1.07–7.30)
Q1 (<2.46)	38	18	6.39 (2.37–17.22)
Former smokers	n=295	n=142	
Q4 (≥ 3.68)	49	33	7.82 (2.93–20.88)
Q3 (3.19–<3.68)	81	37	10.52 (4.05–27.36)
Q2 (2.46–<3.19)	69	36	9.74 (3.74–25.38)
Q1 (<2.46)	96	36	13.31 (5.13–34.55)
Current smokers	n=339	n=128	
Q4 (≥ 3.68)	57	35	10.31 (3.89–27.30)
Q3 (3.19–<3.68)	101	33	22.00 (8.35–60.00)
Q2 (2.46–<3.19)	81	30	17.75 (6.71–46.98)
Q1 (<2.46)	100	30	25.51 (9.61–67.76)

Models were adjusted for age, sex, and region. p for interaction=0.06 and was calculated from stratified model of smoking status (never or ever).

Table 3: Joint effect of hypomethylation and smoking status on risk of bladder cancer

See Online for webtables 1–5

16 of 45 (36%) women were in the highest methylation quartile compared with 84 of 352 (24%) males ($p = 0.12$). Amount of global methylation was not associated with age across the age range of cases or controls in this study (whether considered in quartiles [webtables 2 and 3] or continuously [data not shown]), or smoking status when considered as never, former, current (webtables 2 and 3) or as pack-years (data not shown). To assess modification of global methylation by nutrients that were potentially associated with bladder cancer and folate metabolism, associations with selected nutrients and food groups were assessed. Nutrients included vitamin B₁, B₂, B₃, B₆, B₁₂, folate, total protein, and alcohol. In controls, methylation was weakly associated with protein intake ($p = 0.02$; webtable 2); however, after consideration of multiple comparisons, we attributed this finding to chance. Food groups included total meat, vegetables, and fruits. None of the variables assessed identified new associations that could account for the lower amounts of global methylation recorded in the cases (webtable 3).

To assess the contribution of genetic variation in folate metabolism genes and DNA methylation, 34 SNPs in nine genes (*CBS*, *CTH*, *GSTM1*, *MTHFR*, *MTR*, *MTRR*, *SHMT1*, *SLC19A1*, and *TYMS*) were studied in cases and controls independently. Co-dominant (additive) models were used. Dominant models were only used when the minor allele frequency was less than 10% in the controls (webtables 4 and 5). None of the genotypes assessed, whether considered independently or as haplotypes, explained the lower amounts of DNA methylation noted in cases than in controls, including two functional SNPs in the *MTHFR* gene (A222V and E429A). We attribute the association of methylation quartiles with two SNPs in

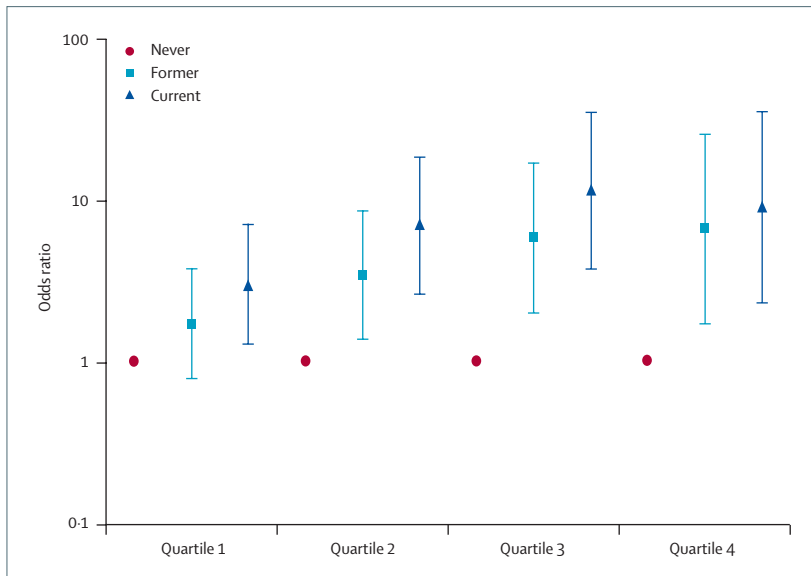


Figure 3: Risk of bladder cancer in former and current smokers compared with never smokers by methylation quartile

CTH ($p=0.05$) and *SHMT1* ($p=0.01$) genes in controls to chance due to the large number of statistical tests. Haplotypes of SNPs genotyped in *CTH* (data not shown), *SHMT1*, and *MTHFR* were not associated with amount of global methylation in cases or controls (webtables 2 and 3).

Since smoking is considered an important risk factor for bladder cancer in Spain,²³ we studied the association between risk of bladder cancer and smoking status jointly, and after stratification by methylation quartiles (table 3). Never smokers in the highest methylation quartile (Q4) had lowest risk of bladder cancer. When this group of never smokers was used as a common referent, risk of bladder cancer was highest in current smokers in the lowest methylation quartile (OR 25.51 [95% CI 9.61–67.76], p for interaction 0.06). The interaction was significant when the analysis was restricted to men (p for interaction 0.02). Risk associated with low methylation (Q1) was strongest for never smokers (OR 6.39 [2.37–17.22], p for trend 0.0007) than for former (approximate OR 13.3/7.8=1.7) or current smokers (approximate OR 25.5/10.3=2.5). In analyses stratified by methylation quartile with never smokers in each quartile as the reference group, risk of bladder cancer associated with former and current smoking was significantly less in participants in Q1 compared with those in Q2–Q4 (figure). Specifically, the risk associated with ever smoking (former and current combined) in participants in Q2–Q4 was greater than two-times that noted in Q1 for the group as a whole (OR_(Q2-Q4) 5.78 [95% CI 3.28–10.28] and OR_(Q1) 2.14 [1.01–4.53], p for interaction 0.12, data not shown) and over four-times greater when the analysis was restricted to men (OR_(Q2-Q4)=8.57 [4.37–16.78] and OR_(Q1) 2.01 [0.90–4.46], p for interaction 0.009; data not shown). A similar association was noted when smoking status was

analysed in pack-years. Whether considered jointly or individually within strata, smoking and hypomethylation seemed to independently contribute to risk of developing bladder cancer (data not shown).

Discussion

In this study, median amounts of global methylation, measured as %5-mC in leucocyte DNA, was significantly lower in cases than controls and lower amounts of DNA methylation (hypomethylation) were independently associated with increased cancer risk. This association was also dose-dependent and not modified by nutritional or polymorphic variants in genes known to be involved in DNA methylation or folate metabolism that were assessed in this study. Although smoking was not associated with amount of DNA methylation, it did modify the association between DNA methylation and risk of developing bladder cancer. After stratification by smoking status, risk was lowest in never smokers in the high methylation quartile and highest in current smokers in the lowest methylation group; the p value for interaction was significant when the analysis was restricted to men. Hypomethylation was a stronger risk factor for the development of bladder cancer in never smokers than in former and current smokers when compared with participants who had higher amounts of methylation (Q4) in each strata. In other words, the comparatively lower risk of developing bladder cancer provided by having highly-methylated DNA (ie, those in Q4) was greater for never smokers than for former or current smokers. After stratification by methylation quartile, the risk of developing bladder cancer associated with smoking was attenuated in participants in the low methylation quartile (Q1) compared with the rest of the study population (Q2–Q4). Again, this association was strengthened when the analysis was restricted to men.

Our findings are consistent with those from a study¹³ of patients with head and neck cancer that noted an inverse association between amounts of global DNA methylation and cancer risk, concluding that global methylation in DNA from blood is an independent risk factor for this type of cancer. By contrast, that study suggested that cigarette smoking and the T variant of the *MTHFR* A222V polymorphism modified amounts of global methylation in controls.¹³ Our findings are also consistent with those of a smaller study¹⁵ of global hypomethylation and a subset of similar genetic polymorphisms in 1-C metabolism genes that did not show that *CBS* (Exon 8, 844INS68), *MTHFR* (V677A, C to T), and *MS* (same as *MTR*; Ex26 -20A>G; rs 1805087 in the current study; D913G, 2756 A to G) polymorphisms increased susceptibility to bladder cancer or affected extent of global DNA hypomethylation. Findings of a study³¹ that noted LINE-1 hypomethylation in DNA from leucocytes was independent of age and sex are in agreement with our findings.

There is uncertainty about whether DNA methylation in leucocyte 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 note that the association between chromosomal aberrations and cancer risk was modified by occupational exposure history or smoking status, although risk of developing bladder cancer was not presented independently in these reports. By contrast, three smaller studies of telomere length have shown that risk of developing bladder cancer was inversely associated with length and that risks were highest in smokers with the shortest telomeres, although the power of these studies to detect interactions was low.^{35–37} Similar to the study by Wu and colleagues³⁵ of several cancer types combined, we also recorded that risk of developing bladder cancer associated with smoking was attenuated in those in the lowest methylation quartile (Q1) compared with the other participants. Although hypomethylation overall was associated with higher risk of developing bladder cancer, in the presence of tobacco carcinogens, cells with low %5-mC might be less genetically stable than cells with well-methylated DNA, and consequently more likely to undergo apoptosis. A survival disadvantage of genetically altered cells might be beneficial to the host when exposed to DNA-damaging agents. Additional studies are needed to understand the association between amounts of global methylation in DNA in blood cells, genomic stability in tissues, and modification by exposures. Bladder cancer provides an excellent model in which to study the association between global methylation in leucocyte DNA and in urothelial tissues in vivo because most bladder tumours are histologically similar (ie, are transitional-cell carcinomas), exfoliated urothelial cells can be non-invasively collected in urine, and risk factors for bladder cancer are well-understood. One study assessed amounts of DNA methylation in exfoliated urothelial cells by use of a specific monoclonal antibody for 5-methylcytosine and detected significantly lower amounts of methylation in cells from patients with cancer compared with 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 note an association between stage and grade of bladder cancer, genomic instability has been reported to increase with bladder-cancer progression.^{3–8,11} However, chromosome 9 instability (measured as LOH, or chromosomal loss) might be an early carcinogenic event and might be specifically associated with amounts of DNA hypomethylation at pericentromeric satellite regions of this chromosome, potentially before cancer development.^{9,11,12}

A limitation of our study is that the Spanish Bladder Cancer Study was hospital-based, did not include healthy controls, and DNA from cases was collected before treatment yet post-diagnosis. To test that specific diseases in controls were not associated with amount of methylation, we compared amounts of methylation between controls according to disease groups and did not note any differences (data not shown). We also did not note differences in amounts of methylation by the tumour stage and grade of cases, which suggested that disease progression would not have affected our findings. To extrapolate the importance of amounts of global methylation and bladder cancer susceptibility in the general population, studies that use prospectively collected samples and are sufficiently powered to study women as a separate group are needed. To apply global methylation as a biomarker in the clinic, sensitive high-throughput methods would probably be applied. The development of new sensitive and quantitative techniques for the analysis of global DNA methylation content, such as pyrosequencing of LINE-1 would be very useful in the systematic implementation and expansion of these studies in larger clinical settings.³⁹

In summary, our findings add to growing evidence that the amount of global methylation in genomic DNA from blood cells might provide an additional phenotypic marker for cancer risk and merit further study in other cancer types.

Contributors

All authors participated in the critical review of this report for its intellectual content. In particular, FR, MK, DS, RC, AT, CS, AC, MD, SC, MGC, NR, and NM participated in the design and undertaking 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, and NR developed the concept, design, analysis, and interpretation of this epigenetic analysis. All authors participated in the undertaking, data analysis, and data interpretation and participated in drafting, revising, and finalising this report. All authors participated in developing the study concept, laboratory analysis, data interpretation, and preparation of the report for publication.

Conflicts of interest

The authors declared no conflicts of interest.

Acknowledgments

This work was supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Division of Cancer Epidemiology and Genetics (Bethesda, MD, USA), and also by Fondo de Investigacion Sanitaria, Spain (G03/174).

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