Quantitative Analysis of DNA Methylation Profiles in Lung Cancer Identifies Aberrant DNA Methylation of Specific Genes and Its Association with Gender and Cancer Risk Factors

Thomas Vaissière,[1] Rayjean J. Hung,[2] David Zaridze,[3] Anush Moukeria,[3] Cyrille Cuenin,[1] Virginie Fasolo,[1] Gilles Ferro,[1] Anupam Paliwal,[1] Pierre Hainaut,[1] Paul Brennan,[1] Jörg Tost,[4] Paolo Boffetta,[1] and Zdenko Herceg[1]

[1]IARC, Lyon, France; [2]Samuel Lunenfeld Research Institute, Toronto, Ontario, Canada; [3]Institute of Carcinogenesis, Cancer Research Center, Moscow, Russia; and [4]Laboratory for Epigenetics-Centre National de Génotypage, CEA-Institut de Génomique, Evry, France

Abstract

The global increase in lung cancer burden, together with its poor survival and resistance to classical chemotherapy, underscores the need for identification of critical molecular events involved in lung carcinogenesis. Here, we have applied quantitative profiling of DNA methylation states in a panel of five cancer-associated genes (*CDH1, CDKN2A, GSTP1, MTHFR*, and *RASSF1A*) to a large case-control study of lung cancer. Our analyses revealed a high frequency of aberrant hypermethylation of *MTHFR, RASSF1A*, and *CDKN2A* in lung tumors as compared with control blood samples, whereas no significant increase in methylation levels of *GSTP1* and *CDH1* was observed, consistent with the notion that aberrant DNA methylation occurs in a tumor-specific and gene-specific manner. Importantly, we found that tobacco smoking, sex, and alcohol intake had a strong influence on the methylation levels of distinct genes (*RASSF1A* and *MTHFR*), whereas folate intake, age, and histologic subtype had no significant influence on methylation states. We observed a strong association between *MTHFR* hypermethylation in lung cancer and tobacco smoking, whereas methylation levels of *CDH1, CDKN2A, GSTP1,* and *RASSF1A* were not associated with smoking, indicating that tobacco smoke targets specific genes for hypermethylation. We also found that methylation levels in *RASSF1A*, but not the other genes under study, were influenced by sex, with males showing higher levels of methylation. Together, this study identifies aberrant DNA methylation patterns in lung cancer and thus exemplifies the mechanism by which environmental factors may interact with key genes involved in tumor suppression and contribute to lung cancer. [Cancer Res 2009;69(1):243–52]

Introduction

Lung cancer is the leading cause of cancer-related malignancy worldwide, accounting for 30% of all cancer-related deaths (1). It is projected that the lung cancer toll by the year 2010 will be 1.5 million deaths, highlighting the lung cancer burden as a major public health issue in the coming years (1). The vast majority of lung cancer cases (80–90%) are due to smoking, with a striking dose-response relationship (2). Despite the fact that the cause of most lung cancer is well known, the disease has proven difficult to diagnose early and treat successfully, reflecting limited advances in our understanding of the molecular mechanisms underlying lung carcinogenesis and individual susceptibility to lung cancer.

In addition to genetic factors such as mutations and susceptibility differences in the form of rare high-penetrance genes and genetic polymorphism (3–5), the role of epigenetic changes has been implicated in lung cancer etiology (6–12). DNA methylation is an epigenetic event whose pattern is altered frequently in a wide variety of human cancers, including promoter-specific hypermethylation as well as genome-wide hypomethylation (13, 14). Aberrant DNA methylation within CpG islands is among the earliest and most common alterations in human cancers, leading to abnormal expression of a broad spectrum of genes (13, 15–17). Although there are now many reports of somatically acquired DNA methylation changes in various genes implicated in lung cancer (13, 14), what triggers these changes is poorly understood.

Various environmental and lifestyle exposures such as tobacco, *Helicobacter pylori*, plutonium, or radon exposure are suspected to be implicated in the development of a wide range of human cancers by eliciting DNA methylation changes (18–20); however, the underlying mechanism and precise epigenetic targets are poorly understood. The major obstacle in establishing a relationship between DNA methylation patterns and exposure to environmental and lifestyle factors in cancer is the fact that case-control studies tend to be too small and lack quantitative measure of methyl-cytosine levels to identify the interactions between DNA methylation changes and specific risk factors.

In the present study, we sought to identify DNA methylation profiles in lung cancer and their association with known or suspected cancer risk factors. For this, we combined the advantages of quantitative measurement of DNA methylation levels in a panel of cancer-associated genes and a large case-control study of lung cancer with adequate statistical power, and identified aberrant DNA methylation of key cellular genes in lung cancer and its association with specific cancer risk factors.

Materials and Methods

Study population. This article is based on a case-control study on lung cancer conducted at the Cancer Research Centre, Moscow (Russia), as part of a larger multicenter case-control study coordinated by the IARC (4, 21). Patients newly diagnosed with lung cancer, and a comparable group of hospital-based control subjects without lung cancer, were recruited between February 1998 and October 2002 (Table 1). All lung cancer cases were confirmed histologically and cytologically. A total of 600 patients with lung cancer and 600 controls were recruited; among these, tumor tissues

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Zdenko Herceg, Epigenetics Group, IARC, 150 cours Albert Thomas, 69372 Lyon cedex 08, France. Phone: 33-4-72-73-83-98; Fax: 33-4-72-73-83-29; E-mail: herceg@iarc.fr.

doi:10.1158/0008-5472.CAN-08-2489

were available for 209 patients with lung cancer. Of these patients, 172 also provided blood samples. In addition, blood samples from 164 control subjects were included in the analysis for comparison. The blood samples from the control group were sampled to match cases by age, sex, and smoking status. Informed consent was obtained from all patients, and the study was approved by the IARC Institutional Review Committee.

Cell lines and culture conditions. Human lung cancer cell lines used for the analysis of DNA methylation and *MTHFR* expression were as follows: A549 (lung carcinoma), H1299 (carcinoma, non–small cell lung cancer), H1975 (adenocarcinoma; non–small cell lung cancer), and H1650 (adenocarcinoma; bronchoalveolar carcinoma, stage IIIB). Cells were maintained in standard medium under conditions recommended by the American Type Culture Collection.

Cancer samples and DNA extraction. Primary tumors of lung and corresponding blood samples were used for the analysis. For each of the lung cancers, one paraffin block was selected, and representative tumor areas were marked on H&E-stained slides from these blocks. Where indicated, samples of lung tissues classified as normal tissues adjacent to the cancerous tissue were also used for the analysis. Adjacent nonmalignant lung tissue was available from 51 cases. Four to five thicker sections (8 μm) per tumor were prepared, and paraffin was removed by incubating slides in xylene (twice for 5 min) followed by incubation in 100%/95%/70% ethanol (3 min each) and water. Tumor areas were carefully scraped and brought into the DNA extraction buffer [TE (pH 9) with 0.1 μg/μL of Proteinase K and 0.25% of Nonidet P40] and incubated at 56°C for at least 24 h. For obtaining DNA from cell lines, cell pellets were directly resuspended in DNA extraction buffer. Samples were then heated for 10 min at 95°C to inactivate Proteinase K, spun and supernatant frozen at −20°C. Buffy coat separated from blood samples was used for extraction of genomic DNA by automated equipment (Autopure LS by Gentra Systems). DNA extraction from paraffin-embedded tumor samples was carried out by EX-WAX DNA extraction kit (Chemicon). DNA concentration was quantified with Quant'iT PicoGreen dsDNA reagent (Molecular Probes), an ultrasensitive assay for fluorescent detection of nucleic acids. Sample DNA concentrations were calculated based on a standard curve established with Lambda DNA. The concentrations were then adjusted to 25 ng/μL.

Bisulfite conversion. For methylation analyses, genomic DNA is modified by treatment with sodium bisulfite, which converts all unmethylated cytosines to uracil, then to thymidine during the subsequent PCR step (22). Briefly, 1 μg of DNA in 50 μL distilled water was incubated with 5.5 μL of 2 mol/L NaOH at 37°C for 10 min, followed by 16 h of treatment at 50°C after adding 30 μL of freshly prepared 10 mmol/L hydroquinone (Sigma) and 520 μL of freshly prepared 10 mmol/L sodium bisulfite (Sigma) at pH 5.0. The bisulfite-treated DNA was purified using a DNA Wizard cleanup kit (Promega) following the manufacturer's instructions. The purified DNA was denatured at room temperature for 5 min with 5.5 μL of 3 mol/L NaOH, followed by ethanol precipitation with 33 μL of 10 mol/L NH_4Ac and 170 μL of ethanol. After washing with 70% ethanol, the DNA pellet was resuspended in 50 μL TE (pH 7.5).

Pyrosequencing assays. To quantify the percentage of methylated cytosine in individual CpG sites, bisulfite-converted DNA was pyrosequenced using a pyrosequencing system (PSQ 96MA, Biotage). This method treats each individual CpG site as a C/T polymorphism and generates quantitative data for the relative proportion of the methylated versus the unmethylated allele. In our selection of relevant genes that are potential targets of DNA hypermethylation associated with lung cancers, we were guided by two criteria, (*a*) genes that may have an association with lung cancer based on their supposed biological function, and (*b*) genes that are proposed to be the frequent targets of hypermethylation in cancer or involved in the DNA methylation process itself.

We have established pyrosequencing assays for quantitative measurement of DNA methylation levels in the promoter region of five genes (*CDH1, CDKN2A, GSTP1, MTHFR*, and *RASSF1A*) in tumor and blood samples (Supplementary Table S1). Quantitative measurement of DNA methylation states are of special importance for establishing the relationship between epigenetic states and environmental/lifestyle factors that are likely to induce subtle and cumulative changes that may culminate in phenotypic traits after repetitive exposure over a long period of time. Pyrosequencing offers a highly reliable, quantitative, and high-throughput method for the analysis of DNA methylation at multiple CpG sites with built-in internal controls for completeness of bisulfite treatment (23). Specific pyrosequencing primers were designed to focus on a series of five to eight "target" CpG dinucleotides in the promoter region of the cyclin-dependent kinase inhibitor 2A (*CDKN2A, $p16^{INK4A}$*), E-cadherin (*CDH1*), glutathione *S*-transferase pi 1 (*GSTP1*), methylenetetrahydrofolate reductase (*MTHFR*), the Ras-association domain family 1 (*RASSF1A*) gene, and for the *LINE-1* repetitive sequence (Supplementary Table S1; Supplementary Fig. S1). We were careful to include a non-CpG cytosine in the region for pyrosequencing, as it provides the internal control of the completeness of bisulfite treatment (Supplementary Table S1), the criteria considered to be of critical importance for the reliability of DNA methylation analysis. Hot-start PCR was performed with HotStarTaq Master Mix kit (Qiagen), and pyrosequencing was carried out in accordance with the manufacturer's protocol (Biotage). The target CpGs were evaluated by converting the resulting pyrograms to numerical values for peak heights (Supplementary Fig. S2). The percentage of methylation was calculated as the mean of all CpG analyzed at a given gene promoter.

Gene expression analysis. Total RNA was extracted using RNeasy mini kit (Qiagen). Between 500 ng and 1.5 μg of total RNA was used for reverse transcription using 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen kit). Two microliters of the reverse transcriptase reaction was subsequently subjected to PCR amplification using PCR primers designed to generate a DNA fragment 150 to 250 bp in length. The sequences of primers were as follows: for the *MTHFR* gene, 5′-CGAACTGCTGAGGGAGCTGT-3′ and 5′-ATGGCCCGTGATCTCCTCCA-3′; for *GAPDH*, 5′-GTCCACTGGCGTCTTCAC-3′ and 5′-CAGGAGGCATTGCTGATG-3′. PCR reaction was performed in a total volume of 20 μL containing 1 μL of cDNA product, 0.15 mmol/L of deoxynucleotide triphosphate, 2.5 mmol/L of $MgCl_2$, 1× PCR buffer, 1.5 units of Taq DNA polymerase (GoTaq; Promega). PCR products were resolved on 2% agarose gel and the intensity of the bands were quantified by Quantity One 4.6.6 (Bio-Rad).

Statistical analysis. All methylation data were generated without knowledge of the exposure status and case-control status of the subjects and histologic features of the samples analyzed. To compare methylation levels in lung tumor samples and blood samples, we used the Wilcoxon rank-sum test that allows the comparison of two groups of independent but continuous samples. Multivariate linear regression analysis was performed to test whether any of the risk factors (smoking, alcohol, and folate status) and demographic or clinical characteristics (sex, age, and histology) were associated with DNA methylation. The statistical significance of the mean differences between the nonsmokers and the other smoking categories were determined by multiple comparison using Dunnett's test. Analyses were performed using SAS software, version 9.1 (SAS Institute, Inc.). $P < 0.01$ was considered statistically significant. Cumulative tobacco consumption was calculated by multiplying smoking duration (in years) by smoking intensity (in the equivalent of cigarette packs) and expressed as pack-years. Folate intake was estimated from the food frequency questionnaire, which included 23 food items as previously described (24). To assess DNA hypermethylation frequency, we calculated the percentage of tumor samples with methylation levels of <95% quantile levels in blood samples. The statistical significance for differential methylation in lung tumors, normal-appearing adjacent lung tissue and blood, was calculated using Newman-Keuls' test.

Results

Patient characteristics. A total of 209 lung cancer cases and 164 control subjects were included in the study (Table 1). Among these (cases and controls), 23% were women and 77% were men. Among the cases of lung cancer, 18% were women and 82% were men. Most smokers were men (96%), whereas most nonsmokers were women (86%). Cases and control subjects were age-matched with an average age of 60 years for both cases and controls. A majority of the cases (74%) were between 51 and 70 years of age.

Table 1. Patients and tumors information

	Tumors (n = 209)	Blood (n = 336)		
		Cases (n = 172)	Controls (n = 164)	Total (n = 336)
Sex				
Male	171	107	115	222
Female	38	65	49	114
Age (y)				
≤40	2	2	1	3
41–50	23	15	26	41
51–60	72	52	53	105
61–70	83	64	63	127
71+	29	39	21	60
Histology				
SCC	121	63	—	63
Adenocarcinoma	58	55	—	55
Mixed	12	12	—	12
Other/unspecified	18	42	—	42
Control	—	—	164	164
Tobacco (packs-years)				
Never	36	63	61	124
0–20	28	8	20	28
20–40	88	22	45	67
40–60	51	50	29	79
60+	6	29	9	38
Folate intake				
Low	119	88	77	165
Medium	50	35	40	75
High	40	49	47	96
Alcohol intake (g/d)				
0–138 (low)	18	35	43	78
139–889 (medium)	90	55	61	116
890+ (high)	50	34	24	58
ND	51	48	36	84

The predominant tumor histology types were squamous cell carcinoma (SCC) (58%) and adenocarcinoma (28%). SCC was predominant in smokers (68%), whereas adenocarcinoma was the main histology type in never-smokers (70%). SCCs were equally distributed among former and current smokers, whereas both SCC and adenocarcinoma were equally distributed over all age groups. We categorized the subjects as never-smokers (26%), 0 to 20 pack-years (13%), 20 to 40 pack-years (36%), 40 to 60 pack-years (21%), and <60 pack-years (4%). Mixed adenosquamous (ADC-SCC) type was identified in 6% of the cases. Based on folate intake, the subjects were categorized into four groups: <4 (25%), 4 to 5.16 (28%), 5.16 to 6.14 (24%), and >6.14 (23%). Alcohol consumption (g/d of ethanol) groups were categorized into low (0–138 g/d, 16%), medium (139–889 g/d, 40%), and high (>890 g/d, 20%).

Methylation profiles of *CDH1, CDKN2A, GSTP1, MTHFR*, and *RASSF1A* genes in lung cancer. Results of quantitative analysis of methylation status in lung tumors and blood samples are shown in Fig. 1 and Supplementary Figs. S2 and S3. Analysis of all the cases for methylation levels showed that one gene (*MTHFR*) exhibited higher levels of methylation (≥25%), two genes (*CDH1* and *GSTP1)* show rather low levels of methylation or were virtually unmethylated (<10%), and the two other genes (*CDKN2A* and *RASSF1A*) exhibited intermediate levels of methylation (Fig. 1). The multivariant pair-wise comparison of DNA methylation revealed no strong correlation between any gene pairs (data not shown). Comparison of mean methylation levels of all CpG sites in tumors and blood samples from cases and controls revealed a highly significant increase in methylation levels in tumors for *CDKN2A* (P < 0.01), *MTHFR* (P < 0.01), and *RASSF1A* (P < 0.01), a moderate but significant decrease for *CDH1* (P = 0.01), whereas methylation states at the *GSTP1* gene were indistinguishable between tumors and blood samples (P = 0.2; Fig. 1; Supplementary Fig. S3). Analysis of DNA methylation frequency (defined as the percentage of tumor samples with methylation levels <95% quantile levels in control blood samples) showed that one gene (*CDKN2A*) was most frequently methylated (91%), two genes (*MTHFR* and *RASSF1A*) exhibited intermediate methylation frequency (39% and 36%, respectively), and the two remaining genes (*GSTP1* and *CDH1*) were virtually unmethylated (≤1%) in lung tumors (Supplementary Table S2; Supplementary Fig. S3). When analyzed individually, 99% of the lung cancer samples had methylation of at least one of these genes; 34% had one gene methylated, 60% had two genes methylated, 14% had three genes methylated, and none had four or more genes methylated.

Association between methylation levels, clinicopathologic features and risk factors exposure. We initially analyzed associations between the methylation of *CDH1, CDKN2A, GSTP1, MTHFR*, and *RASSF1A* genes (both mean levels of all CpG sites or individual CpG sites) separately and available epidemiologic and clinical information including smoking status, folate intake, alcohol

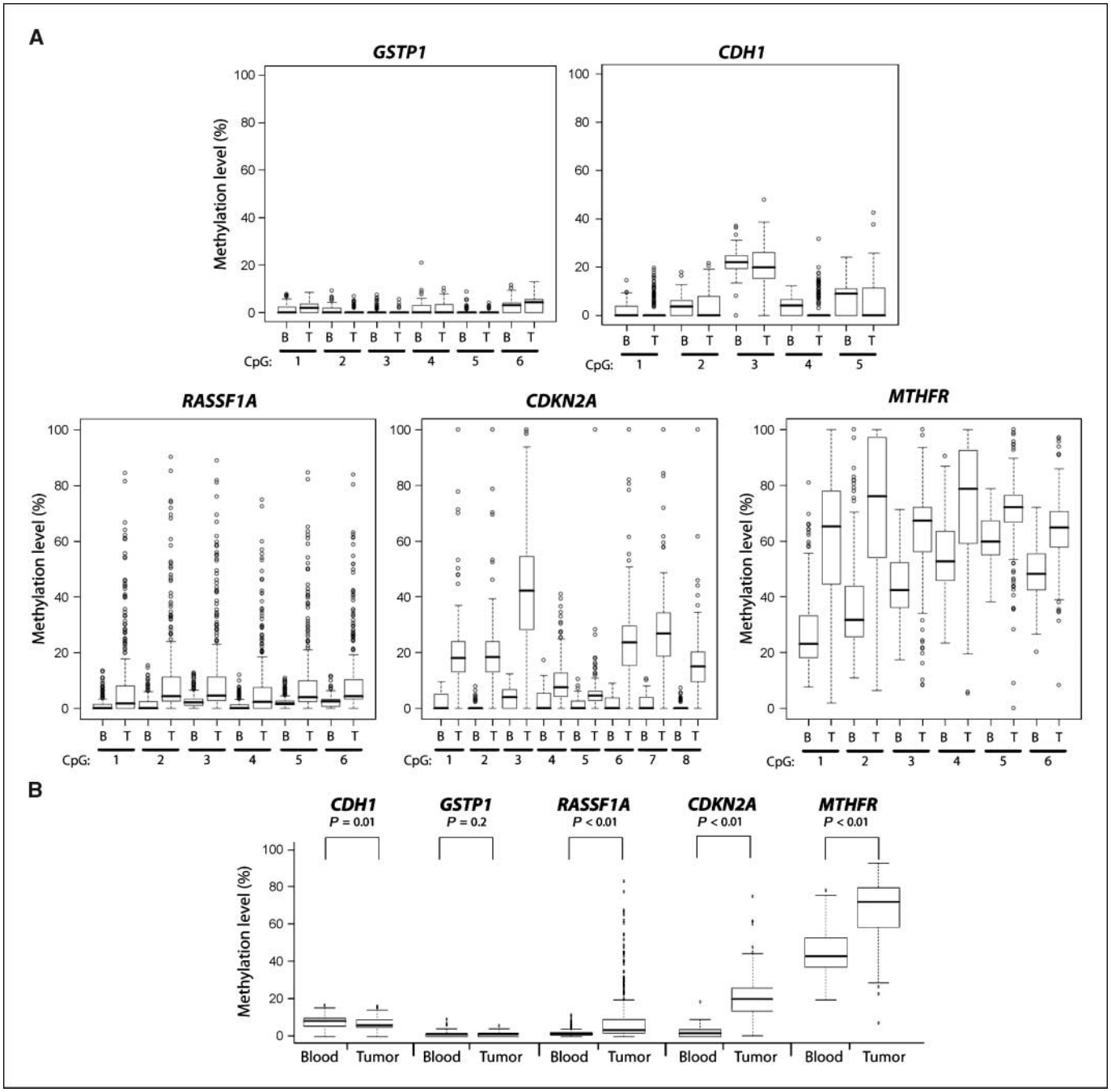

Figure 1. Graphical representation (boxplots) comparing the DNA methylation levels in lung tumors (*T*) and blood (*B*) samples. *A,* boxplots of the results obtained by the analysis of individual genes and CpG sites. *B,* boxplots of the summary results obtained by the analysis of mean levels of all CpG sites for a given gene and the level of statistical significance for differential methylation in tumors compared with blood samples.

consumption, sex, age, and histologic subtype of the tumor. The most statistically significant factors were then included in a multivariable analysis. No association was found between methylation levels (measured as mean levels of all CpG sites) of any gene analyzed in blood samples of cases and controls and any risk factor included in the analysis (data not shown).

Associations between methylation levels of *CDH1, CDKN2A, GSTP1, MTHFR*, and *RASSF1A* in lung cancer and clinical features and risk factor exposures are shown in Table 2. Lung cancers from men exhibited higher methylation levels of *RASSF1A* than those from women (7.5% versus 17.9%, $P < 0.01$), whereas the methylation levels of *CDH1, CDKN2A, GSTP1*, and *MTHFR* were not found to correlate with sex. No association was found between methylation status of any gene analyzed and age or histologic subtypes of the tumor. We found no association between methylation of any gene and alcohol intake, with the exception of *RASSF1A,* which exhibited a significantly lower level of methylation in the intermediate group (5.1%, $P < 0.01$) than in the low-intake (17%) or high-intake (23.3%) group (Table 2).

Although increased levels of DNA methylation of the *CDKN2A* and *RASSF1A* genes were observed in tumors of both smokers and nonsmokers, the methylation level of the *MTHFR* gene was

significantly higher in current smokers (72.1%, $P < 0.01$) and ex-smokers (63.8%, $P < 0.01$) than in never-smokers (51.6%; Table 2). No consistent dose-response association between *MTHFR* methylation levels and the number of cigarettes per day smoked was observed. Smoking may affect the methylation status of some (but not other) CpG sites in the same gene; therefore, we compared smoking status and methylation levels across CpG sites of the *MTHFR* gene, and found that within the six CpGs of *MTHFR* analyzed, CpGs 1 to 4 exhibited significantly greater methylation levels in tumors of smokers than in those who never smoked (Supplementary Table S3). By contrast, methylation levels in the two remaining CpG sites (CpG 5 and 6) did not show appreciable differences between smokers and nonsmokers. Methylation levels of any CpG site of the *MTHFR* gene in blood samples of cases and controls were indistinguishable between smokers and never-smokers (Supplementary Table S3). Therefore, smoking was associated with a significant increase in DNA methylation of specific CpG sites of *MTHFR* in lung cancer.

Methylation of the four CpGs that are differentially methylated in smokers and nonsmokers was not significantly associated with pack-years smoked (Supplementary Table S3). These results indicate that hypermethylation of specific CpG sites of *MTHFR* in lung tumors increases with exposure to tobacco smoke, and that this increase does not occur in a dose-dependent fashion. However, methylation

Table 2. DNA methylation levels at five genes analyzed in lung cancer, stratified by sex, age, histology, tobacco consumption, and alcohol intake

	Gene				
	CDH1	*CDKN2A*	*GSTP1*	*MTHFR*	*RASSF1A*
	Mean (*P*)	Mean (*P*)	Mean (*P*)	Mean (*P*)	Mean (*P*)
Sex					
Women	8.8 (—)	19.0 (—)	1.36 (—)	70.4 (—)	7.5 (—)
Men	5.0 (0.05)	18.0 (0.83)	1.63 (0.56)	64.9 (0.10)	17.9 (<0.01)
Age					
≤40	2.6 (—)	8.35 (—)	1.5 (—)	70.1 (—)	8.3 (—)
40–44	7.2 (0.4)	24.7 (0.10)	2.0 (0.90)	54.0 (0.12)	3.2 (0.79)
45–49	6.9 (0.4)	20.7 (0.17)	0.9 (0.78)	70.3 (1.00)	18.5 (0.38)
50–54	8.5 (0.2)	19.8 (0.18)	1.7 (1.00)	67.5 (0.96)	15.1 (0.61)
55–59	7.2 (0.4)	17.8 (0.32)	1.7 (1.00)	66.7 (0.91)	13.1 (0.79)
60–64	7.5 (0.4)	16.5 (0.43)	1.3 (0.98)	72.2 (0.98)	14.2 (0.70)
65–69	7.8 (0.3)	25.4 (0.03)	1.2 (0.97)	73.1 (0.94)	13.3 (0.78)
70+	7.4 (0.4)	14.4 (0.62)	1.7 (1.0)	67.4 (0.95)	16.1 (0.53)
Histology					
SCC	6.3 (—)	16.4 (—)	1.6 (—)	70.7 (—)	11.5 (—)
Adenocarcinoma	6.9 (0.93)	15.1 (0.82)	1.5 (1.00)	67.8 (0.22)	14.6 (0.25)
Tobacco					
Pack-years					
Never	4.7 (to)	19.5 (—)	1.9 (—)	51.6 (—)	19.8 (—)
0–20	8.1 (0.29)	18.8 (0.99)	1.0 (0.21)	68.0 (<0.01)	8.7 (0.58)
20–40	8.3 (0.21)	19.2 (1.00)	0.9 (0.13)	75.6 (<0.01)	9.3 (0.07)
40–60	7.5 (0.39)	21.3 (0.91)	1.6 (0.91)	64.5 (<0.01)	13.4 (1.00)
60+	7.5 (0.39)	12.5 (0.33)	2.0 (0.99)	78.7 (<0.01)	12.3 (1.00)
Current vs. former					
Never	4.7 (—)	18.9 (—)	1.9 (—)	50.9 (—)	19.9 (—)
Ex-smoker	6.9 (0.47)	19.8 (0.97)	0.7 (0.05)	63.8 (<0.01)	10.4 (0.08)
Current smoker	8.5 (0.13)	19.0 (1.00)	1.2 (0.25)	72.1 (<0.01)	9.9 (0.05)
Smoking intensity (cigarettes/d)					
Never	3.3 (—)	19.5 (—)	1.9 (—)	50.5 (—)	9.4 (—)
0–12	9.0 (0.04)	21.4 (0.82)	0.9 (0.15)	75.9 (<0.01)	5.1 (0.58)
13–16	8.4 (0.06)	19.7 (1.00)	1.0 (0.19)	78.6 (<0.01)	11.1 (0.07)
17–19	8.4 (0.06)	19.8 (1.00)	1.3 (0.45)	66.3 (<0.01)	8.95 (0.99)
20+	6.7 (0.26)	15.4 (0.45)	1.6 (0.81)	68.0 (<0.01)	8.7 (0.99)
Folate intake					
Low	6.8 (—)	17.4 (—)	1.4 (—)	65.4 (—)	9.1 (—)
Medium	6.1 (0.72)	18.2 (0.90)	1.4 (0.53)	65.7 (0.99)	11.9 (0.21)
High	7.8 (0.53)	18.5 (0.86)	1.6 (0.34)	69.0 (0.11)	10.1 (0.91)
Alcohol intake (g/d)					
0–138	7.7 (—)	21.2 (—)	1.1 (—)	71.4 (—)	17.3 (—)
139–889	7.0 (0.89)	19.0 (0.59)	1.5 (0.34)	65.8 (0.03)	5.3 (<0.01)
890–3,119	7.1 (0.92)	19.3 (0.72)	1.5 (0.53)	66.8 (0.12)	4.9 (<0.01)
3,120+	5.8 (0.77)	14.2 (0.17)	1.8 (0.34)	66.6 (0.40)	23.3 (0.33)

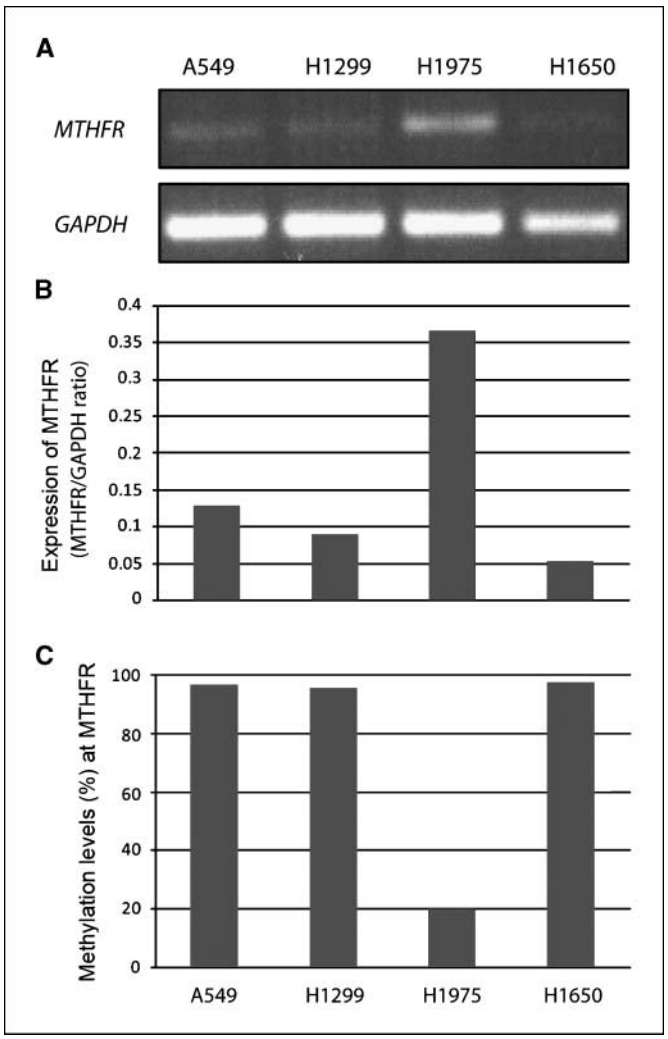

Figure 2. *MTHFR* expression and DNA methylation analysis in lung cancer cell lines. *A,* four lung cancer cell lines (*A549, H1299, H1975*, and *H1650*) were grown under culture conditions recommended by the American Type Culture Collection and total RNA was analyzed by reverse transcription-PCR. Equal RNA loading was controlled by *GAPDH. B,* quantification of *MTHFR* expression levels in *A* was carried out by densitometric analysis and normalization vs. *GAPDH. C,* cells were grown as in *A* and DNA extracted was used for analysis of *MTHFR* methylation. The results are representative of three independent experiments.

levels of the *MTHFR* gene were significantly higher in tumors of current smokers (72.1%, $P < 0.01$) than in ex-smokers (63.8%).

***MTHFR* methylation and its association with expression of the gene and global hypomethylation in lung cancer.** Unscheduled DNA hypermethylation has been associated with gene silencing, therefore, we sought to determine whether the levels of *MTHFR* methylation correlate with expression of the gene. To this end, we took advantage of lung cancer cell lines because of the ease of analysis of gene expression in these cells. To determine *MTHFR* mRNA levels and *MTHFR* methylation, we performed reverse transcription-PCR analysis and pyrosequencing assays, respectively, using identical samples of lung cancer cell lines. The results have revealed that cell lines with high levels of *MTHFR* methylation (A549, H1299, and H1650) exhibit significantly lower levels of *MTHFR* mRNA compared with cell lines with low levels of *MTHFR* methylation (H1975; Fig. 2). These results show an inverse correlation between levels of *MTHFR* methylation and gene

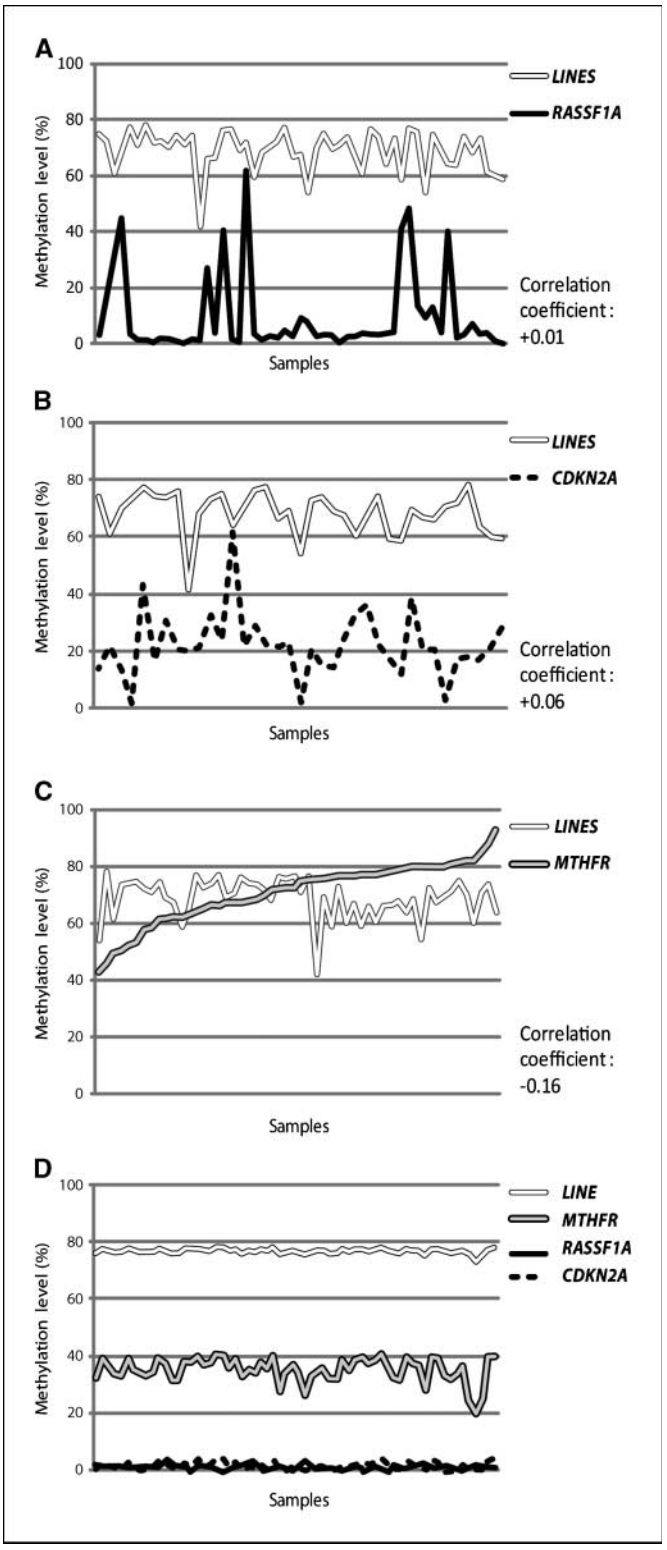

Figure 3. The methylation levels of *LINE-1* and its association with hypermethylation of *MTHFR* in lung tumors. Lung tumor DNA and corresponding blood DNA were evaluated for *LINE-1* methylation (surrogate for global methylation levels). The levels of methylation obtained for *LINE-1* were compared with methylation levels obtained for *RASSF1A* (*A*), *CDKN2A* (*B*), and *MTHFR* (*C*) in the same lung tumor samples and the correlation coefficient was calculated. As a control, methylation levels obtained for *LINE-1, MTHFR, RASSF1A*, and *CDKN2A* in blood samples (*D*). Note a marked inverse correlation (correlation coefficient, −0.16) between *LINE-1* methylation (global methylation) and *MTHFR* methylation in lung cancer (*C*).

expression and thus suggest that hypermethylation of the *MTHFR* gene at the studied CpG sites may have functional significance and may result in partial or complete silencing of the gene.

The comparison between DNA methylation levels in cancer tissue and nontumorigenic adjacent tissues might enforce the association between the exposure to environmental factors and methylation of an individual gene. Therefore, we next examined *MTHFR* methylation levels in normal-appearing adjacent lung tissues. As shown in Supplementary Fig. S4A, the levels of *MTHFR* methylation in normal-appearing adjacent tissues were comparable to those in blood samples, but were significantly lower in comparison to the methylation levels seen in lung tumors. These findings further confirm the notion that *MTHFR* hypermethylation is a tumor-specific event.

The *MTHFR* gene product plays a role in the maintenance of the methionine pool (25, 26), and the inactivation of *MTHFR* in mice results in a significant decrease of global 5-methylcytosine content (27); therefore, down-regulation of the *MTHFR* gene induced by gene hypermethylation may impair DNA methylation reactions leading to global hypomethylation. To test whether *MTHFR* hypermethylation is indeed associated with global hypomethylation, we examined the methylation levels of *LINE-1* sequences, a highly repeated and widely interspersed human retrotransposon commonly used as a surrogate for global demethylation (28–30) in lung tumors and corresponding blood samples. We found that the average level of *LINE-1* methylation in blood samples and adjacent nonmalignant lung tissues was 76% (73–77%) and 73% (67–75%), respectively, whereas it was 63% (41–72%) in lung tumor tissues (Supplementary Fig. S4*B*), an average loss of ~12% of methylation in the tumor, consistent with global demethylation of the tumor cell genome (28). Interestingly, calculation of the correlation coefficient between *LINE-1* methylation and the methylation levels of the genes under study revealed that in tumor samples, a low but consistent inverse correlation is observed between *LINE-1* and *MTHFR* methylation levels (−0.16), whereas no correlation between methylation levels of *LINE-1* and *RASSF1A* or *CDKN2A* genes was found (Fig. 3). These results show a tumor-specific global hypomethylation which inversely correlates with *MTHFR* methylation levels, suggesting that *MTHFR* hypermethylation and associated silencing of the gene may promote genome-wide demethylation in tumor cells.

Discussion

In this study, we have established pyrosequencing assays for the analysis of DNA methylation levels in the promoter region of five genes (*CDH1, CDKN2A, GSTP1, MTHFR*, and *RASSF1A*) and combined these quantitative assays with a large series of lung tumor samples and corresponding blood (control) samples. We found a high frequency of aberrant hypermethylation of *MTHFR, RASSF1A*, and *CDKN2A*, but not *CDH1* and *GSTP1* in lung tumors compared with corresponding normal control samples. These results are consistent with the notion that epigenetic changes mediated by hypermethylation are tumor-specific events (13, 17).

In lung tumors, the CpG island of *CDKN2A* is the most frequently hypermethylated among the genes analyzed (91%), in agreement with previous reports showing a high frequency of *CDKN2A* methylation in a variety of human neoplasia including lung cancers (6–10). The second most frequently hypermethylated CpGs in lung tumors were those of the *MTHFR* gene. *MTHFR* methylation levels were strongly elevated in a large fraction of lung cancer samples (39%). The *MTHFR* gene product is critical in maintaining an adequate methionine pool, and is believed to be important in the process of DNA methylation itself (31). Comparing lung tumors and normal (blood) tissues, there was an increase in hypermethylation of all six CpG sites of *MTHFR* (Supplementary Table S3).

We also observed a high frequency of hypermethylation of the *RASSF1A* gene (36%), a candidate tumor suppressor gene that has been implicated as a pivotal gatekeeper of cell cycle progression (32). These results are consistent with previous studies showing that *RASSF1A* is commonly inactivated by hypermethylation in a broad spectrum of human tumors (33–35). *De novo* methylation of the *RASSF1A* promoter is one of the most frequent epigenetic inactivation events detected in both non–small cell lung cancer and small cell lung cancer types, and leads to silencing of *RASSF1A* expression (34, 36). In contrast, we found no significant increase in methylation levels of *GSTP1* (0.6%) and *CDH1* (1.2%) in lung tumors as compared with blood samples, supporting the notion that unscheduled hypermethylation and associated gene silencing does not occur randomly, but rather, in a gene-specific manner (13, 37). It is believed that the hypermethylation of tumor suppressor genes and other cancer-associated genes would be selected and fixed in tumor cells in accordance with the degree of growth advantage caused by their inactivation. Therefore, hypermethylation of *MTHFR, RASSF1A*, or *CDKN2A* may confer a growth advantage to cancer cells and contribute to the cancer phenotype.

Our analyses that compared tumor histology and methylation levels of any of the genes analyzed showed no significant correlation with histologic subtypes, consistent with previous studies in which the prevalence of hypermethylation was indistinguishable between major histologic subtypes of lung cancer (10, 38). Our study is not in agreement with the studies by van der Weyden and colleagues (36) and Toyooka and colleagues (39), who reported differences in the methylation patterns of SCC and adenocarcinoma. This discrepancy might be due, in part, to differences in exposure and techniques used for analysis of DNA methylation. Furthermore, the pair-wise comparison of DNA methylation levels revealed no strong correlation between any gene pairs, indicating that methylation status of any single gene was largely independent of methylation status of other genes, in agreement with previous studies (39). The absence of concordant hypermethylation in multiple genes also suggests that the methylator phenotype (CIMP) observed in a subset of several cancer types such as colorectal cancer is unlikely to be present in lung cancer, a notion consistent with previous studies on different cancer types (40).

Our data showed that methylation levels in *RASSF1A*, but not the other four genes under study, were correlated with sex and with males showing higher levels of methylation than females. There have been few studies on the age and sex differences in DNA methylation levels and patterns. Quantitative profiling of DNA methylation in a small panel of genes suggested that sex is a strong predictor of methylation levels, with males showing higher methylation levels (41). In contrast, high-resolution methylation profiling of three human chromosomes (6, 20, and 22) did not find a significant attributable effect of age and sex on methylation levels (42). Our results suggest that sex is a strong predictor of methylation levels in some genes (such as *RASSF1A*) in lung tumors, but not in normal tissue (lymphocytes), consistent with the notion that male sex is associated with higher methylation levels in specific genes (41).

To date, there have been few reports on the associations between DNA methylation changes and alcohol consumption in human cancer, notably liver cancer, head and neck cancer, and

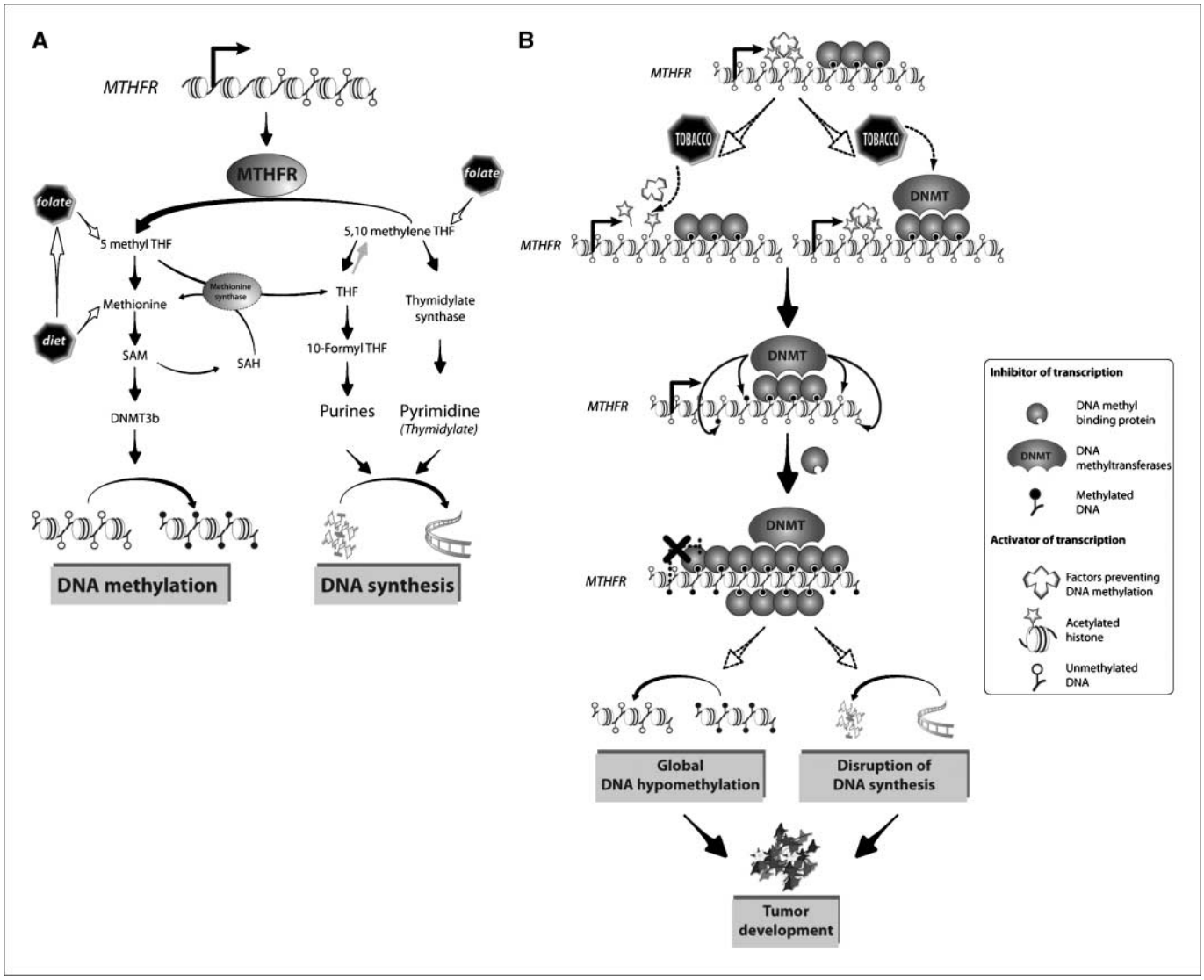

Figure 4. Interaction of tobacco with methyltransfer and DNA methylation. *A,* a simplified representation of the cellular pathways involving *MTHFR* and other key enzymes regulating the metabolism of the methyl group (DNA methylation) and DNA synthesis. *B,* a model for the effect of tobacco exposure on the metabolism of the methyl group and the process of DNA methylation. The pre-existing methylation of *MTHFR* may represent a hotspot for putative tobacco epimutagenes contributing to the differential vulnerability to aberrant methylation. In this model, a higher affinity of methylcytosine for certain tobacco smoke compound(s) deregulates local methylation process through either an enhanced DNMT activity or by removing *cis*-acting elements (e.g., histone acetylation) that block the spread of methylation from neighboring methylated CpG sites. This leads to abnormal hypermethylation of and silencing of the *MTHFR* gene, resulting in the deregulation of methyl group metabolism. Thus, tobacco-mediated hypermethylation of *MTHFR*, and consequent partial or complete silencing of the gene, may trigger global hypomethylation and/or deregulation of DNA synthesis, both of which may contribute to cancer development.

colorectal cancer (19, 43, 44). Our data revealed lower levels of *RASSF1A* methylation in lung tumors from medium alcohol intake groups as compared with those from light or heavy drinkers, whereas no association was found between alcohol consumption and DNA methylation levels of the other four genes under study. These findings suggest that alcohol consumption may be inversely associated with DNA methylation levels in specific genes, although there was no clear dose-response pattern of *MTHFR* methylation across alcohol intake groups.

Importantly, we found a strong association between *MTHFR* methylation levels and tobacco smoking status, whereas methylation levels in *RASSF1A* and *CDKN2A* were not associated with smoking status, indicating that tobacco exposure targets specific genes for hypermethylation. Interestingly, there was no consistent dose-response pattern of *MTHFR* methylation across smoking groups, suggesting that even short periods of smoking are sufficient to induce aberrant methylation of *MTHFR*. It is possible that tobacco smoking and the methylation levels of *MTHFR* in lung cancer are correlated with a certain tobacco consumption threshold above which other changes (such as gene mutations) induced by carcinogens in tobacco smoke may circumvent the need for gene silencing by DNA hypermethylation in tumors. Another noteworthy observation is that the methylation levels of *MTHFR* were significantly higher in tumors of current smokers then in ex-smokers, suggesting that methylation levels may decrease after smoking cessation.

The finding that the methylation of *MTHFR* correlates with tobacco smoking in patients with lung cancer is intriguing. *MTHFR* has been shown to play a critical role in maintaining an adequate

methionine pool as well as ensuring that the homocysteine concentration does not reach toxic levels (31). The enzyme *MTHFR* catalyses the synthesis of methionine and consequently is required for its metabolite, *S*-adenosylmethionine, which is critical for DNA methylation reactions, and its expression and activity may alter DNA methylation states and contribute to cancer risk (25, 26). Consistent with this notion, *MTHFR* hypermethylation in lung cancer cell lines correlates with gene expression and is associated with global hypomethylation in lung cancer (this study), whereas the disruption of the *MTHFR* gene in mice results in global DNA hypomethylation and susceptibility to several disorders (27). Furthermore, the presence of polymorphism in the *MTHFR* gene (C667T, alanine to valine), a common germ line variant associated with lower enzymatic activity, has been implicated in cancer risk (45–49). Therefore, our results, showing higher levels of *MTHFR* methylation in lung cancer among smokers than in never-smokers, suggest that tobacco smoking may target *MTHFR* through an epigenetic mechanism.

Previous studies have unequivocally shown that DNA hypermethylation is associated with gene silencing, and that genes with high levels of methylcytosine in their promoter region are usually transcriptionally silent (13). Therefore, tobacco-mediated deregulation of *MTHFR* gene expression may silence the gene and thus disrupt the DNA methylation process itself. Although further studies are required to test the functional effects of *MTHFR* hypermethylation, it is possible that tobacco-mediated hypermethylation of *MTHFR*, and consequently, partial or complete silencing of the gene, triggers global hypomethylation, a phenomenon almost universally observed in human cancer (13, 17). Consistent with this hypothesis, we found a negative correlation between *MTHFR* methylation and *MTHFR* gene expression and also between *MTHFR* methylation and *LINE-1* methylation, used as a surrogate of genome-wide hypomethylation, whereas no correlation between *LINE-1* methylation and methylation of any other gene studied was observed. However, this does not rule out the possibility that different components of tobacco smoke may induce promoter-specific hypermethylation of genes other than *MTHFR*, and also that other pathways may be involved in tumor-specific global hypomethylation.

The precise mechanism that underlies targeting of *MTHFR* for hypermethylation by tobacco smoke in lung cancer remains unclear. Generally, it has been proposed that several factors related to the DNA methylation process, such as DNMT activity and proximity to a methylation center, as well as locus-specific factors including transcription factor motifs, histone marks, and local chromatin structure, could be involved in the differential susceptibility of methylation among the genes (33). Furthermore, the pre-existing methylation status of CpG islands may also contribute to the differential vulnerability to aberrant methylation (50). In this respect, it is noteworthy that among the genes analyzed in our study, only *MTHFR* exhibited appreciable levels of preexisting methylation in normal tissues. These observations may reflect a higher affinity of methylcytosine for putative tobacco smoke epimutagens compared with unmethylated cytosines. It is thus tempting to speculate that pre-existing methylation of *MTHFR* may represent a hotspot for tobacco compounds that deregulate local methylation processes, possibly through DNMT activity (Fig. 4). An analogous mechanism has been reported for preferential binding of benzo(*a*)pyrene, a highly carcinogenic polycyclic aromatic hydrocarbon present in cigarette smoke, to methylated CpG sites, forming major mutational hotspots in human lung cancer (51, 52). Another possibility is that tobacco smoke compounds may remove *cis*-acting elements that block the spread of methylation from a methylation center, thus exposing neighboring CpG sites to aberrant methylation (Fig. 4). In addition, histone modifications including histone acetylation and methylation maintain local chromatin structure and are believed to play important roles in protecting against unscheduled DNA methylation (53). Therefore, different agents from tobacco smoke may disrupt histone modification patterns and thus expose CpG sites to hypermethylation (19). Further studies are required to elucidate the causal involvement of smoking in the epigenetic inactivation of specific genes in smokers.

In summary, this study provides evidence of gene-specific and sex-specific differences in methylation patterns in lung cancers arising in tobacco smokers and alcohol drinkers, and thus, exemplifies the mechanism by which environmental factors may interact with key genes involved in methyl donors/acceptors and tumor suppression. In light of these findings, it is reasonable to propose that deregulation of *MTHFR* and folate metabolism by carcinogens in tobacco smoke may be an underlying mechanism in lung cancer. Although further studies are required to test the functional effect of *MTHFR* and *RASSF1A* methylation changes in lung cancer, this information could facilitate the development of more accurate risk models. Recent studies have suggested that, in patients with lung cancer, aberrant hypermethylation of specific genes can be detected in samples obtained through noninvasive sampling methods such as plasma or sputum. Therefore, analysis of promoter methylation in specific genes may provide a biomarker valuable for the identification of individuals with an elevated risk of lung cancer.

Disclosure of Potential Conflicts of Interest

The authors of this article are partners of European Cancer Risk, Nutrition, and Individual Susceptibility, a network of excellence operating within the European Union 6th Framework Program, Priority 5: "Food Quality and Safety" (contract no. 513943).

Acknowledgments

Received 7/1/2008; revised 9/22/2008; accepted 9/30/2008.

Grant support: A Ph.D. fellowship from la Ligue National (Française) Contre le Cancer (T. Vaissière). The work in the IARC Epigenetics Group is supported by grants from the U.S. NIH/National Cancer Institute; the Association pour la Recherche sur le Cancer, France; la Ligue Nationale Contre le Cancer, France; the European Network of Excellence Environmental Cancer Risk, Nutrition and Individual Susceptibility; and the Swiss Bridge Award (Z. Herceg).

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We thank Dr. Triantafillos Liloglou for sharing with us his unpublished data on *LINE-1* methylation analysis, and Dr. Rabih Murr for critical reading of the manuscript and helpful discussions.

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Quantitative Analysis of DNA Methylation Profiles in Lung Cancer Identifies Aberrant DNA Methylation of Specific Genes and Its Association with Gender and Cancer Risk Factors

Thomas Vaissière, Rayjean J. Hung, David Zaridze, et al.

Cancer Res 2009;69:243-252.