

# Hypomethylation of *p53* in Peripheral Blood DNA Is Associated with the Development of Lung Cancer

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## Abstract

Alterations in DNA methylation have been associated with cancers at almost all tumor sites and represent one of the most consistent changes in neoplastic cells. The underlying etiological mechanisms for alteration of DNA methylation patterns are not understood, but experimental studies in animals suggest potential environmental and genetic influences. The purpose of this study was to investigate whether DNA hypomethylation in peripheral blood DNA (potentially representing status at the lung) was associated with increased risk for the development of lung cancer. We evaluated genome-wide and *p53* gene-specific hypomethylation in 100 lung cancer cases and controls selected from a large clinical trial of male smokers, the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study. Genome-wide methylation status was assessed using the *in vitro* methyl acceptance capacity assay and *p53* gene-specific methylation status using the *HpaII* quantitative PCR assay. Hypomethylation was evaluated as a risk factor using multivariate conditional logistic regression analyses. Genome-wide methylation status was unrelated to lung cancer risk; the odds ratio was 1.25 and the 95% confidence interval was 0.48–3.21 for those in the highest versus lowest quartile of hypomethylation status. Hypomethylation of the *p53* gene in exons 5–8, the hypermutable region, was associated with a 2-fold increased risk for lung cancer (odds ratio, 2.20; 95% confidence interval, 1.04–4.65), whereas there was no risk increase for hypomethylation at exons 2–4, a region of the gene not known for its mutability or functional significance in cancer. Our results indicate that hypomethylation status within exons 5–8 of *p53* from peripheral lymphocyte DNA may be a relevant predictor of lung cancer among male smokers.

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## Introduction

Methylation of DNA is an epigenetic feature of DNA thought to be integrally involved in the regulation of gene expression as well as in maintaining the stability of the genome and is therefore likely to play an important role in cellular processes, such as development and aging (1–3). DNA is methylated specifically at the 5'-cytosine residue of the dinucleotide CpG by a family of DNA MTases.<sup>2</sup> The methyl group is primarily donated from the compound SAM, which in turn is derived from the labile methyl group of 5-methyltetrafolate, the major circulating form of folate in humans. Many genes have long stretches of unmethylated CpGs in their promoter regions, which is thought to confer an open transcription state allowing for gene expression (4). Conversely, the majority of CpG pairs (~80–90%) found in nontranscribed regions of DNA and within the body of genes are methylated (4). The function of genome-wide CpG methylation is thought to involve chromosomal integrity and stability (5) and gene-specific methylation with regulation of gene expression (6, 7).

Abnormal DNA methylation patterns are among the most consistent cellular alterations found in cancer. Changes in DNA methylation patterns, including hypermethylation of promoter regions as well as genome-wide and gene-specific hypomethylation, often occur simultaneously in neoplastic cells (reviewed in Ref. 8). The mechanism by which such changes contribute to carcinogenesis are thought to be through the aberrant expression of proto-oncogenes and tumor suppressor genes. For example, hypomethylation within the body of genes such as *c-myc*, *fos*, and *p53*, which are normally methylated, are found in rodent neoplasms and correlate with increased gene expression (7). Expression of several tumor suppressor genes, such as *p16* or *Rb*, is turned off by methylation of their promoter regions (9, 10).

Whether abnormal DNA methylation is a consequence or cause of cancer has not been established. Both DNA hypermethylation and hypomethylation occur early in tumorigenesis and are thought to contribute to tumor progression; however, evidence suggests a potentially causal role for DNA hypomethylation in the development of cancer. For example, induction of DNA hypomethylation in mice by dietary restriction of methyl group sources (*e.g.*, folate, methionine, and vitamin B<sub>12</sub>), drugs, carcinogens, or a combination thereof, resulted in the development of lung, colon, and hepatic tumors (7, 11, 12). Studies suggest that DNA hypomethylation precedes DNA hypermethylation in tumor progression and may even stimulate DNA hypermethylation by increasing DNA MTase activity (11). In humans, diminished folate status, a nutritional state that

<sup>2</sup> The abbreviations used are: MTase, methyltransferase; SAM, S-adenosylmethionine; ATBC, Alpha-Tocopherol, Beta-Carotene Cancer Prevention; CV, coefficient of variation; OR, odds ratio; CI, confidence interval.

appears to contribute to DNA hypomethylation, has been associated with colon, lung, and other cancers (13–16).

The purpose of this study was to investigate whether hypomethylation of peripheral blood DNA was associated with lung cancer. We hypothesized that methylation status of peripheral blood DNA would reflect status in lung tissue DNA, and that individual possessing diminished DNA methylation (because of dietary, environmental, or genetic factors) would be at increased risk for lung cancer. We assessed both genome-wide and *p53*-specific hypomethylation among a subset of men who participated in the ATBC study. Alteration of the *p53* gene occurs commonly among lung cancers (16), and this gene has been shown to be sensitive to DNA hypomethylation in animal models (7, 17–19).

## Subjects and Methods

**Study Subjects.** A nested case-control study of 100 lung cancer cases and 100 controls was conducted within the ATBC cohort. The ATBC study was a randomized, placebo-controlled prevention trial that tested the efficacy of 5–8 years of supplementation with  $\alpha$ -tocopherol (50 mg/day),  $\beta$ -carotene (20 mg/day), or both in reducing the incidence of lung, prostate, and other cancers. The trial cohort consisted of 29,133 male smokers, ages 50–69, who smoked at least five cigarettes daily and lived in southwestern Finland. Participants were recruited between 1985 and 1988 and followed up during the active trial period (until April 30, 1993) and postintervention. The trial showed a 16% increase in lung cancer incidence among subjects in the  $\beta$ -carotene-supplemented group and a 32% reduction in prostate cancer in the  $\alpha$ -tocopherol group (20, 21). The ATBC study was approved by the institutional review boards of the National Cancer Institute (United States) and the National Public Health Institute of Finland, and written informed consent was obtained from each participant prior to randomization.

General medical history, diet, smoking, and other background data, along with a fasting blood sample, were collected from all subjects at baseline. Between 1992 and 1993, a whole blood sample was collected from 20,305 men and stored at  $-70^{\circ}\text{C}$  until isolation of genomic DNA.

Eligible cases in this study consisted of men diagnosed with incident primary cancer of lung or bronchus (ICD9-162) diagnosed up to December 31, 1994, identified through the Finnish Cancer Registry and the Register of Causes of Death. The diagnosis of lung cancer was confirmed centrally by one or two study physicians. One hundred cases were randomly selected from the lung cancer cases having a whole blood sample available for DNA analysis ( $n = 362$ ) and matched to 100 controls on age ( $\pm 3$  years), intervention group, and study center. Thirty-two (32%) of the cases were diagnosed before and 68% after the whole blood collection. Twelve % of the cases were adenocarcinomas, 13% were small cell carcinomas, 54% were squamous cell carcinomas, and 21% were of other or unspecified histological subtype. DNA samples for each matched pair were positioned next to each other within batches for analysis. Eight separate, blinded, quality control samples from the same individual were included and randomly inserted into within the batches.

**Genome-wide DNA Hypomethylation Assay.** DNA was isolated from whole blood samples as described previously (22). All DNA preparations were of high molecular weight ( $>20$  kB by gel electrophoresis) and of high purity (260:280 spectrophotometric ratio  $>1.8$ ). Genome-wide DNA methylation was determined by a modification of the *in vitro* methyl acceptance capacity method described previously (23). Briefly, 2  $\mu\text{g}$  of

DNA was incubated with 5  $\mu\text{Ci}$  (184 kBq) of [*methyl*- $^3\text{H}$ ]SAM as a methyl donor, and 4 units of *Escherichia coli* SSI MTase (New England Biolabs) in 50 mM NaCl, 10 mM Tris-HCl, 10 mM EDTA, and 1 mM DTT (pH 8.0), in a total volume of 50  $\mu\text{l}$  for 3 h at  $37^{\circ}\text{C}$ . The reaction was stopped by heating to  $65^{\circ}\text{C}$  for 20 min, and then the incubation mixtures were applied onto Whatman DE-81 anion exchange filters (Fisher Scientific) using a vacuum filtration apparatus. Filters were then dried and counted in a nonaqueous scintillation fluor. The amount of radiolabel that bound to incubations lacking DNA served as a background value and was subtracted from the incubations containing DNA. All samples were run in duplicate. The manner in which this assay is performed produces a reciprocal relationship between the endogenous level of DNA methylation and the observed level of exogenous [ $^3\text{H}$ ]methyl incorporation. Prior studies have demonstrated that this method is a valid means of quantifying genomic DNA methylation (24–26).

***p53* Hypomethylation Assay.** The methylation status of specific loci within the *p53* gene was determined using a quantitative *HpaII*-PCR assay as described (24) but modified for human samples. Briefly, *HpaII* cleaves DNA at CCGG sites and cannot cut if the internal cytosine is methylated. *HpaII*-digested breaks at nonmethylated CCGG sites halts PCR amplification and thereby reduces quantitative recovery of PCR product; the amount of PCR product is therefore directly proportional to the degree of methylation of CCGG sites within the locus defined by the primer pair. This method has been shown previously to be a valid means of assessing locus-specific DNA methylation and in particular, to examine methylation within specific regions of the *p53* gene (27). *HpaII*-digested lymphocyte DNA and undigested control DNA were PCR amplified with primers specific to exons 5–8; 0.25  $\mu\text{g}$  of each digested and undigested DNA was amplified using the sense primer of exon 5 (5'-CTCTTCTGCAGTACTCCCCTGC-3') and the antisense primer of exon 8 (5'-GTCCTGCTGCTTACTCGCTTAGT-3') for an expected fragment of 1661 bp in a 50- $\mu\text{l}$  PCR amplification mixture containing 2 units of AmpliTaq Gold DNA polymerase (Perkin-Elmer, Foster City, CA), 0.4  $\mu\text{M}$  of each primer, 0.2 mM each deoxynucleotide triphosphate, 148 kBq of [ $^{32}\text{P}$ ]dCTP (New England Nuclear, Boston, MA; 370 GBq/l), 2.0 mM  $\text{MgCl}_2$ , and PCR buffer (10 mM Tris-Cl, 50 mM KCl, and 0.01% w/v gelatin, pH to 8.3). The samples were initially denatured at  $95^{\circ}\text{C}$  for 10 min in a thermocycler (PTC-100; MJ Research, Waltham, MA). Thereafter, the PCR amplification mixture was denatured at  $95^{\circ}\text{C}$  for 30 s, annealed at  $66^{\circ}\text{C}$  for 45 s, and extended at  $72^{\circ}\text{C}$  for 2 min for total of 28 cycles. For the PCR of exons 2–4, the sense primer of exon 2 (5'-TCCTCTTGCAGCAGCCAGACTGC-3') and the antisense primer of exon 4 (5'-CTCAGGCAACTGACCGTGCAAG-3') were used for an expected fragment of 716 bp. The PCR conditions of exons 2–4 were the same as those of exons 5–8, except 37 cycles were used.  $^{32}\text{P}$ -labeled PCR amplification product from each DNA sample was separated on 2% NuSieve agarose gel (FMA BioProducts, Rockland, ME). After ethidium bromide staining, the radiolabeled single band was cut from the gel, transferred to scintillation vials in 2 ml of  $\text{H}_2\text{O}$ , melted by microwave heating, and measured by scintillation counting in a nonaqueous scintillation fluor.

The results are expressed as the dpm of  $^{32}\text{P}$  PCR product recovery. The extent of internal cytosine methylation at the CCGG sequences within the exons was assessed by comparing the radioactivity of the *HpaII*-treated product with that of the control (*i.e.*, non-*HpaII*-treated product). Exons 5–8 of the *p53* gene has 43 CpG sites, 6 of which are CCGG and would be

**Table 1** Median and interquartile range of selected factors according to lung cancer case status, ATBC study, Finnish men

	Median and (interquartile range)		<i>P</i> <sup>a</sup>
	Cases ( <i>n</i> = 100)	Controls ( <i>n</i> = 100)	
Age (yr)	59 (56–63)	59 (56–63)	0.88
Body mass index (kg/m <sup>2</sup> )	25.4 (23.4–28.2)	27.1 (23.9–28.8)	0.07
Smoking			
Years smoked	42 (37–45)	40 (35–44)	0.006
Cigarettes/day	20 (19–25)	20 (15–25)	0.12

<sup>a</sup>Based on the Wilcoxon rank sum test of differences.

recognized by *HpaII*; exons 2–4 has 17 CpG sites, 3 of which are CCGG. Demethylation at any one CCGG site would halt PCR amplification. The results are expressed as the ratio of digested:undigested product.

**Statistical Analysis.** All statistical analysis were performed using Statistical Analysis Systems (SAS) software (SAS Corp., Carey, NC). Wilcoxon rank sums were used to test for case-control differences in the distribution of study factors. Genome-wide hypomethylation was evaluated as a continuous variable categorized into quartiles based on the distribution of methylation (dpm/2  $\mu$ g DNA) among the controls. The CV for the genome-wide DNA methylation assay was 15.6%. Given this CV and our samples of 100 cases and controls, we had 80% power (two-sided  $\alpha = 0.05$ ) to detect a difference in genome-wide methylation between cases and controls if the difference was 18% or greater. *p53*-specific hypomethylation was evaluated as a dichotomous categorical variable (high/low) based on a *HpaII*-digested:undigested ratio of 0.75 or less. The ratio cutoff point was determined based upon the assay variability measured among eight separate quality control samples originating from a single individual. A value of twice the SD was used (the mean and SD of the quality control ratios was  $1.00 \pm 0.13$ ). The CV was 12.5% for the *p53*-specific assay. With a sample size of 100 cases and 100 controls and a two-sided  $\alpha = 0.05$ , our study had 80% power to detect an OR of 2.4 The lung cancer-DNA hypomethylation association was evaluated using conditional logistic regression models and is expressed as OR and CI. Potential confounders were assessed by evaluating whether their inclusion in regression models changed the ORs by >15% or led to a significant change in the likelihood ratios ( $P < 0.05$ ). To test for the linear trend of genome-wide methylation, we used the continuous term in the regression models.

Differences in DNA hypomethylation status by tumor characteristic were assessed. The Wilcoxon test was used to compare the differences in the median levels of genome-wide methylation between subgroups. The difference in number of subjects with *p53*-specific hypomethylation between subgroups was evaluated using the  $\chi^2$  test. To determine whether the timing of blood collection relative to case diagnosis (pre- or post-) influenced our risk estimates, we conducted an analysis stratified by whether cases were diagnosed before and after blood collection. Within each stratum, the excluded cases and their matched controls were dropped from the analysis. All *P*s were two-sided and considered statistically significant if  $P < 0.05$ .

## Results

The distributions of established lung cancer risk factors for cases and controls are described in Table 1. The cases had lower body mass index and had smoked for a longer time. Because of

study matching, there were no differences in intervention group assignment or age (data not shown).

The distribution of genome-wide DNA methylation according to case status is presented in Fig. 1. Degree of methylation was measured as incorporation of methyl groups using the *in vitro* methyl acceptance capacity assay expressed as dpm. Using this technique, higher values indicate lower degrees of DNA methylation, or hypomethylation. There was marked variability in DNA methylation among subjects, with up to 40-fold differences were detected. The distribution of DNA methylation status was similar for the cases and controls, although more extreme hypomethylation was prevalent among the cases.

The association between genome-wide and *p53*-specific DNA hypomethylation status and risk of lung cancer is shown in Table 2. In a matched analysis that controlled for smoking, the degree of genome-wide hypomethylation did not appear to be related to lung cancer risk, with no apparent trend across quartiles of hypomethylation status. Because the aberrations in DNA that lead to carcinogenesis are thought to be highly site specific, we also assessed hypomethylation of the *p53* gene (Table 2). Hypomethylation was evaluated as a dichotomous categorical variable of high/low based on the *HpaII*-digested:undigested ratio (see “Subjects and Methods”). Thirty-two (32%) of the cases had *p53* hypomethylation in exons 5–8 compared with 20 (20%) of controls ( $P = 0.04$ ). In a matched analysis also controlling for smoking, hypomethylation of the *p53* gene in exons 5–8 was associated with a >2-fold increased risk for lung cancer (Table 2). We also evaluated hypomethylation of exons 2–4, a region of *p53* considered to be less functionally relevant and having far fewer genetic alterations in cancers compared with exons 5–8. We found that hypomethylation of *p53* exons 2–4 occurred in 26% of the subjects (with no differences between cases and controls), and that there was no association with lung cancer risk (Table 2).

The joint effects of *p53* exon 5–8 and genome-wide or *p53* exon 2–4 hypomethylation status were determined. Genome-wide hypomethylation was categorized into high and low groups with the three lowest quartiles comprising the low status group and the highest quartile comprising the high status group. As shown in Table 3, the joint effect associated with high status for both *p53* exons 5–8 and genome-wide hypomethylation was substantially higher than what would be expected from the combination of the two independent effects, suggesting a synergy between the two. This finding may be attributable to chance alone because the numbers in the high/high cell are small and the interaction is not statistically significant ( $P = 0.21$ ). By contrast, no suggestion of synergy was observed for the joint effect for high *p53* exons 5–8 and 2–4 hypomethylation.

To determine whether DNA hypomethylation status was related to tumor characteristics, we stratified by tumor histological subtype and stage and compared differences in mean levels of genome-wide and proportional differences in *p53*-specific hypomethylation between the case subgroups and controls. We found there were no differences for any of the tumor characteristics evaluated in either type of methylation measure (data not shown). Because the whole blood was collected late in the study, we also assessed whether the *p53* exons 5–8 hypomethylation-lung cancer associations differed between cases diagnosed before (“retrospective”) and after (“prospective”) collection and found no differences in the risk estimates between the two subgroups: OR, 2.50 (95% CI, 0.50–12.89) and OR, 2.13 (95% CI, 0.92–4.92) for “retrospective” and “prospective” cases, respectively.

Fig. 1. Genome-wide DNA methylation according to lung cancer case status, ATBC study, Finnish men. Level of DNA methylation was determined using the *in vitro* methyl acceptance capacity assay and measured as DPM [ $^3\text{H}$ ]SAM/2  $\mu\text{g}$  DNA. Higher numbers indicate lower degrees of methylation, or hypomethylation. Smooth lines, estimated normal distributions and blocks are frequency distribution histograms based on the quartiles of genome-wide methylation among the controls.

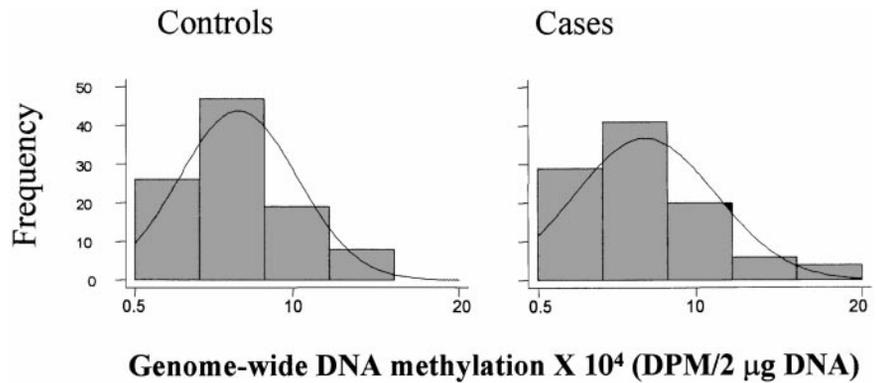


Table 2 Association between peripheral blood DNA hypomethylation and lung cancer, ATBC study, Finnish men

Hypomethylation	Cases n (%)	Controls n (%)	OR (95% CI) <sup>a</sup>
Genome-wide <sup>b</sup>			
0.5–4.1	25 (25)	25 (25)	Reference
4.2–6.6	25 (25)	25 (25)	0.98 (0.43–2.27)
6.7–8.5	21 (21)	25 (25)	0.83 (0.33–2.06)
8.6–20.0	29 (29)	25 (25)	1.25 (0.48–3.21)
<i>p53</i> exon 5–8 <sup>c</sup>			
Low	68 (68)	80 (80)	Reference
High	32 (32)	20 (20)	2.20 (1.04–4.65)
<i>p53</i> exon 2–4 <sup>c</sup>			
Low	74 (74)	74 (74)	Reference
High	26 (26)	26 (26)	1.00 (0.51–1.96)

<sup>a</sup> OR and 95% CI adjusted for age, intervention group assignment, years of smoking, date of blood draw, and study clinic.

<sup>b</sup> Genome-wide hypomethylation expressed as ( $\times 10^4$  dpm/2  $\mu\text{g}$  DNA) determined using the *in vitro* methyl acceptance capacity assay, with the higher numbers representing a greater degree of hypomethylation.

<sup>c</sup> *p53* gene hypomethylation determined using the *HpaII*-PCR assay, with “High” referring to a higher degree and “Low” to a lower degree of hypomethylation based on the *HpaII* digested:undigested ratio of <0.75.

## Discussion

The purpose of this study was to identify whether hypomethylation of DNA, a phenomenon commonly observed early in human carcinogenesis, was related to the development of lung cancer. We hypothesized that methylation status in peripheral lymphocyte DNA would reflect status in lung tissue DNA and that individuals possessing diminished DNA methylation would be at increased risk for lung cancer. To our knowledge, this is the first study that has sought to establish a link between DNA methylation status of peripheral blood lymphocytes with an epithelial cancer. To explore such a relationship, we evaluated both genome-wide and *p53* gene-specific methylation. The latter was evaluated because cancer-associated aberrations are found to be highly site specific and because anomalies of the *p53* gene are strongly associated with carcinogenesis in the lung (16). There was marked interindividual variability in genome-wide methylation status with up to 40-fold differences between the highest and lowest subjects. Nevertheless, there were no significant differences between lung cancer cases and controls, even after controlling for potential confounding factors.

On the contrary, we did observe a 2-fold rise in lung cancer risk in association with exon-specific hypomethylation in a highly conserved region of the *p53* gene (exons 5–8) that is known to contain the majority of the mutations described for

Table 3 Joint effects of *p53* exons 5–8 and genome-wide or *p53* exons 2–4 hypomethylation of peripheral blood DNA on lung cancer, ATBC, Finnish men

Hypomethylation	<i>p53</i> exons 5–8 hypomethylation <sup>a</sup>	
	Low	High
Genome-wide <sup>b</sup>		
Low (Q1–Q3)	1.00 (reference)	1.08 (0.39–3.03)
No. of cases/controls	55/62	13/18
High (Q4)	1.02 (0.39–2.68)	5.13 (1.21–21.80)
No. of cases/controls	16/13	16/7
<i>p53</i> exon 2–4		
Low	1.00 (reference)	2.43 (0.99–5.97)
No. of cases/controls	48/59	26/15
High	1.00 (0.43–2.24)	1.27 (0.31–5.18)
No. of cases/controls	20/21	6/5

<sup>a</sup> Genome-wide hypomethylation expressed as ( $\times 10^4$  dpm/2  $\mu\text{g}$  DNA) determined using the *in vitro* methyl acceptance capacity assay, with the higher numbers representing a greater degree of hypomethylation. Subjects in the lowest three quartiles of hypomethylation were classified as low ( $0.5\text{--}8.5 \times 10^4$  dpm/2  $\mu\text{g}$  DNA) and those in the fourth quartile ( $8.6\text{--}20.0 \times 10^4$  dpm/2  $\mu\text{g}$  DNA) were classified as high.

<sup>b</sup> *p53* gene hypomethylation determined using the *HpaII*-PCR assay, with “High” referring to a higher degree and “Low” to a lower degree of hypomethylation based on the *HpaII* digested:undigested ratio of <0.75.

this gene in human carcinogenesis (28). In contrast, methylation of a region of the gene just a few exons away that uncommonly contains cancer-associated mutations (exon 2–4), and which possesses less functional relevance, was not associated with cancer risk.

Aberrant methylation, including promoter-region hypermethylation and genome-wide or gene-specific hypomethylation, has been demonstrated in tumors at almost all sites, but whether it is a cause or a consequence of cancer has not been established. Experimental evidence from other studies does support a causal role. For example, DNA hypomethylation was induced in hepatic tissue after chronic dietary deficiency of lipotropes (*i.e.*, methyl group donors) and preceded tumor formation in rodents (7, 11); and in other rodent studies, induction of hypomethylation by administration of drugs that inhibit DNA MTase activity, such as 5-aza-cytidine, was associated with increased lung, colon, and other tumors (12). The phenomenon of DNA hypomethylation not only occurs early in animal models of carcinogenesis but often precedes, and perhaps even initiates, DNA hypermethylation. Both genome-wide and *p53*-specific hypomethylation was found in preneoplastic nodules from hepatic tissue in mice fed a prolonged diet deficient of methyl group sources (11). The sites within the *p53*

gene that were unmethylated in preneoplastic tissue were “remethylated” in cancer tissue, and hypermethylation of the promoter region was observed. In related rodent studies, DNA hypomethylation as well as depleted SAM levels appeared to induce DNA MTase activity and DNA hypermethylation (29, 30).

In addition to diet, other environmental factors and genetic components are likely to influence an individual’s DNA methylation status. For example, carcinogens and metals such as arsenic or selenium have been shown to alter DNA MTase activity and DNA methylation in cell lines and in laboratory animals (31, 32). Additionally, a genetic predisposition toward aberrant methylation, which seems to impact on cancer susceptibility, has been demonstrated in several rodent models (11, 33). More recently, preliminary data indicate that a common polymorphism in the human methylenetetrahydrofolate reductase gene, which has been associated with a 40% reduction in colon cancer risk (34), is associated with systemically detectable anomalies in genomic DNA methylation (35).

The biological relevance of *p53* hypomethylation to carcinogenesis has not been established, although several mechanisms have been proposed. The induction of hypomethylation has been associated with the development of DNA strand breaks (24, 36), which in turn are highly related to transcriptional efficiency, cellular transformation, and mutagenesis (37). Another potential mechanism may be through altered *p53* gene expression because methylated CpG sites have been demonstrated to affect DNA-protein interactions (38) and chromosomal conformation (5), both of which would affect gene expression.

It is not difficult to reconcile our observation of an association of lung cancer risk with *p53*-specific but not genome-wide hypomethylation. We postulate that the *p53* assay may be more specific and biologically relevant to the underlying processes, and this is in agreement with several animal models of carcinogenesis. A loss of DNA methylation was shown to occur in a specific manner, with *p53* hypomethylation being an earlier and more sensitive phenomenon among rats fed either a methyl-deficient diet (17, 18) or a diet deplete only in folate (24). Furthermore, in related studies, restoration of adequate levels of methyl group sources after severe deficiency reversed genome-wide but not gene-specific hypomethylation (17). Why we observed an association specifically with exons 5–8 is not clear, but one explanation is that exons 5–8 hypomethylation is a better indicator of methylation status. Differential sensitivity to methylation at CCGG sites within the *p53* gene itself has been shown to occur, with some sites being more prone to demethylation (18). This was thought to be attributable to differences in accessibility of CCGGs to methylation based on DNA conformation and/or the binding of DNA-binding proteins.

The assessment of both *p53* gene-specific as well as genome-wide DNA hypomethylation is a strength of this study, providing important insights regarding the specificity of our observations. The two measures may represent two distinct biomarkers of different methylation functions, regulated by different processes. Our findings suggest a potential interaction between *p53* exons 5–8 and genome-wide hypomethylation. Whether cross-tabulation of the two measures served to better classify hypomethylation status or there is synergistic effect between the two types of hypomethylation is not known. Nevertheless, the evaluation of the methylation status of other genes associated with lung cancer development, such as *c-myc*, would be interesting to pursue and might serve to improve our under-

standing of the mechanisms of carcinogenesis in the lung as well as improve hypomethylation status classification.

It is not possible to tell from these data whether DNA methylation status in lymphocytes reflects what is occurring within the lung. Tissue-specific variation in DNA methylation status in humans has not been adequately explored; however, animal models indicate that DNA methylation status and DNA MTase activity are modulated differentially between various lung cell types. Lung cancer-susceptible mice exposed to tobacco carcinogens exhibited increased DNA MTase activity and a concomitant rise in DNA methylation in alveolar type II cells but not Clara cells (11).

One potential limitation of this study is the inclusion of “retrospective” blood specimens relative to diagnosis of lung cancer and the limited time window between blood collection and diagnosis of the “prospective” cases. The methylation patterns may not reflect a predisease condition in this group, and it is possible that chemotherapeutic drugs and any changes in dietary or smoking habits may have altered the methylation status. However, methylation patterns were not correlated with time to diagnosis, and we observed the association when we analyzed the “prospective” cases separately, suggesting that this is not the case.

The degree to which these findings can be generalized is somewhat limited in that the study population consisted of only older, long-term male smokers. It is likely that DNA hypomethylation may play a role in the development of other cancers (e.g., colon and hepatic cancers); however, our initial interest in this area was in part to explore potential mechanisms that might elucidate why some, but not all, smokers develop lung cancer. Our reasoning for a role of methylation status in smokers was based upon experimental models, suggesting that alterations in methylation patterns are important once the cell is initiated (39, 40), as would most likely be the case in the lung tissue of many smokers. Continued exposure to high levels of carcinogens, however, could override any potential effect, and DNA methylation status may play a stronger role in nonsmokers or light smokers.

In conclusion, we have shown that *p53*-specific hypomethylation is associated with lung cancer risk and may play a role in lung carcinogenesis. Further studies exploring the mechanism behind this association, as well as evaluating the role of DNA methylation status in other populations, would be illuminating.

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