The Relationship between Gene-Specific DNA Methylation in Leukocytes and Normal Colorectal Mucosa in Subjects with and without Colorectal Tumors

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Abstract

CpG island methylation in the promoter regions of tumor suppressor genes has been shown to occur in normal colonic tissue and can distinguish between subjects with and without colorectal neoplasms. It is unclear whether this relationship exists in other tissues such as blood. We report the relationship between estrogen receptor gene (estrogen receptor α) methylation in leukocyte and normal colonic tissue DNA in subjects with and without colorectal neoplasia. DNA was extracted from frozen stored whole blood samples of 27 subjects with cancer, 30 with adenoma, 16 with hyperplastic polyps, and 57 disease-free subjects. DNA methylation in seven CpG sites close to the transcription start of estrogen receptor α was quantitated using pyrosequencing and expressed as a methylation index (average methylation across all CpG sites analyzed). Estrogen receptor α methylation in leukocyte DNA was compared with estrogen receptor α methylation in normal colonic mucosa DNA that had been previously determined in the same subjects. Estrogen receptor α was partially methylated (median, 4.3%; range, 0.0-12.6%) in leukocyte DNA in all subjects, with no significant difference between disease groups (*P* > 0.05). Estrogen receptor α methylation in leukocytes was 60% lower than estrogen receptor α methylation in normal colonic tissue (*P* < 0.001). Estrogen receptor α methylation in colonic tissue (*P* < 0.001) and smoking (*P* = 0.016) were determinants of estrogen receptor α methylation in leukocytes, independent of age, body mass index, gender, and disease status. In conclusion, there was a positive relationship between estrogen receptor α methylation in leukocytes and colonic tissue in subjects with and without colorectal tumors. However, unlike in colonic tissue, estrogen receptor α methylation in leukocytes was unable to distinguish between disease groups. (Cancer Epidemiol Biomarkers Prev 2009;18(3):922–8)

Introduction

CpG island methylation in the promoter regions of tumor suppressor genes is recognized as one of the most common and earliest molecular alterations in colorectal cancer and is associated with transcriptional silencing (1, 2). CpG island methylation in tumor suppresor genes such as estrogen receptor α and insulin-like growth factor-2 (IGF-2; refs. 2-4), human *N33* gene, and mutL homolog 1 (5, 6) also occurs as a function of age in apparently normal mucosa of individuals with neoplasia and in normal mucosa of disease-free individuals. There is evidence that tumor suppresor gene methylation is higher in subjects with colorectal polyps (adenomas and hyperplastic polyps) and cancer than in subjects without disease (7-10).

It is not known whether differences in tumor suppresor gene methylation observed between individuals with and without colorectal neoplasia extend to tissues other than the colon. Tissues such as blood or buccal epithelium may have diagnostic and prognostic potential in cancer management and potentially bypass the need for invasive procedures to obtain biopsy material. Although tumor suppresor gene methylation in a range of body fluids (including serum, urine, bronchioalveolar lavage, and sputum) has been used successfully to classify individuals with and without cancer, the DNA extracted from these samples is mainly from disseminated cancer cells (11-14). Tumor suppresor gene methylation in stool DNA (largely from exfoliated epithelial cells) has been used to identify individuals with adenomas and hyperplastic polyps (15, 16) or cancer (17) from those with no colorectal lesions.

The possibility that DNA methylation anomalies arise systemically and increase the risk for neoplasia by inducing genetic instability has not been explored. Although studies in patients with breast cancer (18) and prostate cancer (19) showed higher tumor suppresor gene methylation in blood compared with controls, it is not clear to what extent disseminated tumor cells contributed to this effect. Several studies have reported decreased genomic DNA methylation in leukocyte DNA from adenoma patients compared with controls (20-22). Decreased genomic DNA methylation is also a marker for neoplasia and coexists with increased tumor suppresor gene promoter methylation in colorectal tumors and normal-seeming colorectal mucosa of subjects with and without neoplasia. Because individuals with adenoma do not have malignant disease, it is more likely that the

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lower genomic DNA methylation observed in the blood of these individuals is the result of a systemic methylation defect.

To date, the relationship between tumor suppresor gene methylation in different tissues has not been explored in individuals with and without neoplasia. The main aim of the present study was to determine whether there is a relationship between tumor suppresor gene methylation in normal-seeming colorectal tissue and leukocytes of subjects with colorectal neoplasia (cancer, adenoma, and hyperplastic polyp) and those without disease. A secondary aim was to determine whether tumor suppresor gene promoter methylation in leukocyte DNA differs between subjects with and without disease. We included subjects with hyperplastic polyps as well as adenoma in our study because, although adenoma is regarded as the precursor lesion for colorectal cancer, recent evidence has implicated some hyperplastic polyps (which were traditionally regarded as lacking malignant potential) in colorectal neoplasia (23). We chose to investigate the estrogen receptor α tumor suppresor gene because increased methylation of the CpG island in its promoter and first exon leads to diminished or absent protein product in colorectal tumors and because it has been shown to display age-dependent increases in methylation in normal-seeming mucosa of subjects with and without neoplasia (2, 4, 7).

Materials and Methods

Study Populations. Subjects involved in this study were participants in a case-control study designed to assess the relationship between folate status and global DNA methylation in colorectal mucosa (22). Subjects were recruited at their clinically indicated colonoscopy between August 2000 and March 2001 (King's College Hospital, London, United Kingdom). Written consent was obtained from all subjects involved, and the study was approved by the research ethics committee at King's College Hospital National Health Service Trust and King's College London.

The classification for subjects with colorectal neoplasms included histologically confirmed cancer (tumor but no distant metastasis), histologically confirmed adenoma (≥1 adenoma; either tubular, tubulovillous, villous, or serrated; at current colonoscopy), and histologically confirmed hyperplastic (≥1 hyperplastic polyps at current colonoscopy, without history of adenoma). Disease-free subjects were included if they had no abnormality on full colonoscopy. Exclusion criteria included subjects with a strong family history of colorectal cancer or adenomatous polyposis coli, inflammatory bowel disease, or any history of gluten-sensitive enteropathy; and clinical and/or laboratory evidence of intestinal malabsorption, pregnancy, alcohol misuse, and the use of medication that antagonizes folate.

Before colonoscopy, information was collected about weight, height, medication, supplement use, and smoking. Fasting venous blood samples for DNA extraction were taken before colonoscopy. During colonoscopy, rectal tissue biopsies were removed from the rectum (about 12 cm from the anal verge) and immediately snap frozen in liquid nitrogen. Three mucosal biopsies were removed and immediately snap frozen in liquid nitrogen. Biopsies were removed at least 5 cm away from any lesion or mucosal abnormality. Blood and tissue samples were kept frozen at -70°C until DNA extraction.

Laboratory Methods

DNA Extraction. DNA was extracted from colorectal mucosal samples in 2001 and stored at -70°C. These were used for measurement of estrogen receptor α promoter methylation as reported in the recent study by Al-Ghnaniem et al. (7). In the present study, leukocyte DNA was extracted from the whole blood samples using the PUREGENE DNA Purification Protocol (Gentra systems). DNA size (>20 kb in all cases) was determined by agarose gel electrophoresis. None of the samples showed degradation. DNA concentration was determined by UV spectrophotometry (ND-1000, Nano Drop Technologies). The 260:280 absorbance ratios were ≥1.7 in all instances.

Bisulfite Modification. Bisulfite modification of DNA (0.5 μg/sample) was done using the EZ DNA Methylation Kit (Zymo Research), according to the manufacturer's protocol. The method involves converting unmethylated cytosine residues to uracil by deamination; the methylated cytosines are left unchanged. The bisulfite modified DNA was stored at -20°C until use.

PCR and Pyrosequencing. PCR was used to amplify sites within the CpG island found in the promoter and first exon of estrogen receptor α, including seven CpG sites close to the transcription start of the gene (+189, +192, +194, +198, +201, +207, +209). To ensure that the PCR product would proportionally represent the methylation characteristics of the template DNA, primers were targeted at CpG-free regions (forward primer, GAGGTGTATTTGGATAGTAGTAAGTT; biotinylated reverse primer, CTATTAAATAAAAAAAAACCCCC-CAA). The PCR was carried out in a 50-μL volume and included 1 μL of bisulfite modified DNA, 10 pmol of forward and reverse primer, 200 μM of each dNTP, 5 μL of 10× PCR buffer (containing 15 mmol/L $MgCl_2$), 1.5 mmol/L $MgCl_2$, 1.25 U HotStarTaq DNA polymerase (Qiagen), and nuclease-free water. The PCR amplification reactions consisted of denaturing at 95°C for 15 mins, followed by 45 cycles of 20 s at 95°C, 20 s at 54°C, and 20 s at 72°C. A final extension of 5 mins at 72°C and 4°C for infinity was done. Forty-five cycles were used to ensure that the biotinylated primers were completely consumed; otherwise, background signals could occur during pyrosequencing. Gel electrophoresis was carried out on all PCR products to ensure that there was no contamination. Ten microliters of PCR product were loaded onto a 1.5% (w/v) agarose gel and visualized using a UV transilluminator (Álpha Imager, Álpha Innotech Corporation 2000). The PCR product for estrogen receptor α was 168 bp.

The PSQ HS System (Biotage AB) pyrosequencing technology was used to quantitatively analyze the methylation at multiple adjacent CpG sites on the estrogen receptor α gene. This method has the ability to produce quantitative and reproducible methylation

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levels at multiple consecutive CpG sites rapidly (24) and allows the measurement of even small changes in methylation levels. The samples were prepared using the Vacuum Prep Tool (Biotage AB) according to the manufacturer's protocol. In brief, 2 μL streptavidin-coated beads [Streptavidin Sepharose HP, GE Healthcare (formerly Amersham Biosciences)] were used to immobilize 10 μL of biotinylated PCR products. The beads were released into the PSQ HS 96 plate containing 10 pmol sequencing primer (GAGGG(C/T)GT(C/T) GTTTA(C/T)GAGTTTA), and annealing was carried out at 80°C for 2 mins before pyrosequencing. A normal human blood donor DNA that showed low methylation (<1%) across all seven CpG sites was used as a negative control. The same DNA was methylated *in vitro* using *Sss*1 (CpG) methylase (New England Biolabs) and served as positive control. A pooled DNA sample from 10 subjects was used as a quality control. The within-run (20 quality control samples assayed on one plate) and between-run (the quality control sample assayed on four plates) coefficient of variance 1.6% and 5.3%, respectively.

Statistical Analysis. SPSS (version 14.0; SPSS, Inc.) was used to analyze the data. Differences in methylation between the seven CpG sites in all disease groups in leukocytes and colonic tissue were compared using the Friedman test. The average methylation value across all CpG dinucleotides was expressed as a methylation index (mean percentage methylation in the seven CpG sites in the sequence). The Wilcoxon signed-rank test was used to compare differences between estrogen receptor α methylation index in leukocytes and colonic tissue.

χ^2 Tests were used to compare gender and smoking status between disease groups. ANOVA with Dunnett's test (to adjust for multiple comparisons) was used to compare differences in age between groups, and Kruskall-Wallis test was used to compare body mass index (BMI) between groups because this variable was not normally distributed. Kruskall-Wallis tests were used to compare differences in estrogen receptor α methylation index in leukocytes and normal colonic mucosa between groups, and wherein a significant difference was found, Mann-Whitney *U* tests were used for subgroup comparisons (with Bonferroni corrections to adjust for multiple comparisons, correcting for the three comparisons of each disease group with the disease-free group).

The relationship between estrogen receptor α methylation index in leukocytes and estrogen receptor α methylation index in normal colonic tissue, age, and BMI were examined by Pearson *r*. A general linear model (analysis of covariance) was used to test the effect of disease status, smoking, gender, age, BMI, and estrogen receptor α methylation index in colonic tissue on estrogen receptor α methylation index in leukocytes. The effects of individual factors as well as their interactions were considered in this analysis. Estimated marginal means were calculated when necessary.

Results

Of the 156 subjects for whom data on estrogen receptor α methylation in normal colonic tissue were available (76 were disease free, 28 had cancer, 35 had adenoma, 17 had hyperplastic polyps), blood samples for DNA extraction were available from only 130 subjects (57 disease free, 27 cancer, 30 adenoma, and 16 hyperplastic polyps); therefore, only these subjects were used in the current analysis. Table 1 shows the baseline characteristics of these subjects by disease group. Subjects with cancer and adenoma were on average older than disease-free subjects ($P < 0.01$), and subjects with hyperplastic polyps were more likely to be smokers ($P = 0.05$).

Table 2 shows the percentage estrogen receptor α methylation in normal colonic tissue and leukocytes by CpG site, and the average percentage methylation across all seven sites (methylation index) by disease status. Average estrogen receptor α methylation in leukocytes was significantly lower ($P < 0.001$) compared with average estrogen receptor α methylation in colonic tissue (disease free, by 43%; cancer, by 50%; adenoma, by 71%; hyperplastic polyps, by 68%). There was heterogeneity in the percentage methylation among the seven sites in all groups for leukocytes and colonic tissue (Figs. 1 and 2). These differences were statistically significant ($P < 0.001$). The medians for the sites show a pattern that is similar in the different patient groups. Subjects with cancer, adenoma, and hyperplastic polyps had higher methylation in colonic tissue compared with disease-free subjects (by 33%, $P = 0.124$; by 99%, $P < 0.001$; and 68%, $P = 0.02$, respectively). There was no significant difference in average estrogen receptor α methylation in leukocytes between disease groups ($P > 0.05$). Subjects with cancer

Table 1. Baseline characteristics of subjects by disease status

	Disease free (n = 57)	Cancer (n = 27)	Adenoma (n = 30)	Hyperplastic (n = 16)	All (n = 130)
Age (y)*	57.5 (54.1-61.0)	68.4 (63.8-72.9)†	65.5 (61.2-69.9)‡	54.4 (47.2-61.6)	61.2 (58.9-63.6)
BMI (kg/m^2)§	24.5 (18.9-35.7)	22.8 (13.4-32.1)	25.2 (19.8-49.4)	25.2 (19.0-35.6)	24.5 (13.4-49.4)
Gender (n)					
Male	24	12	19	6	61
Female	33	15	11	10	69
Smoking (n)					
Nonsmoker	39	17	20	5∥	81
Smoker	18	10	10	11∥	49

*Mean (95% confidence interval).
†$P = 0.001$, significantly different from disease-free group (Dunnett's test).
‡$P = 0.009$, significantly different from disease-free group (Dunnett's test).
§Median (range).
∥$P = 0.05$ (χ^2 test), significantly different from disease-free group.

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Table 2. Estrogen receptor α methylation (%) in normal colonic tissue and leukocytes by CpG site and the average across all sites (methylation index) in subjects by disease status

Site	Disease free (n = 57)		Cancer (n = 27)		Adenoma (n = 30)		Hyperplastic (n = 16)	
	Colonic*	Leukocyte	Colonic †	Leukocyte	Colonic	Leukocyte	Colonic	Leukocyte
1	8.7 (0.0-29.6)	3.7 (0.0-17.4)	11.4 (0.0-41.1)	3.3 (0.0-10.4)	14.2 (3.5-28.0)	3.1 (0.0-8.8)	11.9 (4.8-28.0)	3.1 (0.0-9.1)
2	8.6 (0.0-26.0)	4.8 (0.0-30.5)	9.4 (0.0-41.5)	6.1 (3.7-23.0)	14.3 (5.5-26.5)	4.7 (0.0-16.0)	12.1 (7.7-27.1)	4.1 (2.9-18.4)
3	5.3 (0.0-23.0)	3.0 (0.0-12.9)	8.2 (0.0-33.0)	2.5 (0.0-7.5)	11.1 (3.0-25.0)	3.0 (0.0-12.5)	10.0 (5.0-24.0)	2.3 (0.0-4.3)
4	8.4 (0.0-30.2)	5.2 (0.0-14.7)	11.1 (0.0-39.9)	5.2 (0.0-11.3)	15.8 (5.6-29.8)	5.1 (0.0-9.7)	13.7 (7.2-28.7)	4.8 (0.0-8.2)
5	8.9 (0.0-31.0)	5.3 (0.0-14.7)	11.5 (0.0-43.0)	5.5 (0.0-17.7)	17.0 (6.0-30.0)	5.1 (0.0-13.9)	14.1 (7.0-31.0)	4.5 (0.0-9.1)
6	7.0 (0.0-27.4)	5.7 (0.0-18.3)	9.0 (0.0-39.3)	7.0 (0.0-15.4)	16.8 (6.8-30.3)	5.9 (0.0-15.6)	14.2 (7.8-29.9)	5.4 (0.0-8.9)
7	5.8 (0.0-23.9)	2.7 (0.0-14.7)	7.7 (0.0-33.6)	3.3 (0.0-14.5)	10.2 (3.7-21.8)	2.9 (0.0-11.0)	9.3 (4.4-22.3)	2.7 (0.0-12.5)
MI	7.2 (1.0-27.2)	4.1 (0.0-12.6)	9.6 (0.8-38.7)	4.8 (1.9-10.9)	14.3‡ (4.9-27.2)	4.2 (0.0-7.8)	12.1§ (6.3-27.2)	3.9 (2.7-6.2)

NOTE: All values are median (range).
Abbreviation: MI, methylation index.
*n = 55.
†n = 25.
‡P < 0.001, significantly different from disease-free group in same tissue (Kruskall-Wallis test followed by Mann Whitney U test with Bonferroni correction for multiple comparisons).
§P = 0.02, significantly different from disease-free group in same tissue (Kruskall-Wallis test followed by Mann Whitney U test with Bonferroni correction for multiple comparisons).

had marginally higher estrogen receptor α methylation in leukocytes (by 17%), although this was not significant after adjusting for multiple comparisons (P = 0.171).

There were significant positive correlations between estrogen receptor α methylation index in leukocytes and colonic tissue in all subjects (r = 0.306; P < 0.001; Fig. 3). When analysis was conducted in each group, the correlation between estrogen receptor α methylation index in leukocytes and tissue was significant in cancer (r = 0.570; P = 0.003) and adenoma subjects (r = 0.399; P = 0.029) but not in disease-free (r = 0.135; P = 0.325) and hyperplastic polyp subjects (r = 0.325; P = 0.220). Age and estrogen receptor α methylation index in leukocytes were significantly positively correlated for all subjects (r = 0.280; P = 0.001) but when analyzed by disease group the r was significant for the disease-free group (r = 0.273; P = 0.04) but not the other groups (cancer, r = 0.280, P = 0.158; adenoma, r = 0.165, P = 0.383; hyperplastic, r = 0.217, P = 0.419).

Analysis of covariance was used to assess the effect of disease status, smoking, gender, age, BMI, and estrogen receptor α methylation in colonic tissue on estrogen receptor α methylation in leukocytes. The effects of individual factors as well as their interactions were considered in this analysis. The model showed that estrogen receptor α methylation in colonic tissue (P < 0.001) and smoking (P = 0.016) were determinants of estrogen receptor α methylation in leukocytes. The interaction between smoking and estrogen receptor α methylation in colonic tissue was also associated with estrogen receptor α methylation in leukocytes (P = 0.002). No other significant associations or interactions were noted. The adjusted means of estrogen receptor α methylation index in leukocytes by smoking status are shown in Table 3. There was no difference in estrogen receptor α methylation in leukocytes between smokers and nonsmokers when accounting for estrogen receptor α methylation in colonic tissue.

When the analysis was conducted by individual CpG site, the model showed that estrogen receptor α methylation in colonic tissue at sites 5 and 7 were significant determinants of estrogen receptor α methylation in leukocytes (P < 0.001) and that smoking was only a significant determinant of estrogen receptor α methylation in leukocytes at site 7 (P = 0.004).

Discussion

The main aim of this study was to determine the relationship between tumor suppresor gene methylation in two different tissues in subjects with and without colorectal neoplasia. Seven CpG sites close to the transcription start of estrogen receptor α were investigated for methylation, and the average methylation across the seven sites was expressed as a methylation index, which has been previously used when assessing cytosine methylation in more than one CpG site in the same gene (7, 25). Our results showed that estrogen receptor α is partially methylated (4.3% on average) in leukocyte DNA of subjects with and without colorectal neoplasia. Although estrogen receptor α methylation in

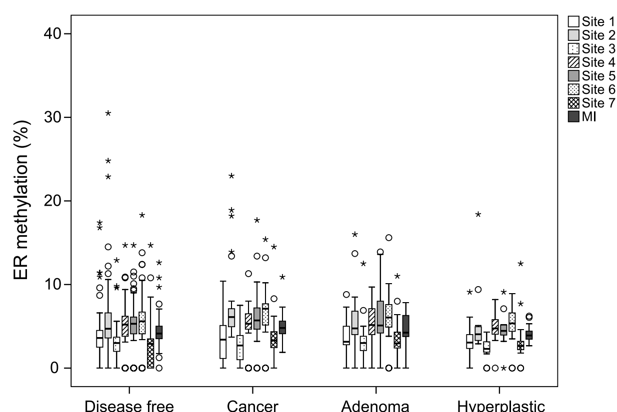

Figure 1. Estrogen receptor α methylation (%) by CpG site and disease status in leukocyte DNA. Boxes, median and interquartile range; whiskers, expected range and outliers.

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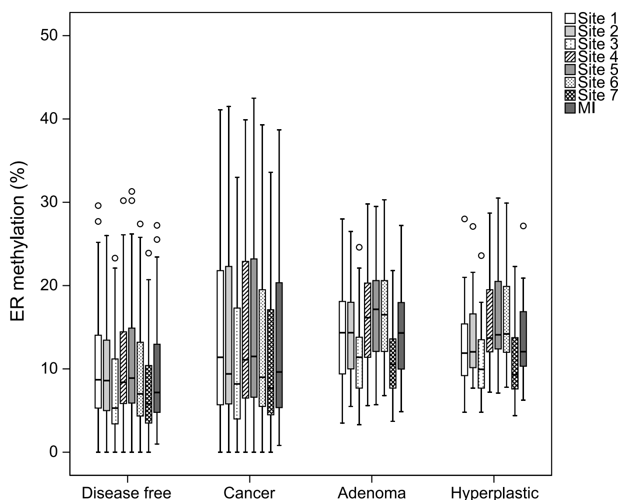

Figure 2. Estrogen receptor α methylation (%) by CpG site and disease status in colonic DNA. Boxes, median and interquartile range; whiskers, expected range and outliers.

colonic tissue was higher than in leukocytes (10.8% on average; $P < 0.001$), there was a positive relationship between methylation in the two tissues, which persisted when adjusting for various factors. This relationship was independent of disease status; therefore, this excludes the possibility that disseminated tumor DNA from subjects with cancer is responsible for the observed effect.

To our knowledge, there have been no studies that have investigated the relationship between DNA methylation in different tissues using robust statistical analysis. Studies that have measured DNA methylation in several tissues were aimed at differentiating between cancer tissues and adjacent normal mucosa and made no comparison between the normal tissues (26, 27) or investigated numerous CpG islands in various human tissues without reporting the relationship between them (28). The only study to have investigated estrogen receptor α methylation in two tissues found higher methylation in the liver than the colon (81% versus 40%) but did not report the relationship between the two (5).

The present study also aimed to determine whether tumor suppresor gene methylation in leukocyte DNA differs between subjects with and without colorectal neoplasia. In the same group of subjects, estrogen receptor α methylation in colonic tissue was higher in subjects with neoplasia compared with disease-free subjects (7). However, despite the positive association with methylation in colonic tissue, estrogen receptor α methylation in leukocytes was unable to distinguish between disease groups. This may be because of the narrower range of methylation values in leukocytes compared with colon (0.0-12.6 versus 0.8-38.7, respectively). A larger sample sizes may be required to detect significant differences between groups because subjects with cancer had marginally higher estrogen receptor α methylation in leukocytes (by 17%) compared with disease-free individuals ($P = 0.06$ before correction for multiple comparisons).

Many studies have shown that, compared with controls, individuals with cancer have higher methylation in cell-free DNA from serum (11, 14) and in leukocyte DNA (18, 19). Generally, the higher methylation in the serum of subjects with cancer has been attributed to disseminated tumor cells, although it is more difficult to extend this to leukocyte DNA methylation. Snell et al. (18) found higher BRCA1 methylation in leukocyte DNA of breast cancer patients (without BRCA1 and BRCA2 germline mutations) compared with age-matched controls. The authors attributed the effect to somatic methylation because the methodology they used to determine methylation was not sufficiently sensitive to detect low levels of disseminated cancer cells.

Somatic methylation defects arising in multiple tissues may predispose to neoplasia. This hypothesis is supported by studies investigating genomic DNA methylation in individuals with preneoplastic disease, which showed lower genomic DNA methylation in leukocytes of subjects with colorectal adenoma (21, 22) and cancer (22) compared with controls. Although studies using cancer subjects cannot exclude the possibility of disseminated tumor cells or the effect the disease itself may have on systemic methylation status, studies that have used subjects with adenoma can be more confident in concluding that the methylation anomalies are systemic and precede the development of disease because it is unlikely that adenomas would lead to disseminated cells or alter methylation levels in leukocytes. Further studies with large sample sizes and investigation of genomic and tumor suppresor gene methylation are required to clarify this issue.

It is unlikely that methylation levels of 4.3% in leukocyte DNA and 10.8% in normal colonic tissue DNA have an effect on transcription of the estrogen receptor α gene, although the range of values was wide. Studies show that expression of tumor suppresor genes is highly affected by the density of methylation rather than by the existence of methylation itself (29, 30). There were clear discernible patterns of methylation for each CpG site in colon and blood DNA that was reproducible across all subject groups. These results suggest that, within a CpG island, successive CpGs display different degrees of methylation. These findings are supported by

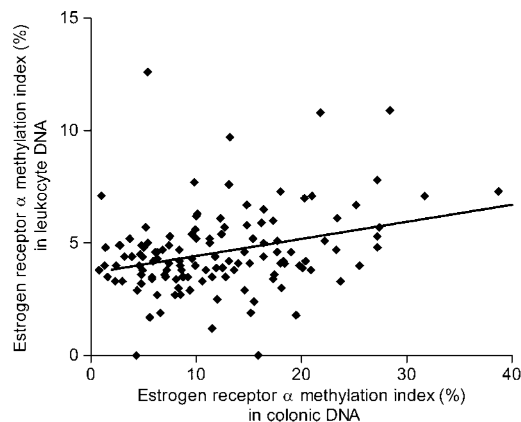

Figure 3. Estrogen receptor α methylation in colonic DNA versus estrogen receptor α methylation in leukocytes in all study participants ($r = 0.306$; $P < 0.001$; $n = 126$).

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Table 3. Adjusted marginal means of estrogen receptor α methylation index (%) in leukocytes by smoking status

Smoking status*	Value
Nonsmoker	4.498 ± 0.195 (4.11, 4.88)
Smoker	4.625 ± 0.245 (4.14, 5.11)

*All values are mean ± SEM (confidence interval). Covariates appearing in the model are evaluated at the following values: average estrogen receptor α methylation in normal colonic tissue = 12.11.

those of (31) who showed highly reproducible patterns of methylation in 61 CpG sites localized in the genomic region harboring the human imprinted genes *IGF-2* and *H19* in leukocyte DNA from four individuals. Shaw et al. (32) also showed this reproducible pattern in 79 oral squamous cell carcinoma patients (paired normal/tumor tissue) in 4 to 5 CpG sites in p16; retinoic acid receptor, beta; E-cadherin; cytoglobin; and cyclin A1. Both the above studies used pyrosequencing to quantitate DNA methylation.

There is currently no explanation for this reproducible variation in methylation in sequential CpG sites in studies that have used pyrosequencing to quantitate methylation. The variation may result from experimental artifact. Alternatively, it is possible that these methylation profiles are real and may play a physiologic role or may be the result of interference between the methylation process and the local chromatin conformation. It has also been suggested that steric hindrance of DNA methylase does not allow equally high methylation of two closely adjacent CpGs (32). In view of the uncertainty over the significance of methylation at each CpG site, it will be important to study as many sites as possible.

The factors that regulate CpG island methylation in normal tissues are largely unknown. Many studies have shown that age has some influence on DNA methylation in colonic mucosa (2, 7, 33). In the present study, there was a positive correlation between age and DNA methylation in leukocytes when all subjects were considered, although r was not significant in the disease groups, possibly because of the narrow age ranges and the smaller sample sizes. However, age was not associated with DNA methylation in leukocytes after adjusting for other factors; although we have previously reported a strong relationship between age and DNA methylation in normal colonic mucosa in these subjects (7). It seems that different factors influence DNA methylation in different tissues, although this needs confirmation in future studies. Although some studies have reported an influence of gender on DNA methylation in colonic mucosa (4) and gastric mucosa (34), our study did not show any differences in estrogen receptor α methylation between men and women. Post et al. (35) found no difference in estrogen receptor α methylation in normal right atrium tissue samples between men and women.

An interesting finding is the influence of smoking on estrogen receptor α methylation in leukocytes, which seemed to be modulated by methylation in colonic tissue. There is some evidence that smoking influences tumor suppresor gene methylation in blood. Russo et al. (36) showed tumor suppresor promoter methylation (E-cadherin, p16, O^6 methylguanine DNA methyltransferase, and death-associated protein kinase) in preneoplastic bronchial epithelium and blood of smokers, which was not present in either tissue in nonsmokers. Other studies have also shown methylation changes of p16 and death-associated protein kinase in bronchial epithelium from current and former smokers (37, 38), which were not observed in those who never smoked. The mechanism for smoking induced methylation is not known, although compounds in cigarette smoke have been shown to activate the aromatic hydrocarbon receptor (39) and activation of this receptor has been linked with methylation of the p16 and p53 promoters in cell lines (40).

In conclusion, the present study has shown a positive association between estrogen receptor α methylation in normal colonic tissue and leukocytes after adjusting for age, gender, disease status, BMI, and smoking. Future studies should assess this relationship in multiple genes using a larger sample size. Estrogen receptor α methylation in leukocytes was unable to distinguish between disease groups, precluding its potential use as a surrogate for the more invasive measurement in colorectal mucosa, although the present study may be underpowered to detect significant differences between estrogen receptor α methylation in leukocytes. Future studies should analyze the subsets of leukocytes because previous studies have found methylation differences between monocytes, granulocytes, and lymphocytes for specific genes (27, 41). From the results of the present study, it also seems that leukocyte DNA should not be used as negative control in methylation studies unless the gene in question has been previously investigated and shown to be unmethylated in leukocytes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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