Reduced Genomic 5-Methylcytosine Content in Human Colonic Neoplasia[1]

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ABSTRACT

DNA methylation appears to play an important role in both physiological and experimentally modified gene expression, and alterations in DNA methylation have been described in animal tumor models and in transformed cells and tumor cell lines. However, there have been comparatively few reports on DNA methylation in primary human malignancies, and these reports are somewhat contradictory. While individual genes have shown hypomethylation in colon cancer and premalignant adenomas as well as in lung cancer, other genes have shown increased methylation, and absolute measures of 5-methylcytosine content have shown decreases in malignancies but not in premalignant adenomas. We have used a sensitive quantitative measurement of 5-methylcytosine content by high performance liquid chromatography revealing an unequivocal hypomethylation of tumor DNA. An average of 8 and 10% reduction in genomic 5-methylcytosine content was seen in apparently all colon adenomas and adenocarcinomas, respectively, and there was no significant difference between benign and malignant tumors. This is a substantial quantitative alteration and suggests a pervasive abnormality in the control of DNA methylation. Surprisingly, three patients with the highest 5-methylcytosine content in their normal colon appear to have a germline predisposition to cancer (Lynch syndrome).

INTRODUCTION

DNA methylation is a covalent modification of cytosine that occurs at CpG doublets in vertebrate DNA (1), and is heritably maintained by a DNA methylating enzyme after DNA replication (2, 3). The pattern of DNA methylation is developmentally and tissue specific, both in overall 5-methylcytosine content (4) and in the sites that are modified at specific genes (5, 6). Methylation is an attractive candidate as a determinant of epigenetic inheritance, both in normal differentiation and in tumorigenesis, for several reasons: (*a*) almost every gene that has been studied is hypomethylated at some sites when it is expressed, despite the fact that techniques for assaying gene methylation using methylcytosine sensitive restriction endonucleases can detect only 15% of methylatable sites (5, 6); (*b*) agents that inhibit DNA methylation induce the expression of undermethylated genes (7), induce differentiation (8, 9), and are tumorigenic even at nonmutagenic concentrations (10–12); (*c*) methylation of mammalian DNA plays an important role in inhibiting gene expression in the inactive X-chromosome (13) and, perhaps, in the stability of autosomes as well (14).

DNA methylation has been measured both quantitatively and qualitatively in numerous experimental systems, including transformed cells, tumor cell lines, and animal tumor models (reviewed in Ref. 15). However, there have been relatively few studies of DNA methylation in primary human cancers, with varying results (Table 1). Gama-Sosa *et al.* demonstrated an approximately 6% overall reduction in average genomic 5-methylcytosine content when comparing a large number of

primary malignancies of various types to many normal tissues and benign neoplasms. DNA from 20 metastases showed an even greater average decrease (11%) in their mean 5-methylcytosine content relative to that of benign tumors or normal tissues (16). There was no difference in that study between benign tumors and normal tissues (16). Later studies of individual, randomly selected genes showed losses of DNA methylation at individual gene sites in colon adenocarcinoma (17, 18), premalignant colonic adenomas (18), and decreases (17) or increases (19) in small cell lung cancer (18), depending upon the genetic locus examined. However, in these studies, a quantitative estimate of overall 5-methylcytosine content by densitometric comparison of ethidium bromide-stained, electrophoresed *Hpa*II and *Hha*I digests showed no significant difference between normal and neoplastic tissue (17, 18). Comparison of 5-methylcytosine content of freshly excised pediatric tumors with that of human fibroblasts showed differences for some tumors and clearly demonstrated that DNA methylation of tumor cells in culture differs from that of primary tumors (20). Finally, some genes in colon (21) and lung (19) tumors show increased DNA methylation (Table 1).

In the present study we looked for a global quantitative alteration in DNA methylation in cancers and matched normal tissue from the same patients. Such analyses can give insights which can be missed in studies limited to specific DNA sequences whose state of methylation may not mirror most of the methylation changes in the genome. In contrast to earlier quantitative studies, we directly compared identically paired samples of human solid tumors and adjacent normal tissue, circumventing the problems of abnormalities associated with cell culture, of mixed cell populations, and of possible individual variation from person to person in 5-methylcytosine content of a given tissue type. Of all the common cancers, only colon cancer is ideally suited for such a comparison. The cell type giving rise to the tumors (adjacent normal mucosa) can be obtained to near purity and is commonly available along with the tumor. Use of the appropriate control tissue is particularly important in studying DNA methylation because of its tissue specificity. Furthermore, by choosing colon tumors the early stages of carcinogenesis (benign adenomas) are also accessible.

MATERIALS AND METHODS

Tissues. We studied 31 specimens from 12 patients, including 8 adenomas, 11 carcinomas, and the 12 matched normal mucosal samples. The patients ranged in age from 43 to 78 years, and the tumors represented both Duke's B and C pathology. Patients 8 and 9 had Gardner syndrome and patients 3, 6, and 12 appeared to have Lynch syndrome. In three cases, adenomas and adenocarcinomas were available from the same patient (Table 2). Tumors were obtained from surgical colectomy specimens or from endoscopic biopsy. The adjacent normal mucosa was stripped from the submucosa and both mucosa and tumor specimens were examined by a pathologist for confirmation of tissue type. In addition, the tumor specimens were sectioned on either side as well as in the interior portions for DNA analysis, and these sections were fixed and stained. Only specimens with 90% or more homogeneous cell populations were analyzed. The specimens were frozen in liquid nitrogen and stored at −70°C until use.

Table 1 *Studies of DNA methylation in human cancer tissues*

Tumor	DNA methylation	Type of analysis	Matched tissues	Ref.
Primary and metastatic epithelial[a] and endocrine carcinomas and lymphoma	Decrease	HPLC	No	16
Benign epithelial[a] and endocrine adenomas	No change	HPLC	No	16
Lung and colon carcinoma	Decrease	Gene probes	Yes	17, 18, 29
Colon carcinoma	No change	Restriction enzyme	Yes	17, 18
Lung carcinoma	Increase	Gene probes	No	19
Embryonal tumors	Variable decrease	HPLC	No	20
Colon carcinoma	Increase	Gene probes	Yes	21

[a] Including colon cancer.

Table 2 *Genomic 5-methylcytosine content of human colon tumors and of adjacent normal mucosa*

Patient	5-Methylcytosine content[a] (mol %)			Decrease[b] (%)	
	Normal	Cancer	Polyp	Cancer	Polyp
1	0.878	0.794		9.57	
2	0.878	0.835		4.90	
3[c]	0.891	0.761		14.59	
		0.748		16.05	
4	0.809	0.734		9.27	
5	0.833	0.814		2.28	
6[c]	0.903	0.814		9.86	
7	0.870	0.774		11.03	
8[d]	0.885		0.809		8.59
			0.830		6.21
9[d]	0.866		0.778		10.16
			0.810		6.47
			0.798		7.85
10	0.860	0.775	0.785	9.88	8.72
11	0.861	0.740	0.821	14.05	4.65
12[c]	0.923	0.840	0.822	8.99	10.94

[a] 5-Methylcytosine content as a fraction of total deoxyribonucleosides. $N_{av} \pm$ 2 SD = 0.871 ± 0.030; P_{av} = 0.807 ± 0.018; C_{av} = 0.784 ± 0.038.

[b] $([N - P]/N)_{av}$ = 7.95 ± 2.10% ($P < 0.001$); $([N - C]/N)_{av}$ = 10.04 ± 4.03% ($P < 0.001$); $([P - C]/P)_{av}$ = 0.82 ± 0.51% (not significant). Paired t test was performed as described (24).

[c] Patients with Lynch syndrome.

[d] Patients with Gardner syndrome.

Feulgen staining of 8-μm frozen sections was performed by the procedure of Davenport and McKeaver (22). Briefly, the sections were allowed to dry on slides precoated with albumin, fixed in 10% buffered formalin, and Feulgen stained with azure A. Histological sections were digitized on an International Imaging Systems Model 75 Image Processor connected to a Leitz Diaplan microscope, MTI Series 68 video monitor, and Masscomp computer.

Reagents. Nuclear P_1 and bacterial alkaline phosphatase were purchased from Boehringer-Mannheim and Sigma. An LC-18 reverse phase column was obtained from Supelcosil, Inc., and chromatography reagents were purchased from Waters.

DNA Digestion and Chromatographic Analysis. A DNA solution (0.5 μg/ml in 3 mM Tris-HCl-0.2 mM EDTA, pH 7) was denatured in a 1.5-ml centrifuge tube placed in a boiling water bath for 2 min. The DNA was quantitatively digested with nuclease P_1 and bacterial alkaline phosphatase, as described (23).

Approximately 10 μg of each tumor or normal DNA digest were chromatographed on a reverse phase column (Supelcosil LC-18 DB) by a modification of the previously described method (24) with 3 ml of 0.05 M KH_2PO_4, pH 4-2.5% methanol (buffer A); 9 ml of a linear gradient of buffer A to 0.05 M KH_2PO_4, pH 4-26% methanol (buffer B); and, finally, 3 ml of buffer B at 30°C. The absorbance at 254 nm as well as that at 280 nm was monitored in order to check the identity and purity of the peaks by their characteristic A_{280}/A_{254} ratio as well as from their retention time relative to that of 8-bromoguanosine, the internal standard. Peak areas and concentrations of deoxyribonucleosides based upon calibrated molar response factors (24) for standards in this chromatography system were determined automatically.

RESULTS AND DISCUSSION

We measured the genomic 5-methylcytosine content directly by HPLC[3] of complete enzymatic digests of DNA. This pro-

vides a most accurate and precise unbiased quantitative assessment of the minor base composition of DNA. With only 5 μg of DNA, the methylcytosine content as well as the major base composition can be determined, and we can detect a 5% decrease in a given tissue's genomic 5-methylcytosine content. This corresponds to the replacement of approximately 2.5 × 10^6 5-methylcytosine residues/haploid genome with cytosine residues (24).

The mean 5-methylcytosine content of normal superficial mucosa was 0.871 ± 0.030 (SD) mol % (Table 2). All 19 tumors examined showed a measurable decrease in 5-methylcytosine content compared to the mucosa from the same patient and to the mean value for all the normal mucosal samples. The decrease was substantial, with a reduction of 10.04 ± 4.03% for cancers and 7.95 ± 2.10% for polyps, relative to the values from matched normal mucosa (Table 2). This degree of reduction was not apparent in ethidium bromide-stained gels of electrophoresed HpaII and HhaI digests of the same samples (data not shown), probably because of the lower sensitivity of this method.

To validate these results, we first performed a statistical analysis of the measured 5-methylcytosine content among the samples. A paired t test (25), comparing variation among the samples of the individual patients (normal, cancer, and polyp) with variation among the samples of a given type, showed a statistically significant decrease from normal to cancer ($P < 0.001$) and from normal to polyp ($P < 0.001$), but no significant difference among the normal samples. Next, a repeat analysis by HPLC of 29 of the samples, representing all of the patients, showed essentially the same result, with an average reduction in 5-methylcytosine content of 8.94 ± 3.48% from normal to cancer and 9.02 ± 3.32% from normal to polyp ($P < 0.001$ by paired t analysis). There were no significant differences between the two analyses.

Finally, in order to determine whether methylation differences between tumor and normal samples could be due in part to contamination of the tumor samples by stroma, quantitative Feulgen microdensitometry was performed on frozen sections bracketing the blocks from which DNA was prepared. The sections were matched with adjacent sections stained with hematoxylin and eosin, in order to identify each cellular component of the section. This analysis revealed that the tumor samples consisted of 91.2 ± 1.6% tumor DNA and the remaining DNA was stromal, about evenly divided between lymphocytes and fibroblasts. Fibroblast DNA is slightly more methylated than colonic mucosa, and lymphocyte DNA slightly less (4). Thus, there should be no net change in our measured DNA content. However, even if the small amount of stromal DNA were due entirely to fibroblasts (or lymphocytes) alone, the total measured change in DNA content is less than 1% of the normal DNA 5-methylcytosine content, based on previously reported measurements of 5-methylcytosine content in these cells (4), and the histological measurements done here.

[3] The abbreviation used is: HPLC, high performance liquid chromatography.

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Previous qualitative analyses of specific gene sites showing hypomethylation were contradicted by other studies showing increases at other sites (Table 1). Quantitative analyses by HPLC showed decreases in methylation in some tumors (16, 20), but no change in others (16, 20), and no change in benign neoplasms (16). The present study shows that these discrepancies are due at least in part to the nature of the control tissue used for comparison. Thus, we observed a substantial decrease in 5-methylcytosine content in benign colonic adenomas when compared to adjacent normal mucosa, but this difference is obscured when samples are not compared directly in the same patient. The degree of reduction in 5-methylcytosine content in these human tumors is greater than the reduction in 5-methylcytosine content in animals fed a tumorigenic diet deficient in metabolic precursors for 5-methylcytosine (26).

We thought that Gardner syndrome patients (patients 8 and 9) might show variation from the mean level of 5-methylcytosine content in normal mucosa because of their genetic predisposition to colon malignancy. In fact there was no significant difference between their normal mucosal 5-methylcytosine content and that of other patients. However, we were surprised to find that the three patients with the highest 5-methylcytosine content in the normal mucosa of any of the patients studied (patients 12, 6, and 3; 0.923, 0.903, and 0.891 mol %, respectively) all appear to have germline mutations predisposing to cancer without polyposis (Lynch syndrome). Two of these patients (patients 12 and 6) were members of non-polyposis cancer families. Such families show apparent autosomal dominant inheritance of non-polyposis single gene mutations with autosomal dominant inheritance and may account for up to 10% of colon cancer in the general population (27). By comparison, polyposis-associated colon cancer, including Gardner syndrome, accounts for less than 1% of all colon cancer. The patient with the third highest normal mucosal 5-methylcytosine content (patient 3), while not a member of a colon cancer family, nevertheless had two primary cancers in his colon, as well as two large villous adenomas (not available for study) independently arising, and these cancers showed the greatest decrease in 5-methylcytosine content among any of the tumors studies (14.59 and 16.05%). Given the large number (four) of primary neoplasms in this patient, it is likely that he was a new mutant for non-polyposis familial colon cancer.

Colon cancer is a heterogeneous disease, and it is thought that there are at least 11 disorders that can predispose to colon cancer and other malignancies in affected individuals (28). Any investigation for possible markers for colon cancer predisposition must be simple and must require only limited amounts of material. A quantitative assay such as that presented here is ideally suited for such a purpose. While the data on familial cancer in the present study are preliminary, we plan to examine large numbers of patients in the hope of identifying subsets at risk for cancer who otherwise could not be identified.

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REFERENCES

1. Bird, A. P. Use of restriction enzymes to study eukayotic DNA methylation. II. The symmetry of methylated sites supports semi-conservative copying of the methylation pattern. J. Mol. Biol., *118:* 49–60, 1978.
2. Pollack, Y., Stein, R., Razin, A., and Cedar, H. Methylation of foreign DNA sequences in eukaryotic cells. Proc. Natl. Acad. Sci. USA, *77:* 6463–6467, 1980.
3. Wigler, M., Levy, D., and Perucho, M. The somatic replication of DNA methylation. Cell, *24:* 33–40, 1981.
4. Ehrlich, M., Gama-Sosa, M. A., Huang, L. H. Midgett, R. M., Kuo, K. C., McCune, R. A., and Gehrke, C. Amount and distribution of 5-methylcytosine in human DNA from different types of cells. Nucleic Acids Res., *10:* 2709–2721, 1982.
5. Doerfler, W. DNA methylation and gene activity. Annu. Rev. Biochem., *52:* 93–124, 1983.
6. Razin, A., and Szyf, M. DNA methylation patterns. Formation and function. Biochim. Biophys. Acta, *782:* 331–342, 1984.
7. Groudine, M., Eisenman, R., and Weintraub, H. Chromatin structure of endogenous retroviral genes and activation by an inhibitor of DNA methylation. Nature (Lond.), *292:* 311–317, 1981.
8. Jones, P. A., and Taylor, S. M. Cellular differentiation, cytidine analogs and DNA methylation. Cell, *20:* 85–93, 1980.
9. Creusot, F., Acs, G., and Christman, J. K. Inhibition of DNA methyltransferase and induction of Friend erythroleukemia cell differentiation by 5-azacytidine and 5-aza-2'-deoxycytidine. J. Biol. Chem., *257:* 2041–2048, 1982.
10. Benedict, W. F., Banerjee, A., Gardner, A., and Jones, P. A. Induction of morphological transformation in mouse C3H/10T1/2 clone 8 cells and chromosomal damage in hamster $A(T_1)C1$-3 cells by cancer chemotherapeutic agents. Cancer Res., *37:* 2202–2208, 1977.
11. Landolph, J. R., and Jones, P. A. Mutagenicity of 5-azacytidine and related nucleosides in C3H/10T1/2 C18 and V79 cells. Cancer Res., *42:* 817–823, 1982.
12. Carr, B. I., Reilly, J. G., Smith, S. S., Winberg, C., and Riggs, A. The tumorigenicity of 5-azacytidine in the male Fischer rate. Carcinogenesis (Lond.), *5:* 1583–1590, 1984.
13. Mohandas, T., Sparkes, R. S., and Shapiro, L. J. Reactivation of an inactive X chromosome evidence for X inactivation by DNA methylation. Science (Wash. DC), *211:* 393–396, 1981.
14. Schmid, M., Haaf, T., and Grunert, D. 5-azacytidine-induced undercondensations in human chromosomes. Hum. Genet., *67:* 257–263, 1984.
15. Hoffman, R. M. Altered methionine metabolism, DNA methylation and oncogene expression in carcinogenesis. A review and synthesis. Biochim. Biophys. Acta, *738:* 49–87, 1984.
16. Gama-Sosa, M. A., Slagel, V. A., Trewyn, R. W., and Ehrlich, M. The 5-methylcytosine content of DNA from human tumors. Nucleic Acids Res., *11:* 6883–6894, 1983.
17. Feinberg, A. P., and Vogelstein, B. Hypomethylation distinguishes genes of some human cancers from their normal counterparts. Nature (lond.), *301:* 89–91, 1983.
18. Goelz, S. E., Vogelstein, B., Hamilton, S. R., and Feinberg, A. P. Hypomethylation of DNA from benign and malignant human colon neoplasms. Science (Wash. DC), *228:* 187–190, 1985.
19. Baylin, S. B., Hoppener, J. W. M., de Bustros, A., Steenburgh, P. H., Lips, C. J. M., and Nelkin, B. D. DNA methylation patterns of the calcitonin gene in human lung cancers and lymphomas. Cancer Res., *46:* 2917–2922, 1986.
20. Flatau, E., Bogenmann, E., and Jones, P. A. Variable 5-methylcytosine levels in human tumor cells lines and fresh pediatric tumor explants. Cancer Res., *43:* 4901–4905, 1983.
21. Vogelstein, B., Fearon, E. R., Hamilton, S. R., and Feinberg, A. P. Use of restriction fragment length polymorphisms to determine the clonal origin of human tumors. Science (Wash. DC), *227:* 642–645, 1985.
22. Davenport, R. D., and McKeever, P. E. DNA content and marker expression in human glioma explants. Acta Neuropathol., *331:* 1–4, 1987.
23. Gehrke, C. W., McCune, R. A., Gama-Sosa, M. A., Ehrlich, M., and Kuo, K. C. Quantitative reversed-phase high performance liquid chromatography of major and modified nucleosides in DNA. J. Chromatogr., *301:* 199–219, 1984.
24. Kuo, K. C., McCune, R. A., Gehrke, C. W., and Ehrlich, M. Quantitative reversed-phase high performance liquid chromatographic determination of major and modified deoxyribonucleosides in DNA. Nucleic Acids Res., *8:* 4763–4776, 1980.
25. Snedecor, G. W., and Cochran, W. C. Statistical methods, Ed. 7, pp. 1183–1206. Ames, IA: Iowa State University Press, 1980.
26. Wilson, M. J., Shivapurkar, N., and Poirier, L. A. Hypomethylation of hepatic nuclear DNA in rats fed with a carcinogenic methyl-deficient diet. Biochem. J., *218:* 987–990, 1984.
27. Lynch, H. T., Kimberling, W. J., Biscone, K. A., Lynch, J. F., Wagner, C. A., Brennan, K., Mailliard, J. A., Johnson, P. S., Soori, J. S., and McKenna, P. J. Familial heterogeneity of colon cancer. Cancer (Phila.), *57:* 2089–2096, 1986.
28. Berini, R. Y., and Kahn, E. Clinical Genetics Handbook, pp. 244–248. New York: National Genetics Foundation, Inc., 1987.
29. Feinberg, A. P., and Vogelstein, B. Hypomethylation of *ras* oncogenes in primary human cancers. Biochem. Biophys. Res. Commun., *111:* 47–54, 1983.

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