Aging and DNA Methylation in Colorectal Mucosa and Cancer

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ABSTRACT

DNA methylation of promoter-associated CpG islands may function as an alternate mechanism of silencing tumor suppressor genes in multiple neoplasias including colorectal cancer. De novo methylation of genes appears to be an early and frequent event in most neoplasias. For the ER and IGF2 genes, we have previously shown that methylation actually begins in the normal colon mucosa as an age-related event and progresses to hypermethylation in cancer. In this study, we have determined the frequency of age-related methylation in normal colonic mucosa among the genes hypermethylated in colorectal cancer. We studied six genes, including N33, MYOD, p16, HIC-1, THBS1, and CALCA. The N33 gene showed partial methylation in normal colon mucosa, which was age-related (r = 0.7; P = 0.003 using regression analysis). Adenomas and cancers showed further hypermethylation at this locus. Similarly, the MYOD gene showed age-related methylation in normal colon mucosa (r = 0.7; P < 0.00001 using regression analysis) and hypermethylation in cancers. Age-related methylation seems to be gene specific, because p16, THBS1, HIC-1, and CALCA were not affected. Furthermore, this process may also be modulated by tissue-specific factors. Our study suggests that aging is a major contributing factor to hypermethylation in cancer.

INTRODUCTION

CGIs are areas rich in CpG dinucleotides that are found within the promoter region of most housekeeping genes (1). These islands are normally unmethylated regardless of the transcriptional status of the gene, except for genes on the inactivated X chromosome in females (2) and certain imprinted genes (3). De novo methylation of promoter-associated CGIs has been associated with the transcriptional inactivation of the gene (4) and seems to be functionally equivalent to an inactivating mutation for the silencing of multiple tumor suppressor genes (5, 6) such as p16, VHL, and RB. Abnormal CpG island methylation seems to be a frequent event in most neoplasias, including CRCs (5, 6).

Aging is one of the most important risk factors for the development of neoplasia (7). The incidence of cancer rises sharply after the age of 60 years (8). For example, CRC, which is one of the common solid human malignancies, appears at a median age of 62 years in both men and women. This risk factor has been attributed partly to the cumulative exposure to carcinogens over time and the multiple hits required for the onset of neoplasia (7). Epigenetic modifications of DNA, such as altered DNA methylation patterns, have long been postulated to play a role in aging and the associated increased incidence of neoplasia (9). However, most of the focus has been on the overall genomic hypomethylation, which has been proposed to function as a "counting mechanism" for cellular senescence (10, 11), or on regional areas of hypermethylation outside the promoter region of genes (12), such as those in c-MYC (13) or c-FOS (14, 15). The functional significance of these events is somewhat unclear at present (16).

However, hypermethylation of promoter-associated CGIs has been more clearly associated with gene silencing (5, 6). We have recently shown that the estrogen receptor (ER) gene on 6q gets hypermethylated at its promoter CGI in all colonic neoplasms (17). This process is associated with decreased ER expression that appears to start in the normal colonic epithelium as a function of age. No methylation is apparent in young individuals, but partial methylation appears in older individuals, and the ER gene is hypermethylated in all colonic adenomas and neoplasms. Thus, aging itself or age-dependent events appear to be a significant risk factor for hypermethylation of this gene. We have subsequently observed that the insulin-like growth factor II (IGF2) gene also shows age-related methylation in the colon, which eventually progresses to hypermethylation in cancers (18). These studies illustrate that aging itself may be a significant risk factor for the hypermethylation of these genes in cancers.

In the current study, we have tried to determine the frequency of age-related methylation in CRC. Our data indicate that aging-related methylation is a common event in CRCs, being present in four of the eight genes known to be hypermethylated in this tumor type, and suggest that aging is a major contributor to hypermethylation in CRC.

MATERIALS AND METHODS

Tissue Samples. Samples of all primary cancers including CRCs, metastatic liver lesions, and normal colon and liver tissues were obtained from surgical resections of patients operated on at the Johns Hopkins Hospital. The samples were immediately frozen in liquid nitrogen after resection and stored at −70°C until processing. For the separation of epithelium from stroma, freshly resected samples were lightly scraped with a razor blade to collect surface epithelium and incubated for 2 h with gentle agitation in HBSS without calcium and phosphate supplemented with 30 mM EDTA (pH 7.0). After incubation, epithelial cells were floating in the solution and collected by centrifugation. The remaining tissue contained stromal cells exclusively. The purity of each fraction was greater than 90% as determined by immunohistochemistry. Specimen collection procedures were approved by the Joint Committee on Clinical Investigation of the Johns Hopkins Hospital in accordance with the policies of the Department of Health and Human Services. Cell lines were obtained from the American Type Culture Collection (Rockville, MD).

Southern Blot Analysis. DNA was extracted using standard techniques. Five μg of genomic DNA were digested with 100 units of the appropriate restriction enzyme as specified by the manufacturer (New England Biolabs). DNA was precipitated, and the DNA fragments were separated on a 1%

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In the current study, we have tried to determine the frequency of age-related methylation for multiple additional genes that are hypermethylated in CRC. Our data indicate that aging-related methylation is a common event in CRCs, being present in four of the eight genes known to be hypermethylated in this tumor type, and suggest that aging is a major contributor to hypermethylation in CRC.

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labeled by random priming (Boehringer Mannheim) using [α-32P]dCTP (Amersham).

Statistics. All statistical analyses were performed using the Microsoft Excel software program.

RESULTS

Genes Studied. We have previously shown age-related methylation of the normal colorectal mucosa for the ER and the IGF2 genes (17, 18). In the present study, we analyzed six additional genes that have previously been shown to be hypermethylated in CRC: (a) N33, a ubiquitously expressed gene on 8p22 that was recently cloned from an area of frequent allelic loss in prostate carcinomas and has been shown to display promoter hypermethylation in several CRC cell lines associated with a lack of expression of the N33 transcript (20); (b) MYOD, a muscle-specific transcription factor located on 11p that was one of the first genes shown to be hypermethylated in immortalized cell lines and has subsequently been shown to be hypermethylated in several cancers, including bladder, breast, and CRCs (23); (c) p16, a cyclin-dependent kinase inhibitor on 9p21 with tumor-suppressor properties that has been shown to be hypermethylated in most common human cancers, including CRCs (24); (d) THBS1, a p53- and Rb-regulated angiogenesis inhibitor on 15q that we have previously shown to be methylated in sporadic CRC with MI (19); (e) HIC-1, a zinc-finger transcription factor on 17p13.3 that was cloned on the basis of the presence of a large CGI in this area that becomes frequently methylated in various cancers, including CRC (25); and (f) CALCA (calcitonin), on 11p15 that shows CGI hypermethylation in lung and CRCs as well as in leukemias (26). Control genes that were not known to be hypermethylated in CRC included c-MYC, IR (insulin receptor), CNP (C-type natriuretic peptide), c-ABL, HASH1 (human achaete scute homologue), and hMSH2.

N33 Methylation in Normal Colon Mucosa and CRCs. The N33 gene has a typical CGI spanning its promoter and first exon with multiple methylation-sensitive restriction sites, including an Eagl site, a BstHII site, and a SacII site (Fig. 1A). We investigated the status of N33 methylation in both normal colon mucosa and in CRCs.

We first examined the methylation of the N33 CGI-associated promoter in 44 normal colorectal mucosa samples from subjects ranging in age from 8 to 90 years. We used Southern blot analysis of genomic DNA that was first restricted with methylation-sensitive enzymes and then hybridized with a 5' region probe. The N33 gene showed a partial methylation in the normal colorectal mucosa that was age-related, being barely detectable in young individuals and progressively increasing in older individuals (see the examples in Fig. 1B). To better study this relationship, we used densitometric analysis to measure the relative ratio of the methylated (1.1-kb) band in each lane. The intensity of methylation was plotted as a function of age (Fig. 1C) and showed that N33 methylation increased as a function of age ($r = 0.7$; $P = 0.003$ using regression analysis). The intensity of methylation averaged 15.1% in patients less than 20 years of age ($n = 4$), 37.1% in patients 20–39 years of age ($n = 10$), and 48.0% in patients 40–59 years of age ($n = 9$), and 58.4% in patients more than 60 years of age ($n = 21$; Fig. 1C). Next we did a subset analysis comparing the extent of N33 methylation with various clinical and pathological parameters.

**Fig. 1.** Methylation status of the N33 gene CGI in normal colorectal mucosa and CRCs. A. Partial restriction map of the N33 gene showing the first exon (■) and flanking region. The CpG density of the area is plotted above the map, and the high density of CpG dinucleotides indicates a CGI. The predicted restriction fragments using HindIII/SacI1 are shown at the bottom: H, HindIII; S, SacII1; E, Eagl; Sm, Smal; B, BstHII. B, partial methylation of the N33 gene in normal colorectal tissues. Representative Southern blots of DNA from normal colorectal mucosa are shown. Left, approximate sizes of the bands (in kb). Top, age of the patient at the time the mucosa sample was obtained. All lanes show patients’ DNA restricted with HindIII, a flanking enzyme, and SacII1, a methylation-sensitive restriction enzyme, except for Lane F, which shows DNA restricted with HindIII alone. The 1.1-kb band reflects the methylation of the SacII1 site. C, methylation of N33 CGI increases with age. The percentage of N33 methylation of DNA from normal colorectal mucosa was plotted against the age of the patient including the 10 patients shown in Fig. 1B and 34 other control patients. A regression analysis of all samples gave a linear fit, as shown by the straight line, with a correlation coefficient of 0.7, which is statistically significant ($P = 0.003$). D, methylation of the N33 CGI in colonic tumors. Left, the size of the various bands (in kb) is shown. The Lane F shows DNA restricted with the HindIII flanking enzyme alone, whereas all other lanes show samples restricted with HindIII/SacII1. Lanes T1–T8 are cancers, whereas Lane T9 is an adenoma. Lanes T1–T3 and T9 are hypermethylated, Lanes T4–T6 appear to be equivalently methylated to normal colonic mucosa, and Lanes T7–T8 are cancers that appear to be hypomethylated at N33. On the right are two colorectal tumor/adjacent mucosa pairs. M, uninvolved mucosa; A, adenoma; C, carcinoma. Note that the small 0.3-kb unmethylated band is visible in only some samples, depending on the length of time the samples were electrophoresed.
There were no differences in the intensity of N33 methylation based on the gender of the patient (means: females = 44% (n = 21) and males = 50% (n = 23); P = 0.3 by t test) or the site of origin of the colorectal mucosa (means: left side = 46% and right side = 48% (n = 22 each); P = 0.7 by t test). Of the 44 cases examined, 22 were from patients without neoplasia. Age-related methylation was also observed in this group (from 10% methylation in patients less than 20 years of age to 43% methylation in patients over 40 years of age). However, control patients were significantly younger than those with neoplasia, precluding an accurate comparison of the two groups.

Next we studied the pattern of N33 methylation in 44 primary sporadic CRCs. The median age of these patients was 68 years. Based on the above data for aging and N33, the normal surrounding colon mucosa of these samples would be predicted to have a high degree of N33 methylation, averaging greater than 50%. The majority of CRCs appeared to either get more hypermethylated (20 of 43 samples) or remain equivalently methylated (19 of 43 samples) when compared to similar age-matched normal colon samples (see the examples in Fig. 1D). However, there are some tumors that are clearly hypomethylated, ranging from 2–20% methylation (4 of 43 samples) at the N33 promoter, but the data on the normal colon mucosa from these particular samples were unavailable (Fig. 1D). To further understand the extent of N33 methylation in CRCs, we studied the pattern of N33 methylation in 11 paired samples containing both cancer and normal mucosa from the same patient. The average age of these 11 paired samples was 65.9 years. As expected, the normal colon tissue from these samples showed a high degree of N33 methylation, averaging 67%. However, the paired tumor tissue from these samples showed a significantly higher amount of N33 methylation, averaging 83% (P = 0.03 using t test; see the examples in Fig. 1D). We also measured N33 methylation in 10 adenomatous colon polypos. These polypos also showed a high degree of N33 methylation, averaging 80% (n = 10; see the examples in Fig. 1D), which was equivalent to that seen in carcinomas. Finally, we had previously observed an association between MI and CGI methylation in CRC for some (P16 and THBS1) but not all (ER) genes (19). MI data were available for 24 of the CRCs analyzed. N33 methylation was not affected by the MI status of the tumors; N33 methylation averaged 68% in 14 MI+ cases and 75% in 10 MI− cases (P = 0.33). Thus, N33 hypermethylation seems to be a very early event in CRC pathogenesis, starting as an age-related event in the normal colorectal mucosa and evolving further in adenomas and carcinomas.

**Age-related MYOD Methylation.** The MYOD gene has a large CGI of about 3.0 kb that spans its promoter as well as the first three exons of the gene. The MYOD CGI contains five SacII sites as well as multiple Smal sites (Fig. 2A). We looked at the methylation status of the MYOD gene CGI in normal colorectal mucosa and CRCs using Southern blot hybridization. There were 37 samples of normal colorectal mucosa of various ages, ranging from 8–90 years. Throughout its entire CGI, the MYOD gene had age-related partial methylation in the normal colorectal mucosa samples as measured by two different methylation-sensitive restriction enzyme sites (see the examples of Southern blots in Fig. 2B). The intensity of MYOD methylation was most dense within the heart of the CGI in exon 1 but also involved the CpG dinucleotides near the transcription start site in an age-related fashion, as indicated by the 1.7-kb band in Fig. 2B. To quantify this relationship, the intensity of methylation at the SacII site closest to the transcription start site, designated as S1 on Fig. 2A, was measured by densitometric analysis and plotted as a function of age. As shown in Fig. 2C, the MYOD gene promoter showed increased methylation of its CGI as a function of age (r = 0.7; P < 0.00001 using regression analysis) at the S1 site. The intensity of methylation averaged 3.5% in patients less than 20 years of age (n = 4), 5.5% in patients 20–39 years of age (n = 6), 11.8% in patients 40–59 years of age (n = 10), and 20.0% for patients more than 60 years of age (n = 17; Fig. 2C). The methylation of the SacII sites within exon 1, namely S2 and S3,
also showed an age-related increase, as demonstrated by multiple higher molecular weight bands on Southern blot analysis (Fig. 2B). In fact, when we considered the methylation of either S1, S2, or S3, the MYOD CGI showed a striking age-related methylation \((r = 0.9; P < 0.00001\) using regression analysis): the intensity of methylation averaged 28.2% in patients less than 20 years of age \((n = 4)\), 39.8% in patients 20–39 years of age \((n = 6)\), 61.7% in patients 40–59 years of age \((n = 10)\), and 70.3% in patients more than 60 years of age \((n = 17)\). On subset analysis, there was no difference in the intensity of MYOD methylation at the S1 site based on gender \((\text{means}: 13.5\% \pm 2.1\%\); for females \((n = 18)\) and 13.7% \pm 2.1%\) for males \((n = 19)\); \(P = 0.9\) by \(t\) test). However, there was a marginally significant higher intensity of MYOD methylation at the S1 site in samples originating from left-sided colorectal mucosa \((\text{mean}, 14.2\%\); \(n = 24)\) compared to right-sided colorectal mucosa \([\text{mean}, 8.6\%\); \(n = 11\) \(P = 0.05\) by \(t\) test]). Of the 37 samples studied, 22 were from controls, and 15 were from patients with CRC. Age-related methylation was equivalent in the two groups.

We also studied the methylation of the Smal sites in the MYOD CGI and found a similar age-related increase in methylation at all of the Smal sites around exon 1 (examples of Southern blot, Fig. 2B). The intensity of methylation at the Smal site closest to the transcription start site, which is designated as Smal in Fig. 2A, was measured by densitometric analysis and plotted as a function of age. As shown in Fig. 2C, the MYOD gene promoter also showed increased methylation at the Smal site as a function of age \((r = 0.8; P < 0.00001\); range, 10–90 years; sample size, 23). The intensity of methylation averaged 0.7% in patients less than 20 years of age \((n = 2)\), 1.1% in patients 20–39 years of age \((n = 4)\), 6.7% in patients 40–59 years of age \((n = 7)\), and 19.9% for patients more than 60 years of age \((n = 10); \text{Fig. 2C})).

To determine the impact of age-related methylation at the MYOD locus on hypermethylation in cancer, we studied 11 cases of paired normal/tumor DNA from the same patients. In all cases, methylation was significantly more extensive in the tumors (including 4 adenomas and 10 carcinomas), with most alleles showing hypermethylation of all of the methylation-sensitive restriction enzyme sites in the area (Fig. 2D). Methylation seemed to be similar in adenomas and carcinomas, suggesting that MYOD hypermethylation is a very early event in CRC pathogenesis. We also studied an additional 20 carcinomas, and all showed high degrees of methylation (>80%), suggesting that MYOD hypermethylation is nearly universal in CRCs (see Fig. 2D). There was no difference in MYOD methylation by MI status because 100% of the tumors were hypermethylated. Finally, six of eight CRC cell lines (RKO, CaCO2, SW480, SW48, DLD1, and HT29) analyzed had more than 95% of the alleles methylated at all SacII sites in the region, whereas the remaining two (Lovo and HCT116) had more than 50% of the alleles similarly methylated. None of the cell lines had evidence of alleles completely devoid of methylation \((i.e., \text{bands at} 0.36\) and 1.1 kb; Fig. 2D). Overall, these data suggest that MYOD methylation starts in the normal-appearing colorectal mucosa as a function of age and becomes more prominent in cancers, suggesting either that the cells with preexisting methylation are selected for in the neoplastic process, or that tumors greatly extend methylation at this locus.

**Age-related Methylation Is Gene Specific.** In contrast to N33 and MYOD, none of the other genes examined showed evidence of age-related methylation using similar Southern blot analysis. In particular, THBS1 was analyzed in 47 samples of normal colon mucosa ranging in age from 20–98 years, and no methylation was observed (see the examples in Fig. 3). Similarly, HIC-1 \((n = 36); \text{range, 20–98 years})\), p16 \((n = 26); \text{range, 20–98 years})\), and CALCA \((n = 23); \text{range, 10–90 years})\) had no evidence of methylation in any normal colonic mucosa examined (Fig. 3; data not shown). Finally, we analyzed the normal colonic mucosa at several gene loci that have shown no hypermethylation in CRC, including IR, c-ABL, c-MYC, CNP, and hMSH2 (Fig. 3; data not shown). These genes were similarly unmethylated in the normal colon in 23 patients ranging in age from 10–90 years.

**Age-related Methylation Is Modulated by Tissue-specific Factors.** Whereas age-related methylation seems to be a common event within the normal colon mucosa, it is unknown whether this process is present to a similar degree in all aging tissues, or whether it is restricted to the colon alone. We therefore examined the methylation

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**Fig. 4. Age-related methylation in various fractions of the colon mucosa.** The mucosa was separated into stroma and epithelial components, and the density of methylation was calculated for each fraction. The two colonic fractions for each of the genes are plotted on the x-axis, and the average density of methylation for each fraction is plotted on the y-axis. Stroma contains primarily stromal components, whereas the epithelium contains epithelial cells only. Both N33 and MYOD show methylation in both the epithelial and stromal components, whereas the ER CGI primarily shows methylation in its epithelial component.
status of the N33 and ER promoter CGIs in different normal tissues from the same patients. In three patients for whom samples of normal liver and colonic mucosa were available, N33 methylation was higher in the colon than in the liver (74 versus 17%), which was statistically significant ($P = 0.01$ by $t$ test). By contrast, ER methylation was much higher in the liver than it was in the normal colonic mucosa (81 versus 40%; $P = 0.02$ by $t$ test). CRC that metastasized to the liver showed high levels of methylation at all loci, as expected. Thus, the age-related methylation of genes such as N33 and ER is modulated by tissue-specific factors.

We next determined the specific patterns of methylation within the colonic mucosa itself. The normal colon is composed of both epithelial and stromal components, and it was unclear which fractions are involved in age-related methylation. Therefore, we separated the epithelial and stromal components of colonic mucosa to determine the differences in methylation patterns between the components in genes showing age-related methylation. The N33 gene showed similar levels of methylation in both epithelium (mean, 60%) and stroma (mean, 75%) in four samples. MYOD also showed equivalent levels of methylation in both the epithelial (mean, 20%) and stromal components (mean, 19%; $P = 0.8$) in two samples. This was in marked contrast to the ER gene, which showed four times higher methylation in the colonic epithelium (mean, 31%) than in the stroma (mean, 8%) in five cases, and this difference was statistically significant ($P = 0.02$; data summarized in Fig. 4).

**Concordancy of Age-related Methylation.** ER, N33, and MYOD all show age-related methylation in the normal colon. Therefore, we looked at the concordancy of methylation of these three genes. Data on the methylation status of all three genes was available for 16 samples of normal colonic mucosa. As shown in Fig. 5, whereas the amount of methylation varied for each gene, the overall pattern of age-related methylation was very similar for all three genes. Of the 16 cases, 4 appeared to have concordant deviations from the average: 1 case (a 30-year-old man) had relatively high methylation levels for all three genes, whereas 3 cases (all of whom were over 60 years of age) had relatively low methylation levels for all three genes.

**DISCUSSION**

In the current study, we have shown a strikingly similar pattern of age-related methylation in normal coloreal mucosa and frequent hypermethylation in CRC affecting the N33 gene on chromosome 8p and the MYOD gene on chromosome 11p. In fact, of eight genes that are known to be hypermethylated in CRC, four (N33, MYOD, ER, and IGF2) display this remarkable pattern of age- and neoplasia-related methylation. Interestingly, all four of these genes are very frequently (>90%) hypermethylated in CRC, whereas the methylation frequency of the other genes ranges from 5–38%. These data suggest that many hypermethylation events observed in cancer are related to the fact that neoplasia begins in aging cell populations.

Although we have limited our observations to CRC in this study, our data should have important implications for all hypermethylation events in cancer. In fact, in a recent study of hypermethylation of multiple genes in glioblastoma multiforme, we found a striking concordance in the methylation status of ER and N33, which were both much more frequently methylated in the tumors of older patients (27). By contrast, just as seen in the colon, p16 and THBS1 methylation was not related to the age of the patients studied. Furthermore, HIC-1, which is frequently methylated in prostate cancer, is also partially methylated in the normal prostate as well, and preliminary observations suggest that this methylation is also age related. An important point to note in these analyses is that the “normal” tissue adjacent to cancer may not always be representative of the cell populations that are predisposed to neoplasia. For example, normal breast tissue is largely composed of supportive stromal cells and is unmethylated at the HIC-1 locus, whereas purified breast epithelium is highly methylated at this CGI. This factor may lead to an underestimation of the contribution of age-related methylation in normal tissues to the ultimate frequency of methylation in neoplasia.

These observations raise the question of whether all hypermethylation events in cancer are related to initial methylation in normal tissues, whether age related or stem cell related. Using the very sensitive methylation-specific PCR (MSP) technique, which can detect a methylated cell diluted with $10^7-10^8$ normal cells (28), we were unable to find methylation of the p16 gene in coloreal mucosa; similarly, we found no evidence of p15 gene methylation in purified hematopoietic progenitor cells (29). Thus, there seem to be two distinct types of CGI methylation events in cancer: (a) methylation that can be detected in both normal aging cells and neoplastic cells; and (b) methylation that can only be detected in the neoplastic cells.

At present, it is unclear why only certain genes show age-related methylation changes. This process seems to be tissue-specific and involves both genes that are expressed in the colon (ER and N33) and genes that are not expressed or are expressed at low levels (IGF2 and MYOD). Age-related methylation of the ER gene may provide a selective growth advantage for the normal colonic cells, but this does not seem to hold true for MYOD or N33. MYOD is not expressed in the normal colon, and N33, which seems to be an oligo-saccharyl-transferase enzyme, has not been shown to affect the growth of cancer cells in vitro. Thus, it is not readily apparent why the loss of N33 gene expression would provide a selective advantage for tumor growth. Furthermore, age-related methylation does not seem to be a simple random event with selection of affected cells (based on a growth advantage), because hypermethylation is not seen for the p16 locus in normal colon. It seems possible, therefore, that different CGIs have different intrinsic rates of de novo methylation and protection against

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methylation, and that the weakly protected islands are those that display age-related methylation. Thus, age-related methylation of genes may initially arise from local triggering factors (chromatin structure or the proximity of Alu sequences) and be modulated by trans-acting factors such as described in the APRT gene promoter, where the CGI is protected from methylation by a cluster of binding sites for the Sp1 transcription factor (30). It is also possible that for some genes, this protection against de novo methylation may be lost during aging. Additionally, several factors have been shown to modulate CGI methylation in cancers such as defects in DNA repair systems (19) or different types of carcinogenic exposure (31). There may also be genetic differences in the rate of age-related methylation between different individuals, as suggested perhaps by the fact that a few patients in our study appeared to have concordant differences in methylation for all three genes when compared with the age-adjusted average. Age-related methylation of promoter-associated CGI sites may then be related to both endogenous triggering factors and exogenous modulating factors, such as carcinogen exposure.

Overall, our data suggest that many genes become methylated as a function of age in the normal colon. Whereas some of the genes affected are not expressed in the normal colon and may be of little physiological relevance, others, such as the ER gene, seem to modulate growth and differentiation in the normal colon (17). Thus, methylation and the loss of expression of these latter genes may impair a growth-selective advantage to the affected cells, which then become more susceptible to acquiring further genetic defects that ultimately lead to neoplasia. Therefore, we propose that age-related methylation constitutes a type of field-defect in the colon, and that it partially explains the dramatic age-related increase in CRC incidence. This hypothesis predicts that patients with high levels of methylation in their colorectal mucosa may be at higher risk for developing colorectal adenomas and cancer, and that normal mucosa from CRC patients would have higher levels of methylation than mucosa from patients without cancer. In the current study, the limited number of patients studied precludes such an analysis, which will require a carefully designed epidemiological investigation. This hypothesis also provides a potential explanation for the important finding that the reduction of DNA methyltransferase in a mouse model of CRC results in a marked reduction in polypl incidence (32). Thus, reducing DNA-methyltransferase levels may inhibit or slow the development of age-related methylation, thus limiting the extent of the field defect and ultimately decreasing the formation of tumors.

In summary, our study shows that age-related methylation is a frequent event among genes that are hypermethylated in cancer. Age-related methylation may be one of the key events, accounting for the fact that aging is the most important risk factor for most of the common types of cancers in humans.

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