Published in final edited form as:

Cancer Prev Res (Phila). 2009 September ; 2(9): 814–822. doi:10.1158/1940-6207.CAPR-09-0054.

Concordant DNA Methylation in Synchronous Colorectal Carcinomas

Kazuo Konishi^{1,3}, Lanlan Shen¹, Jaroslav Jelinek¹, Yoshiyuki Watanabe^{1,5}, Saira Ahmed¹, Kazuhiro Kaneko³, Mari Kogo⁴, Toshihumi Takano⁶, Michio Imawari³, Stanley R. Hamilton², and Jean-Pierre J. Issa¹

¹Department of Leukemia, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

²Department of Pathology, The University of Texas M.D. Anderson Cancer Center, Houston, Texas

³Department of Gastroenterology, Showa University School of Medicine, Tokyo, Japan

⁴Promotion Center of Pharmaceutical Education, Showa University School of Pharmacy, Tokyo, Japan

⁵Division of Gastroenterology and Hepatology, Department of Internal Medicine, St. Marianna University School of Medicine, Kawasaki, Japan

⁶Department of Diagnostic Pathology, St. Marianna University School of Medicine, Kanagawa, Japan

Abstract

Epigenetic changes have been proposed as mediators of the field defect in colorectal carcinogenesis, which has implications for risk assessment and cancer prevention. As a test of this hypothesis, we evaluated the methylation status of eight genes (MINT1, 2, 31, MLH1, p16, p14, MGMT, and ESR1), as well as BRAF and KRAS mutations, in 57 multiple colorectal neoplasias (M-CRN) and compared these to 69 solitary colorectal cancers (S-CRC). There were no significant differences in methylation between M-CRNs and S-CRCs except for p14 and MGMT that was significantly higher in M-CRNs than S-CRCs (16.1% versus 9.3%; 26.5% versus 17.3%, respectively; $P < 0.05$). We found significant ($P < 0.05$) correlations for MINT1 ($r = 0.8$), p16 ($r = 0.8$), MLH1 ($r = 0.9$), and MGMT ($r = 0.6$) methylation between tumors pairs of the same site (proximal/proximal and distal/distal). KRAS showed no concordance in mutations. BRAF mutation showed concordance in proximal site pairs but was discordant in different site pairs. Histologically, eight of 10 paired cancers with similar locations were concordant for a cribriform glandular configuration. We conclude that synchronous colorectal tumors of the same site are highly concordant for methylation of multiple genes, BRAF mutations, and a cribriform glandular configuration, all consistent with a patient-specific predisposition to particular subtypes of colorectal cancers. Screening for and secondary prevention of colon cancer should take this fact into account.

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Requests for reprints: Jean-Pierre J. Issa, Department of Leukemia, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 428, Houston, TX 77030. Phone: 713-745-2260; Fax: 713-745-2261; jpiissa@mdanderson.org..

Supplementary data for this article are available at Cancer Prevention Research Online (<http://cancerprevres.aacrjournals.org/>).

Disclosure of Potential Conflicts of Interest No potential conflicts of interest were disclosed.

The adenoma-carcinoma sequence is well-accepted as a major pathway for the development of colorectal cancers (CRC; ref. 1). Genetic alterations in the adenoma-carcinoma sequence comprise two groups (2): the major group is characterized by a mechanism associated with loss of heterozygosity, which accounts for a significant proportion of tumor suppressor gene inactivation, and another group exhibits a high frequency of DNA microsatellite instability. Recent studies have proposed that epigenetics, which is distinct from genetic alterations, is an alternative mechanism in carcinogenesis (3). Sporadic CRCs frequently show epigenetic abnormalities such as DNA promoter methylation (4). CpG island methylation-associated-transcriptional silencing is a frequent and early event in the tumorigenesis of sporadic CRCs. The CpG island methylator phenotype (CIMP) represents a subset of sporadic CRCs that is characterized by a high degree of cancer-specific methylation and leads to epigenetic inactivation of tumor suppressor genes (5). CIMP-positive CRCs have significant associations with proximal location, *BRAF* and *KRAS* mutations, wild-type *p53*, and microsatellite instability (6, 7). Evidence suggests that some CIMP-positive cancers evolve along a pathway distinct from the adenoma/carcinoma sequence, which involves serrated polyps instead (8).

A field defect (field effect or field cancerization) is considered to underlie multicentricity of cancer in some patients who have multiple tumors of the same tissue type but no apparent familial predisposition to cancer (9). This concept has important implications for risk assessment and secondary prevention, as shown in studies of oral leukoplakia (10). Previous studies suggest that precursor cells that are adjacent to cancer have some of the genetic and/or epigenetic alterations that are present in cancer (11–14). Synchronous CRCs are present in 2% to 11% of patients with this disease but the molecular explanation for this finding is unknown (15). Epigenetics can be affected by aging, exposures, and inflammation and has been proposed as a mechanism underlying the field defect in CRCs (5). Indeed, MGMT methylation has been observed to mark the field defect in some patients (14). We hypothesized that a field effect may be observed more frequently in patients with multiple synchronous CRCs than solitary CRCs (S-CRC) and that this would be reflected by concordant changes in the tumors. To test this hypothesis, we evaluated methylation and mutation status in patients with multiple colorectal neoplasias (M-CRN).

Materials and Methods

Patients and samples

We examined a total of 57 synchronous primary colorectal neoplasias (44 cancers and 13 adenomas) from 28 patients. All adenomas were >10 mm in size and showed high-grade dysplasia. Synchronous multiple neoplasias were defined on the basis of modified Moertel's criteria (16) as follows: (a) Each lesion must show pathologically proven advanced neoplasia (cancer or adenoma with high grade dysplasia). (b) All lesions must be distinctly separated by intervals of normal bowel wall. (c) The possibility that one of the lesions represents a local extension or metastasis must be ruled out beyond any reasonable doubt. Of 57 tissue specimens, 50 were obtained from patients who had undergone surgery at the M.D. Anderson Cancer Center ($n = 38$) and at the Showa University Hospital ($n = 12$), whereas the remaining tissue specimens ($n = 7$) were taken using colonoscopic biopsy at the Showa University Hospital before patients received chemotherapy for stage IV CRCs. Corresponding adjacent normal-mucosa tissues were collected from each case. A series of previously reported (17) 69 S-CRCs from 69 patients was used as controls. These included 31 samples of normal mucosa adjacent to tumors. We excluded patients who have human familial predisposition to cancers such as familial adenomatous polyposis or hereditary nonpolyposis CRC. Tumors were selected solely on the basis of availability. Tissue collection was approved by patients according to institutional guidelines.

Tissue samples and DNA preparation

We used 67 frozen (45 neoplasias and 22 adjacent normal) and 18 formalin-fixed, paraffin-embedded tissue samples (12 neoplasias and 6 adjacent normal) from 28 patients with synchronous M-CRNs. Frozen tissue samples were harvested postoperatively and stored at -80°C . H&E-stained slides from frozen tissue blocks were reviewed by a senior pathologist (S.R.H) to evaluate the distribution of tumor cells. Representative tumor samples contained a minimum of 80% tumor cells. When colonic biopsy specimens were obtained from patients, we used chromoendoscopy with pit pattern classification to accurately distinguish between neoplastic and non-neoplastic area in the lesion (18, 19). DNA was extracted from the frozen tissue samples using standard proteinase K-phenol-chloroform methods.

Serial slides were obtained from archival blocks of formalin-fixed, paraffin-embedded tumor tissue. One slide was stained with H&E for microdissection. After microdissection, DNA was extracted using the QIAamp DNA mini kit (QIAGEN, Inc.).

Histologic evaluation

An H&E-stained slide from each sample was histologically examined for architectural features of the carcinomas at low-power magnification. We previously examined four distinctive glandular configurations (cribriform, corkscrew/serrated, small, and large-dilated glands) invading into the stroma of 87 CRCs and reported that the cribriform gland configuration was significantly more frequently found in CIMP-positive microsatellite-stable CRCs (20). Definition of cribriform glands was described in detail previously (20). Tumor location was classified into two groups: (a) distal, corresponding to descending and sigmoid colon and rectum; (b) proximal, corresponding to cecum, ascending and transverse colon. Other histologic findings were reviewed based on pathologic records.

Methylation-related genes and definition of CIMP

We studied eight genes overall (*MINT1*, *MINT2*, *MINT31*, *p16*, *p14*, *MLH1*, *MGMT*, and *ESR1*). We previously reported that S-CRCs can be classified into two groups, CIMP-positive and CIMP-negative by the frequency of methylation of five promoter CpG islands (*MINT1*, *MINT2*, *MINT31*, *p16*, *p14*, *MLH1*; refs. 21, 22). This required a quantitative tool, and methylation positivity was defined by a methylation density, $>15\%$ (17). CIMP was defined using six genes (*MINT1*, *MINT2*, *MINT31*, *p16*, *p14*, and *MLH1*) as described previously (17). A tumor was considered CIMP positive if two or more of the CIMP markers showed methylation. All others were defined as CIMP negative.

Bisulfite PCR and pyrosequencing analysis of DNA methylation

Bisulfite treatment was done as previously described (17). Two or 3 μL of bisulfite-treated DNA were used as a template for the bisulfite PCR. Primers and PCR conditions used for amplifying specific DNA fragments of various target genes except *MGMT* have been reported previously (17). Primer sequences and PCR condition for *MGMT* are shown in Supplementary Table S1. The protocol for pyrosequencing was described in detail previously (23). Pyrosequencing measures the methylation status of several CpG sites in a given promoter. Usually, these different sites show highly concordant methylation. Therefore, for each gene, we averaged the methylation percentage of all CpGs measured.

KRAS and BRAF gene mutations

We respectively used mutant allele-specific PCR amplification and pyrosequencing of genomic DNA to analyze samples activating mutations in codon 12 of *KRAS* and in codon 600 of *BRAF* as previously described (7, 24).

Data analysis and statistics

Pyrosequencing provides a methylation level (%), which was analyzed as a continuous variable for comparison of each gene with clinicopathologic variables, and we computed mean, ranges, and 95% confidence interval (95% CI). Z-score analysis was used to normalize the methylation data of multiple genes and allow the derivation of a mean methylation score. When analyzing multiple genes, we used the average of the Z-score for each gene. Differences in promoter methylation between two groups or associations between methylation and clinicopathologic characteristics were analyzed by the Mann-Whitney test. Correlation between methylation levels of synchronous colorectal tumors was analyzed by Spearman RANK correlation analysis. Logistic regression analysis was used to evaluate the relationship between presence of synchronous colorectal neoplasias and DNA methylation. The odds ratio (OR) and 95% CI were determined for a variety of factors. The incidence of CIMP or gene mutation and patient's characteristics were compared between tumor groups using the χ^2 test or using Fisher's exact test when testing small samples. All tests were two sided, and *P* value of <0.05 was considered statistically significant.

Results

Clinicopathologic characteristics of patients

Table 1 shows the clinicopathologic characteristics of studied patients with M-CRNs and S-CRCs. There was no significant difference in any clinicopathologic characteristics between M-CRNs and S-CRCs. Of 28 patients with M-CRNs, we identified 13 (46%) patients with two proximal, 8 (29%) patients with two distal, 6 (21%) patients with proximal and distal CRNs, and 1 patient with a proximal cancer and two adenomas that were of unknown tumor location (4%).

Methylation status and genetic alterations in multiple and solitary colorectal neoplasias

Methylation status of eight genes was studied by bisulfite/pyrosequencing. All assays were designed to study regions within 200 bp upstream or downstream of transcription start sites. These assays except MGMT were previously described (17). The results are shown in Fig. 1. There was no significant difference in methylation or mutations between carcinomas and adenomas (all of which were advanced), and they were therefore considered together. Methylation levels of p14 and MGMT were significantly higher in M-CRNs than in S-CRCs [16.1% (95% CI, 11.4–20.9%) versus 9.3% (95% CI, 5.7–12.8%); *P* = 0.0004; 26.8% (95% CI, 19.1–34.6%) versus 17.3% (95% CI, 12.7–21.9%); *P* = 0.0370, respectively]. There were no significant differences in methylation levels for all other genes between M-CRNs and S-CRCs. We classified tumors as CIMP positive or negative based on methylation of two or more CIMP-related genes (*MINT1*, *MINT2*, *MINT31*, *p16*, *p14*, and *MLH1*), and we observed no significant difference in the incidence of CIMP between M-CRNs and S-CRCs (16 of 57, 28% and 26 of 69, 38%; *P* = 0.34). When we used Z-score analysis to normalize the data of CIMP-related genes, there was no significant difference in the average of Z-scores for CIMP-related genes between M-CRNs and S-CRCs [−0.20 (95% CI, 0.81 to −1.21) and 0.17 (95% CI, 1.29 to −0.95); *P* = 0.93]. We found no significant differences in the incidence of BRAF and KRAS mutations between M-CRNs and S-CRCs (7 of 57, 12% and 6 of 69, 9%; *P* = 0.72 for BRAF mutations; 22 of 57, 39% and 36 of 69, 52%; *P* = 0.18 for KRAS mutations]. BRAF and KRAS mutations were mutually exclusive in both M-CRNs and S-CRCs. Finally, to examine the relationship between presence of M-CRNs and clinicopathologic, genetic, and epigenetic variables, we used a multivariate analysis. Logistic regression analysis using step wise method was used to calculate the relationship between synchronous colorectal neoplasias and gender, age, tumor location, methylation densities of eight genes (*MINT1*, *MINT2*, *MINT31*, *MLH1*, *p16*, *p14*, *MGMT*, and *ESR1*), and genetic alterations (KRAS and BRAF mutations). In this analysis, gender, tumor

location, and genetic alterations were shown as categorical variables, whereas age and methylation densities were as continuous variables. The odds ratio and 95% CI for the multivariate analysis, together with the P_{trend} value in methylation of each gene are shown in Table 2. This analysis revealed that p14 and MGMT methylation were associated with an increased risk of M-CRNs, whereas methylation of MINT31 was a factor for decreased risk of M-CRNs (Table 2).

Promoter methylation in normal-appearing colorectal mucosa adjacent to tumors

We examined methylation status of eight genes in normal-appearing mucosa of M-CRNs and found no samples with >10% methylation for any gene except MGMT and ESR1. Thus, we compared methylation status of MGMT and ESR1 in normal mucosa adjacent to M-CRNs with S-CRCs. There were no significant differences in mean methylation of MGMT or ESR1 of normal-appearing mucosa between patients with M-CRNs and S-CRCs [3.7% (95% CI, 2.7–4.7%) and 4.1% (95% CI, 2.2–6.0%); $P = 0.16$ for MGMT; 16.6% (95% CI, 13.3–20.0%) and 14.0% (95% CI, 11.4–16.6%); $P = 0.26$ for ESR1, respectively; Fig. 1]. When analyzed by a sensitive assay for MGMT, we observed no significant difference in the methylation level of MGMT between M-CRNs and S-CRCs [18.0% (95% CI, 11.4–24.6%) and 25.7% (95% CI, 18.0–33.5%); $P = 0.18$; Fig. 1].

Methylation status and genetic alterations in synchronous colorectal neoplasias

Figure 2 shows the methylation status of seven cancer-specific genes and genetic alterations of *KRAS* and *BRAF* in 27 patients with M-CRNs (we excluded a patient with proximal cancer and advanced adenomas that were of unknown tumor location). When we evaluated only paired tumors with the same location, mean methylation levels of p16 and p14 were higher in proximal tumor pairs than distal tumor pairs (10.6% and 1.8%, $P = 0.0061$; 22.1% and 4.8%, $P = 0.0048$, respectively). We also found a significant difference in the incidence of CIMP (10 of 26, 38% and 0 of 16, 0%; $P = 0.0067$) and the average of Z-scores for CIMP-related genes [1.11 (95% CI, 2.70 to -0.49) and -2.29 (95% CI, -1.71 to -2.87); $P = 0.0004$] between proximal and distal tumor pairs. There were remarkable correlations in methylation levels of MINT1, p16, MLH1, and MGMT between synchronous neoplasias in the same patients ($r = 0.8$, $P = 0.0100$; $r = 0.8$, $P = 0.0058$; $r = 0.9$, $P = 0.0001$; $r = 0.6$, $P = 0.0193$, respectively; Fig. 3A–D). MINT31 was rarely methylated in these tumors, which precluded concordance analysis. MINT2 and p14 showed no evidence of concordance ($r = 0.3$, $P = 0.34$; $r = 0.4$, $P = 0.12$, respectively). When we looked at only paired tumors with proximal location, we still observed significant correlations in methylation of p16, MLH1, and MGMT between proximal synchronous neoplasias in the same patients ($r = 0.8$, $P = 0.0336$; $r = 0.9$, $P = 0.0031$; $r = 0.7$, $P = 0.0273$, respectively; Fig. 3F–H). *KRAS* showed no evidence of concordant mutations between tumors. *BRAF* was only mutated in two cancers, both occurring in the same patient. We next analyzed synchronous tumors discordant for location. In these, only proximal tumors showed *BRAF* mutation and promoter methylation of all genes except MINT1 and MGMT (Fig. 2). There was no concordance in methylation or mutation in patients with tumors of different sites.

To confirm that the concordant methylation changes in tumors observed in M-CRNs is not a random event related to site or age, we turned to S-CRCs and selected 28 paired cancers from 69 patients with S-CRCs dependent on gender, age, and tumor location as shown in Supplementary Fig. S1. There was no significant correlation in methylation levels of any gene between these paired cancers (Fig. 3I–L). When we analyzed only paired proximal cancers with similar CIMP status, we still observed no significant correlation in DNA methylation of any gene between these pairs (Fig. 3M–P).

Relationship of methylation status and histologic characteristics

We have recently reported an unusual cribriform glandular configuration as a histologic characteristic of methylated microsatellite-stable CRCs (20). We were able to assess histologic findings in a total of 36 synchronous cancers (20 proximal and 16 distal cancers, 14 patients with paired cancers, and 8 patients with paired cancer/adenoma). Of these CRCs, the cribriform glandular configuration was observed in nine proximal cancers and one distal cancer (9 of 20, 45% and 1 of 16, 6%; Fig. 4). Cribriform glands were more frequently observed in cancers with CIMP than without CIMP (8 of 13, 62% and 3 of 23, 13%; $P=0.0064$; Fig. 4). However, there was no significant association between CIMP and other histologic findings such as tumor stage, mucinous histology, or poor differentiation. When we evaluated the 10 paired cancers with the same location, eight pairs showed concordant histology of cribriform gland architecture (8 of 10, 80%). Of four paired cancers with different location, however, one pair had concordant histology (1 of 4, 25%).

Discussion

Synchronous CRCs have been reported to occur in 2% to 11% of sporadic CRCs (16, 25–32). According to the literature, patients with synchronous CRCs are older than those with solitary cancers (15). Synchronous CRCs show a higher incidence of proximal location than solitary cancers (15). These features of synchronous CRCs were also found in our series. Many investigators have reported that the incidence of benign adenomatous polyps is higher in patients with synchronous CRCs than S-CRCs (15, 31, 33, 34). Also, patients with CRC and adenomatous polyps have a higher incidence of metachronous CRCs than those without polyps (1, 35). Considering that some, but not all, polyps progress to cancer, patients with multiple CRCs may have a field defect characterized by occurrence of synchronous or metachronous CRCs. Recently, we have reported on a field effect that is molecularly defined by epigenetic inactivation of MGMT in colorectal carcinogenesis (14). However, it remained unknown whether promoter methylation in other relevant genes such as MLH1 and p16 (CDKN2A) are involved in field effects for CRC development. Although uncommon, occurrence of multiple CRCs in single individuals is a unique model to investigate a field effect in colorectal carcinogenesis, and we have now found evidence of concordant methylation and BRAF mutations in these.

Our data are consistent across several genes but not all, perhaps because of the limited sample size. Importantly, they are unlikely to be explained by chance. Although chance findings due to multiple comparisons are always an issue, the primary goal of the study was to look for concordant events, and the findings were not a chance observation in the setting of a different study. Indeed, if the associations were found by chance alone, we would have expected some positive findings from the S-CRC mock pairing. Some of the associations are also very strong (R values of 0.8 or 0.9) despite the relatively small sample size. Although our results are consistent with a patient-specific predisposition that is reasonably explained by a field defect, they could in principle also be explained by genetic predisposition to specific colon cancer subtypes. However, we excluded patients with a clear family history from the study, and the median age of the patients was similar to that of sporadic colorectal tumorigenesis. We cannot exclude a familial predisposition component in some patients, but this seems unlikely to explain the high degree of concordance observed.

In this study, we divided colorectum into two subsites: proximal and distal colon. There is ample evidence that proximal and distal colon cancers are molecularly and etiologically distinct, thus justifying this division (36). M-CRNs with the same location showed significantly concordant methylation for MINT1, p16, MLH1, and MGMT, whereas M-CRNs with different location had discordant methylation. We also observed significantly concordant methylation for p16, MLH1, and MGMT between M-CRNs with the proximal

location. Proximal M-CRN pairs showed significantly higher methylation levels of p16 and p14 than distal M-CRN pairs. These observations support the idea that field effects defined by epigenetic inactivation of multiple genes may be implicated in the pathogenesis of proximal multiple CRNs, whereas a separate process that does not involve frequent DNA methylation events (perhaps chromosomal instability) is implicated in distal multiple CRNs.

Sporadic CRCs with CIMP have significant associations with proximal tumor location. In this study, all but one CIMP tumors were observed in proximal M-CRNs, suggesting that CRCs of different sites (proximal/distal) have different molecular pathways leading to tumorigenesis, resulting in discordant molecular phenotypes. Ogino et al. (37) examined DNA methylation status in 14 synchronous colorectal neoplasias (six cancer pairs and eight cancer/adenoma pairs) using MethyLight. They showed that one proximal site pair showed concordance of CIMP positivity but two different site pairs (proximal/distal) revealed CIMP in only the proximal tumor. Norrie et al. (38) found that one patient showed concordant methylation of MLH1 in 14 patients with synchronous CRCs, whereas five patients had discordant methylation. However, four of these five patients had proximal and distal CRCs, and showed MLH1 methylation in proximal CRCs but not in distal CRCs. On the other hand, distal CRCs show allelic losses more than twice as often as proximal CRCs do (39). Moreover, Eguchi et al. (40) reported higher incidence of p53 mutations in distal synchronous CRCs than in proximal ones (80% and 43%, respectively). Also, Jernvall et al. (41) indicated that mutations in the conserved regions of p53 accumulate in distal but not in proximal CRCs. It is therefore likely that the mechanism of synchronous CRCs may differ between proximal and distal CRCs in the same patients. By extension, patients with synchronous tumors of different locations (proximal/distal) likely have a distinct mechanism for their tumor predisposition.

Recently, we have reported that a cribriform glandular configuration is one pathologic characteristic of methylated microsatellite stable CRCs (20). These cancers also share some characteristics such as proximal locations, mucinous histology, and poor differentiation with microsatellite instability cancers (7, 42–44). Here, we confirmed a significant correlation between the presence of CIMP and a cribriform glandular configuration. Moreover, paired cancers with the same tumor location more frequently showed concordant histology of cribriform architecture. These results also histologically support a field defect in patients with synchronous CRCs.

The concept of field effects defined by epigenetic changes as early events predisposing to CRC predict that methylation in normal colon could correlate with risk of development of colorectal tumors. We previously reported that MGMT methylation was significantly frequently detected in colon mucosa adjacent to sporadic CRCs with MGMT methylation than those without MGMT methylation, and suggested that MGMT methylation may serve as a potential source of the field defect in colorectal carcinogenesis (14). Consistent with this, there was significant correlation in methylation level of MGMT between synchronous neoplasias in the same patients. Our results extend this concept to MINT1, p16, and MLH1, although no substantial methylation of these genes was seen in normal-appearing colon. It is possible that a more sensitive test would pick up a field defect based on these genes. However, it is also possible that the field defect is a hypothetical predisposition to developing tumors with high degrees of methylation, which would explain concordance in cancer without much methylation in normal-appearing colon mucosa. Another possibility is that synchronous neoplasias with similar tumor location arise from a single clone of precancerous cells that colonizes large portions of the colon through seeding. This has been shown in bladder cancer, for example. However, this is unlikely to explain most cases of synchronous colon cancer given that RAS mutations were often discordant in our case

series, and others have noted discordant genetic lesions when synchronous tumors were relatively far from each other (45).

Our study showed that several methylation markers showed concordant methylation in synchronous colorectal tumors but other markers did not. Some markers may be better able to show concordant methylation than others because of frequency and sensitivity. However, we also found that methylation of p14, MGMT, and lack of MINT31 methylation could be independent risk factors for M-CRNs in the multivariate analysis. Whether these methylation markers are useful for screening for M-CRNs needs to be further investigated.

Molecular concordance has important implications for cancer screening and prevention. Molecular tests are being developed to screen for colon cancer in stool or serum DNA (46). In patients with a history of colorectal tumorigenesis (cancer or advance polyp), it would seem appropriate to tailor screening for those molecular events that characterize the first tumor identified. Similarly, as we are moving toward active cancer prevention, it is appropriate to consider tumor subtype specific interventions for secondary prevention. For example, if a patient has shown a propensity to develop a tumor with a high degree of epigenetic changes, one could consider testing epigenetic-based prevention (47) after resection. On the other hand, if the first tumor is characterized by a p53 mutation, targeting that pathway for prevention might be more appropriate. Indeed, research into individualized chemoprevention should proceed in parallel to individualized therapy for cancer.

Acknowledgments

We thank Dr. Toshinori Kurahashi for reviewing clinical data and Nicole Sieffert and Hisako Nozawa for their assistance in identifying samples for this study.

Grant support: NIH grants CA098006 and CA105346. J.P. Issa is an American Cancer Society Clinical Research Professor supported by a generous gift from the F. M. Kirby Foundation.

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Fig. 1.

Distribution of methylation levels of eight genes/CpG islands in cancer (A) and colonic mucosa adjacent to cancer (B). Each dot represents the methylation level of an individual sample. Horizontal lines, mean methylation levels for each group. #, $P = 0.0004$; ¶, $P = 0.037$. M, M-CRN; S, S-CRCs. *, regular assay; **, sensitive assay.

Case No	Histology	Location	Methylation							Mutation		Histology		
			MINT1	p16	hMLH1	MGMT	MINT2	p14	MINT31	KRAS	BRAF	CGC	Muc	Type
1	C	P										-	-	MD
	C											-	-	MD
2	C	P										-	+	MD
	C											+	+	MD
3	C	P										+	+	MD
	C											+	+	MD
4	C	P										NA	-	MD
	C											NA	-	WD
5	C	P										-	-	MD
	C											-	-	MD
6	A	P										NA	NA	TV
	C											+	-	MD
7	A	P										NA	NA	TV
	C											-	+	MD
8	C	P										NA	-	MD
	A											NA	NA	AD
9	C	P										-	-	WD
	A											NA	NA	AD
10	A	P										NA	NA	TV
	C											+	-	WD
11	C	P										+	-	PD
	A											NA	NA	AD
12	C	P										+	-	MD
	C											-	-	MD
13	A	P										NA	NA	T
	C											+	-	MD

Case No	Histology	Location	Methylation						Mutation		Histology			
			MINT1	p16	hMLH1	MGMT	MINT2	p14	MINT31	KRAS	BRAF	CGC	Muc	Type
14	C	D										NA	-	MD
	C	D										NA	-	MD
15	A	D										NA	NA	T
	C	D										NA	-	MD
16	C	D										-	-	MD
	C	D										-	-	MD
17	C	D										-	-	WD
	C	D										-	-	WD
18	C	D										-	+	PD
	C	D										-	+	PD
19	C	D										-	+	MD
	C	D										-	+	MD
20	C	D										NA	-	MD
	A	D										NA	NA	TV
21	C	D										-	-	WD
	C	D										-	-	MD

Case No	Histology	Location	Methylation						Mutation		Histology			
			MINT1	p16	hMLH1	MGMT	MINT2	p14	MINT31	KRAS	BRAF	CGC	Muc	T type
22	C	P										-	-	MD
	C	D										-	+	MD
23	A	P										NA	NA	TV
	C	D										-	-	MD
24	A	P										NA	NA	AD
	C	D										-	-	MD
25	C	P										+	-	PD
	C	D										-	-	MD
26	C	P										-	-	MD
	C	D										+	-	MD
27	C	P										+	-	MD
	C	D										-	-	MD

Fig. 2.

Methylation status of seven CpG islands, mutation status of KRAS and BRAF, and histologic findings in synchronous colon tumors. Each column represents a separate gene locus indicated on top. Each row represents a synchronous colorectal neoplasm. *White*, average methylation densities less than <15%; *gray*, 15–50% methylation; *black*, methylation greater than 50%. *Black squares*, presence of mutations; *white squares*, absence of mutations. The top group is patients with two proximal CRCs, the middle group is patients with two distal CRCs, and the bottom group is patients with proximal and distal CRCs. *C*, cancer; *A*, adenoma; *P*, proximal colon; *D*, distal colon; *CGC*, cribriform glandular configuration; *WD*, well-differentiated adenocarcinoma; *MD*, moderately differentiated adenocarcinoma; *PD*, poorly differentiated adenocarcinoma; *TV*, tubulovillous adenoma; *T*, tubular adenoma; *AD*, adenoma; *NA*, not applicable.

Fig. 3.

Correlations in methylation levels of MINT1 (A, E, I, and M), p16 (B, F, J, and N), MLH1 (C, G, K, and O), and MGMT (D, H, L, and P) in synchronous tumors and sporadic cancers. We observed significant correlations in methylation of these four genes in synchronous tumors with the same location (A–D) but not in paired tumors selected from S-CRCs (I–L). Moreover, there were significant correlations in p16, MLH1, and MGMT methylation between proximal synchronous tumors in the same patients (F–H). However, we observed no significant correlations between proximal S-CRCs (M–P). PP+DD, case with two proximal tumors or two distal tumors; PP, case with two proximal tumors.

Fig. 4. Histopathology in synchronous CRCs with/without CIMP. *A*, *B*, and *C*, cribriform glandular architecture as follows: a gland with a comedo-like pattern and bridging of cells across the lumen has spaces filled with necrotic debris. These tumors (*A*, *B*, and *C*) showed CIMP, whereas a tumor without cribriform glandular architecture did not (*D*). *A* and *B*, histologic findings of a patient with two proximal CRCs; *C* and *D*, histologic findings in a patient with a proximal and a distal CRC, respectively.

Table 1

Clinicopathologic characteristics of patients with multiple and solitary colorectal carcinomas

	M-CRNs (n = 57)	S-CRCs (n = 69)
Gender		
Male	14 (50%)	42 (61%)
Female	14 (50%)	26 (38%)
Missing data	0	1 (2%)
Mean age		
(y)	68.0	63.6
(range)	(29–90)	(32–86)
Location		
Distal	22 (39%)	33 (48%)
Proximal	33 (58%)	29 (42%)
Missing data	2 (3%)	7 (10%)
Stage		
Adenoma *	13 (23%)	NA
Cancers		
I–II	23 (40%)	28 (41 %)
III–IV	21 (37%)	39 (57%)
Missing data	0	2 (2%)
Histology		
Cancer/cancer	16 (57%)	
Cancer/adenoma *	12 (43%)	NA
Missing data	0	
Number of tumors		
Two	27 (96%)	NA
Three	1 (4%)	

Abbreviations: CRNs, colorectal neoplasias; distal, descending and sigmoid colon and rectum; proximal, cecum, ascending, and transverse colon; NA, not applicable.

* All adenomas showed advanced histology (size of all lesions was >10 mm and histologic findings showed-high grade dysplasias and/or villous histology).

Table 2

Synchronous colorectal neoplasias in relation to DNA methylation (multivariate analysis)

Risk factor	Mean methylation density		Multivariate analysis odds ratio (95% CI)	P
	M-CRNs (n = 57)	S-CRCs (n = 69)		
MINT31	5.6%	12.3%	0.93 (0.84–0.97)	0.003
p14	16.1%	9.3%	1.05(1.01–1.10)	0.010
MGMT	26.8%	17.3%	1.02 (1.00–1.04)	0.036