

Aberrant Methylation of $p16^{INK4a}$ in Anatomic and Gender-specific Subtypes of Sporadic Colorectal Cancer¹

John K. Wiencke,² Shichun Zheng, Amalia Lafuente, Maria Jose Lafuente, Corita Grudzen, Margaret R. Wrensch, Rei Miike, Antonio Ballesta, and Manuel Trias

Laboratory for Molecular Epidemiology, Department of Epidemiology and Biostatistics, University of California San Francisco, San Francisco, California 94143-0560 [J. K. W., S. Z., C. G., M. R. W., R. M.]; Institut Salut Publica de Catalunya, Pavello Central, Campus de Bellvitge, Universitat de Barcelona, Ciutat Sanitaria de Bellvitge, Feixa Llarga s/n, 08907 Hospitalet de Llobregat, Barcelona, Spain [A. L.]; Department of Surgery, Hospital St. Pablo, Barcelona, Spain [M. J. L., M. T.]; and Department of Clinical Biochemistry, Hospital Clinic i Provincial, University of Barcelona, Spain [A. B.]

Abstract

Colorectal cancer (CRC) occurring in the proximal colon and among women may represent a distinct subtype of the disease. In the present study of 120 sporadic CRCs, we used methylation-specific PCR to test whether methylation of the CpG island in the 5' region of the $p16^{INK4a}$ tumor suppressor gene was associated with anatomical location, gender, or other clinicopathological characteristics. Overall, 18.3% of the tumors had detectable $p16^{INK4a}$ methylation. A marked preponderance of methylated tumors occurred within the proximal colon; cancers occurring proximal to the sigmoid colon were 13.1 times more likely to contain methylated $p16^{INK4a}$ compared with distal tumors. In addition, female patients were 8.8 times more likely than males to have methylation-positive cancers, and $p16^{INK4a}$ methylation was also associated with poorly differentiated tumors. The localization of tumors with $p16^{INK4a}$ methylation within the proximal colon and among female patients specifically adds to a growing database of molecular alterations that define important subtypes of sporadic CRC. The potentially reversible nature of CpG methylation may provide novel therapeutic opportunities for this increasing subtype of the disease, which, due to anatomical location, presents a great challenge for early detection.

Received 12/4/98; revised 3/15/99; accepted 4/7/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported by the Foundation Marato (A. L., M. J. L.) and the National Institutes of Environmental Health Sciences NIH Grant P42-ESO4705 with funds provided by the Environmental Protection Agency (J. K. W., S. Z., C. G.).

² To whom requests for reprints should be addressed, at Laboratory for Molecular Epidemiology, Department of Epidemiology and Biostatistics, University of California San Francisco, 500 Parnassus Avenue, MU-W 420, San Francisco, CA 94143-0560.

Introduction

CRC³ is the third most common form of internal malignancy in Westernized countries. In the United States, 143,000 people were diagnosed with CRC in 1995 (1, 2). Epidemiological studies of migrant populations show that environmental factors play an important role in the etiology of this form of cancer. Persons migrating from low to high CRC risk countries acquire the risks of the high-risk population within a single generation (3, 4). The incidence of CRC worldwide is very dynamic; CRC rates are increasing in traditionally low-risk countries that are adopting a globalized Western-style diet high in fat and meat protein and low in fiber, vegetables, and fruit (5) and a more sedentary lifestyle. Within high-risk countries such as the United States, CRC rates are rising rapidly among African-American (6), Hispanic, and American Indian populations (7). Whereas a dominant role has been ascribed to diet and lifestyle in the etiology of CRC, a striking and perplexing aspect of the epidemiology of CRC is the variation in risk associated with gender and anatomical location. Numerous studies have implicated hormonal and reproductive influences in the etiology of CRC in women (5). Recent examples include studies that found reduced risks for CRC (8, 9) and adenomatous polyps (10, 11) in women receiving postmenopausal hormone replacement therapy. Moreover, a female excess of right-sided CRC at all ages and an excess of left-sided cancers among males have been documented repeatedly (12–15). These gender associations with anatomical subsite parallel the risk observed in low- versus high-risk countries (16). A notable feature of the abovementioned increase in CRC within United States minority groups is the pronounced increase in right-sided CRC among older women (6, 7). These observations indicate differential environmental influences in women and men and in specific locations in the large bowel. The role of female hormones and their effects on bile acid metabolism were previously proposed to explain gender influences on the anatomical subsite distribution of CRC (17, 18).

Creating an etiological model of CRC that integrates these epidemiological features of the disease with the manifold molecular and genetic aspects of CRC that have recently been discovered is a formidable challenge. The well-described model of colorectal carcinogenesis envisions a stepwise accumulation of early and late genetic alterations in the progression of adenoma to carcinoma (19). Epidemiological clues to the etiology of CRC have been sought by studying the mutational patterns within these loci (e.g., the $p53$ gene; Ref. 20). Identification of mutations within the familial APC (21) and within HNPCC genes (22) has helped to explain the inherited forms of CRC. Interestingly, loci involved in the heritable forms of CRC may

³ The abbreviations used are: CRC, colorectal cancer; MSPCR, methylation-specific PCR; APC, adenomatous polyposis coli; HNPCC, hereditary nonpolyposis colorectal cancer; OR, odds ratio.

also be altered somatically in sporadic CRC (23), providing a mechanistic bridge between familial and sporadic CRC.

In addition to mutational changes, epigenetic mechanisms play a prominent role in CRC. Aberrant DNA methylation has long been noted in colorectal carcinogenesis; imbalances in methylation are thought to occur early in the process and are characterized by genome-wide hypomethylation and regional and locus-specific hypermethylation (24). Among the common targets for aberrant DNA methylation is the 5' region of the *p16^{INK4a}* tumor suppressor gene (25, 26). The *p16^{INK4a}* protein plays an important role in the passage of cells through the G₁ phase of the cell cycle by binding to cyclin-dependent kinases and inhibiting their interaction with cyclin D1 and reducing phosphorylation of the retinoblastoma protein (pRB; Ref. 27). Methylation of cytosine residues at CpG sites in *p16^{INK4a}* is common in CRC cell lines and is associated with transcriptional silencing of the gene (28, 29). Epigenetic alterations that suppress gene expression are potentially reversible, offer unique opportunities for the development of new therapies, and could also be the focus of preventive strategies.

In the present study, we measured the occurrence of *p16^{INK4a}* methylation in primary CRCs using a recently developed MSPCR method and explored the relationship of methylation to demographic and clinicopathological characteristics of the patients. Because of the known epidemiological correlates of gender and anatomical location, we stratified our population by gender and location to test whether *p16^{INK4a}* methylation is involved in a unique pathway for CRC development.

Materials and Methods

Patient Population and Clinicopathological Information.

Patients were ascertained through the University of Barcelona Hospital Clinic. The Hospital Clinic is a 900-bed institution with about 33,000 patient admissions per year and serves a source population of approximately 600,000 inhabitants of Catalonia, Spain. The Hospital Clinic is a teaching institution and tertiary care facility. During 1996–1997, 275 consecutive CRC patients undergoing surgery were identified through the clinic's two surgical services as potential subjects for a hospital-based case-control study of CRC. Patients who were ≥ 35 years of age and < 90 years were invited to participate in the study. Eighteen subjects refused to participate, leaving a total of 249 enrolled subjects (132 men and 117 women). A consecutive case-series subset of 120 patients within one surgical service also provided tissue specimens (uninvolved colon and tumor) for the current molecular study. Patients provided signed informed consent; all procedures were approved by the Hospital Clinic's institutional review board (Comite Etico de Investigacion Clinica).

Primary tumors were surgically dissected and immediately frozen at -80°C . A questionnaire was administered to each patient by an interviewer; the questionnaire elicited demographic information and data on occupation, diet, and personal medical history. Clinicopathological data were collected and merged with patient questionnaires. A modified version of the Dukes' staging system was used. Surgical records identified eight subsites: (a) cecum; (b) ascending colon; (c) transverse colon; (d) descending colon; (e) sigmoid colon; (f) rectosigmoid junction; (g) rectum; and (h) anal canal. Proximal tumors were defined as including the right side of the colon and descending colon up to the sigmoid colon. Additional analyses were carried out that included the transverse and descending colon tumors with the distal category of CRCs.

DNA was isolated from tumor specimens using standard methods involving RNase, proteinase K, chloroform/isoamyl

alcohol extraction, and ethanol precipitation. DNA was quantitated by Hoescht 33258 fluorometry (Hoeffer Scientific).

MSPCR. Detection of methylated CpG sites within the 5' region of the *p16^{INK4a}* gene was carried out using MSPCR (30, 31). Briefly, 1.0 μg of purified DNA was diluted in 36 μl of H₂O to which 4 μl of 3.0 M NaOH were added, and DNA was denatured at 37°C for 15 min. The sample was then treated with 416 μl of 3.6 M sodium bisulfite solution (pH 5.0) and 24 μl of 10 mM hydroquinone. Both bisulfite and hydroquinone solutions were prepared fresh for each analysis. Samples were incubated at 55°C for 16 h; 100 μl of mineral oil were layered on top of the solution to prevent evaporation. After incubation, the solution was cooled to -80°C for 10 min, after which the unfrozen mineral oil was removed without disturbing the bisulfite-DNA solution.

Bisulfite-modified DNA was purified with the Wizard DNA Clean-UP System and Vacuum Manifold (Promega), according to the manufacturer's instructions. DNA was eluted twice with a total volume of 100 μl of 10 mM Tris-1 mM EDTA buffer (pH 7.8). The final step of the cytosine to uracil conversion reaction was achieved with alkali treatment (NaOH, final concentration = 0.3 M) at 37°C for 15 min followed by ammonium acetate (pH 7.0; final concentration, 3.0 M) neutralization and ethanol precipitation. For each set of tumors assayed, two cell line DNA samples were also treated with bisulfite and amplified by MSPCR. Both a methylation-positive CRC cell line (SW-480; American Type Culture Collection) and a methylation-negative cell line (SK-N-SH; American Type Culture Collection) served as controls for the bisulfite conversion, DNA recovery, and PCR reactions.

For each tumor, a multiplex PCR amplification of bisulfite-treated DNA was carried out with primers (Life Technologies, Inc.) specific for methylated (M primer) and nonmethylated (U primer) CpG sites within the *p16^{INK4a}* promoter region (31); (M primer, 5'-TTATTAGAGGGTGGGGCGGATCGC and 5'-GACCCCGAACCGCGACCGTAA), and nonmethylated (U2 primer, 5'-TTATTAGAGGGTGGGGTGGATTGT and 5'-CCACCTAAATCAACCTCCAACCA). The PCR mixture contained GeneAmp PCR buffer (Perkin-Elmer Corp.), MgCl₂ (1.5 mM), deoxynucleotide triphosphates (200 μM each), primers (0.4 μM for each primer), modified DNA templates (50 ng), and 2.5 Units of AmpliTaq (Perkin-Elmer Corp.) in a total volume of 50 μl . The PCR reaction was repeated for 35 cycles on a GeneAmp 9600 thermal cycler (Perkin-Elmer Corp.) under the following conditions: preheat at 94°C for 1 min, 94°C for 30 s, 65°C for 10 s, 72°C for 30 s, and a final extension at 72°C for 10 min. Aliquots (15 μl) of PCR products were loaded onto 2.5% agarose gels, stained with ethidium bromide, and visualized under UV light.

To confirm the specificity of the method, PCR product from the positive and negative controls and several CRC tumor specimens were sequenced using purified PCR product and an ABI 377 automated sequencer with dye primer chemistry. To assess the sensitivity of the MSPCR method, methylated and unmethylated cell line DNA samples were mixed in different ratios; a single unambiguous *p16^{INK4a}* methylated band was detectable when methylated template was present at greater than 1:32 (3%) of the total DNA. All analyses were carried out on coded samples without knowledge of the patient's clinical status.

Statistical Analysis. Data were analyzed using statistical analysis software. Means and SDs were computed for continuous variables. The ORs and 95% confidence intervals were calculated to detect associations of variables with the *p16^{INK4a}* methylation status of patients' tumors. Multivariate analyses (logistic regression) were used to estimate the association of

Table 1 Demographic and clinical characteristics of the University of California, San Francisco-University of Barcelona CRC Study

Variable	Male (n = 60)	Female (n = 60)	Total (n = 120)
Mean age (\pm SD)	69.4 (11.0)	72.5 (11.9)	70.9 \pm 11.5
Stage			
A	1 (1.7) ^a	1 (1.7)	2 (1.7)
B	28 (46.6)	21 (35.6)	49 (41.1)
C	16 (26.7)	19 (32.2)	35 (29.5)
D	15 (25.0)	18 (30.5)	33 (27.7)
Differentiation (poor)	4 (7.1)	8 (14.6)	12 (10.8)
Location			
Cecum	2 (3.3)	9 (15.0)	11 (9.2)
Ascending	5 (8.3)	6 (10.0)	11 (9.2)
Transverse	4 (6.7)	5 (8.7)	9 (7.5)
Descending	4 (6.7)	8 (13.3)	12 (10.0)
Proximal	15 (25.0)	28 (46.7)	43 (35.9)
Sigmoid	24 (40.0)	13 (21.7)	37 (30.8)
Sigmorectal	6 (10.0)	8 (13.3)	14 (11.7)
Rectal	15 (25.0)	11 (18.3)	26 (21.7)
Distal	45 (75.0)	32 (53.3)	77 (64.2)

^a Number of cases (percentage in parentheses).

variables that were identified in univariate analyses with *p16^{INK4a}* methylation. Significance levels of 0.05 were used for hypothesis testing.

Results

Patient Population and Molecular Approach for Detecting *p16^{INK4a}* Methylation. Patients in the present study were 120 consecutive incident cases of CRC ascertained through the University of Barcelona surgical services. As shown in Table 1, equal numbers of male and female patients were included in the series (50% male), with female patients showing an older age at presentation. The proportion of male cases from the larger hospital series (*i.e.*, 53%) was somewhat, although not significantly, greater than the *p16^{INK4a}* series. Population data from the Tarragona cancer registry, which is the closest regional registry to Barcelona, also indicate a greater proportion of male CRC; 57% of all incident CRCs in this region were male (1982–1995). The somewhat lower male:female ratio of CRC in our study may be due to the fact that men are less likely to undergo surgery; resection rates for rectal cancer, which is more common in men, are lower than for proximal CRC disease.

Approximately 57% of the patients presented with Dukes' stage C or D disease; this is similar to the proportion of such patients (50%) reported in a recent population-based case-control study in Majorca, Spain (32). A higher proportion of women presented with advanced disease, which could represent a diagnostic delay. Histopathology results identified 12 (10%) cases whose tumors were classified as poorly differentiated. None of the patients reported a positive family history of early-onset CRC or multiple familial cases that might indicate a dominantly inherited CRC gene.

The multiplex MSPCR method was found to be highly specific and sensitive in the detection of methylated *p16^{INK4a}*. DNA sequencing (data not shown) indicates that for methylated cell line DNA, all CpG sites within the amplified fragment were resistant to bisulfite conversion, whereas all cytosines at non-CpG sites were converted to thymidine residues. Only methylated PCR product was detected in the SW-480 cell line. Conversely, in the unmethylated cell line, all cytosines were converted to thymidine. Only unmethylated PCR product was detected in the SK-N-SH cell line. The MSPCR method, using

Table 2 Clinical correlations of *p16^{INK4a}* methylation in CRC in the University of California, San Francisco-University of Barcelona CRC Study

Variable	<i>p16^{INK4a}</i> status		<i>P</i> ^a
	Unmethylated	Methylated	
Location			
Proximal	25/43 (58%)	18/43 (42%)	
Distal	73/77 (95%)	4/77 (5%)	0.001
Age (yrs)	69.8 \pm 11.7	75.8 \pm 9.4	0.03
Gender			
Female	41/60 (68%)	19/60 (32%)	
Male	57/60 (95%)	3/60 (5%)	0.001
Differentiation ^b			
Poor	5/12 (42%)	7/12 (58%)	
Moderate to well	86/99 (87%)	13/99 (13%)	0.001
Stage			
<Dukes' C ^c	45/51 (88%)	6/51 (12%)	
\geq Dukes' C	52/68 (76%)	16/68 (24%)	0.10

^a *P*s shown are from Student's *t* test for age and χ^2 tests for other variables.

^b Differentiation data are missing on nine subjects.

^c Stage information is missing on one subject.

35 rounds of amplification, was capable of detecting 3% methylated template within a background of unmethylated DNA. Other studies (33) have validated the MSPCR approach by showing that it yields results that are fully concordant with other assays of *p16^{INK4a}* methylation (*e.g.*, restriction digest and Southern blot). Overall, 22 tumors were found to contain methylated *p16^{INK4a}*. An unmethylated PCR product was detected in all tumors.

Clinicopathological Correlations with *p16^{INK4a}* Methylation. We used univariate analyses to explore correlations of clinical factors with *p16^{INK4a}* methylation. Variables that had been previously identified as risk factors for anatomical and gender subtypes of CRC or that have prognostic importance were examined. Table 2 shows the strong association of several of these factors with methylated *p16^{INK4a}*. These factors were proximal location, advanced age, female gender, and poor histological differentiation. Stage was not associated with *p16^{INK4a}* methylation status. To determine which of these factors was independently associated with *p16^{INK4a}* methylation, a logistic regression analysis was carried out with the variables identified in the univariate comparisons. As shown in Table 3, proximal location, poor differentiation, and female gender remain statistically significant when included in this model; however, age was not a significant predictor. Fig. 1 shows that women were much more likely than men to have methylated tumors and that proximal tumors were more likely to contain methylated *p16^{INK4a}*. In Fig. 2, the *p16^{INK4a}* methylation data are stratified according to the anatomical subsite of the CRCs. Although methylated tumors were dominant in cecal tumors and were common in cancers occurring in the ascending, transverse, and descending segments of the colon, methylation was very uncommon in tumors arising in the sigmoid, rectosigmoid colon, and rectum. Only 4 of 77 distal cancers had detectable *p16^{INK4a}* methylation (Table 2). The association of methylation with proximal location remained highly significant in the logistic regression when distal cases were defined as those occurring beyond the hepatic flexure (location, OR = 10.7; *P* < 0.002; female gender, OR = 6.5; *P* = 0.01).

Discussion

Methylation of *p16^{INK4a}* is now recognized as a widespread epigenetic alteration in a variety of human cancers. We found that 18% of sporadic primary CRCs had detectable methylation

Table 3 Logistic regression of *p16^{INK4a}* methylation in the University of California, San Francisco-University of Barcelona CRC Study

Variable ^a	OR	95% Confidence interval	<i>p</i> ^b
Location	9.1	2.4–34.2	0.001
Differentiation	10.1	1.7–59.8	0.01
Age (yrs)	1.05	0.98–1.12	0.16
Gender	6.2	1.4–26.8	0.015

^a A total of 110 cancers. Location, proximal *versus* distal; differentiation, poorly differentiated *versus* moderately and well differentiated; age at diagnosis; gender, female *versus* male.

^b *P*s shown are for the variables in models, adjusting for all other variables.

of the 5' region of the *p16^{INK4a}* gene within a population of surgical CRC patients in Catalonia, Spain. A dramatic association of *p16^{INK4a}* methylation was observed with proximal anatomical location of CRC in our series. Overall, proximal CRCs were 13-fold more likely to exhibit *p16^{INK4a}* methylation than distal lesions. The highest prevalence of *p16^{INK4a}* methylation was 64% among cecal tumors. An earlier study (26) reported that 20–60% of CRCs tested contained methylated *p16^{INK4a}*; methylation was found to be more common in tumors exhibiting microsatellite instability compared with those that did not. It is not possible to compare the present results with those of the earlier report directly because that study included tumors selected for microsatellite instability. Our results are not dissimilar from that report because microsatellite instability was found in predominantly right-sided tumors (26). In addition, our study found that female gender and poor histological differentiation were independently associated with *p16^{INK4a}* methylation.

Taken together, the current results and previous epidemiological, clinical, and molecular evidence strongly indicate that distinct anatomical and gender-related subtypes of sporadic CRC exist (5, 34, 35). It is of interest to compare our results on *p16^{INK4a}* methylation with other molecular features of these subtypes; we note both overlap and divergence among the different genetic pathways. As shown here, an epigenetic pathway involving *p16^{INK4a}* methylation dominates in proximal CRC in older women. The observation that microsatellite instability in sporadic CRC is far more common in the proximal *versus* distal colon and also shows a gender and age bias toward older women is similar to our observations (36). Furthermore, high-grade microsatellite instability, which was recently observed in 16% of a large series of CRCs, is strongly associated with a loss of *hMLH1* expression (36, 37). Although mutations in the *hMLH1* gene are the most commonly known genetic change in HNPCC, they are not frequent (<25%) in sporadic CRC with microsatellite instability. Hence, methylation silencing is now thought to be the dominant mechanism for affecting *hMLH1* in sporadic CRC (37–40) and has an anatomical distribution similar to *p16^{INK4a}* methylation. The consequence of deficient DNA mismatch repair, whether caused by mutation or transcriptional silencing, is thought to be the accumulation of mutations in critical genes containing simple repetitive sequences within their coding regions (*e.g.*, transforming growth factor β receptor type II and insulin-like growth factor receptor type II). Although a large series of CRC patients has not yet been studied, preliminary evidence indicates that most type II transforming growth factor β and insulin-like growth factor receptor mutations occur in proximal colon tumors containing microsatellite instability (41–43).

Another distinct subtype of CRC involves mutations in the *p53* gene that occur dominantly in the distal colon and rectum

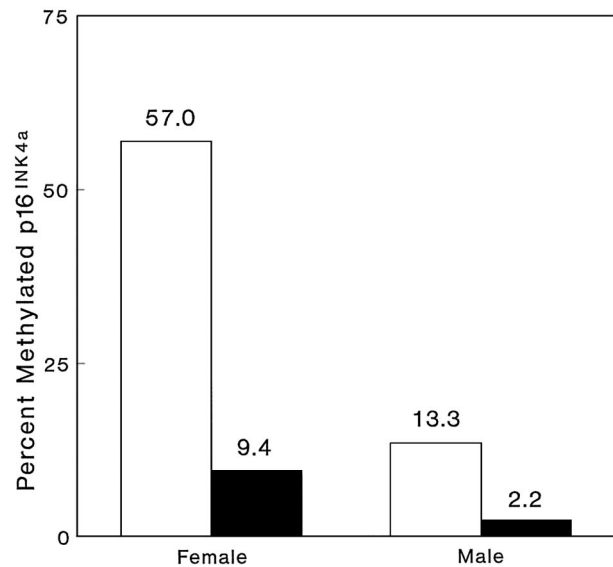


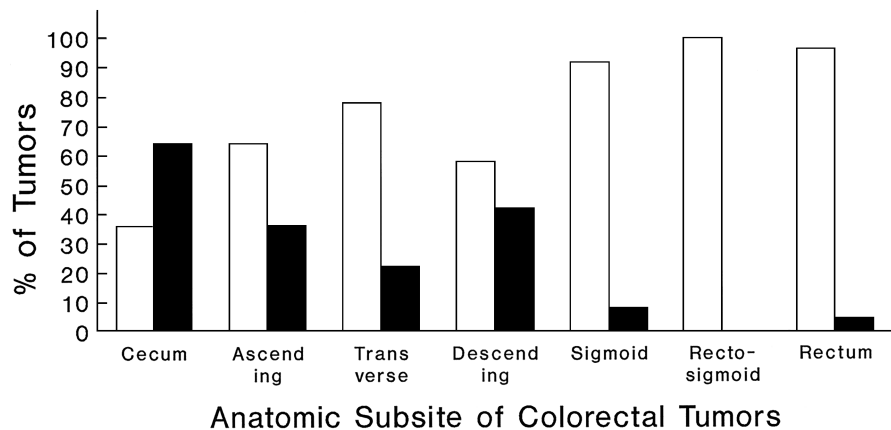
Fig. 1. Anatomical subsite distribution of *p16^{INK4a}* methylation in CRC by gender. The histogram shows the percentage of tumors (*n* = 120) exhibiting *p16^{INK4a}* methylation in the proximal (□) and distal colon (■) by gender. Numbers on bars are the percentage of methylated tumor for each subgroup. Proximal CRC includes the cecum, ascending, transverse, and descending segments; distal CRC includes the sigmoid, rectosigmoid, and rectal segments.

(44). A gender bias in mutant *p53* has not been documented, but distal and particularly rectal CRC are well known to favor male gender. Mutations in codons 12 and 13 of the *K-ras* gene are also a common feature of CRC that may define yet another pathway in CRC genesis; the influences of anatomical location and gender in this pathway are not entirely clear. Although a meta-analysis of 2721 CRCs did not reveal an association of *K-ras* mutation with tumor location or gender (45), other studies have reported a higher incidence of *K-ras* mutation in proximal cancers (46) and in proximal disease in younger women (<40 years) and older men (>90 years; Refs. 44 and 47).

Perhaps the most common alterations in CRC are those affecting the *APC* and *DCC* genes; these occur with high frequency (*e.g.*, 70%) and have not been noted to occur preferentially in any anatomical location or gender group (48). Thus, these alterations may also overlap several mechanisms of colon carcinogenesis. Interrelationships between methylation-driven mechanisms and those involving *APC* may be anticipated; however, based on observations in transgenic mice, tumorigenesis related to the *APC* gene is modified significantly by DNA methylation (49). In addition, the *APC* gene promoter itself is a target of hypermethylation (50).

The present results raise once again the intriguing and unresolved question of how female gender influences a specific pathway for CRC development. One of the earliest hypotheses addressing the role of anatomical subsite and gender in CRC postulated an influence of estrogen on bile acid secretion (17, 18). According to this hypothesis, estrogen, through its effect on serum cholesterol levels, affects the concentrations of secondary bile acids (*e.g.*, deoxycholate), which have toxic, trophic, and promoting effects on the colonic epithelium. Diets high in animal fat and low in vegetables and fiber also increase bile secretion. A recent case-control study found that lower intake of vegetables and dietary fiber is a risk factor for proximal cancer specifically in older women and men (51). In contrast, only limited evidence has been found to support a role

Fig. 2. Anatomical subsite distribution of $p16^{INK4a}$ methylation in CRC for individual segments of the colorectum. Absolute numbers of tumors for each location are given in Table 1. ■, percentages of subsite location of methylated tumors; □, percentages of subsite location of unmethylated tumors.



for dietary factors thought to affect methylation (*e.g.*, folate, B₁₂, alcohol, and methionine intake; Ref. 52). There are clinical observations, however, that add to the biological plausibility of an etiological pathway operating within the proximal colon in women that involves aberrant DNA repair. For example, it has been noted that in HNPCC families, young women have about half the risk of men of developing CRC (53). Our results indicate that the MSPCR method provides a powerful marker that future molecular epidemiological investigations can use to test specific hypotheses about estrogen, reproductive factors, diet, and CRC risk.

The unique anatomical distribution and gender bias found for $p16^{INK4a}$ methylation in our study indicate a distinct molecular pathway in CRC and provide important clues to the etiology of this subtype of sporadic CRC. Our *a priori* hypothesis concerning the point of anatomical division of proximal *versus* distal CRC (*i.e.*, between the descending and sigmoid colon) was based on a large (38,931 cases) descriptive study of CRC that found this specific anatomical division to be correlated with secular increases in CRC risk within gender and ethnic subgroups (54). We are aware, however, that several definitions of right- *versus* left-sided CRC have been proposed by different investigators. Among the considerations is the fact that the proximal and distal segments of the large intestine have different embryological origins (midgut *versus* hindgut, respectively) and vascular supplies (superior *versus* inferior mesenteric arteries). Interestingly, our data indicate an increased prevalence of $p16^{INK4a}$ methylation in CRCs up to the sigmoid colon and hence may provide a molecular correlate to the observed epidemiological differential in risk by gender. When we analyzed our data by redefining proximal CRC at the hepatic or splenic flexures, the location and gender associations with methylation remained highly significant. In addition, the anatomical distribution of CRC, and perhaps the associated methylation abnormalities, may be influenced by the source population of CRC. Spain has historically been a relatively low CRC risk country compared with other European countries or the United States. In recent decades, however, CRC incidence and mortality have increased markedly in Spain and have been accompanied by changes in dietary habits (55).

In addition to being of significance in understanding the etiology of CRC, our results also have implications for the clinical management of CRC. The association of $p16^{INK4a}$ methylation with poorly differentiated tumors may indicate a more aggressive form of the disease. Also, it has been noted that as life expectancy increases in Westernized countries and the location of CRC is shifted toward more proximal cancers in

older individuals, an increasing number of CRCs may not be detected with current screening practices (56). Furthermore, these problems may be exaggerated in certain minority populations (6, 7). Future studies of the pathobiology of methylation silencing in colon carcinogenesis could help elucidate new screening and diagnostic approaches to target populations at high risk for anatomical and gender-specific subtypes of CRC. Finally, the aberrations of DNA methylation that are so common in some forms of CRC are potentially reversible alterations; hence, drug development targeting methylation could specifically aid in the treatment of populations now identified by anatomical location, gender, and ethnic characteristics (57).

References

- American Cancer Society. Cancer Facts and Figures, 1995. Atlanta, GA: American Cancer Society, 1995.
- Shottenfeld, D., and Winewar, S. J. Cancers of the large intestine. In: D. Shottenfeld and J. F. Fraumeni (eds.), *Cancer Epidemiology and Prevention*, pp. 813–840. New York: Oxford University Press, 1996.
- Haenszel, W. Cancer mortality among the foreign born in the United States. *J. Natl. Cancer Inst.*, 26: 37–132, 1961.
- McMicheal, A. J., and Giles, G. G. Cancer in migrants to Australia: extending the descriptive epidemiologic data. *Cancer Res.*, 48: 751–756, 1988.
- Potter, J. D. Epidemiologic, environmental, and lifestyle issues in colorectal cancer. In: G. P. Young, P. Rozen, and B. Levin (eds.), *Prevention and Early Detection of Colorectal Cancer*, pp. 23–44. London: WB Saunders, 1996.
- Demers, R. Y., Severson, R. K., Shottenfeld, D., and Lazar, L. Incidence of colorectal adenocarcinoma by anatomic subsite: an epidemiologic study of time trends and racial differences in the Detroit Michigan area. *Cancer (Phila.)*, 79: 441–447, 1997.
- Chao, A., Gilliland, F., Hunt, W. C., Bulterys, M., Becker, T. M., and Key, C. R. Increasing incidence of colon and rectal cancer among Hispanics and American Indians in New Mexico, 1969–1994. *Cancer Causes Control*, 9: 137–144, 1998.
- Kampman, E., Potter, J. D., Slattery, M. L., Caan, B. J., and Edward, S. Hormone replacement therapy, reproductive history, and colon cancer: a multi-center case-control study in the United States. *Cancer Causes Control*, 8: 146–158, 1997.
- Grodstein, F., Martinez, M. E., Platz, E. A., Giovannucci, E., Colditz, G. A., Kautzky, M., Fuchs, C., and Stampfer, M. J. Postmenopausal hormone use and risk for colorectal cancer and adenoma. *Ann. Intern. Med.*, 128: 705–712, 1998.
- Potter, J. D., Bostick, R. M., Grandits, G. A., Fosdick, L., Elmer, P., Wood, J., Grambsch, P., and Louis, T. A. Hormone replacement therapy is associated with lower risk of adenomatous polyps of the large bowel: the Minnesota Cancer Prevention Research Unit Case-Control Study. *Cancer Epidemiol. Biomark. Prev.*, 5: 770–784, 1996.
- Chen, M. J., Longnecker, M. P., Morganstern, H., Lee, E. R., Frankl, H. D., and Haile, R. W. Recent use of hormone replacement therapy and the prevalence of colorectal adenomas. *Cancer Epidemiol. Biomark. Prev.*, 7: 227–230, 1998.

12. Butcher, D., Hassanein, K., Dudgeon, M., Rhodes, J., and Holmes, F. F. Female gender is a major determinant of changing subsite distribution of colorectal cancer with age. *Cancer (Phila.)*, 56: 714–716, 1985.
13. Vobecky, J., Leduc, C., and Devroede, G. Sex differences in the changing anatomic distribution of colorectal carcinoma. *Cancer (Phila.)*, 54: 3065–3069, 1984.
14. Alley, P. G., and McNee, R. K. Age and sex differences in right colon cancer. *Dis. Colon Rectum*, 29: 227–229, 1986.
15. Dubrow, R., Bernstein, J., and Holford, T. R. Age-period-cohort modeling of large-bowel-cancer incidence by anatomic sub-site and sex in Connecticut. *Int. J. Cancer*, 53: 907–913, 1993.
16. Correa, P., and Haenszel, W. The epidemiology of large bowel cancer. *Adv. Cancer Res.*, 26: 2–141, 1978.
17. McMichael, A. J., and Potter, J. D. Do intrinsic sex differences in lower alimentary tract physiology influence the sex-specific risks for bowel cancer and other biliary and intestinal diseases? *Am. J. Epidemiol.*, 118: 620–627, 1983.
18. McMichael, A. J., and Potter, J. D. Host factors in carcinogenesis: certain bile-acid metabolic profiles that selectively increase the risk of proximal colon cancer. *J. Natl. Cancer Inst.*, 75: 185–191, 1985.
19. Fearon, E. R., and Vogelstein, B. A genetic model for colorectal tumorigenesis. *Cell*, 61: 759–767, 1990.
20. Lasky, T., and Silbergeld, E. P53 mutations associated with breast, colorectal, liver, lung and ovarian cancer. *Environ. Health Perspect.*, 104: 1324–1331, 1996.
21. O'Sullivan, M. J., McCarthy, T. V., and Doyle, C. T. Familial adenomatous polyposis: from bedside to benchside. *Am. J. Clin. Pathol.*, 109: 521–526, 1998.
22. Marra, G., and Boldand, C. R. Hereditary nonpolyposis colorectal cancer: the syndrome, the genes and historical perspective. *J. Natl. Cancer Inst.*, 87: 1114–1125, 1995.
23. Olschwang, S., Hamelin, R., Laurent-Puig, P., Thuille, B., De Rycke, Y., Li, Y. J., Muzeau, F., Girodet, J., Salmon, R. J., and Thomas, G. Alternative genetic pathways in colorectal carcinogenesis. *Proc. Natl. Acad. Sci. USA*, 94: 12122–12127, 1997.
24. Zingg, J. M., and Jones, P. A. Genetic and epigenetic aspects of DNA methylation on genome expression, evolution, mutation and carcinogenesis. *Carcinogenesis (Lond.)*, 18: 869–882, 1997.
25. Herman, J. G., Merlo, A., Mao, L., Lapidus, R. G., Issa, J. P. J., Davidson, N. E., Sidransky, D., and Baylin, S. B. Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Res.*, 55: 4525–4530, 1995.
26. Ahuja, N., Mohan, A. L., Li, Q., Stolker, J. M., Herman, J. G., Hamilton, S. R., Baylin, S. B., and Issa, J. P. Association between CpG island methylation and microsatellite instability in colorectal cancer. *Cancer Res.*, 57: 3370–3374, 1997.
27. Serrano, M. The tumor suppressor protein p16^{INK4a}. *Exp. Cell Res.*, 237: 7–13, 1997.
28. Merlo, A., Herman, J. G., Mao, L., Lee, D. J., Gabrielson, E., Burger, P. C., Baylin, S. B., and Sidransky, D. 5' CPG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nat. Med.*, 1: 686–692, 1995.
29. Gonzalez-Zulueta, M., Bender, C. M., Yang, A. S., Nguyen, T., Beart, R. W., Van Tomout, J. M., and Jones, P. A. Methylation of the 5' CpG island of the p16/CDKN2 tumor suppressor gene in normal and transformed human tissues correlates with gene silencing. *Cancer Res.*, 55: 4531–4535, 1995.
30. Clark, S. J., Harrison, J., Paul, C. L., and Frommer, M. High sensitivity mapping of methylated cytosines. *Nucleic Acids Res.*, 22: 2990–2997, 1994.
31. Herman, J. G., Graff, J. R., Myohanen, S., Nelkin, B. D., and Baylin, S. B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci. USA*, 93: 9821–9826, 1996.
32. Bautista, D., Obrador, A., Moreno, V., Cabeza, E., Canet, R., Benito, E., Bosch, X., and Costa, J. Ki-ras mutation modifies the protective effect of dietary monounsaturated fat and calcium on sporadic colorectal cancer. *Cancer Epidemiol. Biomark. Prev.*, 6: 57–61, 1997.
33. Gonzalvo, M. L., Bender, C. M., You, E. H., Glendening, J. M., Flores, J. F., Walker, G. J., Hayward, N. K., Jones, P. A., and Fountain, J. W. Low frequency of p16/CDKN2A methylation in sporadic melanoma: comparative approach for methylation analysis of primary tumors. *Cancer Res.*, 57: 5336–5347, 1997.
34. Bulfill, J. A. Colorectal cancer: evidence for distinct genetic categories based on proximal or distal tumor location. *Ann. Intern. Med.*, 113: 779–788, 1990.
35. Distler, P., and Holt, P. R. Are right and left-sided colon neoplasms distinct tumors? *Dig. Dis.*, 15: 302–311, 1997.
36. Thibodeau, S. N., French, A. J., Cunningham, J. M., Tester, D., Burgart, L. J., Roche, P. C., McDonnell, S. K., Schaid, D. J., Vockley, C. W., Michels, V. V., Farr, G. H., Jr., and O'Connell, M. J. Microsatellite instability in colorectal cancer: different mutator phenotypes and the principal involvement of hMLH1. *Cancer Res.*, 58: 1713–1718, 1998.
37. Cunningham, J. M., Christensen, E. R., Tester, D. J., Kim, C. Y., Roche, P. C., Burgart, L. J., and Thibodeau, S. N. Hypermethylation of the hMLH1 promoter in colon cancer with microsatellite instability. *Cancer Res.*, 58: 3455–3460, 1998.
38. Kane, M. F., Loda, M., Gaida, G. M., Lipman, J., Mishra, R., Goldman, H., Jessup, J. M., and Kolodner, R. Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Res.*, 57: 808–811, 1997.
39. Veigl, M. L., Kasturi, L., Olechnowicz, J., Ma, A. H., Lutterbaugh, J. D., Periyasamy, S., Li, G. M., Drummond, J., Modrich, P. L., Sedwick, W. D., and Markowitz, S. D. Biallelic inactivation of hMLH1 by epigenetic gene silencing, a novel mechanism causing human MSI cancers. *Proc. Natl. Acad. Sci. USA*, 95: 8698–8702, 1998.
40. Herman, J. G., Umar, A., Polyak, K., Graff, J. R., Ahuja, N., Issa, J. P., Markowitz, S., Willson, J. K., Hamilton, S. R., Kinzler, K. W., Kane, M. F., Kolodner, R. D., Vogelstein, B., Kunkel, T. A., and Baylin, S. B. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. *Proc. Natl. Acad. Sci. USA*, 95: 6870–6875, 1998.
41. Akiyama, Y., Iwanaga, R., Ishikawa, T., Sakamoto, K., Nishi, N., Nihei, Z., Iwama, T., Saitoh, K., and Yuasa, Y. Mutations of the transforming growth factor- β type II receptor gene are strongly related to sporadic proximal colon carcinoma with microsatellite instability. *Cancer (Phila.)*, 78: 2478–2485, 1996.
42. Togo, G., Toda, N., Kanai, F., Kato, N., Shiratori, Y., Kishi, K., Imazeki, F., Makuuchi, M., and Omata, M. A transforming growth factor β type II receptor gene mutation common in sporadic cecum cancer with microsatellite instability. *Cancer Res.*, 56: 5620–5623, 1996.
43. Souza, R. F., Appel, R., Yin, J., Wang, S., Smolinski, K. N., Abraham, J. M., Zou, T. T., Shi, Y. Q., Lei, J., Cottrell, J., Cymes, K., Biden, K., Simms, L., Leggett, B., Lynch, P. M., Frazier, M., Powell, S. M., Harpaz, N., Sugimura, H., Young, J., and Meltzer, S. J. Microsatellite instability in the insulin-like growth factor II receptor gene in gastrointestinal tumours. *Nat. Genet.*, 14: 255–257, 1996.
44. Breivik, J., Loth, R. A., Melin, G. I., Rognum, T. O., Borresen-Dale, A. L., and Gaudernack, G. Different genetic pathways to proximal and distal colorectal cancer influenced by sex-related factors. *Int. J. Cancer*, 74: 664–669, 1997.
45. Andreyev, H. J. N., Norman, A. R., Cunningham, D., Oates, J. R., and Clarke, P. A. Kirsten ras mutation in patients with colorectal cancer: the multicenter "RASCAL" study. *J. Natl. Cancer Inst.*, 90: 675–684, 1998.
46. Elnatan, J., Goh, H. S., and Smith, D. R. c-Ki-ras activation and the biological behavior of proximal and distal colonic adenocarcinoma. *Eur. J. Cancer*, 32A: 491–497, 1996.
47. Breivik, J., Meling, G. I., Spurkland, A., Rognum, T. O., and Gaudernack, G. K-ras mutation in colorectal cancer: relations to patient age, sex and tumour location. *Br. J. Cancer*, 69: 367–371, 1994.
48. Gryfe, R., Swallow, C., Bapat, B., Redston, M., Gallinger, S., and Couture, J. Molecular biology of colorectal cancer. *Curr. Probl. Cancer*, 21: 233–300, 1997.
49. Laird, P. W., Jackson-Grusby, L., Fazeli, A., Dickinson, S. L., Jung, W. E., Li, E., Weinberg, R. A., and Jaenisch, R. Suppression of intestinal neoplasia by DNA hypomethylation. *Cell*, 81: 197–205, 1995.
50. Hiltunen, M. O., Alhonen, L., Koistinaho, J., Myohanen, S., Paakkonen, M., Marin, S., Kosma, V. M., and Janne, J. Hypermethylation of the APC (adenomatous polyposis coli) gene promoter region in human colorectal carcinoma. *Int. J. Cancer*, 70: 644–648, 1997.
51. Slattery, M. L., Potter, J. D., Coates, A., Ma, K. N., Berry, T. D., Duncan, D. M., and Caan, B. J. Plant foods and colon cancer: an assessment of specific foods and their related nutrients. *Cancer Causes Control*, 8: 575–590, 1997.
52. Slattery, M. L., Schaffer, D., Edwards, S. L., Ma, K. N., and Potter, J. D. Are dietary factors involved in DNA methylation associated with colon cancer? *Nutr. Cancer*, 28: 52–62, 1997.
53. Gaglia, P., Atkin, W. S., Whitelaw, S., Talbot, I. C., Williams, C. B., Norhover, J. M., and Hodgson, S. V. Variables associated with the risk of colorectal adenomas in asymptomatic patients with a family history of colorectal cancer. *Gut*, 36: 385–390, 1995.
54. Nelson, R. L., Doller, T., Freels, S., and Persky, V. The relation of age, race, gender to the subsite location of colorectal carcinoma. *Cancer (Phila.)*, 801: 193–197, 1997.
55. Lopez-Abente, G., Pollan, M., Vergara, A., Moreno, C., Moreo, P., Ardanaz, E., and Aragonés, N. Age-period-cohort modeling of colorectal cancer incidence and mortality in Spain. *Cancer Epidemiol. Biomark. Prev.*, 6: 999–1005, 1997.
56. Cooper, G. S., Yuan, Z., Landefeld, C. S., Johanson, J. F., and Rimm, A. A. A national population-based study of incidence of colorectal cancer and age. Implications for screening older Americans. *Cancer (Phila.)*, 75: 775–781, 1995.
57. Zingg, J. M., and Jones, P. A. DNA methylation as a target for drug design. *Pharm. Res. (NY)*, 15: 175–187, 1998.

BLOOD CANCER DISCOVERY

Aberrant Methylation of *p16^{INK4a}* in Anatomic and Gender-specific Subtypes of Sporadic Colorectal Cancer

John K. Wiencke, Shichun Zheng, Amalia Lafuente, et al.

Cancer Epidemiol Biomarkers Prev 1999;8:501-506.

Updated version Access the most recent version of this article at:
<http://cebp.aacrjournals.org/content/8/6/501>

Cited articles This article cites 51 articles, 17 of which you can access for free at:
<http://cebp.aacrjournals.org/content/8/6/501.full#ref-list-1>

Citing articles This article has been cited by 18 HighWire-hosted articles. Access the articles at:
<http://cebp.aacrjournals.org/content/8/6/501.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://cebp.aacrjournals.org/content/8/6/501>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.