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Colorectal Cancer ''Methylator Phenotype'': Fact or Artifact?[1]

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Abstract

It has been proposed that human colorectal tumors can be classified into two groups: one in which methylation is rare, and another with methylation of several loci associated with a ''CpG island methylated phenotype (CIMP),'' characterized by preferential proximal location in the colon, but otherwise poorly defined. There is considerable overlap between this putative methylator phenotype and the well-known mutator phenotype associated with microsatellite instability (MSI). We have examined hypermethylation of the promoter region of five genes (*DAPK*, *MGMT*, *hMLH1*, $p16^{INK4a}$, and $p14^{ARF}$) in 106 primary colorectal cancers. A graph depicting the frequency of methylated loci in the series of tumors showed a continuous, monotonically decreasing distribution quite different from the previously claimed discontinuity. We observed a significant association between the presence of three or more methylated loci and the proximal location of the tumors. However, if we remove from analysis the tumors with *hMLH1* methylation or those with MSI, the significance vanishes, suggesting that the association between multiple methylations and proximal location was indirect due to the correlation with MSI. Thus, our data do not support the independent existence of the so-called methylator phenotype and suggest that it rather may represent a statistical artifact caused by confounding of associations.

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Keywords: CpG methylation, phenotype, colorectal, cancer, microsatellite instability.

Introduction

Two different major pathogenetic mechanisms have been proposed for the development of colorectal cancers (CRCs) [1]. The first, so-called ''classic pathway,'' seems to be the most common and depends on multiple additive mutational events (germline and/or somatic) in tumor-suppressor genes and oncogenes, frequently involving chromosomal deletions in key genomic regions [2]. However, the ''mutator pathway,'' operationally recognizable by the presence of microsatellite instability (MSI), depends on early mutational loss of the mismatch repair system (germline and/or somatic), leading to accelerated accumulation of gene mutations in critical target genes and progression to malignancy. The distinction between these pathways seems to be more than academic because there is evidence that the tumors emerging from the mutator pathway have a specific ''mutator phenotype'' that includes preferential localization in the right colon, undifferentiated histology, lymphocyte infiltration, a better prognosis, and resistance to adjuvant therapy with 5-fluorouracil [3–5].

Recently, it has been discovered that in either pathogenetic pathways, loss of activity of key genes may occur through epigenetic, rather than genetic, means [6]. Indeed, although lack of expression of mismatch repair genes is generally found in sporadic tumors with MSI, the majority of such tumors does not show mutations in these DNA repair genes [7–9]. In fact, methylation of *hMLH1* is the single most common recognizable form of MSI in sporadic colorectal tumors [10]. Recent work has shown that loss of tumor-suppressor and/or DNA repair gene function by promoter methylation can occur in many different genes in sporadic CRCs [11]. In 1999, Toyota et al. studied human CRC with a technique which they called ''methylated CpG island amplification'' and observed that tumors could be classified in two very distinct groups: one with simultaneous methylation of several loci, and another in which methylation of these loci was very rare. Moreover, they noted that a large proportion of proximal tumors belonged to the former group, and they proposed the existence of a ''CpG island methylated phenotype (CIMP).'' They also observed that $CIMP^+$ tumors often also exhibited *hMLH1* methylation and MSI, and they did remark that MSI was also correlated with proximal tumors but failed to point out that this chain of associations might lead to confounding of variables [12].

Abbreviations: CRC, colorectal cancer; CIMP, CpG island methylated phenotype; MSI, microsatellite instability; MSP, methylation-specific PCR

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We have studied promoter methylation of the tumor-suppressor genes *p16^{INK4a}* and *p14^{ARF}*, the apoptosis-associated gene death–associated protein kinase (*DAPK*), and the DNA repair genes *hMLH1* and *O^6*-methylguanine-DNA-methyltransferase (*MGMT*), and also analyzed MSI in 106 human colorectal adenocarcinomas. We did not find any discontinuities in the distribution of the number of methylated genes in CRC. Moreover, after we removed tumors that had MSI from the statistical analysis, there was no longer a significant association between multiple methylated loci and proximal tumor location. Thus, our data do not support the existence of the so-called methylator phenotype and suggest that it rather represents a statistical artifact caused by confounding of associations.

Materials and Methods

Sample Collection and Nucleic Acid Isolation

Primary tumor samples from 106 patients diagnosed with CRC were collected at the A. C. Camargo Cancer Hospital (São Paulo, Brazil). Informed consent was obtained from all patients, and this research was approved by the Research Ethics Committee of the A. C. Camargo Hospital and the Ludwig Institute for Cancer Research (São Paulo, Brazil). In 30 of the patients, we also obtained matching normal colon tissue. To avoid selection bias, the samples were collected on sequential surgical cases of CRC. Immediately after collection, the samples were snap-frozen in liquid nitrogen and kept as part of a tumor bank. For this study, H&E–stained sections from each tumor sample were histologically examined, and only those that were microdissected to contain more than 70% neoplastic cells were used for analysis. DNA was prepared from microdissected tissue by digestion with pronase in 1% SDS, followed by standard phenol–chloroform extraction and ethanol precipitation [13]. In all patients, we obtained medical information on the nature of the cancer, patient sex and age, tumor location, histologic features, and clinical evolution.

Bisulfite Treatment and Methylation-Specific PCR (MSP)

MSP is based on the chemical modification of genomic DNA with sodium bisulfite, which converts unmethylated cytosines (but not methylated cytosines) to uracil. Specific primers are then designed to distinguish between the sequence differences produced with methylated and unmethylated DNA in MSP [14]. We studied the methylation status of the following loci: *DAPK*, *MGMT*, *hMLH1*, *p16^{INK4a}*, and *p14^{ARF}*. Briefly, 1 μg of genomic DNA was denatured with NaOH (final concentration, 0.2 M), and 10 mM 6-hydroquinone (Sigma, St. Louis, MO). Sodium bisulfite, pH 5.0 (Sigma), was added to a final concentration of 3 M and the mixture was incubated at 50°C for 16 hours. The modified DNA was then purified using the Wizard DNA purification kit (Promega Corporation, Madison, WI), followed by precipitation with ethanol.

The primers and thermal cycle conditions for MSP of *DAPK*, *MGMT*, *hMLH1*, *p16^{INK4a}*, and *p14^{ARF}* were as detailed elsewhere [14–17]. The PCR mixture contained bisulfite-modified DNA, specific primers (final concentration, 0.6 μM each per reaction), 1 U of Taq polymerase (Phoneutria, Belo Horizonte, Brazil), and deoxynucleotide triphosphates (1.25 mM) in 1% Triton X-100, 500 mM KCl, 15 mM $MgCl_2$, and 100 mM Tris–HCl (pH 8.4). Reactions were maintained at 950°C for 5 minutes before the addition of polymerase. Amplification was carried out using a PTC100 MJ Research, Inc. Thermal Cycler (Watertown, MA). About 10 μl of the amplified products was electrophoresed on 6% acrylamide gels and visualized by silver staining.

MSI

The Bethesda consensus panel, composed of two mononucleotide repeat microsatellites (*BAT25* and *BAT26*) and three dinucleotide repeat microsatellites (*D2S123*, *D5S346*, and *D17S250*), was used to evaluate MSI [18]. The mononucleotide microsatellite BAT-26, which is part of the panel, has been reported to have close to 100% sensitivity and specificity as a marker of this phenomenon [19,20]. As an additional criterion for MSI, we also utilized a battery of nine tetranucleotide microsatellite loci and one trinucleotide microsatellite [21].

Statistical Analysis

The 2 × 2 cross-categorized frequency data were tested by Fisher's exact test using the online facility at *http://faculty.vassar.edu/lowry/VassarStats.html*. A probability value of $< .05$ was considered significant, and we applied the Bonferroni correction for multiple comparisons [22].

Results

Frequency of Methylation in Primary Colorectal Tumors and Corresponding Nonmalignant Tissues

We used MSP to determine the frequency of methylation of *DAPK*, *MGMT*, *hMLH1*, *p16^{INK4a}*, and *p14^{ARF}* in 106 microdissected primary CRCs. These loci were chosen because they are among the most frequently methylated in CRC [23]. No aberrant methylation of any of these loci was detected in 30 samples of nonmalignant colon tissues. However, a total of 109 methylation events was detected in 106 tumors. In addition, the unmethylated form of all genes was detected in 100% of samples in both tumors and nonmalignant tissues. This was not unexpected because, inevitably, all tumor specimens contain a small proportion of normal cells. Moreover, some loci may be heterozygous for methylation and thus possess a nonmethylated allele. As shown in Figure 1, the most frequently methylated locus was *MGMT* (32/109; 29.4%), followed by *DAPK* (21/109; 19.3%), *p16^{INK4a}* (20/109; 18.3%), *hMLH1* (19/109; 17.4%), and *p14^{ARF}* (17/109; 15.6%). We identified at least one methylated promoter region in 58.5% (62/106) of the tumors (Figure 1). Overall, 41.5% (44/106) of the tumors had no methylated genes, 30.2% (32/106) had only one methylated gene, 16.0% (17/106) had two methylated genes, 8.5% (9/106) had three methylated genes, and 4% (4/106) had four methylated

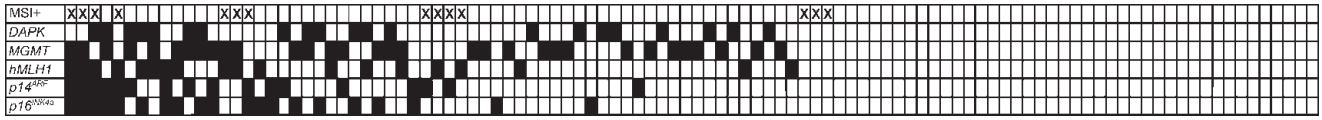

Figure 1. *Hypermethylation of the promoter region of five genes (DAPK, MGMT, hMLH1, $p16^{INK4a}$, and $p14^{ARF}$) in 106 microdissected primary CRCs. Each column is a different tumor. Back squares indicate methylated loci. The top row shows (marked with an X) tumors with MSI (MSI^+).*

genes (Figure 2). We checked all loci for pairwise association using Fisher's exact test. The only significant association found, following application of the Bonferroni correction for multiple comparisons, was between the methylation of $p16^{INK4a}$ and $p14^{ARF}$. However, this finding was not pursued further because numerous other studies have previously tested the possibility of this association and ruled it out [17,24,25].

MSI

We scored tumors for MSI (MSI^+) following the Bethesda guidelines [18] (i.e., if there were alterations in two or more of the mononucleotide repeat microsatellites *Bat25* and *Bat26*, or the dinucleotide repeat microsatellites *D2S123*, *D5S346*, and *D17S250*). As expected from previous reports [19,20], deletions in the *BAT26* were seen in all tumors with instability. Moreover, we also looked for extra alleles in a battery of one trinucleotide and nine tetranucleotide microsatellite loci [21]. There was complete concordance between the two criteria. Fourteen of 106 tumors (13.2%) displayed MSI. This value is compatible with that found in other studies [26,27]. As expected, the presence of MSI was very highly associated with the location of the tumor proximal to the splenic flexure of the colon (Table 1). However, it showed no significant correlation with recurrence within 3 years of diagnosis (Table 1).

Association between Methylation and Clinical Features

We searched for associations between the number of loci found to be methylated and some clinical characteristics of the tumors (i.e., we tested if a "methylator phenotype" could be recognized). Because we had not found any discontinuities in the distribution of the number of methylated loci per tumor (Figure 1) as previously claimed by Toyota et al. [12,28], we lacked a clear criterion for defining a "high-methylation group." Thus, we did the analysis using as "high-methylation group" the category of three or more methylated loci (criterion 1) and repeated it with the category of two or more methylated loci (criterion 2). We used Fisher's exact test to assess, for each of the categories of ≥ 2 and ≥ 3 methylated loci, an association with location of the tumor (distal *versus* proximal colon), recurrence within 3 years of diagnosis, and MSI. We found no significant association of the level of methylation with recurrence rate or with MSI (Table 1). However, tumor location was significantly associated with three or more methylated loci ($P = .011$) and also with two or more methylated loci ($P = .029$), although the former was no longer significant after applying the Bonferroni correction for multiple comparisons (Table 1). This led us to use Fisher's exact test to ascertain whether there was any association between location and methylation at each of the five loci in isolation. There was no association of proximal location with *DAPK*, *MGMT*, $p14^{ARF}$, or $p16^{INK4a}$, but there was a highly significant association with the methylation of *hMHL1* that persisted after application of the Bonferroni correction for multiple contrasts (Table 1). As expected, methylation of *hMLH1* was also highly associated with MSI and with the categories of ≥ 2 and ≥ 3 methylated loci (Table 1).

Discussion

CRC is a common malignancy that is expected to afflict approximately 106,000 people and to cause 57,000 deaths in the United States in 2004 [29]. There is an urgent need for markers that can be used in the establishment of a prognosis and that can guide in choosing the most appropriate treatment. In this sense, the discovery of the "mutator pathway" of CRC, operationally signaled by the presence of MSI (MSI^+), was a major development. MSI^+ CRC has characteristic biologic properties that include preferential proximal location, undifferentiated histology, and a relatively better prognosis [3,4]. Recent data suggest that fluorouracil-based adjuvant chemotherapy is of no benefit to patients with MSI^+ CRC [5]. If these findings are confirmed, there will be a strong case for testing all CRCs for MSI [30].

Hypermethylation of the promoter region of specific genes can be profitably used as a molecular marker of cancer cells in the detection of micrometastases, diagnosis of recurrences, and even as a screening tool for discovering primary tumors [6]. An advantage of DNA methylation is that it constitutes a positive and stable marker that cannot be masked by the presence of normal tissues and thus offers

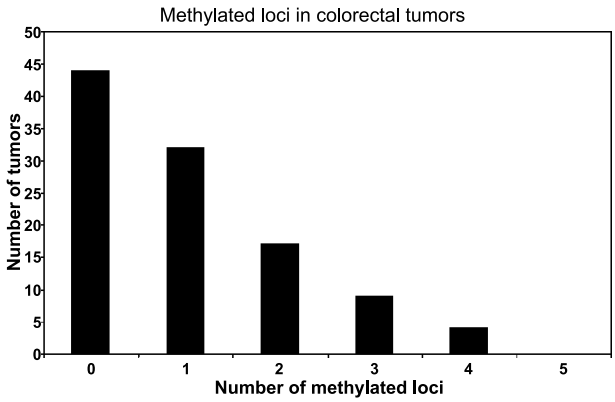

Figure 2. *Bar graph of the proportion of methylated loci in 106 CRCs.*

Table 1. Values of *P* (Two-Tailed) from Several Pairwise Comparisons Using Fisher's Exact Test.

	hMLH1 Methylation			MSI			Location			Recurrence within 3 Years		
		+	−		+	−		Proximal	Distal		+	−
MSI	+	8	7									
	−	11	80									
	P = .0008*											
Location	Proximal	9	13	Proximal	9	13						
	Distal	8	62	Distal	5	65						
	P = .004*			*P* = .0005*								
Recurrence within 3 years	+	1	12	+	2	11	+	3	10			
	−	14	60	−	12	62	−	19	54			
	P = .295			*P* = .652			*P* = .563					
≥2 Methylated loci	+	14	16	+	7	23	+	10	15	+4	21	
	−	5	71	−	8	68	−	12	55	−	9	66
	P = .000006*			*P* = .086			*P* = .029			*P* = .415		
≥3 Methylated loci	+	8	5	+	4	9	+	6	4	+	0	7
	−	11	82	−	11	82	−	16	66	−	13	80
	P = .0002*			*P* = .083			*P* = .011*			*P* = .365		

Proximal = proximal to the splenic flexure; Distal = distal to the splenic flexure.
*Statistically significant after Bonferroni correction for multiple comparisons.

extraordinary sensitivity in cancer detection through the use of MSP. Moreover, methylation might provide a new therapeutic target in CRC [6]. It is less clear whether multiple methylations have prognostic value. Toyota et al. [12,28] proposed the existence of a "CIMP" in human CRC, which included simultaneous methylation of several genes, preferential proximal location in the colon, and also an association with MSI. Although several other authors have supported this concept [31–33], the exact nature of such methylator phenotype is still poorly defined [27]. In particular, there is considerable overlap between the well-known phenotype associated with MSI and the proposed methylator phenotype. Because there exists a correlation between multiple methylations and MSI, the possibility of statistical confounding must be considered.

We have examined here the hypermethylation of the promoter region of five genes (DAPK, *MGMT*, *hMLH1*, $p16^{INK4a}$, and $p14^{ARF}$) in 106 microdissected primary CRCs. A histogram depicting the frequency of methylated loci in the series of tumors showed a continuous, monotonically decreasing distribution (Figure 2) that was quite different from the discontinuity that had been previously described by Toyota et al. [12]. Our results agree well with other authors who examined large numbers of tumors and who also did not find any discontinuities [27,33]. There was a significant association between the presence of three or more methylated loci and the tumor location proximal to the splenic flexure (Table 1). However, if we removed from analysis the tumors that display *hMLH1* methylation or those with MSI, the significance of association of "high methylation" with location vanishes ($P = .32$ and $P = .26$, respectively). Likewise, the data of Yamashita et al. [27] also show that the significant association observed between "high methylation" and right-side location becomes nonsignificant (after correction for multiple testing) on removal of the MSI^+ tumors. Thus, it appears that the association of "high methylation" with proximal location is not direct, but indirect, due to correlation with MSI. However, the association of MSI with location is known to occur very strongly in hereditary nonpolyposis colorectal cancer (HNPCC), in which 70% of tumors are right-sided [3] and in which methylation is uncommon [10].

In conclusion, our data do not support the existence of a methylator phenotype and suggest that it may represent a statistical artifact caused by confounding with the phenotype of tumors displaying MSI.

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