Association between CpG Island Methylation and Microsatellite Instability in Colorectal Cancer

Nita Ahuja, Avinash L. Mohan, Qing Li, Joshua M. Stolker, James G. Herman, Stanley R. Hamilton, Stephen B. Baylin, and Jean-Pierre J. Issa


Abstract

De novo methylation of promoter region CpG islands has been increasingly associated with transcriptional inactivation of important genes in neoplasia. To study the potential mechanisms underlying aberrant methylation in cancer, we have determined the methylation patterns of selected genes in colorectal cancers with and without microsatellite instability (MI), which results from defects in one of several base mismatch repair (MMR) genes, whether or not cancer-associated microsatellite instability (MI+) cancers, compared to only 22% in MI− cancers (P = 0.02). Similarly, hypermethylation of the thrombospondin-1 (TSP-1) gene, an angiogenesis inhibitor, was increased in MI+ cancers (27% versus 0%; P = 0.008). Extensive methylation of insulin-like growth factor II (IGF2) and hypermethylated in cancer-1 (HIC-1) genes was observed in 60 and 80% of MI+ cancers, respectively, as compared with 6 and 38% of MI− cancers (P = 0.0002 and 0.01, respectively). Furthermore, 60% of the MI+ cancers displayed the hypermethylation of two or more loci in a concordant manner compared to only 9% of the MI− cancers (P < 0.001). These results demonstrate a strong link between promoter hypermethylation and genetic instability due to deficient DNA repair.

Introduction

CpG islands are areas rich in CpG dinucleotides, which are found within the promoters of about 60% of human genes. These CpG islands normally lack DNA methylation, regardless of the expression status of the gene (1). Promoter methylation, when present, is usually associated with irreversible inhibition of gene transcription (2), as is seen with genes on the inactive X chromosome (3) and with imprinted genes (4). Abnormal CpG island methylation is a common and early event in human neoplasia (5, 6) as well as being an age-related phenomenon in a subset of genes in selected tissues (7, 8). In recent years, de novo methylation has emerged as an important alternate mechanism to coding region mutational events for inactivating tumor suppressor genes during neoplasia (9—12). A subset of CRCs have a novel type of genetic instability characterized by alterations within simple repeated nucleotide sequences termed microsatellites (13). Such MI has been seen in patients with HNPCC syndrome as well as some (about 15%) sporadic CRCs (14, 15). A large body of literature in recent years has demonstrated that the majority of HNPCC patients have a germ-line defect in one of several genes involved in DNA base MMR, and some of the sporadic CRCs exhibiting MI (MI+) also have a somatic defect in these MMR genes (16—18). Both the HNPCC tumors as well as the sporadic CRCs with MI are thought to exhibit a faster rate of tumor progression from adenoma to carcinoma (16) and are associated with an improved clinical outcome (14). In addition to these differences in clinical and progression characteristics, MI+ CRCs appear to have somewhat different underlying molecular events than CRC without MI (MI−). These include a marked reduction in frequency of chromosome loss and loss of heterozygosity (15, 16), a high rate of mutation of the TGFβRII gene (19) and a low rate of p53 mutations (13, 20). Recently, Lengauer et al. (21) have also observed an association between MMR deficiency and enhanced inactivation of foreign DNA sequences in CRC cell lines, which is accompanied by de novo methylation of these exogenous sequences (21).

In the present study, we sought to determine whether the MI+ CRCs also differ from MI− CRCs with respect to their ability to hypermethylate promoter-associated CpG islands as an alternate mechanism of gene inactivation. We determined the methylation status of selected genes in primary CRCs with MI compared with MI− cancers. Remarkably, we found a strong association between MI and promoter-associated CpG island methylation, which provides possible clues into the mechanism of neoplasia-related hypermethylation.

Materials and Methods

Tissue Samples. The study was restricted to primary CRCs to minimize the effects of clonal selection that occurs during in vitro cell culturing. Samples of human CRC tissue and native mucosa were obtained from surgical resection specimens of patients operated on at the Johns Hopkins Hospital. All samples were frozen in liquid nitrogen immediately after resection and stored at −70°C until processing. Specimen collection procedures were approved by the Joint Committee on Clinical Investigation (Institutional Review Board) of the Johns Hopkins University School of Medicine.

MI Analysis. DNA was purified from microdissected histopathological sections as discussed previously (22). Mutations in the polyadenine tract of the fourth exon of TGFβRII were identified as described (19). A second polyadenine tract located within the fifth intron of the hMSH2 gene was analyzed for MI using the marker BAT-26, as described (23). Allelic shifts in dinucleotide repeat markers D18S55, D18S58, D18S61, D18S64, and D18S69 (Research Genetics, Huntsville, AL) on chromosome 18q were detected as described previously (22). Primers were designed to produce a PCR product less than 180 bp in size, because longer fragments did not amplify consistently with DNA purified from paraffin-embedded sections. PCR assays were carried out in 96-well plates for 38 cycles using PCR Master (Boehringer Mannheim, Mannheim, Germany) in a 10-μl volume with 10 ng of each oligonucleotide, with each cycle carried out at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min with an initial 3 min at 94°C and a final 72°C step for 10 min. The 5′-primers were end-labeled using T4 polynucleotide kinase (New England Biolabs) and

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2 To whom requests for reprints should be addressed, at The Johns Hopkins Oncology Center, 424 North Bond Street, Baltimore, MD 21231. Phone: (410) 955-8506; Fax: (410) 614-9884.

3 The abbreviations used are: CRC, colorectal cancer; MI, microsatellite instability; SOX-18, simian virus 40 tumor; p53, mismatch repair; MMR, mismatch repair; TGFβRII, transforming growth factor-β type II receptor; MTase, DNA methyltransferase; IGF2, insulin-like growth factor II; HIC-1, hypermethylated in cancer-1; TSP-1, thrombospondin 1.
[γ-32P]ATP (DuPont NEN: 3000 Ci/μmol). An equal volume of stop buffer (95% formamide, 0.05% bromphenol blue, and 0.5 M EDTA) was added at the end of the amplification, and the samples were loaded onto 6% polyacrylamide gels containing 32% formamide and 5.6 M urea. Tumors were classified as MI+ if at least two of the five 18q dinucleotide markers revealed new PCR fragments not found in control mucosal DNA of the same patient, as well as changes in a polyadenine tract, either a mutation in TGFβRII or allelic shift in BAT-26.

Southern Blots. DNA was extracted using standard techniques. Five μg of genomic DNA was digested with 100 units of the appropriate restriction enzyme for 16 h as specified by the manufacturer (New England Biolabs). DNA was then precipitated, electrophoresed on a 1% agarose gel, transferred to a nylon membrane (Zetaprobe; Bio-Rad), and hybridized with the appropriate probe. The blots were then exposed on a phosphor screen for 2 to 5 days and developed using a PhosphorImager (Molecular Dynamics). All image analysis was carried out using the ImageQuant software (Molecular Dynamics). Probes used for the study included a 0.3-kb 5′ ER probe (7), a 0.6-kb 5′ IGFl probe (8), a 0.65-kb 5′ c- abl probe (8), a 0.35-kb 5′ p16 probe (10), and the highly polymorphic probe pYNZ22.1 (Ref. 24; American Type Culture Collection) as described previously, as well as a 0.6-kb 5′ TSP-I probe obtained by PCR amplification of genomic DNA using primers 5′-ATACAA-CACCTCCCCGCAAAGAAA (upper) and 5′-TATCCAGTTTCCACAGC-CAA (lower).

Statistical Analysis. Statistical comparisons were performed using Fisher’s exact test.

Results

We analyzed 40 randomly selected primary sporadic CRCs and 7 cancers that had been determined previously to display MI. Of the 40 random samples, 8 (20%) were found to have MI (examples in Fig. 1). Therefore, in total, 15 samples (32%) were MI+ and 32 samples were MI−. All of the MI+ tumors were found to show allelic shifts in at least two 18q dinucleotide markers as well as one polyadenine tract [either TGFβRII (19) or BAT-26 (23)].

De novo CpG island methylation has been shown to affect the promoter region of several genes in CRC. To test whether MI affects de novo promoter methylation in CRC, we analyzed p16 and TSP-I for methylation changes in all 47 tumors. The p16 tumor suppressor gene, a cyclin-dependent kinase inhibitor, has a typical CpG island in chromosome 15q) is a p53-regulated angiogenesis inhibitor (25) with tumor suppressor properties (26). Using Southern blot analysis, the TSP-I promoter is unmethylated in native colorectal mucosa (data not shown). In CRC, all MI− tumors studied had no evidence of methylation at this locus (Figs. 2 and 3). By contrast, 4 of 15 (27%) MI+ tumors exhibited a 3.3-kb band (Fig. 3), indicating hypermethylation of the TSP-I promoter. Thus, similar to p16, MI+ tumors exhibited more frequent hypermethylation of TSP-I compared to MI− tumors, (Fig. 4; F = 0.008).

Although de novo promoter methylation of p16 and/or TSP-I are relatively rare events in CRCs, other genes are very frequently methylated in these tumors. To determine whether MI affects methylation at these other sites, we examined the extent of promoter methylation of the IGFl (8) and HIC-I (27) genes. The IGFl CpG island, which encompasses the imprinted P2−4 promoters, contains multiple Sacll sites (Fig. 2), which we have shown previously to be hypermethylated in CRC (8). Using Southern blot analysis (Figs. 2 and 3), all CRCs showed some evidence of hypermethylation at this locus. However, complete methylation of this CpG island, as indicated by the presence of the 8.5-kb band, was detectable in 9 of 15 (60%) MI+ CRCs, compared to only 2 of 32 (6%) MI− tumors (Fig. 4; F = 0.0002). Thus, the IGFl CpG island is much more extensively methylated in MI+ compared to MI− tumors. We next analyzed the candidate tumor suppressor gene HIC-I on 17p13.3, which is known to display frequent hypermethylation in CRC (24, 27). The HIC-I gene is encompassed in a CpG island that has five methylation-sensitive Norl sites in an 8-kb region (Fig. 2) and which is unmethylated in native
colorectal mucosa. Most CRCs have some evidence of methylation at this locus (Fig. 3). However, complete methylation of all of the NotI sites around the HIC-1 gene, as indicated by the presence of the 12.0-kb band, was seen in 12 of 15 (80%) MI+ CRCs compared to only 12 of 32 (38%) MI—tumors (Figs. 3 and 4; P < 0.01). Thus, similar to IGF2, MI+ cancers showed significantly more extensive methylation at the HIC-1 locus when compared to MI—tumors.

One of the most important differences between MI+ and MI—cancers emerges when the concordance for the above methylation events (de novo methylation at p16 and TSP-1, extensive methylation at IGF2 and HIC-1) is examined. Of the 15 MI+ tumors, 9 (60%) showed hypermethylation at two or more loci simultaneously, and furthermore, 4 of these (27%) were concordant for increased methylation at all four loci. By contrast, of the MI—tumors, only three (9%; P < 0.001) showed hypermethylation at two or more loci, and none (0%, P = 0.008) were concordant for all four events (Fig. 4). Interestingly, 11 of the MI+ tumors were right-sided, and 9 (82%) of these were hypermethylated at two or more loci, whereas 4 of the MI+ tumors were left-sided, and none of these displayed hypermethylation at two or more loci. There were no similar left/right-sided differences in the MI—tumors. Thus, there is a subset of CRC tumors with MI that show methylation at multiple loci in a concordant fashion and are located predominantly in the proximal colon.

By contrast to the above genes, no difference in methylation between MI+ and MI—CRCs was observed at control loci. Thus, as expected based on our previous observations (7), all CRC tumors were similarly hypermethylated at the ER gene locus (data not shown), suggesting that the differences observed are not due simply to a failure to methylate in MI—tumors. In addition, all tumors were completely unmethylated at the promoter of the c-abl oncogene (data not shown).

Fig. 3. Methylation patterns in MI— and MI+ CRCs as demonstrated by representative Southern blot analysis for selected loci. Left, approximate size of the bands in kilobases. In each case, the arrowhead depicts the band size associated with complete methylation of all of the methylation-sensitive restriction sites. A, Southern blot analysis of the p16 gene. DNA is restricted with HindIII and SacII. The 6.0-kb high molecular weight band reflects complete methylation of the two SacII methylation-sensitive restriction sites, whereas the 3.9-kb fragment is seen if the SacII sites are unmethylated. B, methylation analysis of the TSP-1 gene with EcoRI and NotI digestion. As above, the highest molecular weight 3.3-kb band is seen only when the methylation-sensitive NotI restriction site is completely methylated, and this occurs only in some of the MI+ samples. The 1.0-kb band is a constant band resulting from the presence of two adjacent EcoRI sites with no NotI site. C, Southern blot analysis of the IGF2 gene with HindIII/SacII digestion. Most CRCs methylate some of the SacII sites in this region, resulting in the 3.6-kb band. The 8.5-kb band represents methylation of all of the SacII sites and is seen almost exclusively in the MI+ tumors. D, Southern blot analysis of the HIC-1 gene digested with EcoRI/NotI. The lower molecular weight 4.0–5.0-kb bands are polymorphic due to the variable number of tandem repeats near the 5' region of the gene (Fig. 2). Most CRC samples show partial methylation represented by a series of slightly larger bands. However, the 12.0-kb band representing complete methylation of all NotI sites is rarely seen in MI— samples but is prominent in MI+ samples.

Fig. 4. Summary of the methylation analyses. A, percentage of methylation in MI+ and MI— colorectal tumors at selected loci. The MI+ CRCs (■) exhibit more frequent hypermethylation at all these loci compared to MI— tumors (□), as shown by Fisher's exact test (see text). B, concordancy of hypermethylated sites in MI+ cancers compared to MI—cancers. The percentage of tumors displaying hypermethylation of none, two or more, and all four loci, shown in Fig. 3, is depicted by ■ (MI+) or □ (MI—) columns. The MI+ cancers frequently display hypermethylation (60%) at multiple loci in a concordant manner, whereas only three of the MI— samples display hypermethylation at two or more loci (9%). Note that the samples displayed with the 4 loci heading are a subset of this group.
suggesting that the pattern of more frequent and extensive methylation of MI+ tumors is not a generalized phenomenon but rather selectively involves specific loci in CRC.

Previous studies have suggested a possible role for increased MTase activity in hypermethylation in cancer (28). We had previously measured MTase activity in a panel of 16 CRCs (29). To determine whether MI impacts on MTase activity, we measured the presence of MI in these samples. Of the 16 cancers, 4 were MI+ (25%). In this small series, MTase activity averaged 4665 ± 2563 dpm/µg protein in MI+ tumors versus 5703 ± 629 dpm/µg in MI− tumors (P = 0.25). Thus, based on these early analyses, the increased methylation in MI+ CRCs cannot be simply attributed to differences in the level of the MTase enzyme between the tumor types.

Discussion

In this study, we have shown that sporadic MI+ colorectal tumors demonstrate: (a) more frequent de novo methylation of the promoter-associated CpG islands of the p16 and TSP-I genes; (b) more extensive methylation patterns in the promoters of the IGFI2 and HIC-1 genes; and (c) a high level of concordance for abnormal CpG island methylation at these multiple loci. These gene methylation changes appear to be selective because genes such as c-abl remain unmethylated in MI+ tumors. Finally, these MI+ tumors with increased methylation tend to be predominantly in the proximal colon. Overall, our results show a strong association between CpG island methylation and MI in sporadic CRC. Most of these tumors probably harbor a defect in the MMR system (17, 18), suggesting a link between MMR deficiency and aberrant methylation in CRC.

Promoter region hypermethylation of CpG islands is known to be associated with transcriptional inactivation (10–12). In this report, we did not directly address the correlation between promoter-region methylation and expression of the selected genes. However, we have shown previously an excellent correlation between the extent of methylation described here and expression of the p16 (10) and HIC-1 (27) genes in CRC, including some of the same tumors used for the present study. Furthermore, we have demonstrated recently a similar association between methylation and lack of expression in CRC for the TSP-I gene.4 Furthermore, in these same studies, we found that p16, HIC-1, and TSP-I can all be reactivated using the demethylating agent 5-deoxyazacytidine in CRC cell lines. For IGFI2, however, it is difficult to determine the impact of the methylation differences observed on gene expression because all colorectal tumors have some hypermethylation at this locus, and in the present study, we correlated the extent of methylation of the entire CpG island with MMR status of the cancers. Overall, our data would suggest important differences in gene expression profiles based on MMR status in colorectal tumors, and this prediction needs to be confirmed in future studies.

The mechanisms responsible for such aberrant patterns of methylation in tumorigenesis are not well understood. For some gene loci such as the ER and IGF2 loci (7, 8), this CpG island methylation actually begins in normal cells as a function of aging, and these “aged” cells may be selected for during tumor formation. In addition, recent data have suggested that hypermethylation can be modulated by different types of carcinogen exposure (30, 31). The results of our current study provide new evidence suggesting that, in CRC, CpG island methylation is increased in the setting of genetic instability due to deficient DNA MMR.

There are several possible mechanisms that may be responsible for this association between cellular mismatch repair activity and altered patterns of DNA methylation. The enzyme MTase may play a role in this process because its levels show progressive increases during CRC development (29), and experimentally, overexpression of MTase results in de novo methylation and increasing tumorigenicity (28). However, we have found no differences in MTase levels between MI+ and MI− CRCs. Still, it is possible that mutations in MTase may have arisen in MI+ cancers that change its substrate specificity and would not be detected by enzyme activity levels. MTase is also known to bind more efficiently to DNA substrates containing mismatched bases than to normal DNA (32, 33), raising the possibility that it preferentially methylates at these mismatched sites. This possibility now deserves further experimental study.

Another possible mechanism linking hypermethylation and MMR deficiency may relate directly to changes in chromatin structure caused by the alterations in microsatellite sequences seen in MI+ tumors. An analogous situation can be seen in the fragile X mental retardation syndrome. Most carriers of this syndrome have an expansion of a CGG triplet repeat in the promoter region of the FMR1 gene, located on the X chromosome. The disease phenotype appears only when the expansion reaches a certain length and then becomes associated with abnormal methylation in and around the triplet repeat sequences, with resultant loss of FMR1 expression (34, 35). Thus, it has been postulated that expansion of the FMR1 microsatellite may change the local chromatin structure such that the area becomes susceptible to hypermethylation. It may, therefore, be possible that a similar phenomenon exists in the tumors with MMR deficiency, whereby alterations in microsatellites repeats trigger promoter methylation.

Methylation is part of the strand recognition process in MMR in bacteria (36). Mammalian systems also display strand recognition during mismatch, and it has been suggested that methylation is also important to MMR in mammalian cells (37). If this is the case, it is possible that mutations in the MMR complex may somehow alter DNA methylation patterns directly. One additional possibility is that the MMR system is responsible for repairing unwanted de novo methylation events in CpG islands, and that MMR deficiency results in increased methylation in this way. Thus, when such an aberrant methylation event does occur in a MMR-deficient system, the enzyme MTase now preferentially binds to such hemimethylated strands and extends the methylation to both strands. Finally, MMR-deficient tumors are known to have unique mutations (16), and it is possible that a mutation in a methylation modifier gene may account for the observed results.

Intriguingly, Kane et al. (38) have reported recently that some sporadic MI+ CRCs, themselves, have underlying aberrant methylation in one of the MMR genes (hMLH1), accounting possibly for the MI phenotype. This finding, in association with our current results, raises the question of whether the hMLH1 methylation precedes the MI phenotype or whether it is a consequence of MI. One possible explanation is that these tumors demonstrate a “hypermethylator phenotype,” which precedes the genetic instability seen in these cancers. This phenomenon might also explain the findings of Lengauer et al. (21), in which they were unable to reverse the methylated status of exogenous sequences by correcting for the MMR defect.

Whatever the mechanism, our data illustrate a potentially critical link between deficiencies in DNA repair and gene promoter-region CpG island methylation. This link promises to shed new light on the interplay between cytosine methylation and MMR in mammalian cells and may be a key mechanism underlying promoter methylation in cancer.

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4 Q. Li, N. Ahuja, P. C. Burger, and J-P. J. Issa, Methylation associated inactivation of thrombospondin-1 in human cancers, manuscript in preparation.
References


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