Complete Genetic Suppression of Polyp Formation and Reduction of CpG-Island Hypermethylation in Apc<sup>Min/+</sup> Dnmt1-Hypomorphic Mice

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Abstract

Promoter CpG island hypermethylation of critical genes is thought to play an important role in human colorectal tumorigenesis. In this study, we show that low levels of CpG island methylation occur in the normal intestinal mucosa of Apc<sup>Min+</sup> mice and are increased in Multiple Intestinal Metaplasia (Min) polyps. We examined the interaction between CpG island hypermethylation and tumorigenesis by genetically modulating expression levels of the predominant DNA methyltransferase, Dnmt1, in Apc<sup>Min</sup> mice. We show that a combination of Dnmt1 hypomorphic alleles results in the complete suppression of polyp formation and an accompanying reduction in the frequency of CpG island methylation in both the normal intestinal mucosa and intestinal adenomas. These results suggest that sufficient DNA methyltransferase expression is a prerequiste for poly formation and that hypomorphic alleles of Dnmt1 are not merely genetic modifiers but the first identified true genetic suppressors of the Min phenotype.

Introduction

Promoter CpG island hypermethylation leading to transcriptional silencing of critical genes has been documented widely in many types of human cancer (1–4). Three functional DNA methyltransferases have been described in mammals, of which Dnmt1 appears to be the predominant enzyme (5, 6). Colorectal cancer is the second leading cause of death by cancer in the United States (7). The APC tumor-suppressor gene is implicated in the majority of colorectal tumors (8). Min<sup>−/−</sup> mice carry a germ-line mutation in the murine Apc ortholog and are predisposed to the development of intestinal neoplasia (9, 10). We have shown previously that the combined effects of heterozygosity for a null mutation of the Dnmt1 gene and treatment with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine could reduce poly multiplicity in Apc<sup>Min−/−</sup> mice (11). In this previous study, we could not rule out that the reduction in poly number by 5-aza-2'-deoxycytidine was because of drug toxicity unrelated to its demethylating capability (12). Here, we report that hypomorphic alleles of Dnmt1 lead to the complete suppression of intestinal polyp formation. Furthermore, we use the sensitive MethyLight assay to show that CpG island hypermethylation occurs at low frequency in the histologically normal mucosa of Apc<sup>Min−/−</sup> mice and is increased in intestinal polyps. Finally, we show that Dnmt1 hypomorphic alleles reduce the frequency of CpG island methylation in the normal mucosa and intestinal polyps.

Materials and Methods

Dnmt1-Hypomorphic Mice. The Dnmt1<sup>N</sup> allele has been described previously (5, 13). The Dnmt1<sup>R</sup> allele was generated as follows. A 320-bp PvdI-EcoRV fragment from pL1–2LacO (14) was inserted into the unique EcoRV site of pPGKTKNeo (15) resulting in an insertion-type targeting vector called pPLW532. This insertion-type vector was digested at the unique HindIII site and transfected by electroporation into J1 murine embryonic stem cells (5). Gene-targeted clones were subject to counterselection of the TKNeo gene by FIAU as described (15). ES cell clones that had undergone excision of the vector backbone and TKNeo gene but had retained the LacO insertion were selected for injection into blastocysts. Chimeric mice with verified germ-line transmission capabilities were bred with 129/svJae animals to obtain pure 129/svJae mice carrying the LacO insertion. This allele was designated the Dnmt1<sup>−/−</sup> allele for the EcoRV site insertion in the third intron.

Genotype Analysis. DNA was isolated from tail biopsies as described previously (16). The genotypes of the Dnmt1 alleles were determined by multiplex-PCR analysis using primers OL106 (5'-GGGACTCTTCCAGATGAGGG-3'), OL168, (5'-CCCAAACACCATGTGCTCCTG-3'), OL173, (5'-CCAGGTTCCTCAGAAGACTTAC-3'), and OL369 (5'-CAATTTCCACACATCAGGGC-3'). Reactions were carried out in a 15-μl volume with 20 mU Taq enzyme (Promega), 1× PCR buffer, 0.2 mM each dNTP, and 0.08 μM each primer. Cycling conditions were 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min 30 s, followed by 72°C for 5 min. OL168 and OL173 produced both a 342-bp wild-type-specific band and a 661-bp Dnmt1<sup>−/−</sup> allele-specific band. OL106 and OL173 produced a 430-bp Dnmt1<sup>−/−</sup> allele-specific band. OL137 and OL369 produced a second 211-bp Dnmt1<sup>−/−</sup> allele-specific band. The Apc genotype was performed by fluorescence-based, allelic discrimination-PCR (TaqMan; Refs. 17 and 18). The primer and probe sequences are listed below. Forward primer (5'-GCCACAGTTCCCTTCTCTT-3'), Reverse primer (5'-GATGTGATAGCCTGAGCCCAAATAC-3'). Apc<sup>+/−</sup>-specific probe (6FAM-TCTTCCCAAACCTCTGTCTTTCT-TAMRA), and Apc<sup>−/−</sup>-specific probe (6VIC-TCTTCTTCCTCTAACTTCTGTTTCT-TAMRA). Both probes were synthesized as TurboTaq probes.

Intestinal Polyp Scoring and Size Determination. The entire intestine was removed immediately after euthanasia, washed with 70% ethanol, and fixed in RNAlater (Ambion, Austin, TX). The entire length of the intestine was measured (cm) and subjected to a careful microscopic screen. Adenomas of at least the size of two villi were included in the counting. The investigator was blind to the genotype while counting. Adenomas and mucosal tissue samples selected for DNA analysis were microdissected from the middle third (cm) of the intestine of male mice. Polyp sizes were determined by measuring the maximum diameter of polyps found in the middle third (cm) of the intestine using calipers with an accuracy of 0.05 mm (MECHANIC Type 6901; Fine Science Tools, Inc., Foster City, CA). Male mice only were used for the size determinations to exclude gender-based differences in polyp size.
DNA Methylation Analysis. Genomic DNA isolation and sodium bisulfite conversion were performed as described previously (16, 19). Agarose beads were incubated for 14 h at 50°C to ensure complete bisulfite conversion. Methylation analysis was performed using the MethyLight assay (20–22). Parallel TaqMan PCR reactions were performed with primers specific for the bisulfite-converted methylated sequence for a particular locus and with two reference primers (Lhx1 and Gucac2). The ratio between the values obtained using these two reference primers (GEn/hLhx1 and GEn/Gucac2) was averaged. The PMR at a specific locus was calculated by dividing the \( \text{GEn/REFERENCE} \) ratio for the sample by the \( \text{GEn/REFERENCE} \) averaged ratio for the \( \text{SssI} \) treated (\( \text{SssI} \) enzyme; New England Biolabs) control J1 ES cell DNA and multiplying by 100 (22). CpG islands were defined as being methylated in a particular sample if the PMR value \( > 1.0 \). The MethyLight primer and probe sequences are listed below. In all cases, the first primer listed is the forward PCR primer, the second is the Taqman probe, and the third is the reverse PCR primer: Apc, GGGGCGTAGGTATACGTGTACGGA; Mgmt, CGACAGCTTACTGCAACAT; Cdkn2a, CCACTTTCCTTCGGAAGAATAAC; Timp3, GAGAGGGCTGGTACCATTGAG; Itga4, TAAATGCTACATCAGAGGC; Lhx1, AGAGTGTTTGGAAGTTAGGTGAAGGT.

Results and Discussion

In previous work, we have shown that Apc\(^{Min/+}\) Dnmt1\(^{+/+}\) heterozygous mice treated with 5-azaCdR develop fewer intestinal polyps than untreated, Dnmt1\(^{+/+}\) Apc\(^{Min/+}\) (11). This result was substantiated subsequently and expanded on by others (24). One explanation for this suppression of polyp multiplicity by reduced levels of functional Dnmt1 expression could be that CpG island hypomethylation is an important step in polyp formation and requires sufficient Dnmt1 expression. However, CpG island hypomethylation, though widely studied in human colorectal tumors, has never been documented in mouse intestinal tumors. Therefore, we used the sensitive MethyLight assay (20–22) to investigate whether CpG island methylation occurs at all in the mouse intestine and whether it is associated with the genes that are sufficiently frequent methylation in the samples analyzed and were consequently selected for further analysis. These were the CpG islands associated with the genes Itga4, Timp3, and Mgmt. The CpG island associated with Cdkn2a (p16) was hypermethylated in a single polyp sample but was not included for additional study because of its very low frequency of methylation. Subsequently, we screened nine normal mucosal samples and 10 intestinal polyps with each of these three MethyLight reactions.

Fig. 1A shows an overview of the frequency of CpG island methylation for each of these genes in the 19 samples analyzed. Fig. 1B shows the mean percentage of genes methylated for the mucosal samples versus the polyp samples. The percentage of these three CpG islands that were methylated in the normal mucosal tissue was 41%, whereas the polyp tissues showed an average of 77% methylation for these same genes. We found substantial variation among mice, e.g., mouse 3 was unmethylated, and mice 5 and 7 were methylated at all three genes in both mucosa and polyp. This is consistent with the variation in methylation profiles observed in human colorectal tumors (20). It is interesting to note that CpG island methylation was detectable in the normal mucosa. This has also been reported for human colorectal tissue and has been shown to increase with age and predispose to subsequent malignancies for some genes (25–27). These results indicate that CpG island methylation occurs in normal mucosa and suggest that cells with an increased frequency of CpG island methylation may be predisposed to tumorigenesis.

If a reduction in CpG island methylation is the molecular mechanism by which decreased functional expression of Dnmt1 suppresses polyp formation in Min mice (11, 24), then one would expect to see a reduced frequency of CpG island methylation in the normal mucosa or polyp tissue of Min mice with reduced Dnmt1 expression. To avoid the use of drug inhibitors, we developed a hypomorphic allele referred to as the Dnmt1\(^{Sss}\) allele (see "Materials and Methods") and combined this allele with the previously described Dnmt1\(^{R}\) allele (5) to generate mice with varying levels of Dnmt1 expression. Fig. 2A shows a schematic representation of the three Dnmt1 alleles used in this study. Fig. 2B shows the relative expression of these alleles in embryonic stem cells. The Dnmt1\(^{N}\) allele does not show detectable levels of gene expression by Northern blot analysis (Fig. 2B and Ref. 13) but has been shown to produce low levels of a truncated protein by alternative splicing (13). Quantitation of the normalized RNA expression level of Dnmt1\(^{Sss}\) cells versus Dnmt1\(^{+/+}\) cells indicated that the Dnmt1\(^{Sss}\) is expressed at \( \sim 60\% \) of that of the wild-type allele (Fig. 2B). This intermediate level of expression is consistent with the observation that the Dnmt1\(^{Sss}\) allele resulted in reduced viability in conjunction with the null Dnmt1\(^{S}\) allele but was fully viable in combination with the previously described hypomorphic Dnmt1\(^{R}\) allele (data not shown). We tested whether the combination of these two hypomorphic alleles resulted in detectable hypomethylation \( \text{in vivo} \). Fig. 2C shows that the centromeric minor satellite repeat sequence is significantly hypomethylated in the intestinal mucosa of Dnmt1\(^{N/R}\) mice but not in that of any of the other three allelic combinations. Therefore, we conclude...
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that both Dnmt1R and Dnmt1N are hypomorphic alleles with reduced functional expression of Dnmt1 and together result in significant hypomethylation in vivo. Nevertheless, the degree of hypomethylation achieved in these mice is not sufficient to cause an overt phenotype. Dnmt1N/R mice are comparable in size and weight to their wild-type littermates and are fertile.

ApcMinN/Dnmt1 hypomorphs were generated from crosses between C57BL/6 ApcMinN Dnmt1N/N+ males and 129/SvJae Apc+Dnmt1R/R females. Mice were euthanized at 180 days and analyzed for polyp multiplicity and polyp size as described in “Materials and Methods.” Apc+ offspring were euthanized on weaning, because we had shown previously that Dnmt1 mutant mice do not develop intestinal tumors in an Apc+ background (11). We found a gradual decline in polyp number with diminishing Dnmt1 expression levels with a drop to 0 in the ApcMin+/Dnmt1N/R mice, which have the lowest levels of Dnmt1 expression (Fig. 3A). The number of polyps was reduced significantly in ApcMin+/Dnmt1N+ mice (P = 0.001) and ApcMin+/Dnmt1N/R mice (P < 0.0001) compared with wild-type ApcMinN mice. We did not observe a single polyp in any of the 14 ApcMin+/Dnmt1N/R mice that we analyzed. This suggests that Dnmt1 hypomorphic alleles act as genetic suppressors of the ApcMinN phenotype. Other studies have reported genetic modifiers of the Min phenotype that reduce the number of polyps (9, 11, 28–31), but this is the first documentation of complete genetic suppression, to our knowledge.

We also examined the effect of reduced Dnmt1 expression on polyp size. Polyps from Dnmt1R/R (P = 0.0021) and Dnmt1N/R (P < 0.0001, unpaired t test) were significantly smaller than those from ApcMin+/+ Dnmt1+/+ siblings at 180 days (Fig. 3B), indicating that reduced Dnmt1 expression affects not only polyp formation but also the growth rate of the polyps. This is consistent with the findings of Cormier and Dove (24), who reported a reduced net growth rate and multiplicity of intestinal adenomas in Dnmt1N/R ApcMin+/+ mice.

If CpG island hypermethylation is an essential feature to polyp formation, and if this is the basis for the suppressive effects of reduced levels of Dnmt1 expression, then we would expect to see a lower CpG island methylation frequency in tissues with reduced DNA methylation levels. The results shown in Fig. 4 show that this is indeed the case. The level (Fig. 4A) and frequency (Fig. 4B) of CpG island methylation in the intestinal mucosa is substantially diminished in Dnmt1 hypomorphic mice compared with Dnmt1+/+ mice. The most striking reduction is seen in Dnmt1N/R mucosa, where we did not observe a single instance of CpG island methylation >1 PMR threshold (P = 0.0057, unpaired t test). Dnmt1N+ mice also showed a statistically significant reduction in the frequency of CpG island

![Fig. 2. Structure and characterization of Dnmt1 hypomorphic alleles. A, schematic representation of the structure of the Dnmt1 hypomorphic alleles, which are described in detail in “Materials and Methods.” B, Northern blot of total RNA extracted from embryonic stem cells with different hypomorphic alleles, as indicated above the lanes. Left panel, a hybridization with an α-Tubulin control probe; right panel, a hybridization with a Dnmt1 cDNA probe. C, Southern blot analysis of intestinal DNA derived from 180-day-old mice and from ES cells (Dnmt1N), hybridized with a centromeric minor satellite repeat probe (23). Dnmt1N ES cells are homozygous for a mutation in the catalytic domain of the Dnmt1 gene and, thus, can be considered nullizygous for Dnmt1 (13). The Dnmt1 genotypes and restriction digests are indicated above each corresponding lane.](https://cancerres.aacrjournals.org/content/full/62/22/1298/F2.large.jpg)

![Fig. 3. The mean number and size of intestinal polyps in ApcMinN+ mice with different Dnmt1 genotypes. Mice were euthanized at 180 days of age, and polyps were counted and sized as described in “Materials and Methods.” The Dnmt1 genotype is shown below each bar. Error bars, SE. In A, the mean number of polyps for each group is shown as a bar with the mean value indicated within the bar. Polyps were counted throughout the entire small intestine and colon. n, the number of mice in each genotype analyzed. In B, the mean polyp size (mm) for each genotype is shown as a bar with the mean value indicated within the bar. Polyps were measured from the middle third of the intestine of male mice. n, the number of polyps analyzed for each genotype.](https://cancerres.aacrjournals.org/content/full/62/22/1298/F3.large.jpg)
methylated in the normal mucosa (P = 0.025) and polyps (P = 0.0002; Fig. 4C). We could not measure the CpG island methylation frequency in Dnmt1<sup>N/R</sup> adenomas, because these mice did not develop any polyps.

These results are consistent with a model in which CpG island methylation occurs in the normal intestinal mucosa in a small minority of cells, which then become predisposed to undergo neoplastic transformation. We do not suggest that the three individual genes that we analyzed are necessarily implicated in tumorigenesis but just that they contribute to polypl formation and additional growth. This model is supported by the observation that substantially reduced expression of Dnmt1 in the intestinal mucosa results in the complete suppression of detectable polyp formation. One caveat to our results is that we used a binocular stereo microscope to scan the intestine for adenomas, rather than relying on serial sections analyzed with an upright microscope. Therefore, microadenomas or aberrant crypt foci in Dnmt1<sup>N/R</sup> mice may have been missed. Nevertheless, if such early stage adenomas did arise in these mice, they did not progress further to a stage at which they would be detectable using a dissecting microscope. We found that the RNAAfter fixative that we used to allow for subsequent nucleic acid analysis rendered the intestines too fragile for subsequent embedding and analysis at high magnification. With these caveats in mind, we tentatively conclude that CpG island hypermethylation, mediated at least in part by Dnmt1, is an essential and rate-limiting step in intestinal adenoma formation in Apc<sup>Min</sup>/ mice and that Dnmt1 hypomorphic alleles are the first identified true genetic suppressors of the Apc<sup>Min</sup> phenotype.

References
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