Suppressive effect of global DNA hypomethylation on gastric carcinogenesis

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Global DNA hypomethylation and concomitant site-specific gene hypermethylation are among the most common molecular alterations in human neoplasia. Although site-specific DNA hypermethylation has been shown to be associated with the development of various tumors accompanied by transcriptional silencing of target genes, the functional significance of global DNA hypomethylation in tumorigenesis remains unclear. Previous studies have revealed that a genetic reduction of the DNA methylation levels leads to opposing effects on tumor development, depending on the tumor cell type and the stage of tumorigenesis. In the present study, we investigated the effect of DNA hypomethylation on gastric carcinogenesis in mice. The genetic reduction of DNA methylation levels suppressed the incidence, number and size of gastric tumors in two different mouse models for gastric tumorigenesis: the Min+/− mouse model that spontaneously develops gastric tumors with aging, Histological analyses revealed DNA hypomethylation to completely inhibit the development of invasive gastric tumors. These findings indicate that the reduction of DNA methylation levels suppresses gastric carcinogenesis and suggest that DNA methylation is closely associated with gastric tumorigenesis.

Introduction

Changes in the DNA methylation status, including global DNA hypomethylation and site-specific gene hypermethylation, are concomitantly found in tumors and are the most common molecular alterations in human neoplasia (1). Site-specific DNA hypomethylation has been extensively analyzed and a number of genes have been shown to be hypermethylated and transcriptionally silenced in various tumors. However, the functional significance of global DNA hypomethylation remains unclear, although this alteration was discovered in a wide variety of human cancers >20 years ago. Global DNA hypomethylation, which is frequently observed at the early stages of tumorigenesis in human cancer (2,3), promotes chromosomal instability in vitro and accelerates tumor development in several mouse models (4–6). Although the consequences of global hypomethylation and site-specific hypermethylation have been mechanistically connected to chromosome instability and transcriptional silencing, respectively, the cause of aberrant DNA methylation patterns remains unclear.

Abbreviations: CdH1, Cadherin 1; Cdkn2a, Cyclin-dependent kinase inhibitor 2A; LINE, long interspersed nuclear element; Mafa2, Melanoma antigen family A 2; MNU, N-methyl-N-nitrosourea; Sfrp, Secreted frizzled-related protein.

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Materials and methods

Mice

Two mutant alleles of Dnmt1 were used: the null Dnmt1−/− allele in the C57BL/6 background (23) and the hypomorphic Dnmt1+/−hypo allele in the 129Sv4 background (6). Dnmt1+/− mice (C57BL/6) were crossed with female Dnmt1+/−hypo mice (129Sv4) to generate experimental mice in an isogenic F1 hybrid (C57/129). A previous study reported that Dnmt1+/−hypo mice have the same levels of genomic methylation as Dnmt1+/+ mice, whereas Dnmt1+/−hypo mice have reduced DNA methylation contents at pericentromeric satellite repeats (18). We therefore analyzed Dnmt1+/−hypo mice as a control cohort and Dnmt1−/− mice as a DNA hypomethylated cohort in this study. All mice were maintained under specific-pathogen-free conditions with isolated ventilation cages in an air-conditioned room with a 12 h light–dark cycle. They were bred and maintained on a basal diet, CE-2 (CLEA Japan, Tokyo, Japan), until the termination of the study.

Methylation analysis of gastric mucosa

The DNA methylation levels in gastric mucosae were examined by methylation-sensitive Southern blot analysis and bisulfite methylation analysis. Genomic DNA were extracted from the homogenized gastric mucose of Dnmt1−/− and Dnmt1+/− mice. Firstly, DNA were digested with the methylation-sensitive enzyme HpaII (New England Biolabs, Ipswich, MA) and the digests were analyzed by Southern blotting using a centromeric...
satellite repeat probe as previously reported (6,18,24). Next, the methylation pattern in 5′-noncoding region of Line-1 was analyzed by bisulfite sequencing in accordance with the previous report (25). Bisulfite treatment of the genomic DNA was performed using EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA). After polymerase chain reaction amplification using primers for 5′-noncoding region of Line-1, the products were cloned into the TOPO vector (Invitrogen, Carlsbad, CA). The inserted polymerase chain reaction fragments of the individual clones obtained from each sample were sequenced with primer for T7 promoter using the ABI Prism Dye Terminator Cycle Sequencing Kit and an ABI Prism 3100 DNA Sequencer. The primers for Line-1 are shown in supplementary Table 1, available at Carcinogenesis Online.

MNU treatment

MNU (Sigma Chemical, St Louis, MO) was dissolved in distilled water at a concentration of 240 p.p.m. and freshly prepared thrice per week for administration in drinking water in light-shielded bottles ad libitum. Dnm1Δallopc and Dnm1Δallop mice (C57BL/6), and male ApcMin+/c; Dnm1Δallop mice (C57BL/6) were subsequently crossed with female Dnm1chip/c mice (C57SvJ) to generate experimental mice in an isogenic F1 hybrid (C57:129) background. We analyzed 30 Dnm1Δallop+/c; ApcMin+/c mice and 21 Dnm1Δallop−/−; ApcMin+/c mice to quantify gastric lesions at 30-32 weeks of age (Figure 1B). In addition, 9 Dnm1Δallop+/c; ApcMin−/c and 15 Dnm1Δallop−/−; ApcMin−/c mice were analyzed as controls without the ApcMin allele. All mice were maintained under the same conditions as those described above.

Preparation of tissue samples for tumor counting and histological analysis

All mice underwent a thorough postmortem examination at the time of killing. The stomach was removed and opened along the greater curvature. The number and the lengths of the major and minor axes of the gastric tumors were determined using a dissecting microscope at ×7 magnification. Tumors >0.5 mm in long axis length were mapped and counted. The sizes of the tumors were determined by multiplying the major axis by the minor axis. To eliminate interobserver error, all counts were performed by a single observer blinded to the genotype of the mice. In addition, all the cases were counted by a second observer to confirm the results of the first observer. After counting the tumors, all the excised stomachs, including the neoplastic nodules, were fixed for 24 h in neutral-buffered 10% formalin and were subsequently cut into eight strips. These strips were processed by standard methods, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. The defining characteristics for adenoma and adenocarcinoma were adapted from both the consensus guidelines on murine models of intestinal cancer (26) and previous reports in the literature (20,27).

Immunohistochemistry

The avidin–biotin–peroxidase complex technique was used for immunohistochemical studies. Five micrometers thick sections were cut, deparaffinized, rehydrated in phosphate-buffered saline, placed in 10 mmol/l citrate buffer (pH 6.0) and heated in a 750 W microwave four times for 6 min. The endogenous peroxidase activity was blocked by incubation for 30 min in 0.3% H2O2. After washing three times with phosphate-buffered saline, the sections were then pre-incubated with normal blocking serum for 20 min at room temperature and then were incubated with Ki-67 (1:200; DAKO Corporation, Carpinteria, CA) and cleaved caspase-3 (1:400; Cell Signaling Technology, Danvers, MA) antibody overnight at 4°C. Subsequently, the sections were incubated with biotinylated secondary antibodies (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) for 30 min, followed by incubation with avidin-coupled peroxidase (Vector Laboratories) for 30 min. The sections were developed with 3,3′-diaminobenzidine using DAKO liquid 3,3′-diaminobenzidine Substrate-Chromogen System (DAKO Corporation) and then counterstained with hematoxylin. No specific staining was observed in the negative control slides prepared without primary antibody. The number of Ki-67-positive cells per gland was calculated as the Ki-67-labeling index.

Methylation analysis of MNU-induced gastric tumors

In order to examine the involvement of aberrant site-specific methylations in the development of MNU-induced mouse gastric cancer, the methylation status of the promoter regions of Cyclin-dependent kinase inhibitor 2A (Cdkn2a), Cadherin 1 (Cdh1), Secreted frizzled-related protein 1 (Sfrp1), Sfrp2 and Melanoma antigen family A, 2 (Magea2) were analyzed by bisulfite sequencing. Genomic DNA were extracted from the gastric tumors and the surrounding gastric mucosae of MNU-treated wild-type mouse and bisulfite analysis were performed as described above. The primers for bisulfite sequencing are shown in supplementary Table 1, available at Carcinogenesis Online.

Statistical analysis

The incidence of gastric tumors was analyzed using Fisher’s exact test. Differences in the number and the size of gastric tumors and Ki-67-positive cell ratio in non-cancerous mucosa were assessed with Mann–Whitney U-test.
Fig. 2. DNA hypomethylation suppresses gastric tumorigenesis in MNU-treated mice. (A) Macroscopic photographs of the glandular stomach in MNU-treated Dnmt1chip/+ and Dnmt1chip/c mice at 52 weeks of age. (B) Incidence of gastric tumors in MNU-treated Dnmt1chip/+ and Dnmt1chip/c mice (n = 24 and n = 18). The incidence of MNU-treated Dnmt1chip/c mice with gastric tumors was significantly lower than that of MNU-treated Dnmt1chip/+ mice. *P < 0.05, by Fisher's exact test. (C) Number of gastric tumors per mouse in MNU-treated Dnmt1chip/c and Dnmt1chip/+ mice. MNU-treated Dnmt1chip/c mice developed significantly fewer gastric tumors than MNU-treated Dnmt1chip/+ mice. Columns, mean; bars, SD. **P = 0.0001, by Mann–Whitney U-test. (D) Sizes of gastric tumors in MNU-treated Dnmt1chip/+ and Dnmt1chip/c mice. The tumor size of gastric tumors in the MNU-treated Dnmt1chip/c mice was significantly smaller than that of the MNU-treated Dnmt1chip/+ mice. Columns, mean; bars, SD. ***P < 0.0001, by Mann–Whitney U-test.

Fig. 3. DNA hypomethylation suppresses the development of gastric tumors in ApcMin/+ mice model. (A) Incidence of gastric tumors in Dnmt1chip/+; ApcMin/+ mice and Dnmt1chip/c; ApcMin/+ mice (n = 17 and n = 10). The incidence was significantly smaller in Dnmt1chip/c; ApcMin/+ mice than in Dnmt1chip/+; ApcMin/+ mice. *P < 0.05, by Fisher’s exact test. (B) Number of gastric tumors per mouse in Dnmt1chip/+; ApcMin/+ mice and Dnmt1chip/c; ApcMin/+ mice. Dnmt1chip/c; ApcMin/+ mice developed significantly fewer gastric tumors than Dnmt1chip/+; ApcMin/+ mice. Columns, mean; bars, SD. **P < 0.05, by Mann–Whitney U-test. (C) Histopathology of the gastric tumors in ApcMin/+ mice. (a) Adenoma in Dnmt1chip/c; ApcMin/+ mice; bar, 200 μm. (b) Higher magnification in (a); bar, 100 μm.
Spearman’s rank correlation test was used to determine the relationship between Dnmt1 genotype and tumor malignancy.

**Results**

**Dnmt1 hypomorphic alleles induce global DNA hypomethylation in gastric mucosa**

To determine whether the mutant Dnmt1 alleles definitely affect DNA methylation in the gastric mucosa, the DNA methylation levels in the gastric mucosae were examined. The methylation-sensitive Southern blot analysis revealed that the gastric mucosa of the Dnmt1 chip/c mouse was significantly hypomethylated at pericentromeric regions in comparison with that of the Dnmt1 chip/+ mouse (Figure 1C), consistent with both our previous findings and the present observations in the colonic mucosa (18). Additionally, bisulfite analysis showed the reduced CpG methylation of the Line-1 regulatory region in the Dnmt1 chip/c mouse gastric mucosa, whereas this region was highly methylated in that of the Dnmt1 chip/+ mouse (Figure 1C). When compared the methylation frequency at each CpG in the Line-1 regulatory region, the values in the Dnmt1 chip/c mouse were lower than those in the Dnmt1 chip/+ mouse at almost all CpGs except for the second CpG. These findings indicate that Dnmt1 hypomorphic alleles lead to the global DNA hypomethylation in the mouse gastric mucosa.

**DNA hypomethylation suppresses gastric tumorigenesis in MNU-treated mice**

We administered MNU, an alkylating agent that induces the formation of adenomas and adenocarcinomas in murine glandular stomach tissue, to Dnmt1 chip/c and Dnmt1 chip/+ mice, which express different levels of DNA methyltransferase Dnmt1. The MNU-treated Dnmt1 chip/c and Dnmt1 chip/+ mice (n = 24 and n = 18, respectively) were examined for gastric lesions at 52 weeks of age. Macroscopically, most tumors developed in the pyloric antrum and showed a sessile and/or polypoid morphology in both Dnmt1 chip/c and Dnmt1 chip/+ mice (Figure 2A). Both the incidence and the multiplicity of macroscopic gastric tumors in the MNU-treated Dnmt1 chip/c mice were significantly decreased as compared with those in the MNU-treated Dnmt1 chip/+ mice (Figure 2B and C). The incidence of gastric tumors was 77.8% (14/18) in Dnmt1 chip/c mice, whereas it was 100% (24/24) in Dnmt1 chip/+ mice (*P* = 0.0005, by Spearman’s rank correlation test). Further, the tumor size in the MNU-treated Dnmt1 chip/c mice was significantly smaller than that of the MNU-treated Dnmt1 chip/+ mice (*P* < 0.0001, Figure 2D). Large tumors, namely >5 mm in the longest diameter, were only observed in MNU-treated Dnmt1 chip/+ mice (data not shown). These results clearly indicate that a genetic

Fig. 4. DNA hypomethylation suppresses the progression of gastric carcinogenesis. (A) Histopathologic features of gastric tumors in MNU-treated Dnmt1 chip/+ and Dnmt1 chip/c mice at 52 weeks of age. (a) Adenoma in MNU-treated mice. (b) Intramucosal adenocarcinoma. (c) Invasive adenocarcinoma. (d–f) Higher magnification in (a–c), mm, muscularis mucosae; bars, 200 μm in (a–c) and 100 μm in (d–f). (B) Histologic grade of the most advanced tumor in tumor-bearing MNU-treated Dnmt1 chip/+ and Dnmt1 chip/c mice (n = 24 and n = 14). The development of malignant gastric cancer was significantly decreased in Dnmt1 chip/c mice as compared with Dnmt1 chip/+ mice. Columns, mean; bars, SD. *P* = 0.0005, by Spearman’s rank correlation test.

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DNA hypomethylation suppresses gastric carcinogenesis

DNA hypomethylation reduces the persistent increase in the cell proliferative activity in the non-cancerous gastric mucosa exposed to MNU

In order to clarify the inhibitory mechanisms of reduced DNA methylation levels on gastric tumorigenesis, we assessed cell proliferative activity in the non-cancerous gastric mucosa of MNU-treated Dnmt1chip/c and non-treated Dnmt1chip/c mice by immunostaining for Ki-67, a marker for proliferating cells (Figure 5A). The Ki-67-positive cell ratio in MNU-treated Dnmt1chip/c mice was equivalent to that in non-treated Dnmt1chip/c mice, whereas MNU-treatment significantly elevated the Ki-67-positive cell ratio in Dnmt1chip/c mice. Columns, mean; bars, SD. *P < 0.0005 and **P < 0.0005, by Mann–Whitney U-test.

Fig. 5. DNA hypomethylation suppresses abnormal cell proliferation in the non-cancerous gastric mucosa induced by MNU treatment. (A) Ki-67 immunostaining of the non-cancerous gastric mucosa in MNU-treated Dnmt1chip/+ (a) and Dnmt1chip/c (b) mice at 52 weeks of age. (B) Percentage of Ki-67-positive cells in non-cancerous gastric mucosa of non-treated and MNU-treated Dnmt1chip/+ and Dnmt1chip/c mice (n = 10 for each group). The Ki-67-positive cell ratio in MNU-treated Dnmt1chip/c mice was equivalent to that in non-treated Dnmt1chip/c mice, whereas MNU-treatment significantly elevated the Ki-67-positive cell ratio in Dnmt1chip/c mice. Columns, mean; bars, SD. *P < 0.0005 and **P < 0.0005, by Mann–Whitney U-test.

DNA hypomethylation reduces the progression stage of gastric carcinogenesis

DNA-induced gastric lesions were evaluated for their histopathologic features and classified as hyperplasia, adenoma, intramucosal adenocarcinoma or invasive adenocarcinoma (Figure 4A). Histopathological analyses clearly demonstrated significant decreases in the development of intramucosal and invasive adenocarcinomas in MNU-treated Dnmt1chip/c mice as compared with Dnmt1chip/c mice (Figure 4B), thus indicating that the reduced DNA methylation levels suppressed the progression into advanced gastric tumors. It is noteworthy that the MNU-treated Dnmt1chip/c mice often developed invasive adenocarcinomas, whereas the MNU-treated Dnmt1chip/c mice did not (Figure 4B). These findings suggest that DNA methylation may play a role in the progression stage of gastric tumorigenesis.

DNA hypomethylation suppresses the development of gastric tumors in ApcMin/+ mice

We also investigated the effect of reduced DNA methylation levels on gastric carcinogenesis using a different mouse model. We have previously reported that ApcMin/+ mice spontaneously develop gastric tumors with aging (21). ApcMin/+ mice were crossed with Dnmt1 hypomorph mice to generate ApcMin/+ mice that express different levels of Dnmt1. Consistent with the results in the MNU-induced gastric tumor model, both the incidence and the number of the gastric tumors were significantly smaller in Dnmt1chip/c, ApcMin/+ mice than in Dnmt1chip/+; ApcMin/+ mice (Figure 3A and B, P < 0.05 for the incidence and P < 0.005 for the number). As previously reported, the gastric tumors were histopathologically classified as adenomas in the ApcMin/+ mice (Figure 3C) (21). These results provide additional evidence that a genetic reduction of DNA methylation levels suppresses gastric tumorigenesis. Although we assessed the cell proliferative activities in non-cancerous gastric mucosa of Dnmt1chip/+; ApcMin/+ and Dnmt1chip/c, ApcMin/+ mice, genetic reduction of the DNA methylation level did not affect the cell proliferative activities in the gastric mucosa of ApcMin/+ mice. The Ki-67-positive cell ratios in Dnmt1chip/+; ApcMin/+ and Dnmt1chip/c, ApcMin/+ mice (n = 10 for each animal) were 22.3 ± 5.8 and 19.4 ± 5.4 (average ± SD), respectively.

The aberrant site-specific methylations are not found in MNU-induced mouse gastric tumors unlike in the case of human gastric cancers

We cannot rule out the possibility that the genetic reduction of DNA methylation suppress gastric tumorigenesis by blocking the aberrant site-specific methylation of specific genes that play a crucial role in gastric tumorigenesis, but there is no available information on hypermethylated or hypomethylated genes in MNU-induced mouse gastric tumors. In order to examine the involvement of aberrant site-specific methylations in the development of MNU-induced mouse gastric tumors, the methylation status of the promoter regions of Cdkn2a, Cdh1, Sfrp1, Sfrp2 and Magea2 were analyzed. Though it has been reported that the CpG islands in the promoter regions of Cdkn2a, Cdh1, Sfrp1 and Sfrp2 are highly methylated and those of Magea2 are frequently demethylated in human gastric tumors (7–12), altered methylation patterns were not found in MNU-induced mouse gastric
CpG dinucleotide and open circles, unmethylated CpG dinucleotide.

Relative to the transcription start site. TSS, transcription start site. Circles, CpG dinucleotide (potential target of methylation); closed circles, methylated CpG dinucleotide.

tumors (Figure 6), indicating that the pattern of the site-specific aberrant methylations in mouse gastric tumors is distinctly different from that in human gastric cancers.

Discussion

Previous studies have revealed that a genetic reduction of the DNA methylation levels results in opposing effects on tumor development, depending on the tumor cell type and the stage of tumorigenesis. Consistent with earlier reports concerning tumorigenesis in the intestine (17,18) and the upper digestive tract (19), we herein demonstrated that the genetic reduction of DNA methylation levels suppresses gastric tumorigenesis in two different models of gastric cancer: the MNU-induced model and the ApcMin/+ mouse model. Although further long-term observations would be required to determine whether DNA hypomethylation completely suppresses the progression to the invasive tumors or not, it is noteworthy that DNA hypomethylation inhibited the development of invasive adenocarcinoma, suggesting that DNA hypomethylation suppresses the malignant transformation of gastric tumors. This notion is also consistent with previous findings that genetic reduction blocked the development of invasive squamous cell carcinoma in the tongue and esophagus (19). These findings may shed some light on the involvement of epigenetic modification in the acquisition of invasive properties of tumor cells.

The presence of cancer-predisposed mucosa was initially described in the setting of oral carcinogenesis, and the concept of ‘field cancerization’ (28) has been widely accepted in regard to various organs, including the stomach (29,30). Recent studies suggest the involvement of epigenetic alterations in field cancerization in the human stomach (29–31). In the present study, the administration of MNU induced abnormal cell proliferation in non-cancerous gastric mucosa, which supported the concept of field cancerization. It is important that global DNA hypomethylation significantly suppressed the induction of abnormal proliferation in the MNU-exposed non-cancerous gastric mucosa. Because increased cell proliferation is obviously associated with the risk of cancer development, DNA hypomethylation suppresses the development of gastric tumors by preventing the gastric mucosa from being transformed into a favorable environment for tumor development.

In human gastric cancer, several tumor suppressor genes are activated more frequently by epigenetic silencing associated with site-specific DNA hypermethylation than by mutations (7). In addition, higher methylation levels at seven CpG islands in human gastric mucosa have also been reported to significantly correlate with a higher risk of gastric cancer development (29). Therefore, one may hypothesize that the genetic reduction of DNA methylation blocks the epigenetic silencing of the candidate genes that play a crucial role in MNU-induced gastric tumorigenesis. However, it remains controversial whether DNA hypermethylation plays a major role in gene silencing in rodent tumorigenesis. Although previous studies have indicated the presence of genomic hypermethylation in the tumor suppressor genes observed in rodent gastric tumors (32,33), another study revealed no involvement of DNA hypermethylation in the specific genes that are frequently hypermethylated in human gastric cancers (34). In the present study, we also examined the methylation status of the promoter regions of the genes that are frequently hypermethylated or hypomethylated in human gastric tumor (8–12,30) but found no altered DNA methylation patterns. Other epigenetic mechanisms, which are independent of the activation of the silenced genes, might therefore be associated with the suppression of gastric tumorigenesis by the genetic reduction of DNA methylation levels. Further analyses are therefore required to identify the target genes and/or sites of genomic hypomethylation responsible for the tumor suppression in this model.

Our data suggest that a pharmacological modification of the epigenetic status may be a potent strategy for the prevention and treatment of gastric cancers, and this idea is supported by the findings that Helicobacter pylori-infected human gastric mucosa harbors genomic hypermethylation (29,31) and several tumor suppressor genes are inactivated by promoter hypermethylation in human gastric cancer (7,9–12). However, possible side effects must be carefully taken into consideration for clinical application of DNA hypomethylating agents because the reduction of genomic methylation levels promotes the development of other types of tumors as previously reported (5,6,18).

In summary, we herein demonstrated that a reduction of the DNA methylation levels consistently suppresses gastric tumorigenesis, thus suggesting that DNA methylation is closely associated with gastric tumorigenesis.

Supplementary material

Supplementary Table 1 can be found at http://carcin.oxfordjournals.org/

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