PRDM5 Identified as a Target of Epigenetic Silencing in Colorectal and Gastric Cancer

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Abstract

Purpose: PR (PRDI-BF1 and RIZ) domain proteins (PRDM) are a subfamily of the kruppel-like zinc finger gene products that play key roles during cell differentiation and malignant transformation. The aim of the present study was to begin to examine the involvement of epigenetic alteration of PRDM expression in gastric and colorectal cancer.

Experimental Design: We used real-time PCR to assess expression of PRDM1-17. In addition, we used bisulfite PCR to assess DNA methylation and chromatin immunoprecipitation to assess histone modification in colorectal and gastric cancer cell lines lacking PRDM5 expression.

Results: Among the 17 PRDM family genes tested, we found that PRDM5 is the most frequently silenced in colorectal and gastric cancer cell lines. Silencing of PRDM5 was mediated by either DNA methylation or trimethylation of Lys27 of histone H3. Introduction of PRDM5 into cancer cells suppressed cell growth, suggesting that it acts as a tumor suppressor in gastrointestinal cancers. Methylation of PRDM5 was detected in 6.6% (4 of 61) of primary colorectal and 50.0% (39 of 78) of primary gastric cancers but not in noncancerous tissue samples collected from areas adjacent to the tumors.

Conclusions: Our data suggest that epigenetic alteration of PRDM5 (e.g., methylation of its 5′-CpG island or trimethylation of Lys27 of histone H3) likely plays a key role in the progression of gastrointestinal cancers and may be a useful molecular marker.

Epigenetic inactivation of tumor suppressor genes is frequently associated with the development and progression of human cancers (1, 2). One such epigenetic change is cytosine methylation, which leads to recruitment of transcriptional repressors and chromatin modification. During the development and progression of gastrointestinal cancer, genes involved in cell cycle regulation (3, 4), apoptosis (5, 6), DNA repair (7, 8), immune function (9), and signal transduction (10–12) are all silenced by aberrant methylation of the 5′-regions of CpG islands. Recent studies also have shown that gene silencing by DNA methylation is linked to histone modification. For example, deacetylation of histones H3 and H4 and dimethylation of lysine (K) 9 residues of histone H3 play important roles in DNA methylation–mediated gene silencing (13–15). Another histone modification involved in gene silencing is methylation of Lys27 of histone H3 (H3K27). This reaction is often catalyzed by the histone methyltransferase EZH2 (16), overexpression of which has been linked to various cancers, including cancers of the prostate, breast, and stomach (17–19). Little is known about the role of H3K27 methylation in cancer-related gene silencing, however.

PR (PRDI-BF1 and RIZ) domain proteins (PRDM) are a subfamily of the kruppel-like zinc finger gene products and play key roles during cell differentiation and malignant transformation. PRDM1/BLIMP1 was originally identified as a transcript that was rapidly induced during the differentiation of B lymphocytes into immunoglobulin secretory cells; its expression is characteristic of late B and plasma cell lines (20, 21), and it is mutated in diffuse large B-cell lymphoma (22). PRDM2/RIZ1 has histone methyltransferase activity (23), and its aberrant methylation has been reported in various types of cancers (24, 25). PRDM3/EVI1 and PRDM16/MEL1 are associated with translocation in hematopoietic malignancies...
and aberrant methylation and silencing of PRDM5 have been detected in both breast and liver tumors (28). In the present study, therefore, we used quantitative real-time PCR to examine the expression profile of 17 PRDM family genes in panels of gastric and colorectal cancer cell lines and primary cancer specimens. Taken together, our results suggest that epigenetic silencing of PRDM5 is a frequent event in gastrointestinal cancer, particularly in gastric cancer, and could be a useful molecular target for diagnosis and therapy.

**Materials and Methods**

**Cell line and specimens.** Eight colorectal (CaCo2, RKO, SW48, DLD1, LoVo, Colo320, HT29, and SW480) and six gastric cancer cell lines (MKN7, MKN45, MKN74, AZ521, NUGC4, and SNU638) were obtained from the Japanese Collection of Research Bioresources or the American Type Culture Collection. In addition, samples of 61 primary colorectal and 78 primary gastric cancers and 44 samples of stomach mucosa collected from areas adjacent to tumors were obtained from the Department of Surgery, Sapporo Keiyukai Hospital and the Division of Gastroenterology and Hepatology, Department of Internal Medicine, St. Marianna University School of Medicine Hospital after acquisition of informed consent from each patient. All cell lines were cultured in the appropriate medium. DNA was extracted using the phenol-chloroform method, and total RNA was extracted using bogen (Nippon Gene) according to the manufacturer’s instructions. To assess restoration of PRDM5 expression, cell lines were incubated for 72 h with 2 μmol/L 5-aza-2′-deoxycytidine (5-aza-dC, Sigma), a methyltransferase inhibitor. The cells were then harvested, and total RNA was extracted for further analysis.

**Reverse transcription-PCR.** First-strand cDNA was prepared by reverse transcription of 5 μg samples of total RNA using SuperScript III reverse transcriptase (Invitrogen). The primer sequences used were 5′-ATGTGGGAGGTTCGTGGGAGTAAG-3′ (PRDM5-forward) and 5′-TTTCTGCCCGCTGTTGATTGTCT-3′ (PRDM5-reverse). The PCR protocol for reverse transcription-PCR was 95°C for 5 min, followed by 35 cycles of 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C, and then a 7-min final extension at 72°C. Controls consisted of DNA treated identically but without the addition of reverse transcriptase. The integrity of the cDNA was confirmed by amplifying glyceraldehyde-3-phosphate dehydrogenase as described previously (29). Samples (10 μL) of the amplified products were subjected to 2.5% agarose gel electrophoresis and stained with ethidium bromide.

**Real-time quantitative reverse transcription-PCR.** First-strand cDNA was prepared by reverse transcription of 5 μg samples of total RNA using Taqman Gene Expression Assays [PRDM1, Hs00153357_m1; PRDM2, Hs01030716_m1; PRDM3, Hs00602795_m1; PRDM4, Hs00097363_m1; PRDM5, Hs00218855_m1; PRDM6, Hs01372996_m1; PRDM7, Hs00364862_m1; PRDM8, Hs00220274_m1; PRDM9, Hs00360639_m1; PRDM10, Hs00999748_m1; PRDM11, Hs00220293_m1; PRDM12, Hs0022080_m1; PRDM13, Hs0022082_m1; PRDM14, Hs01119056_m1; PRDM15, Hs01565177_m1; PRDM16, Hs00223161_m1; PRDM17, Hs01119928_g1; EZH2, Hs_00544830_ml; and glyceraldehyde-3-phosphate dehydrogenase, Hs_00266705_gl (Applied Biosystems)] with a 7900HT Fast Real-time PCR System (Applied Biosystems) according to the manufacturer’s instructions. ‘SDS2.1 software
(Applied Biosystems) was used to do comparative ΔCt analysis. Glyceraldehyde-3-phosphate dehydrogenase served as an endogenous control.

Combined bisulfite restriction analysis and bisulfite sequencing. Genomic DNA was initially treated with sodium bisulfite (Sigma) as described previously (30). Combined bisulfite restriction analysis (COBRA), a semiquantitative methylation assay, was then carried out as described previously (31). Touchdown PCR was then carried out as follows. After denaturing at 94°C for 3 min, the cycling protocol entailed 3 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s; 4 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s; 5 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s; and 26 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 30 s. Primers were designed based on the nucleotide sequence obtained from Genbank. The primers used for COBRA were 5'-GGTTYGGAT-TYGTTTTTGTTAT-3' (forward) and 5'-CCRCATTTAAAACCCTAAAA-TCA-3' (reverse; PRDM5 GM1-R). The PCR products were digested with the restriction endonuclease Hinf (TaKaRa) and precipitated with ethanol, after which the resultant DNA fragments were subjected to 2.5% agarose gel electrophoresis and stained with ethidium bromide.

To sequence the bisulfite PCR products, amplified fragments were cloned into pDNA2.1 vector using a TOPO-TA cloning kit (Invitrogen). The primer sequences used were 5'-GGTTYGGAT-TYGTTTTTGTTAT-3' (forward) and 5'-AAACAAACRAAACTCCCTCA-3' (reverse). The cycle sequencing reaction was then carried out using a BigDye terminator kit (Applied Biosystems), and the DNA was sequenced using an ABI 3100 automated sequencer (Applied Biosystems).

Chromatin immunoprecipitation analysis. Chromatin immunoprecipitation (ChIP) analysis was carried out as described previously (32). Briefly, cells were harvested and their proteins were cross-linked to DNA by incubation in 1.0% formaldehyde for 10 min at 37°C. The formaldehyde-fixed cells were allowed to settle on ice for 10 min and then spun down by brief centrifugation, after which the supernatant was carefully aspirated. The cells were then washed with ice-cold PBS containing protease inhibitors and resuspended in lysis buffer [1% SDS, 10 mmol/L EDTA, 50 mmol/L Tris-HCl

Fig. 1 Continued. C, bisulfite sequence analysis of PRDM5 in the indicated colorectal and gastric cancer cell lines. Ten clones were sequenced for each cell line. White and black circles, unmethylated and methylated alleles, respectively.

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(pH 8.0) and protease inhibitor]. The nucleoprotein complexes were sonicated to reduce the size of the DNA fragments to 200 to 1,000 bp and immunoprecipitated for 16 h at 4 °C with 10 μL anti-K9 acetylated histone H3, anti-K9 dimethylated histone H3, anti-K27 monomethylated histone H3, or anti-K27 trimethylated histone H3 (all from Upstate Biotechnology) or anti-histone H3 antibody (as an internal control; Abcam). The resultant immune complexes were collected using protein G-Sepharose beads, after which the DNA was purified by phenol–chloroform extraction, precipitated with ethanol and resuspended in distilled water. Approximately 1:100 of the precipitated DNA was used for PCR, and 1:100 of the solution before adding antibody was used as an internal control of the quantitative accuracy of the DNA. Quantitative PCR (qPCR) was carried out using the following primers: PRDM5, 5′-CTCTCCCTGCACCGTCTTCT-3′ (PRDM5-CHIP-forward), 5′-AGCGGGCCATCGAAATT-3′ (PRDM5-CHIP-reverse), and 5′-TC-TACCCTGCACCCCAA-3′ (PRDM5-qPCR probe); p21, GCCAACCT-CATCTCCTCAAGTTAAA (p21-Chip-forward), GTCGGCTGAGCA-ATTCC (p21-Chip-reverse), and CCAGATTGGTGCTCAC (p21-qPCR probe); p16, GGCGGATTTCTTTTTAACAGA (p16-Chip-forward), TTGAGCTGAGCAGAAAGG (p16-Chip-reverse), and TGACGG-CATCTCACC (p16-qPCR probe); and RASSF1, GACACCCGAGTAC (RASSF1-Chip-forward), GACACCGAGGAGA-TACC (RASSF1-Chip-reverse), and TGACCCCTCAAGAAC (RASSF1-qPCR probe).

Colony formation assays. Cells (1 × 10^5) were plated in 100-mm culture dishes for 24 h before transfection with the expression vector pcDNA3.1-PRDM5 or empty vector (5 μg each) using a Cell Line Nucleofector kit R and V (Amaxa) and a Nucleofector I electroporation device (Amaxa) according to the manufacturer's instructions. After transfection, cells were selected for 14 days in medium containing 0.6 mg/mL G418 and stained with Giemsa. The resultant colonies were then stained with crystal violet, and cells were counted in triplicate cultures using NIH Image software.

Luciferase assay. Three constructs (P1-P3) that covered different sized regions upstream of PRDM5 were created. All three were amplified by PCR and cloned into pCR2.1 TOPO (Invitrogen). After verification of the sequences, the fragments were ligated into pGL3-Basic vector (Promega), and cells (5 × 10^4 cells in 24-well plates) were transfected using LipofectAMINE 2000 (Invitrogen). A pGL3-Basic vector without the insert served as a negative control. Forty-eight hours after transfection, luciferase activities were measured using a Dual-Luciferase Reporter Assay (Promega).

Knockdown of EZH2 by RNA interference. We designed two retroviral vectors (RNAi-Ready pSIREN-RetroQ Vector, BD Biosciences) encoding a small hairpin RNA directed against EZH2 in SW480 cells (target sequences, 5′-ATATGACTGCTTCCTACAT-3′ and 5′-CATGTAGACAGGTGTATGA-3′). Both constructs reduced EZH2 expression by ~90%. As controls, we used small hairpin RNAs for the luciferase (Luc) gene synthesized by BD Biosciences or small hairpin RNA vector without the hairpin oligonucleotides (mock). Expression of EZH2 was examined using real-time PCR, after which two independent clones were used for further studies.

Immunohistochemical analysis. Tissue sections were deparaffinized, soaked in 10 mmol/L sodium citrate buffer, and boiled in an electronic oven for 15 min at 500 W to retrieve cell antigens. The sections were then immunohistochemically stained using the streptavidin-biotin peroxidase method (Universal DakoCytomation LSAB kit, DAKO) with primary antibodies against EZH2 (mouse anti-Ezh2 monoclonal antibody, Cell Signaling Technology, Inc.) and PRDM5 (rabbit polyclonal antibody, Abgent). Briefly, the sections were blocked in 3% H2O2 for 5 min and then incubated overnight with primary antibody at 4 °C. The samples were then washed with TBS buffer and incubated with secondary antibody for 30 min.

Results

Epigenetic silencing of PRDM5 in colorectal and gastric cancer cell lines. To examine the expression profile of PRDM family genes, we initially carried out real-time PCR using cDNA from normal colon and stomach tissues, two colorectal cancer cell lines (RKO and SW48), and a gastric cancer cell line (SW638). As can be seen in Fig. 1A, we detected expression of PRDM1, PRDM2, PRDM3, PRDM4, PRDM10, PRDM15, PRDM16, and PRDM17 in both normal tissues and cancer cell lines; conversely, we detected little or no expression of PRDM7, PRDM9, PRDM12, PRDM13, and PRDM14 in either normal tissue or cancer cell lines. On the other hand, we detected expression of PRDM5, PRDM6, PRDM8, and PRDM11 in normal tissues but that expression was down-regulated in at least two of the three cancer cell lines tested. To further evaluate expression of these five PRDM genes, we carried out real-time PCR in a larger panel of normal tissues and colorectal and gastric cancer cell lines (Fig. 1B; Supplementary Fig. S1). We detected expression of PRDM5 in normal colon, stomach, liver, and pancreatic tissue and in six of the cell lines tested. Little or no expression was detected in eight cell lines. Treating those eight cell lines with the methyltransferase inhibitor 5-aza-dC
restored PRDM5 expression in five. In addition, levels of PRDM6, PRDM8, PRDM11, and PRDM16 expression were lower in a subset of cancer cell lines than in normal tissues, but their expression was not restored by treatment with 5-aza-dC, indicating that DNA methylation is likely not the primary mechanism by which these genes were silenced (Supplementary Fig. S1).

**Aberrant DNA methylation of PRDM5 is associated with gene silencing.** We next used bisulfite sequencing to assess the methylation of the 5'-CpG island of PRDM5 in a panel of cancer cell lines and normal tissues (Figs. 1C and 2A). Although normal stomach tissue and MKN45 cells showed very little methylation, dense methylation in the region around the transcription start site was detected in SW48, SN1U638, MKN74, and MKN7 cells, which expressed little or no PRDM5. SW480 cells showed relatively sparse methylation, and LoVo cells showed very little methylation.

We then used luciferase assays to compare the PRDM5 promoter activity in cell lines that did (MKN45) or did not (SW480, MKN74, and SN1U638) express PRDM5. Three different constructs were created that covered different sized regions upstream from the transcription start site (Fig. 2A). Promoter activity was detected in all of the cell lines tested, regardless of gene expression, with most of the luciferase activity residing in the construct that contained the largest sequence (-205 to -502 bp; Fig. 2B). Thus, the silencing of PRDM5 does not seem to reflect the absence of a transcriptional regulator.

**Trimethylation of H3K27 is involved in silencing PRDM5 in SW480 cells.** Although SW480 cells express PRDM5 at only barely detectable levels, bisulfite sequencing revealed only sparse methylation (Fig. 1C). In addition, treatment with 5-aza-dC did not restore PRDM5 expression in SW480 cells. To determine whether another epigenetic mechanism, histone modification, was involved in silencing PRDM5 in SW480 cells, we examined the acetylation and methylation status of histone H3 on PRDM5 promoter activity (Fig. 3 A and B). Acetylation of histone H3K9 was well correlated with gene expression in p16, p21, and RASSF1 cells, which is consistent with earlier reports (29, 32). By contrast, dimethylation of H3K9 and monomethylation of H3K27 were inversely related to gene expression. In MKN7 cells, where PRDM5 expression was restored by 5-aza-dC treatment, levels of histone H3K9 acetylation were reduced, whereas levels of H3K9 dimethylation and H3K27 monomethylation were increased. Interestingly, we observed elevated levels of H3K27 trimethylation in SW480 cells, which suggests an alternative mechanism for silencing PRDM5 expression there. Because trimethylation of H3K27 is catalyzed by EZH2, one of the polycomb repressor complex 2 proteins, we examined the effect of knocking down EZH2 on expression of PRDM5 in SW480 cells. We found that PRDM5 gene expression was restored in SW480 cells when EZH2 was stably knocked down by either of two different small hairpin RNA constructs targeting EZH2 (data were averaged and shown in Fig. 4A), which suggests trimethylation of H3K27 plays a key role in silencing PRDM5 expression in SW480 cells.

Because overexpression of EZH2 has been seen previously in cancer (17–19), we also carried out an immunohistochimical analysis of EZH2 expression in a panel of primary gastric cancers. We found that 9 of the 10 specimens tested expressed higher levels of EZH2 than adjacent noncancerous stomach tissues (Fig. 4B), which suggests that overexpression of EZH2 is a frequent event in gastrointestinal cancer that is not always associated with down-regulation of PRDM5.

**Expression of exogenous PRDM5 suppresses cell growth in gastric cancer lines.** We next used colony formation assays to determine whether PRDM5 has tumor suppressor activity (Fig. 5). When PRDM5 was introduced to two gastric cancer cell lines that do not otherwise express the gene,
there was a significant reduction of colony formation, suggesting that PRDM5 does indeed function as a tumor suppressor gene.

**Epigenetic silencing of PRDM5 in primary colorectal and gastric cancers.** Finally, to assess the extent to which PRDM5 is methylated in primary colorectal and gastric cancers, we initially used COBRA to detect methylation of the region around the transcription start site of the gene (Fig. 6A). We found that 4 of 61 primary colorectal and 39 of 78 primary gastric cancers showed such methylation. To examine the methylation status of PRDM5 in more detail, we then carried out bisulfite sequencing using DNA from three primary gastric cancers (Fig. 6B). In the specimens shown by COBRA to be methylated (WG313 and WG314), all of the CpG sites in the region analyzed were densely methylated. Moreover, high quality RNA obtained from 15 of the primary gastric cancers showed diminished expression of PRDM5 in 11 (73%), although expression of PRDM5 also was diminished in a subset of tumors without methylation (Fig. 6C).

**Discussion**

In the present study, we identified PRDM5 as a target of epigenetic inactivation in both colorectal and gastric cancers; in fact, PRDM5 seems to be the most frequently altered PRDM family gene in gastrointestinal cancer. We also found that expression of PRDM6, PRDM8, PRDM11, and PRDM16 was down-regulated in a subset of colorectal and gastric cancer cell lines, but the fact that their expression was not significantly restored by treating cells with 5-aza-dC indicates that DNA methylation was likely not the principle mechanism by which they were silenced. Earlier studies showed that PRDM2/RIZ1 is silenced in gastrointestinal cancer (25) and that it has an alternative transcript, RIZ2, which lacks the SET domain (24). Our strategy failed to detect down-regulation of PRDM2/RIZ1 in cancer cells because the Taqman primer/probe set we used amplified both PRDM2/RIZ1 and RIZ2. Several other PRDMs, including PRDM1, PRDM3/EVI1, and PRDM16, also express alternative transcripts that lack the SET domain. Epigenetic inactivation of
PRDMs derived from alternative promoters should be examined further.

It was reported previously that DNA methylation of the region around the transcription start site of PRDM5 is involved in silencing the gene in breast and liver cancer cells (28). Consistent with that report, we found that levels of PRDM5 expression in MKN7 cells were correlated with DNA methylation, deacetylation of histone H3, and...
methyltion of H3K9. By contrast, SW480 cells showed sparse DNA methylation in the region of the PRDM5 promoter, and trimethylation of H3K27 was correlated with gene silencing. Recently, Abhosh et al. (33) reported that introduction of a dominant-negative H3K27 mutant into cancer cells restores expression of RASSF1, indicating that such histone modification is involved in certain types of gene silencing in cancer. Methylation of H3K27 is catalyzed by EZH2, and overexpression of EZH2 has also been reported in cancers of the prostate, breast, and stomach (17–19). In the breast, for example, overexpression of EZH2 is an early event during tumorigenesis and is involved in preneoplastic progression (34). In the present study, we found that 90% of the gastric cancers tested expressed higher levels of EZH2 than adjacent noncancerous stomach tissues (Fig. 4B), suggesting that down-regulation of PRDM5 cannot be explained solely as the result of overexpression of EZH2. Indeed, Cha et al. (35) reported recently that phosho-AKT can phosphorylate EZH2, thereby preventing its interaction with histone H3, which suggests that overexpression of EZH2 may not always lead to H3K27 trimethylation of genes. Because SW480 cells do not carry a PIK3CA mutation and do not show the phosphorylated form of AKT, we would expect that EZH2 may be active in this cell line (data not shown).

The molecular mechanism by which PRDM5 suppresses cell growth remains unknown. Deng et al. (28) reported that introducing PRDM5 into cancer cells using an adenoviral vector increased the fractions of G2-M and sub-G1 cells, suggesting that PRDM5 is involved in cell cycle arrest and apoptosis. Although it remains unclear whether the PR domain of PRDM5 has histone methyltransferase activity, PRDM5 may be involved in transcriptional regulation of genes associated with the cell cycle and apoptosis. Moreover, PRDM5 contains 16 zinc finger domains, which often show sequence-specific DNA binding activity. Thus, a full understanding of the role played by PRDM5 in gene transcription, cell growth, and apoptosis awaits further study.

In summary, we have shown for the first time that PRDM5 expression is often epigenetically silenced in colorectal and gastric cancers. Such silencing of PRDM5 was mediated by either DNA methylation or trimethylation of H3K27. That introduction of PRDM5 into cancer cells suppressed cell growth suggests PRDM5 acts as a tumor suppressor in gastrointestinal cancers. Understanding the precise role played by PRDM5 in gene transcription will not only increase our understanding of the biology of gastrointestinal cancer but may also enable epigenetic silencing of PRDM5 to serve as a useful molecular target for diagnosis and therapy.

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