DNA damage-inducible gene, Reprimo functions as a tumor-suppressor and is suppressed by promoter methylation in gastric cancer

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Running title: Reprimo in gastric cancer


Conflict of Interest: All authors of this manuscript have no commercial associations that might pose a conflict of interest in connection with the submitted article.
Abstract

In several types of human cancer, the gene expression of Reprimo, a highly glycosylated protein, is frequently silenced via methylation of its promoter. The aim of this study was to characterize the epigenetic inactivation of Reprimo and its biological function and clinical relevance in gastric cancer (GC). The correlation between Reprimo methylation and clinical relevance was assessed in 83 primary human GC tissues. The effects of Reprimo expression were also examined using in vitro and in vivo assays. Reprimo methylation was cancer-specific and frequently observed. In two GC cell lines without Reprimo methylation, we observed faint or weak Reprimo expression under normal conditions and high expression under DNA-damaging conditions. In four GC cell lines with Reprimo methylation, however, Reprimo expression remained faint even under DNA damaging conditions, with expression being restored in combination with agents that induce demethylation. Enforced Reprimo expression robustly inhibited cell proliferation and anchorage-independent colony formation and enhanced DNA damage-induced apoptosis. Inverse effects were observed via small interfering RNA-mediated knockdown of endogenous Reprimo. Reprimo expression inhibited tumorigenesis in vivo. Reprimo methylation was also associated with a poor response in GC patients treated with chemotherapy ($P=0.028$), and a poor prognosis in advanced GC patients ($P=0.03$). In conclusion, Reprimo expression is normally induced in response to DNA damage, acting as a novel tumor suppressor in GC. However, Reprimo methylation abrogates its expression and effects. The clinical assessment of Reprimo promoter methylation may serve not only as a predictive marker for chemotherapy, but also as a marker for tumor aggressiveness.
Introduction

Gastric cancer (GC) remains a major clinical challenge, being both the fourth most common cancer and the second leading cause of cancer-related death worldwide (1). Globally, a total of 989,600 new cases and 738,000 deaths are estimated to have occurred in 2008. The case-fatality ratio of GC is higher than that of other common malignancies, such as colorectal, breast, or prostate cancer (2). However, while recent improvements in diagnostic tools and methods have facilitated the detection of GC at an early stage, advanced GC is still associated with a poor clinical outcome due to the limited success of surgery and chemotherapy.

Accumulating evidence suggests that cancer is caused by both epigenetic and genetic abnormalities (3). The epigenetic pathway involved in the development of cancer is determined by chromatin structure, including DNA methylation, histone modifications, or non-coding regulatory RNAs (4). DNA methylation occurs at the fifth carbon of the cytosine, termed a dinucleotide CpG, which precedes a guanine. When methylation occurs within dinucleotide CpG-rich regions—"CpG islands"—of a gene promoter, it is associated with a compact chromatin structure and is accompanied by transcriptional silencing of the affiliated gene, especially tumor-suppressor genes (5). DNA methylation occurs more frequently than genetic mutations in GC and has recently opened the exciting doors for developing cancer biomarkers and therapeutic targets (6-8).

Reprimo is a highly glycosylated protein localized predominantly in the cytoplasm, which was identified by differential display screening of genes distinctly expressed between X-ray irradiated wild-type and p53-deficient mouse embryonic fibroblast cells (9).
Transiently enforced Reprimo expression induces G2 arrest of the cell cycle by inhibiting Cdc2 activity and nuclear translocation of the Cdc2-cyclin B1 complex in various cell lines such as DLD1, HeLa and MCF7 cells (9), whereas loss of Reprimo expression is associated with an increase in uterine sarcoma incidence in mutant p53 transgenic mice treated with the carcinogen 1,2-dimethylhydrazine (10). These findings suggest that both the expression and function of Reprimo as a tumor-suppressor gene are regulated in a p53-dependent manner. In contrast, Reprimo promoter methylation has been reported to be involved in an alternative mechanism that regulates Reprimo expression in various types of tumors (11-13). Further, the regulation of Reprimo expression is independent of p53 status in neuronal cells treated with copper, with neuronal damage being associated with Wilson’s disease (14). In GC, Reprimo promoter methylation is a frequent event as we, and others, have previously reported (11, 13, 15). The relationship between Reprimo and GC, however, has never been examined from a mechanistic and functional point of view.

In the present study, we examined the clinical significance of the Reprimo gene, especially for its promoter methylation in primary GC and assessed the functional change associated with its abnormality.

**Materials and Methods**

**Cell Lines and Tissue Samples**

The GC cell line MKN7 was provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University in December 2006. Seven other GC cell lines (GCIY, AZ521, KatoIII, SH10, H111, MKN74, and
NUGC4) were purchased from RIKEN BioResource Center (Ibaraki, Japan) in December 2006. All cell lines were grown in RPMI 1640 medium (GIBCO, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS). We confirmed the authenticity of the cell lines using short tandem repeat profiling in June 2013. UV irradiation (10 mJ/cm²) was performed without medium using a Stratalinker UV Crosslinker (Agilent Technologies, Santa-Clara, CA, USA) to cause DNA damage. The cells were then incubated in medium with 10% FBS for 24 h, and analyzed. The DNA-damaging agents cisplatin (CDDP) and 5-fluorouracil (5-FU) were purchased from Kyowa Hakko (Tokyo, Japan).

Ten pairs of frozen primary human GC tissues and corresponding normal mucosal tissues, obtained at least 5 cm from the tumor edge, were analyzed as the preliminary data set for expression and methylation status. Formalin-fixed, paraffin-embedded tissue samples were collected from 83 patients with primary GC who underwent a gastrectomy at the Kitasato University Hospital in accordance with the gastric cancer treatment guidelines in Japan (16). Informed consent was obtained from all patients before sample collection. All histopathological findings were examined in accordance with the 14th edition of the Japanese Classification of Gastric Carcinoma (JCGC) (17). Advanced gastric cancer is defined as cancer invading the muscularis propria or deeper, irrespective of the presence of lymph node metastases in JCGC. TNM classification was made accordance with the 7th edition of the American Joint Committee on Cancer/International Union Against Cancer (AJCC/UICC) staging system. Detailed information on the 83 patients is summarized in Table 1. This study was performed with approval of the Ethics Committee of Kitasato University.
Bisulfite Treatment of DNA and Sequencing Analysis

Tissue sections from the tumor and the corresponding normal mucosa were sharply dissected on hematoxylin-and-eosin-stained slides, and genomic DNA was subsequently extracted using a QIAamp DNA FFPE Kit (QIAGEN Sciences, Hilden, Germany). Genomic DNA from cell lines and frozen tissues were extracted using a QIAamp DNA Mini Kit (QIAGEN). Bisulfite treatment was performed using an EpiTect Bisulfite Kit (QIAGEN) and was subsequently amplified via polymerase chain reaction (PCR). The PCR conditions and sequences of primers and probes used in this study are shown in Supplementary Table 1. Primer sequences for Reprimo promoter region were designed to recognize DNA alterations caused by bisulfite treatment. The PCR products were purified and then inserted into a pCR®-4-TOPO® vector using a TOPO TA Cloning® Kit for sequencing (Invitrogen, Carlsbad, CA, USA). Ten clones were selected for each sample and then sequenced using a Big Dye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). For analysis of $p53$ mutation, genomic DNA was also sequenced using primers for exons 4 to 9 of the $p53$ gene (18).

Quantitative-Methylation-Specific-Polymerase Chain Reaction (Q-MSP)

To quantify the methylation level of Reprimo, quantitative-methylation-specific-PCR (Q-MSP) was performed via an iQ™ Supermix (Bio-Rad Laboratories, Hercules, CA, USA) in triplicate on the iCycler iQ™ Real-Time PCR Detection system (Bio-Rad Laboratories). Serial dilutions of CpGenome™ universal methylated DNA (Chemicon International, Inc.,
Temecula, CA, USA) were used to construct the calibration curve for each plate. CpGenome™ universal methylated DNA and unmethylated DNA (Chemicon) were used as a methylation positive and a negative control, respectively. The methylation value was defined by the ratio of fluorescence intensity emitted from the amplified bisulfite DNA of the CpG islands of Reprimo gene divided by that of β-actin and multiplied by 100 (Q-MSP value). The optimal cut off value for Q-MSP was determined using a receiver operating characteristic (ROC) curve.

**Quantitative-Reverse Transcriptase-Polymerase Chain Reaction (QRT-PCR)**

Total RNA was isolated using TRIzol Reagent (Invitrogen) in accordance with the manufacturer’s protocol and reverse-transcribed with a SuperScript III Reverse Transcriptase kit (Invitrogen). Quantitative-reverse transcriptase-PCR (QRT-PCR) was performed to quantify the expression level of Reprimo gene using the iCycler iQ™ Real-Time PCR Detection System (Bio-Rad). Relative quantitative analysis normalized to β-actin was performed via the comparative cycle threshold method (19).

**5-Aza-2′-Deoxycytidine (5-Aza-dC) Treatment**

Cells (1×10^6/T-75 flask) were treated with 5 µmol/L of the demethylating agent 5-aza-2′-deoxycytidine (5-Aza-dC; Sigma-Aldrich, St. Louis, MO, USA) dissolved in 50% acetic acid or were mock-treated with PBS including the same amount of acetic acid every 24 h for 5 days (20).
Western Blot Analysis

Whole cell lysates were extracted in RIPA buffer (Pierce, Rockford, IL, USA) supplemented with 10 µL/mL Halt™ Protease Inhibitor Cocktail Kit (Pierce) and Halt™ Phosphatase Inhibitor Cocktail Kit (Pierce). The protein concentrations were determined using a Coomassie Plus-The Better Bradford™ Assay Kit (Pierce), separated on NuPAGE® 4-12% Bis-Tris Gel (Invitrogen) with anti-flag-V5 mouse monoclonal antibody (Sigma-Aldrich) and anti-β-actin mouse monoclonal antibody (Sigma-Aldrich).

Plasmid Transfection and Small Interfering RNA Transfection

A full-length complementary DNA (cDNA) of Reprimo was isolated from GCIY cells using PCR with Platinum Pfx® DNA Polymerase (Invitrogen). The plasmid expressing Reprimo (pcDNA™ 3.1-Reprimo) was obtained by cloning the PCR products into the pcDNA™ 3.1D/V5-TOPO® vector via a pcDNA™ 3.1 Directional TOPO® Expression Kit (Invitrogen). The sequence of the cloned Reprimo cDNA was verified by sequencing analysis, and an empty vector with self-ligation (pcDNA™ 3.1-mock) was used as a control. Cells were transfected using Lipofectamine™ 2000 Reagent (Invitrogen) in OPTI-MEM medium (Invitrogen). Stable clones with Reprimo or mock were established by G418 (GIBCO) selection. For small interfering RNA (siRNA) transfection, cells (2x10^5) were seeded in 6-well plates 24 h before transfection. The cells were transfected with 1 µmol/L Accell SMARTpool, siRNA-Reprimo (Thermo Fisher Scientific, Lafayette, CO, USA) mixed with Accell siRNA Delivery Media (Thermo Fisher Scientific) according to the Thermo Scientific Dhharmacon® Accell™ siRNA Delivery Protocol. The Accell Non-
targeting Pool (siRNA-ctr) and Accell siRNA Delivery Media alone (siRNA-mock) were used as a control for non-sequence-specific effects and as a mock-treatment, respectively.

**Anchorage-Independent Colony Formation Assay**

Anchorage-independent cell growth was analyzed by plating 0.36% top agarose (Bacto™ Agar; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing $1 \times 10^5$ cells on a surface of 0.72% bottom agarose in the 6-well plates. Cells were fed weekly by overlying fresh soft-agar solution, and colonies were photographed after 2 weeks of incubation. The experiment was performed in triplicate.

**Proliferation Assay and Invasion Assay**

The effects of Reprimo expression on cell proliferation and viability ($2 \times 10^5$ cells/well) were measured using the Premix WST-1 Cell Proliferation Assay System (Takara Bio, Co., Tokyo) in 96-well plates. The invasion assay was performed in the 24-well BD BioCoat™ Matrigel™ Invasion Chamber (BD Biosciences Discovery Labware, Bedford, MA, USA). Forty-eight hours after transfection, the lower chamber was filled with 750 µL DMEM (GIBCO) supplemented with 10% FBS as a chemoattractant, and cells ($5 \times 10^5$/well) were then seeded into an upper chamber in 500 µL of serum-free DMEM. After incubation for 22 h, the membrane of the upper chamber was fixed and stained using Diff-Quik reagent (Sysmex, Co., Kobe, Japan). Cells that had invaded through the membrane were counted in four separated fields per well. Both experiments were performed in triplicate.
Cell Cycle and Apoptosis Assays

Cell cycle and apoptosis assays were performed using the Guava PCA System (Guava Technologies, Inc., Hayward, CA, USA). In the cell cycle assay, after synchronization for 48 h in serum free medium, the cells were then cultured in complete medium. After 24 h, the cells (2×10^5) were then fixed in 75% ethanol, and stained with propidium iodide (Guava Cell Cycle Reagent). In the apoptosis assay, the harvested cells (1x10^5) were stained with Annexin V and 7-AAD (Guava Nexin Reagent) for discrimination of early and late apoptotic cells, respectively. Both experiments were performed in triplicate and analyzed using CytoSoft 2.1.5 software (Guava Technologies).

Mouse Xenograft Assay

Female BALB/cAJcl nude mice aged 5 weeks were purchased from CLEA Japan Inc. (Tokyo, Japan). Mice were subcutaneously injected with AZ521 patient cells or AZ521 stable cells transfected with pcDNA™ 3.1-Reprimo or pcDNA™ 3.1-mock on both flanks (2.5 × 10^6 cells per flank). In the treatment combined with CDDP and 5-FU, mice with tumors approximately 5 mm in diameter received intraperitoneal administration of CDDP (0.1 mg/kg) and 5-FU (10 mg/kg) once daily for 7 days (21). Each group consisted of three mice (n=3). Tumor volumes were calculated from caliper measurements of two orthogonal diameters (larger [x] and smaller [y] diameters) using the following formula: volume = xy^2/2. All animal experiments were performed in strict accordance with the guidelines for animal experiments of the Saitama Cancer Center.
Statistical Analysis

Categorical variables were analyzed via Chi-square test or Mann-Whitney U-test, and continuous variables were analyzed via Student’s t-test. For continuous variables, the data are expressed as mean ± standard deviation (SD). The disease-specific survival (DSS) was measured from the date of surgery to the date of death or the last follow-up. DSS was estimated via the Kaplan-Meier method, and values were compared using the log-rank test. \( P < 0.05 \) was considered to indicate statistical significance. All statistical analyses were conducted with the Statistical Analysis System (SAS) software package (SAS Institute, Cary, NC, USA).

Results

Cancer-Specific Reprimo Promoter Methylation in GC

The location of CpG islands in the 5’-flanking region of Reprimo genomic sequencing is shown in Figure 1A. The Reprimo promoter harbors CpG islands encompassing the transcription start site, and we previously reported that Reprimo harbored its promoter methylation in 75% (6/8) of GC cell lines, 80% (8/10) of primary human GC tissues, and 10% (1/10) of corresponding normal tissues in the preliminary data set of 10 matched pairs by bisulfite sequencing analysis (15). Q-MSP can achieve a much more objective and specific assessment of a small amount of genomic DNA through PCR amplification using methylation-specific primers and fluorescent probes (22). Q-MSP analysis was therefore performed using the same samples (15) to confirm the above findings on bisulfite
sequencing analysis (Figure 1B). The Q-MSP value was significantly higher in GC cell lines (5.25 ± 1.73, \( P=0.006 \)) or primary human GC tissues (3.05 ± 0.88, \( P=0.014 \)) than in the corresponding normal tissues (0.51 ± 0.34). Next, the bisulfite sequencing with cloned PCR products was performed to assess the correlation between the Q-MSP values and methylation status of the individual CpG sites (Figure 1C and 1D). Higher Q-MSP values tended to indicate denser methylation.

We next examined the correlation of Reprimo methylation to clinical relevance using Q-MSP in 83 primary tumors and corresponding normal mucosa tissue specimens with human GC patients. The optimal cut-off value for distinguishing between tumor and normal tissues was calculated using a ROC analysis, and the best cut-off value of 0.42 showed a sensitivity of 69% and specificity of 82% (Figure 2A). Further, all cell lines or human samples with a Q-MSP value higher than 0.42 showed denser methylation on bisulfite sequencing with cloned PCR products (i.e. hypermethylation, Figure 1D). Reprimo promoter hypermethylation was found in 69% (57/83) of tumor tissues and 18% (15/83) of corresponding normal tissues, with both differences being statistically significant (\( P<0.0001 \), Figure 2B). In addition, the Q-MSP values in tumor tissues were significantly higher than those in normal tissues, not only for different tumor stages, but also in individual patients (\( P<0.0001 \), Figure 2C). These findings suggest that Reprimo promoter hypermethylation might be a frequent and early alteration that occurs specifically in the development of cancer.

Because some genes show age-related methylation alterations (23), the clinicopathological characteristics of Reprimo promoter hypermethylation were assessed.
Although Reprimo promoter methylation was marginally associated with vascular invasion ($P=0.058$), no correlation was found between Reprimo promoter hypermethylation and clinicopathological factors, including aging and stage of tumor development (Table 1).

**Transcriptional Silencing by Reprimo Promoter Methylation**

Reprimo was classified as a $p53$-mediated gene in embryonic fibroblast cells (9). However, Reprimo promoter methylation is an alternative mechanism in the regulation of Reprimo expression in various tumor types (11-13). Further, the regulation of Reprimo expression is independent of the $p53$ gene status in neuronal cells treated with copper (14). Reprimo expression was recently reported to be repressed by a ligand-activated estrogen receptor via a mechanism involving histone deacetylase-7 and Fork head A1 in breast cancer (24). Thus, $p53$ gene status is unlikely to be the only determining factor in the regulation of Reprimo expression.

To clarify whether or not promoter methylation or $p53$ gene status results in transcriptional silencing of Reprimo in GC, the expression level was evaluated by RT-PCR in eight GC cell lines and the preliminary data set of five matched pairs (Figure 3A). In Reprimo promoter methylation, Reprimo mRNA expression was undetectable in four of six GC cell lines, all four tumor tissues, and one of the corresponding normal tissues by RT-PCR. Further, similar results were observed with Q-RT-PCR. Reprimo promoter methylation showed a trend towards the decreased expression, while Reprimo expression was undetectable even in SH10 cells without methylation. Next, we examined the correlation between $p53$ gene status and Reprimo expression. The mutation of $p53$ was documented in five of eight GC cell lines and two of tumor tissues (Supplementary Figure
1). In the *p53* mutation, Reprimo mRNA expression was undetectable in four of five GC cell lines and one of the two tumor tissues. Overall, Reprimo expression was observed at a weak or faint level, regardless of the promoter methylation or *p53* gene status. We were unable to assess the protein level because of the absence of a commercially available antibody. In addition, we examined the correlation between *p53* gene status and Reprimo promoter methylation in 83 primary human GC tissues. Mutation of *p53* was found in 34.9% (29/83) of tumor tissues, which were missense mutations clustered in the core DNA-binding domain (residues 94-312) (25) and have been documented as biologically inactive via the transcriptional activity on the WAF1 promoter in the Universal Mutation Database (UMD) TP53 mutation databases (26) (Supplementary Table 2). Reprimo promoter methylation was unrelated to *p53* gene status (Table 1).

Given that Reprimo expression was induced by X-ray irradiation as the DNA damage in embryonic fibroblast cells with *p53* wild type (9), we examined whether or not Reprimo expression was altered in response to forms of genotoxic stress, such as UV irradiation, 5-FU (fluoropyrimidine class), or CDDP (platinum-based class). SH10 cells without Reprimo methylation increased Reprimo expression under DNA-damaging conditions despite the presence of the *p53* mutation (Figure 3B), speculating that DNA-damage-inducible Reprimo expression may be abrogated due to methylation of the Reprimo promoter in a *p53*-independent manner. To substantiate this hypothesis, six cell lines with different *p53* gene and Reprimo methylation statuses were treated with the demethylating agent 5-Aza-dC in the presence or absence of DNA-damaging agents (Figure 3C). In GCIY cells without Reprimo promoter methylation, Reprimo expression
was weak under normal conditions, but was induced by UV irradiation despite presence of p53 mutation, in line with findings in SH10 cells. On the other hand, in four cell lines (AZ521, NUGC, KatoIII, and MKN74 cells) with Reprimo promoter methylation, the expression of Reprimo could be slightly restored by demethylation alone, but not UV irradiation. Notably, in combination with demethylation and UV irradiation, these cell lines were restored regardless of p53 gene status. Similar trends were also observed in the treatment with 5-FU or CDDP (data not shown). We examined Reprimo mutations via direct sequencing analysis using primer sequences including the entire open reading frame according to previous study (13) in eight GC cell lines and 83 primary tumors, and no mutations were found (data not shown).

**Tumor-Suppressive Activity after Transfection of Reprimo**

Transcriptional silencing of Reprimo due to its frequent promoter methylation prompted investigation into the potential role of Reprimo as a tumor-suppressor gene in GC. AZ521 cells with methylation were stably transfected with pcDNA™ 3.1-Reprimo containing the full-length cDNA of Reprimo gene (Figure 4A). The AZ521 cells transfected with pcDNA™ 3.1-Reprimo (AZ-r10 and AZ-r16 cells) resulted in an increase of mRNA levels, unlike those with pcDNA™ 3.1-mock (AZ-mock cells). Reprimo protein levels were also confirmed by western blotting using an anti-flag-V5 antibody. An important characteristic in the growth of aggressive tumors is the ability of cancer cells to grow under anchorage-independent conditions. We therefore examined whether or not the exogenous expression of Reprimo could suppress the growth of cells in soft agar. The AZ-r10 and AZ-r16 cells
showed significantly decreased cell size and colony number (Figure 4B), but no decrease in matrigel invasive ability (Supplementary Figure 2A) when compared to AZ-m cells. We also observed a decrease in cellular proliferation under normal conditions or CDDP exposure in AZ-r10 and AZ-r16 cells (Figure 4C).

Reprimo has been shown able to act as a G2 phase cell cycle break in cell lines such as DLD1, HeLa, and MCF7 cells (9), but not in LβT2 and GH3 pituitary cells (27), indicating that it may have different functions depending on the type of cell. To examine the role of Reprimo in regulating cell growth in GC, a cell cycle assay was performed (Supplementary Figure 2B). Analysis of the cell cycle distribution showed a significantly increased sub-G1 population along with minimally affected S and G2/M populations in AZ-r10 and AZ-r16 cells. H111 cells transfected with pcDNA™ 3.1-Reprimo also showed similar results. To verify these results, an apoptosis assay was performed under exposure to the DNA-damaging agents 5-FU or CDDP (Figure 4D). Both early and late apoptosis were significantly enhanced in AZ-r10 and AZ-r16 cells compared with AZ-m cells.

To further confirm the tumor-suppressive activity of Reprimo, knockdown of endogenous Reprimo was performed using siRNA. SH10 cells without methylation were transfected with siRNA-Reprimo (SH-si cells) or siRNA-ctr (SH-ctr cells). SH-si cells, but not SH-ctr cells, resulted in decreased Reprimo expression induced by CDDP exposure when compared to mock-treatment cells (Figure 4E) and showed a dramatic repression of 5-FU or CDDP-mediated apoptosis (Figure 4F). We observed similar results in GCIY cells (data not shown).
Tumor-Suppressive Activity of Reprimo in Mouse Xenograft Assay

To examine the effect of Reprimo on tumorigenesis in vivo, AZ521 patient (AZ-p), AZ-m, AZ-r10, or AZ-r16 cells were subcutaneously injected into both flanks of nude mice. A remarkable reduction of tumor volume was observed in mice injected with Reprimo-transfected cells (AZ-r10 or AZ-r16 cells) when compared to AZ-p or AZ-m cells. (Figure 5A and 5B). Tumor weight was also significantly decreased in mice injected with AZ-r10 or AZ-r16 cells (Figure 5C). As Reprimo promoter methylation was marginally associated with vascular invasion in 83 patients with GC (Table 1), angiogenesis, visualized as a marked red color with the formation of a dense surrounding vascular network, was more greatly observed in the tumors of mice injected with AZ-p or AZ-m cells than those injected with AZ-r10 or AZ-r16 cells (Figure 5D).

To examine the anticancer effect of treatment combined with CDDP and 5-FU via Reprimo expression in vivo, mice with tumors approximately 5 mm in diameter received intraperitoneal administration of CDDP and 5-FU for 7 days. No anticancer effect was observed in mice injected with AZ-p or AZ-m cells, but an anticancer effect was observed in the mice injected with AZ-r10 or AZ-r16 cells (Supplementary Figure 3).

Potential Utility of Reprimo Methylation as a Predictive Marker

The most promising candidate epigenetic marker reported thus far for the prediction of responses to chemotherapy is O⁶-methylguanine-methyltransferase (MGMT). MGMT reverses the addition of alkyl groups to the guanine base of DNA and protects DNA damage caused by the alkylating agents, such as temozolomide, thus generating
chemoresistance. Therefore, the methylation of MGMT is a predictor of a favorable response to temozolomide in gliomas (28). In the present study, we found that Reprimo could enhance 5-FU or CDDP-mediated apoptosis in GC. The combination of CDDP and fluoropyrimidine class, including 5-FU, capecitabine, or S-1, was the most standard regimen of chemotherapy against GC (29, 30). We therefore analyzed 83 human GC samples to evaluate whether or not assessment of Reprimo methylation could be a predictive marker for chemotherapy combined with CDDP and fluoropyrimidine class. Of the 49 patients who received a combination regimen for metastatic or recurrent GC, 41 had measurable lesions. The tumor response was assessed according to Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1 (31). Reprimo promoter hypermethylation, previously defined as a value greater than 0.42 on Q-MSP, was inversely associated with the response rate ($P=0.028$, Table 1), and showed a trend towards a worsening prognosis, compared with absence of Reprimo promoter methylation (Figure 5E).

We next examined whether or not the assessment of Reprimo hypermethylation could be used to predict patient prognosis and thereby serve as a marker for tumor aggressiveness in 68 patients with advanced GC. Reprimo promoter hypermethylation had a significantly poor outcome ($P=0.031$, Figure 5F). In multivariate analysis, Reprimo promoter hypermethylation was independently associated with a poor prognosis, with a hazard ratio of 2.148 ($P=0.026$; Supplementary Table 3).

**Discussion**
We previously reported that Reprimo promoter methylation was one of the most frequent cancer-specific alterations, using bisulfite sequencing analysis in primary GC tissues (15). Of note, many of the methylation alterations are tissue-specific or associated with the field of cancerization, but are not cancer-specific (32). As such, we examined the epigenetic inactivation, biological function, and clinical relevance in GC in the present study.

In GC cell lines without Reprimo promoter methylation, we observed an unexpectedly faint or weak Reprimo expression under normal conditions but a robust expression under DNA-damaging conditions. In GC cell lines with Reprimo promoter methylation, however, we observed faint expression even under DNA-damaging conditions, with expression being restored only in combination with the demethylating agent. In 83 human GC samples, Reprimo promoter methylation was a cancer-specific alteration that frequently occurred at an early stage in the development of cancer and was not related to p53 status. Thus, Reprimo promoter methylation may be the predominant mechanism for loss of DNA damage-inducible Reprimo expression, regardless of p53 status.

Although promoter methylation alterations are common events in human cancers, most are merely passengers that have no effect per se on the process of carcinogenesis. In contrast, driver methylation mainly inactivates tumor-suppressive activity, leading to the processes of tumor initiation, progression or metastasis. Thus, the identification of driver methylation will be of key importance for cancer etiology, development of biomarkers, and therapeutic targets (33). Enforced Reprimo expression robustly inhibited tumor cell growth, including cell proliferation and anchorage-independent colony formation. In addition, the cell-cycle distribution showed a significantly increased sub-G1 population, but not G2
arrest that has been reported as a function of Reprimo (9). Our findings are in line with those in pituitary tumors (27), and Reprimo may therefore have differential roles depending on the type of tumor. Apoptosis assays also showed that enforced Reprimo expression enhanced DNA damage-induced apoptosis, indicating that Reprimo might suppress cell growth through the induction of apoptosis but not cell-cycle arrest in GC. Inverse effects were observed on siRNA-mediated knockdown of endogenous Reprimo. Further, Reprimo expression inhibited tumorigenesis in a mouse xenograft model. Taken together, these findings suggest that Reprimo possesses bona fide tumor-suppressive activity, and its methylation may be a driver methylation in GC. Further investigation of the Reprimo-regulated molecules is required to clarify the mechanisms by which Reprimo exerts tumor-suppressive activity.

Three major clinical oncology areas can potentially benefit from DNA methylation-based biomarkers: cancer detection, tumor prognosis and prediction of treatment responses (34). The cancer-specific Reprimo promoter methylation that occurs at an early stage in disease development can serve as a potential biomarker for early detection in GC. Indeed, Reprimo promoter methylation was frequently detected in plasma from patients with GC (11). Further, Reprimo promoter methylation is significantly lower in chemoradiotherapy-responders than in non-responders in esophageal cancer (35), and is predictive of a poor prognosis in pancreatic ductal carcinoma (36). Reprimo promoter methylation was inversely associated with response to the treatment combined with CDDP and the fluoropyrimidine class as a standard chemotherapy. In addition, Reprimo expression enhanced CDDP or 5-FU-mediated apoptosis in vitro, and anticancer effect of treatment
combined with CDDP and 5-FU in vivo. We were unable to assess any association between Reprimo protein level and response to chemotherapy due to lack of a commercially available antibody. However, Reprimo expression is induced by DNA damage, and as such, potential functionality of Reprimo as tumor-suppressive activity may not be reflected by expression level of mRNA or protein at diagnosis. At present, the assessment of Reprimo promoter methylation may be useful in predicting response to chemotherapy with CDDP and the fluoropyrimidine class. Further, Reprimo promoter methylation in advanced GC patients was an independent prognostic factor reflecting an aggressive tumor phenotype, indicating that Reprimo promoter methylation is one of the most critical alterations in cancer progression and is also a potential marker for tumor aggressiveness.

In the last decade, a number of landmark trials have demonstrated efficacy of multimodality therapy in resectable GC, such as perioperative chemotherapy (37), adjuvant chemoradiotherapy (38), adjuvant chemotherapy (39, 40), and neoadjuvant chemoradiotherapy (41). However, the therapeutic strategies by which clinicians distinguish between the various treatment options remain a contentious issue. One point of clinical importance is that, although preoperative therapy has the potential to improve the therapeutic outcome for responders (42), it runs the risk of shifting the status of non-responders from potentially resectable to unresectable, despite that radical resection is the only curative treatment modality, requiring the predictive marker for treatment response. Therefore, information on Reprimo promoter methylation may provide much-needed guidance in determining the therapeutic strategies, such as the selection of the most suitable patients for preoperative therapy in GC.
In conclusion, Reprimo expression is normally induced in response to DNA damage, robustly inhibiting tumorigenesis through enhancing apoptosis as a novel tumor-suppressor in GC. Methylation of the Reprimo promoter is a frequent and cancer-specific event that abrogates its expression and functions in \( p53 \) independent manner. In addition, clinical assessment of Reprimo promoter methylation may serve not only as a predictive marker for chemotherapy consisting of CDDP and the fluoropyrimidine class, but also as a marker for tumor aggressiveness. Further research will be necessary to validate the clinical potential of Reprimo promoter methylation as a biomarker in GC.

Acknowledgements

Grant Support: This work was supported in part by the Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan and by the Japanese Foundation for Multidisciplinary Treatment of Cancer. The funding agencies had no role in the design of the study, data collection, analysis, interpretation of the results, preparation of the manuscript, or in the decision to submit the manuscript for publication.
References

**Figure Legends**

**Figure. 1.** Schematic diagram of the 5’-flanking region of the Reprimo gene, and the Reprimo promoter methylation status in eight GC cell lines and preliminary data set. (A) Schematic diagram of CpG islands (gray area) in the 5’-flanking region of the Reprimo gene. Vertical bars indicate dinucleotide CpGs. Solid and dotted lines indicate the sequences for bisulfite sequencing analysis or Q-MSP, respectively. TSS, transcription start site; ATG, start codon; P, probe for Q-MSP. (B) Q-MSP analysis in eight GC cell lines and preliminary data set. Q-MSP values are expressed as mean values and SD. *, P<0.05. (C) Representative results of methylation status in the dinucleotide CpG within the promoter region by bisulfite sequencing with cloned PCR products. Arrowhead indicates dinucleotide CpGs. (D) Methylation status in 30 individual CpG sites within promoter region by bisulfite sequencing with 10 cloned PCR products. Cloned PCR products from cell lines and human tissue samples were examined based on the Q-MSP results. White and black circles denote unmethylated and methylated CpG sites, respectively.

**Figure. 2.** Quantitative methylation analysis in 83 primary human GC tumors and the corresponding normal samples. (A) ROC curve of Reprimo promoter methylation for distinguishing between tumor and normal samples. When the optimal cutoff value is 0.42, the area under the curve (AUC) is 0.77 and represents the accuracy in distinguishing tumor from normal samples in terms of sensitivity and specificity (P<0.0001). (B) Frequency of Reprimo promoter methylation by Q-MSP. Number in parentheses indicates patient number.
Dashed line indicates the optimal cutoff value (0.42). Q-MSP values are expressed as mean values and SD. (C) Q-MSP value of Reprimo in each stage. Data are expressed as mean values and SD within primary tumor and the corresponding normal samples in each stage, respectively. N, human normal mucosa; T, human primary tumor; *, P<0.05.

Figure 3. DNA damage-inducible Reprimo expression and its silencing by promoter methylation. (A) Expression level of Reprimo by RT-PCR (top panel) and Q-RT-PCR (Reprimo/β-actin × 1000, bottom panel) in eight GC cell lines and preliminary data set. N, human normal mucosa; T, human primary tumor; W, p53 wild type; M, p53 mutation. (B) Expression level of Reprimo after genotoxic stress in SH10 cells without Reprimo methylation by RT-PCR (top panel) or Q-RT-PCR (bottom panel). SH10 cells were incubated for 24 h followed by UV irradiation (10 mJ/cm²). Cells were treated with 5-FU (10 µmol/L) or CDDP (20 µmol/L) for 24 h. With mock-treatment cells as 1.0, the relative expression level of Reprimo was shown by Q-RT-PCR. (C) Expression level after treatment with demethylating agent 5-Aza-dC alone or in the presence or absence of UV irradiation by Q-RT-PCR. Cells were treated with 5-Aza-dC (5 µmol/L) for 5 days. When combined with the UV irradiation, UV irradiation was performed on Day 4 of 5-Aza-dC treatment. 5-Aza, 5-Aza-dC; m, mock including the same volume of acetic acid; *, P<0.05; Error bars; SD.

Figure 4. Tumor-suppressive activity of Reprimo. (A) Expression level of Reprimo in AZ521 cells transfected with pcDNA™ 3.1-mock or pcDNA™ 3.1-Reprimo. The stable
cells were established by G418 selection after transfection with pcDNA™ 3.1-Reprimo (AZ-r10 or r16). AZ521 cells transfected with pcDNA™ 3.1-mock (AZ-m) were used as a control. Reprimo mRNA and protein level was confirmed by RT-PCR (top panel) and western blotting with an anti-flag-V5 antibody (bottom panel), respectively. Reprimo protein (12 kD) is detected as 38 kD due to heavy glycosylation, eventually resulting in approximately 40 kD by adding the recombinant fusion protein containing a V5 epitope (3.6 kD). (B) Anchorage-independent colony formation assay. Colonies were photographed (left and middle panels) and counted under a microscope (right panel) after 3 weeks of cell culture. With colony number in AZ-m cells as 1.0, the relative rates of colony number were shown. *, P<0.05; Error bars; SD. (C) Proliferation assay. Proliferation on Day 1, 2, or 3 was shown as absorbance at 450 nm (top panel). Cells were treated with CDDP at concentrations ranging from 0 to 25 µmol/L for 3 days. Cells with chemical solution alone (0 µmol/L CDDP) were as 1.0, and the relative proliferative rate 3 days after treatment are shown in the bottom panel. *, P<0.05; Error bars, SD. (D) Apoptosis assay by Annexin V and 7-AAD staining 24 h after treatment with 5-FU (10 µmol/L) or CDDP (20 µmol/L). The level of apoptosis was compared in AZ-r10 or AZ-r16 cells versus AZ-m cells. The percentage and SD of early apoptosis (bottom right quadrant) and late apoptosis (top right quadrant) are shown in each panel. *, P<0.05. (E) RT-PCR 72 h after transfection with siRNA in SH10 cells without Reprimo promoter methylation. CDDP (20 µmol/L) was added to the medium for the final 24 h. SH-m, mock-treated SH10 cells; SH-ctr, SH10 cells transfected with siRNA-ctr; SH-si, SH10 cells transfected with siRNA-Reprimo. (F)
Apoptosis assay 24 h after transfection with siRNA. 5-FU (10 µmol/L) or CDDP (20 µmol/L) was added to the medium for the final 24 h.

**Figure. 5.** Effect of Reprimo expression on tumorigenesis *in vivo* and Kaplan-Meier analysis in advanced GC patients. (A) Mouse xenograft assay. AZ-p, AZ-m, AZ-r10, or AZ-r16 cells were subcutaneously injected into both flanks of nude mice (2.5 × 10⁶ cells per flank), respectively. Twenty-eight days after injection, mice were sacrificed, and pictures were taken. (B) Time course of tumor growth. Tumor volumes were calculated from measurements of two orthogonal diameters (larger \( x \) and smaller \( y \) diameters) using the following formula: volume = \( xy^2/2 \). *, \( P < 0.05 \); Error bars; SD; NS, not significant. (C) Tumor weight in mice injected with Reprimo-transfected cells (AZ-r10 or AZ-r16 cells) when compared to AZ-p or AZ-m cells. *, \( P < 0.05 \); Error bars; SD; NS, not significant. (D) Typical appearance of tumors excised on day 28. Angiogenesis was visualized as a marked red color with the formation of a dense surrounding vascular network. (E) Kaplan-Meier analysis in 41 patients who received a combination regimen (CDDP and fluoropyrimidine class) (F) Kaplan-Meier analysis in 68 patients with advanced GC patients.
Figure 1

A

GC percentage (%)

CpG: 1

20 bp

TSS

ATG

bisulfite sequencing

Q-MSP

P

B

* P=0.006

* P=0.014

Q-MSP value

0.51 ± 0.34

3.05 ± 0.88

5.25 ± 1.73

N

T

cell-lines

C

Positivity

Negativity

D

KatoIII

(11.66)

AZ521

(9.27)

H111

(1.46)

GCIY

(0.00)

10T

(7.70)

10N

(0.13)

5T

(1.32)

5N

(0.01)
Figure 2

A

Methylation value: 0.42
AUC: 0.77
Sensitivity: 0.69, Specificity: 0.82
P < 0.0001

B

P < 0.0001

C

Tumor (T): 2.61 ± 0.31
Corresponding normal (N): 0.39 ± 0.10
P < 0.0001

T: 2.54 ± 0.62
N: 0.25 ± 0.13
P = 0.0003

T: 1.60 ± 0.48
N: 0.39 ± 0.21
P = 0.0035

T: 2.74 ± 0.54
N: 0.34 ± 0.13
P = 0.0004

T: 3.54 ± 0.79
N: 0.58 ± 0.30
P < 0.0001

Stage I Stage II Stage III Stage IV
**Figure 3**

A. Western blot analysis showing Reprimo and β-actin expression in tumor and normal tissues.

B. Western blot analysis showing β-actin expression in tumor and normal tissues.

C. qRT-PCR analysis showing relative Reprimo expression in different cell lines with varying methylation status and p53 status. The results indicate significant differences between groups, with p-values indicating statistical significance.
**Figure 4**

A. Reprimo (384bp)

B. AZ-m AZ-r10 AZ-r16

C. AZ-m AZ-r10 AZ-r16

D. AZ-m AZ-r10 AZ-r16

E. Reprimo

F. SH-m SH-ctr SH-si
Figure 5

A: Photographs of mice with tumors.

B: Graph showing tumor volume over time (day).

C: Bar graph comparing tumor weight among groups.

D: Photos of excised tumors.

E: Kaplan-Meier survival curve for patients treated with 5FU+CDDP (n=41).

F: Kaplan-Meier survival curve for advanced gastric cancer (n=68).
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Abbreviations: m, mucosa; sm, submucosa; mp, muscularis propria; ss, subserosa; se, serosa-exposed; si, serosa-infiltrating; AJCC/UICC, 7th edition of the American Joint Committee on Cancer/International Union Against Cancer; DSS, disease specific survival; CR, complete response; PR, partial response; NS, not significant.

*, Mann-Whitney U-test; **, Logrank test; the remainig variables, Chi-square test; §, assessment for fluoropyrimidine class and cisplatin.
DNA damage-inducible gene, Reprimo functions as a tumor-suppressor and is suppressed by promoter methylation in gastric cancer

Akira Ooki, Keishi Yamashita, Kensei Yamaguchi, et al.

Mol Cancer Res  Published OnlineFirst August 27, 2013.

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