Frequent Loss of Brm Expression in Gastric Cancer Correlates with Histologic Features and Differentiation State

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Abstract

The mammalian SWI/SNF chromatin remodeling complex, an essential epigenetic regulator, contains either a single Brm or BRG1 molecule as its catalytic subunit. We observed frequent loss of Brm expression but not of BRG1 in human gastric cancer cell lines. Treatment with histone deacetylase inhibitor rescued Brm expression, indicating epigenetic regulation of this gene, and an RNA interference–based colony formation assay revealed antioncogenic properties of Brm. Brm immunostaining of 89 primary gastric cancers showed an obvious reduction in 60 cases (67%) and a severe decrease in 37 cases (42%). Loss of Brm is frequent in the major gastric cancer types (well- or moderately-differentiated tubular adenocarcinoma and poorly-differentiated adenocarcinoma) and positively correlates with the undifferentiated state. Among the minor gastric cancer types, Brm expression persists in signet-ring cell carcinoma and mucinous adenocarcinoma, but a marked decrease is observed in papillary adenocarcinoma. Intestinal metaplasia never shows decreased expression, indicating that Brm is a valid marker of gastric oncogenesis. In contrast, BRG1 is retained in most cases; a concomitant loss of BRG1 and Brm is rare in gastric cancer, contrary to other malignancies. We further show that Brm is required for *villin* expression, a definitive marker of intestinal metaplasia and differentiation. Via regulating such genes important for gut differentiation, Brm should play significant roles in determining the histologic features of gastric malignancy. [Cancer Res 2007;67(22):10727–35]

Introduction

The incidence and mortality of gastric cancer has gradually fallen over the past few decades. Nevertheless, gastric cancer remains a major public health issue as the fourth most common cancer and the second leading cause of cancer death worldwide (1). The development and progression of many cancers have now been shown to involve a number of epigenetic disorders in addition to genetic defects. In this regard, it has been well established that promoter hypermethylation of many tumor suppressor genes is frequently observed in gastric carcinomas (2). Moreover, global DNA hypomethylation is also thought to occur during the early stages of tumor development in gastric as well as other tissues (3). However, the roles of other factors involved in epigenetic regulation, such as histone modification and chromatin remodeling, remain largely unknown in relation to the development of gastric cancer.

The SWI/SNF complex facilitates gene expression by remodeling nucleosomes and plays many important roles in epigenetic regulation during tumorigenesis, differentiation, and development (4, 5). Through some of its components, SWI/SNF interacts with various oncogenic and antioncogenic proteins, such as c-Fos, c-Jun (6), c-Myc (7), Rb (8), BRCA1 (9), β-catenin (10), and p53 (11). These interactions suggest that SWI/SNF is involved in multiple processes associated with the formation and suppression of tumors. In addition, it has been reported that several subunits of the SWI/SNF complex exhibit tumor-suppressive effects. Ini1 (12–15) and BRG1 (16–19) are designated as tumor suppressor genes based on analyses of cancer cell lines, primary tumors, and knockout mice. BAF53 and BAF57 are also thought to play important roles in some malignancies (20–22).

Both Brm and BRG1 are catalytic ATPase subunits, and each mammalian SWI/SNF complex includes either a single Brm or BRG1 molecule (23). Brm has been reported to be deficient in various cancer cell lines and primary tumors (4, 8, 18), and we have previously shown that *Brm* has antioncogenic properties (24, 25). We have also shown that the loss of Brm is the result of its posttranscriptional suppression in various cancer cell types, wherein Brm is actively transcribed as determined by nuclear run-on transcription assay (24, 25). Although the mechanisms underlying *Brm* repression still remain unresolved, we have shown in a previous report that the suppression of both *Brm* mRNA and protein levels is alleviated by treatment with histone deacetylase (HDAC) inhibitors, such as FK228, CHAP31, and Trichostatin A (24, 25).

In our present study, we have screened 27 human cell lines originating from the alimentary tract for the expression of Brm, BRG1, and Ini1 and found that the epigenetic suppression of *Brm* occurs frequently in gastric cancer cells. We further did immunostaining of Brm and BRG1 in 89 primary gastric cancer tissues and observed that Brm expression is frequently suppressed in gastric cancer and that the expression pattern of this gene has an obvious correlation with the histologic classification of gastric tumors. Finally, through a screening of several epithelial and intestinal markers, we provide evidence that Brm has a significant influence on the differentiation status and histologic typing of gastric malignancies.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cell culture. Six gastric cancer cell lines (KE-39, KE-97, HuG1-N, HuG1-PI, ECC-10, and ECC-12) were incubated in RPMI 1640 with 10% FCS (Life Technologies/Invitrogen Corp.) at 37°C. Other tumor cell lines were maintained in DMEM with 10% FCS (Life Technologies/Invitrogen Corp.) at 37°C. FK228 (a gift from Fujisawa Corporation) was used at concentrations of 1.0 to 2.5 ng/mL.

Plasmids and transfection. pCAGF1-IG (empty vector), pCAGF1-IG-Brm, pCAGF1-IG-Brm-KR, pCAGF1-IG-BRG1, and pCAGF1-IG-BRG1-KR were prepared as described previously (26). All transfections were done using Lipofectamine Plus (Invitrogen Corp.).

Western blotting. An anti-Brm antibody (Transgenic, Inc.), anti-BRG1 antibody (H-88; Santa Cruz Biotechnology), and anti-BAF47/Ini1 antibody (BD Transduction Laboratories) were used, and immunostaining was done as described previously (24).

Immunohistochemistry. Cells were fixed with 4% paraformaldehyde at 37°C for 1 h. After treatments with 0.2% Triton-X100 for 20 min at room temperature, blocking with 2% normal goat serum for 2 h at 37°C was done. The cells then were incubated overnight at 4°C with anti-human Brm antibody (Abcam) at a 1:200 dilution or antihuman BRG1 antibody (Santa Cruz Biotechnology) at a 1:200 dilution. Primary antibody binding was visualized with Alexa Fluor 488–conjugated antirabbit IgG antibody (Molecular Probes, Inc.) for immunofluorescence detection.

Formalin-fixed and paraffin-embedded tissues were deparaffinized thrice in xylene for 1 min, followed by 1 min each in serial dilutions of ethanol (100%, 100%, 95%, 95%) and followed by three changes of water. Endogenous peroxidase was inactivated by 0.3% hydrogen peroxide in methanol for 30 min. Villin immunostaining was done as described previously (27). For Brm and BRG1 immunostaining, hydrated heating in 10 mmol/L citrate buffer (pH 6.0) at 120°C was done in a pressure cooker (Delicio 6L; T-FAL) for 10 min for antigen retrieval. The sections were then incubated for 1 h at room temperature with antihuman Brm antibody (Abcam) at a 1:3,000 dilution or antihuman BRG1 antibody (Santa Cruz Biotechnology) at a 1:1,000 dilution. For the amplification of signals, antirabbit (for Brm, Dako) or antimouse (for BRG1, Dako) antibody, both conjugated to horseradish peroxidase (HRP), was applied to the slides for 1 h at room temperature, followed by three washes in TBS. This was followed by incubation with FITC-conjugated phenol (fluorescyl-tyramide; Dako) for 30 min at room temperature and incubation with anti-FITC antibody conjugated to HRP (Dako) for 30 min at room temperature was then done. Finally, the reaction products were visualized in 20 mg/dL 3,3′-diaminobenzidine tetrahydrochloride (Dako) solution containing 0.006% hydrogen peroxide. Negative control studies were done without applying the primary antibody, and sections known to be stained were included with each run as a positive control.

The immunostained sections were evaluated independently by two pathologists, together with H&E-stained sections from the same lesions. For Brm and BRG1, the immunostaining of cancer cells in gastric tumors was evaluated in comparison with normal epithelial cells of digestive tracts, and values ranging from 1 to 5 were decided as follows: 5, each of the cancer cells shows same immunoreactivity as normal epithelial cells; 4, percentage of cancer cells with significantly lower immunoreactivity ranges from 0% to 10%; 3, percentage of cancer cells with significantly lower immunoreactivity ranges from 10% to 50%; 2, percentage of cancer cells with significantly lower immunoreactivity ranges from 50% to 90%; 1, percentage of cancer cells with significantly lower immunoreactivity is >90%.

Reverse transcription–PCR. Total cellular RNAs were prepared with Isogen RNA isolation reagent (Wako Pure Chemical Industries). Semiquantitative reverse transcription–PCR (RT-PCR) was done with Superscript One-Step RT-PCR with Platinum Taq kit (Invitrogen Corp.). In Supplementary Table S1, primer pairs used in the RT-PCR are shown with annealing temperature (°C) and amplification cycles.

Retrovirus vectors. Vesicular stomatitis virus G protein–pseudotyped MuLV-based retrovirus vectors were prepared with the prepackaging cell line PLAT as described previously (24). The short hairpin RNAs (shRNA) targeting nt 5,425 to nt 5,445 of the *Brm* sequence (shBrm) and shRNAs targeting *GFP* (shGFP) virus were prepared as described previously (24).

Colony formation assay in soft agar. Cells were seeded into 60-mm dishes as a suspension of 0.4% Noble agar (BD Company) in high-glucose DMEM supplemented with 10% FCS (upper agar layers). The upper agar layers were placed on top of beds of 0.5% Bacto agar (BD Company) in DMEM supplemented with 10% FCS (bottom agar layers). Cultures were incubated at 37°C for 28 days, and the resulting colonies were counted. The averages of two experiments were calculated with SD.

5′ rapid amplification of cDNA ends. Using 1 μg of total RNA derived from villin-positive NUGC-4 cells, 5′ rapid amplification of cDNA ends (RACE) was done based on the SMART_RACE cDNA amplification system (Clontech). 5′ RACE PCR reaction was done using ExTaq (TaKaRa Bio, Inc.) with *villin* gene specific primer (5′-ccagtagtggatgtcataggacagg-3′). PCR products were cloned into TA vector (PCR2.1, Invitrogen Corp.) and sequenced.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) analysis was done using ChIP assay kit (Upstate Biotechnology, Inc.) according to the manufacturer's instruction. AGS cells (1×10^6) were used, and immunopreciptation was done overnight at 4°C with 2 μg of anti-Brm antibody (Abcam) or nonimmunized rabbit IgG whole molecule (Santa Cruz). After reverse crosslinking, DNA was purified with QIAquick PCR purification kit (Qiagen) from the immunoprecipitates. Each DNA sample was amplified after an initial denaturation at 95°C for 5 min using ExTaq (TaKaRa Bio, Inc.) as follows; for the first exon of *villin*, 36 cycles of 95°C for 30 s, 62°C for 1 min, and 72°C for 1 min with 5′-acaattccctgagatctcccaggt-3′ and 5′-tgaggtcagggagaccaactag-3′ oligonucleotides; for the second exon of *villin*, 32 cycles of 95°C for 30 s, 62°C for 1 min, and 72°C for 1 min with 5′-aaatggtccctgagtggggagt-3′ and 5′-tggtgatgttgagagagccttt-3′ oligonucleotides.

Tumor samples. Eighty-nine tumor tissues were randomly selected from a list of advanced gastric cancer patients from 1998 to 2005, which were banked at the Fujita Health University School of Medicine. The patients were 36 to 92 years old (mean age, 70.0 ± 11.4 years) and included 60 males and 29 females. The clinical stage distribution was stage I in 7 patients (7.9%), stage II in 13 patients (14.6%), stage III in 46 patients (51.7%), and stage IV in 23 patients (25.8%) according to International Union Against Cancer classification. This study was approved by the institutional ethical review board for human investigation at Fujita Health University.

Results

Brm expression is frequently lost in gastric cancer cells, but not in colorectal cancer cells. Many cancer cell lines and primary tumor types from various tissues have been screened for the expression of SWI/SNF complex subunits (4), but the relationship between SWI/SNF and malignancies of the alimentary canal has not been fully assessed. We examined the expression of three major components of SWI/SNF (Brm, BRG1, and Ini1) in 27 gastric and colorectal cancer cell lines and found that 7 of 17 of the gastric cell types were strongly deficient in Brm (Fig. 1*A*). In contrast, all of the colorectal cancer cell lines tested were positive for Brm expression (Fig. 1*B*).

A concomitant down-regulation of Brm and BRG1 was previously reported in primary non–small lung carcinoma (18) and in a variety of cancer cell lines (4, 24, 28). However, among the 27 cell line examined in our present screen, only AZ521, which lack Brm expression, was found to be deficient in BRG1 expression (Fig. 1). Comparing the previous reports analyzed by Western blotting (19, 28) or immunostaining (18, 19), our results suggest that a simultaneous loss of Brm and BRG1 is not so frequent in gastric cancer. But we cannot exclude the possibility that the full-length BRG1 detected in these cell lines has some point mutations, as has been reported in tumor cell lines originated from lung cancer (17). As for Ini1, we found no cells that were deficient in the

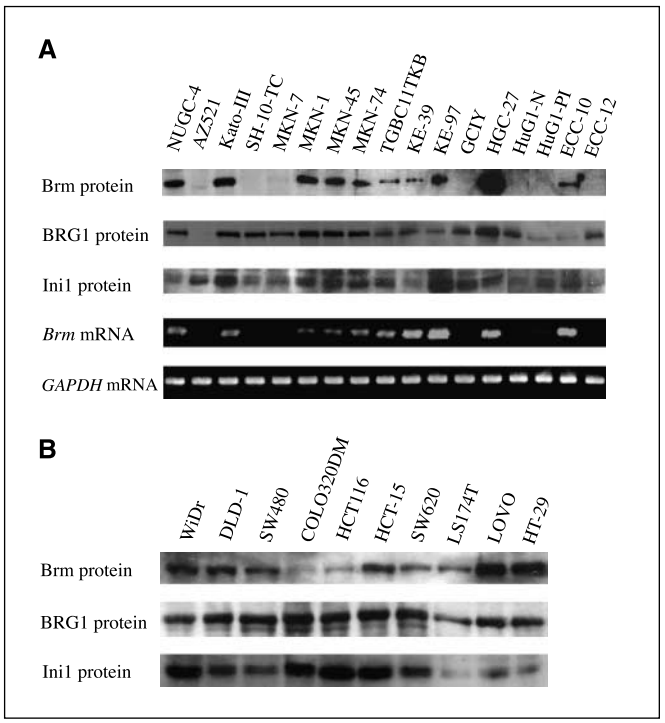

Figure 1. *A,* detection of Brm, BRG1, and Ini1 expression by Western blotting of 17 gastric cancer cell lines. Protein bands corresponding to Brm (190 kDa), BRG1 (190 kDa), and Ini1 (47 kDa) were detected by immunostaining with the appropriate antibodies. *Brm* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in the same gastric cancer cell lines analyzed by RT-PCR are also shown. *B,* detection of Brm, BRG1, and Ini1 protein by Western blotting of 10 colorectal cancer cell lines.

expression of this gene (Fig. 1). It has been well established that the Ini1-deficiency leads to a rhabdoid phenotype (13–15), but this is quite rare in gastrointestinal malignancy (29) and was not associated with any of the 27 cell lines examined.

Treatment with HDAC inhibitors rescues the Brm deficiency in gastric cancer cells and thereby exerts tumor-suppressive effects. Whereas *Brm* mRNA levels were not fully proportional to Brm protein levels among the cell lines examined, *Brm* mRNA is not detected without exception when Brm protein is undetectable (Fig. 1*A*). These results suggest that loss of Brm expression occurred before the production of mature mRNA. We verified that treatment with an HDAC inhibitor (FK228) induced *Brm* mRNA and Brm protein expression in all of the Brm-deficient gastric cancer cell types examined (Fig. 2*A*).

To next examine whether the loss of Brm is primarily responsible for the oncogenicity of gastric cancer, the AZ521 gastric cell line was transfected with shRNAs targeting *Brm* (designated as AZ521-shBrm). We chose these cells as they also lack BRG1, which is thought to be able to partially substitute for Brm (30), and HDAC inhibitor treatment does not induce endogenous BRG1 in AZ521 (Fig. 2*B*). Neither AZ521-shBrm nor AZ521-shGFP express Brm, but after the HDAC inhibitor treatment, only the AZ521-shGFP cells showed Brm induction (Fig. 2*C*). After these cells were treated with FK228, we did a colony formation assay in soft agar in the absence of an inhibitor. Without any treatment, AZ521-shBrm and AZ521-shGFP showed no differences in anchorage-independent growth. However, after the transient treatment with FK228, the colony-forming activity of AZ521-shBrm was significantly higher than that of AZ521-shGFP (Fig. 2*D*).

The loss of Brm expression is frequently observed in primary gastric cancers, whereas a concomitant deficiency in Brm and BRG1 is rare in these tumors. The discrimination between Brm and BRG1 by immunohistochemistry is potentially problematic because they have high-sequence homology and resemble each other in terms of both their protein structures and localization. We tested all of the available Brm and BRG1 antibodies and succeeded in avoiding such cross-reaction issues using human cancer cell lines (Supplementary Figs. S1 and S2). Formalin-fixed and paraffin-embedded normal colon mucosal samples were next immunostained together with colorectal cancer samples, as colon mucosa has been reported to be positive for both Brm and BRG1 expression (31). By the use of a biotin-free tyramide

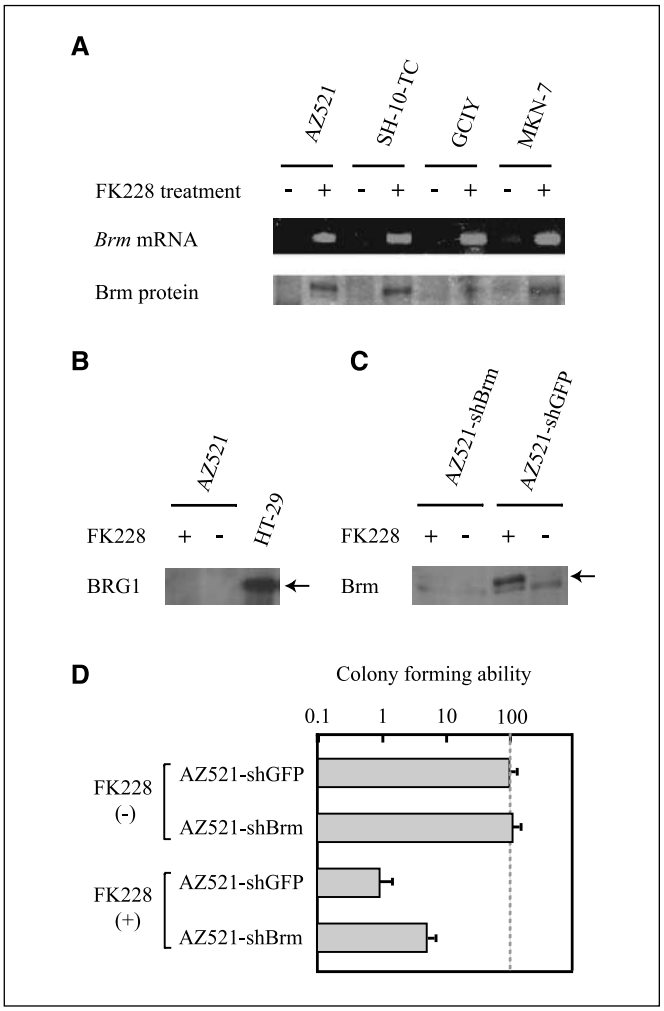

Figure 2. *A,* expression patterns of the *Brm* gene in four Brm-deficient gastric cancer cell lines with or without HDAC inhibitor treatment. Cells were grown in DMEM medium in the absence (−) or presence (+) of FK228 (1.3 ng/mL for AZ521 and SH-10-TC, 2.5 ng/mL for GCIY, and 1.6 ng/mL for MKN-7) for 3 d and were subjected to RNA and protein extraction. Total cellular lysates (30 μg each) were immunoblotted with anti-Brm antibody, and total RNAs (200 ng each) were analyzed by RT-PCR. *B,* Western analysis of BRG1 in AZ521 cells grown for 3 d in the presence or absence of FK228. Total cellular extracts in each case (30 μg) were immunoblotted with anti-BRG1 antibodies, and HT-29 cells were used as a positive control. *C,* Western analysis of Brm in AZ521 cells expressing shRNAs directed against the *Brm* or *GFP* gene (AZ521-shBrm and AZ521-shGFP, respectively), which were grown for 3 d in the presence or absence of FK228. Total cellular extracts in each case (30 μg) were immunoblotted with anti-Brm antibodies. *D,* effects of induced endogenous *Brm* upon colony formation in soft agar. Colony-forming ability was defined as the percentage of colony numbers compared with untreated AZ521-shGFP cells.

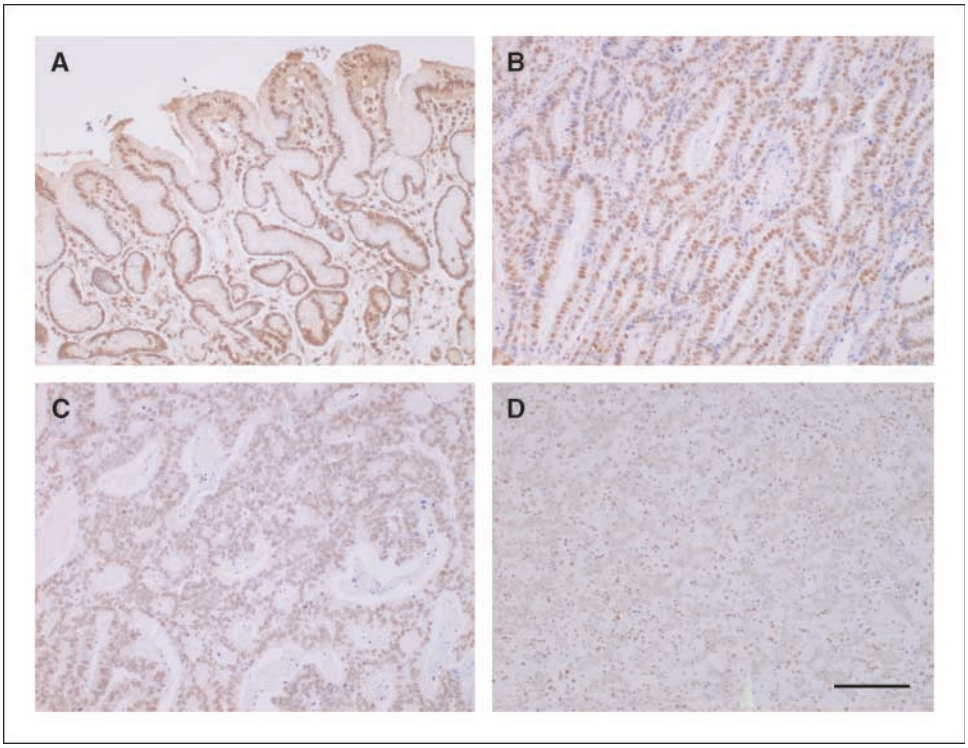

Figure 3. Immunostaining of Brm in normal gastric mucosa and three major types of gastric cancer according to the Japanese histopathologic classification system. Nuclear Brm proteins are stained brown, and the cellular nuclei are counterstained pale blue with hematoxylin. *Scale bar,* 80 μm. *A,* normal gastric mucosa. *B,* well-differentiated tubular adenocarcinoma (tub1). *C,* moderately-differentiated tubular adenocarcinoma (tub2). *D,* poorly-differentiated adenocarcinoma (por).

signal amplification system and antigen retrieval via a pressure cooker, we succeeded in clearly detecting intranuclear signals for both proteins (Supplementary Fig. S3).

We then immunostained for Brm and BRG1 in 89 primary gastric cancer tissues (Fig. 3; Supplementary Table S2; Supplementary Figs. S4 and S5), and the degrees of their expressions were divided into five classes (see Materials and Methods). As expected, an obvious reduction of Brm was observed in 67% (60 of 89) of the samples, and a severe Brm decrease (more than half of the malignant cells in the tumor were deficient in Brm) was observed in as much as 42% (37 of 89) of the gastric cancer cases (Table 1). Based upon our current findings, the deficiency of Brm in gastric cancers is far higher than previously reported in non–small cell lung cancer (10%; ref. 18). Precancerous intestinal metaplasia sectioned on the same slides never showed decreased Brm expression, indicating that the loss of Brm is a definitive marker of gastric malignancy. In contrast, BRG1 expression was found to be retained in most of the tumor samples tested (Table 1), consistent with the result of gastric cancer cell lines. In gastric malignancy, it seems that Brm-deficient cell lines and primary cancers are mostly BRG1 positive, further suggesting that it is the Brm and not BRG1 that plays the significant roles in gastric tumorigenesis.

The Brm expression status is indicative of the histologic classification of gastric tumors. We next investigated the relationship between the histologic features of gastric cancer and the Brm expression profile. Among our study cohort of 89 samples, a firm histopathologic diagnosis was difficult to make in 12 cases, as these cases showed various manifestations in a single tumor mass (e.g., an almost equal mixture of poorly differentiated adenocarcinoma and signet-ring cell carcinoma). We thus excluded these 12 cases and scrutinized the remaining 77 cases only.

According to the Lauren classification (32), the most widely used for the histologic typing of gastric carcinoma, we divided our 77 cases into intestinal and diffuse type cancers. However, we could not find any correlation between the loss of Brm and the Lauren histopathologic diagnoses (Table 2; Supplementary Table S2),

Table 1. Evaluation of the reduction in Brm and BRG1 expression in gastric cancer

	Evaluation of protein expression					Total
	5	4	3	2	1	
Brm	6	23	23	24	13	89
BRG1	32	33	19	4	1	89

NOTE: Values assigned to the Brm and BRG1 staining patterns (from 1 to 5) were decided as follows: 5, each of the cancer cells shows same immunoreactivity as normal epithelial cells; 4, percentage of cancer cells with significantly lower immunoreactivity ranges from 0% to 10%; 3, percentage of cancer cells with significantly lower immunoreactivity ranges from 10% to 50%; 2, percentage of cancer cells with significantly lower immunoreactivity ranges from 50 to 90%; 1, percentage of cancer cells with significantly lower immunoreactivity is greater than 90%.

Table 2. Summary of the association between the Brm deficiency values and the Lauren classification of gastric cancer

Histologic typing	Evaluation of Brm expression					Total
	5	4	3	2	1	
Intestinal type	2	12	13	10	7	44
Diffuse type	3	8	5	12	5	33
Total	5	20	18	22	12	77

NOTE: The Brm and BRG1 staining values were estimated using the criteria shown in Table 1.

probably because of the inherent oversimplification of this system. We thus classified our 77 samples using the more detailed Japanese classification of gastric cancer (33, 34), which conforms largely to the current WHO classification system (35). Each of the samples could be classified as three major types (tub1, tub2, por) and three minor types (pap, sig, muc). Significantly, a clear correlation between the Brm-deficiency status and the histologic appearance of gastric malignancy could then be observed (Table 3; Supplementary Table S2).

Tubular adenocarcinoma (tub) is the most major type of gastric cancer and is known to account for most of the intestinal type gastric carcinomas by the Lauren classification (33). Based upon the conservation of tubular structure (Supplementary Fig. S6), tubular adenocarcinoma is divided into two categories: a well differentiated type (tub1) and a moderately differentiated type (tub2). A deficiency in Brm was frequently observed in gastric tubular adenocarcinoma (10 of 32). Furthermore, the degree of Brm reduction was much higher in tub2 (8 of 20) than in tub1 (2 of 12) lesions, suggesting that the loss of Brm expression significantly correlates with the extent of undifferentiation in gastric cancer (Fig. 3; Table 3). Poorly differentiated adenocarcinoma (por), another type of major gastric cancer, is representative of most of the diffuse type gastric tumors according to the Lauren classification (33). The por classification is generally interpreted to be more anaplastic than both tub1 and tub2, as the intestinal tubular morphology of such lesions is almost completely disrupted (Supplementary Fig. S6). A tendency toward a Brm decrease was found to be more prominent in por cases (16 of 27) compared with tub2 (8 of 20), further suggesting that Brm is an important factor for determining the differentiation status of gastric cancer (Fig. 3; Table 3).

With regard to the three minor gastric cancer types, a correlation between Brm deficiency and histology is noticeable. Signet-ring cell carcinoma (sig) and mucinous adenocarcinoma (muc), both of which are categorized as a Lauren's diffuse type gastric cancer (33), are characterized by abundant mucus secretion within the tumor, which causes a specific morphologic appearance (Supplementary Fig. S6). Sig is defined by isolated or small groups of malignant cells containing intracytoplasmic mucin, whereas muc is defined as an adenocarcinoma within which a substantial amount of extracellular mucin is retained (35). As is shown in Table 3 and Supplementary Fig. S4, the expression levels of Brm were found to be mostly preserved in both the sig (five of six) and muc (four of four) cases. Additionally, in the excluded sections that were difficult to definitively diagnose, Brm was clearly stained in the cancerous lesions, wherein signet-ring or mucinous morphologies were observed (data not shown). Taken together, we conclude that the loss of Brm occurs seldomly in the sig-type and muc-type gastric cancers.

In contrast to sig and muc, gastric papillary adenocarcinoma (pap), a rather rare histologic entity among the gastric cancers, showed a very high incidence of Brm deficiency in our cohort (seven of eight; Table 3; Supplementary Fig. S4). Pap is characterized by papillary epithelial processes with thin fibrous cores (Supplementary Fig. S6) and is also typified by biological behavior that includes a proximal tumor location, frequent liver metastasis, and a poor surgical outcome (36, 37). A decrease in Brm expression showed the highest occurrence in papillary

Table 3. Summary of the association between the Brm deficiency and Japanese classification of gastric cancer

Histologic typing		Evaluation of Brm expression					Total
		5	4	3	2	1	
Major types	Well-differentiated tubular adenocarcinoma (tub1)	0	3	7	2	0	12
	Moderately-differentiated tubular adenocarcinoma (tub2)	1	6	5	7	1	20
	Poorly-differentiated adenocarcinoma (por)	2	5	4	11	5	27
Minor types	Papillary adenocarcinoma (pap)	0	1	0	1	6	8
	Signet-ring cell carcinoma (sig)	1	3	1	1	0	6
	Mucinous adenocarcinoma (muc)	1	2	1	0	0	4
Total		5	20	18	22	12	77

NOTE: The Brm and BRG1 staining values were estimated using the criteria shown in Table 1.

adenocarcinoma, and thus, we surmise that a lack of Brm expression plays a significant role in the development of its characteristic structure.

Expression of villin requires Brm in gastric cancer. By RT-PCR analysis of gastric cell lines, we further examined the possible role of Brm in regulating the expression of gastrointestinal differentiation markers including *villin, MUC5AC, MUC6*, and *MUC2* (Fig. 4*A*). Villin is a major component of microvilli in both intestinal epithelial cells and kidney proximal tubule cells. This cytoskeletal protein is also known to be a calcium-regulated actin-binding protein and to display strict tissue-specific expression (38). Villin is never expressed in the normal gastric mucosa, but is frequently induced in the gastric mucosa in connection with intestinal metaplastic change, a well characterized precancerous condition of the stomach (39). Consequently, villin is often ectopically expressed in stomach malignancies, particularly in gastric adenocarcinoma (38, 40). As for three kinds of mucin, MUC5AC, MUC6, and MUC2, their expression patterns are often used for the typing of not only intestinal metaplasia but also gastric cancer (41, 42).

The expression of these mucin genes seemed not to have any direct association with the pattern of Brm expression, but we observed that *villin* mRNA was undetectable in all the four Brm-deficient cell lines tested (Fig. 4*A*). We thus expected that Brm might be necessary for the *villin* gene expression and treated our established AZ521-shBrm and GCIY-shBrm cells with FK228 to examine the effect upon *villin* expression. We confirmed that the shRNA knockdown of *Brm* worked in the GCIY-shBrm cells as efficiently as in the AZ521-shBrm cells (Fig. 4*B*). Although the inductions were not so strong, the levels of *villin* mRNA induced in the shGFP-expressing cells were higher compared with the shBrm-expressing cells in both AZ521 and GCIY (Fig. 4*B*).

The transfection of *Brm, Brm-KR* (a dominant negative *Brm*), *BRG1, BRG1-KR* (a dominant negative *BRG1*), or empty vector into AZ521 cells was further done (26). Two days later, the transfected cells were disputed to quantitate the levels of both *CD44* and *villin* mRNA by RT-PCR. As shown in Fig. 4C, we observed an obvious induction of *villin* mRNA only in AZ521 cells transfected with wild-type *Brm*, indicating that ATPase activity of Brm is essential for *villin* expression. On the contrary, strong induction of *CD44* mRNA

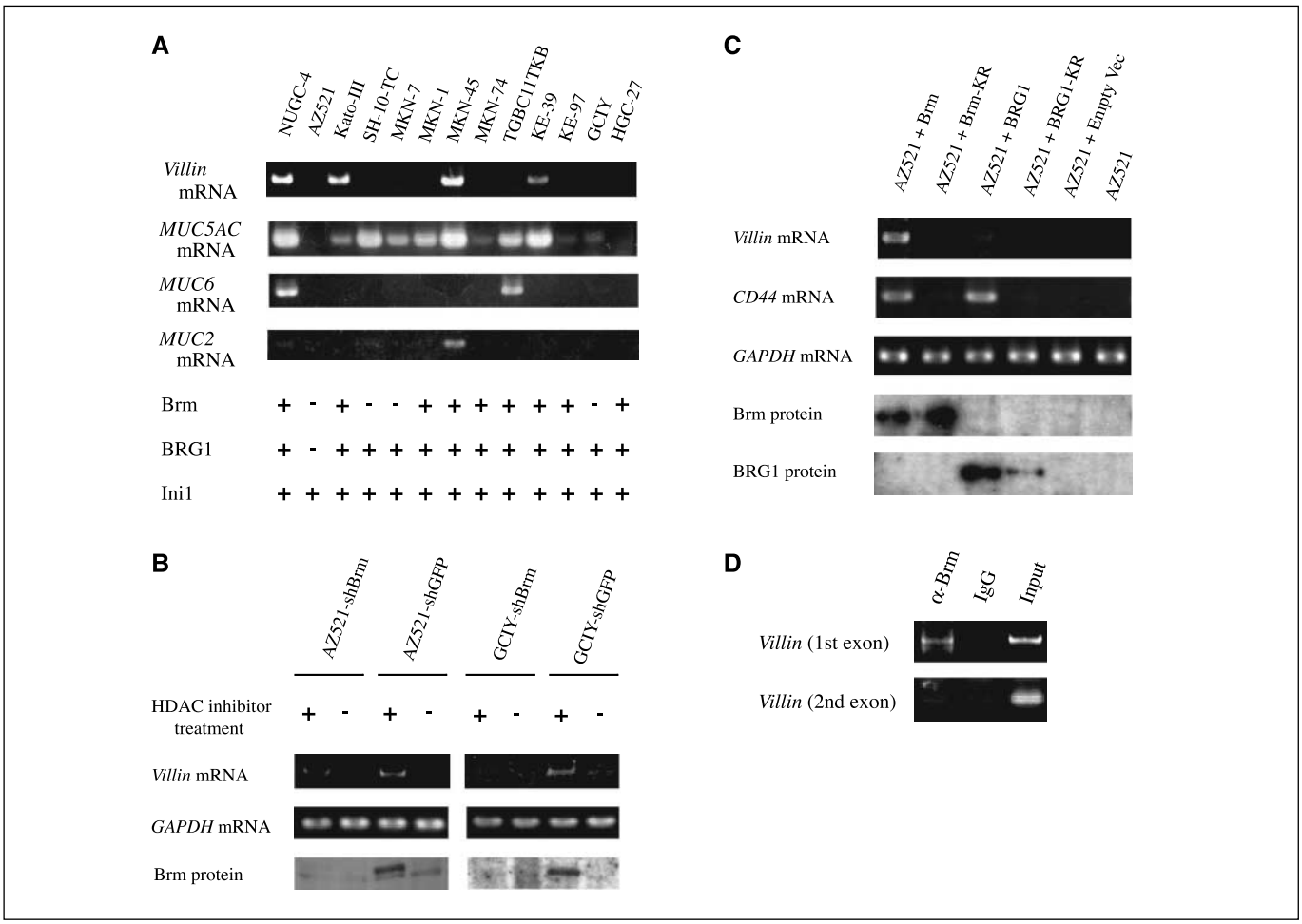

Figure 4. *A,* expression patterns of some genes related to gastrointestinal differentiation and malignancy. Total RNAs from 13 gastric cancer cell lines were analyzed by RT-PCR, and the expression levels of Brm, BRG1, and Ini1 are denoted with (+) and (−) symbols according to the results shown in Fig. 1*A*. *B, villin* mRNA expression in AZ521-shBrm and GCIY-shBrm cells treated with FK228 analyzed by RT-PCR. AZ521-shGFP and GCIY-shGFP cells were used as controls, and Western blotting shows efficient shRNA targeting of endogenous *Brm* induced by FK228. *C,* expression of *villin, CD44*, and *GAPDH* mRNA analyzed by RT-PCR using the AZ521 cell transfected with Brm, Brm-KR (lacking ATPase domain of Brm), BRG1, BRG1-KR (lacking ATPase domain of BRG1), or the empty vector. *GAPDH* was used as an internal control. Western blotting analysis of Brm and BRG1 was also done to verify efficient transduction. *D,* ChIP analysis of human *villin* gene promoter region. DNA isolated from chromatin immunoprecipitation with anti-Brm antibody (*α-Brm*) or nonimmunized rabbit IgG whole molecule (*IgG*) was used, and PCR was done with primer pairs detecting first or second exon of human *villin* gene.

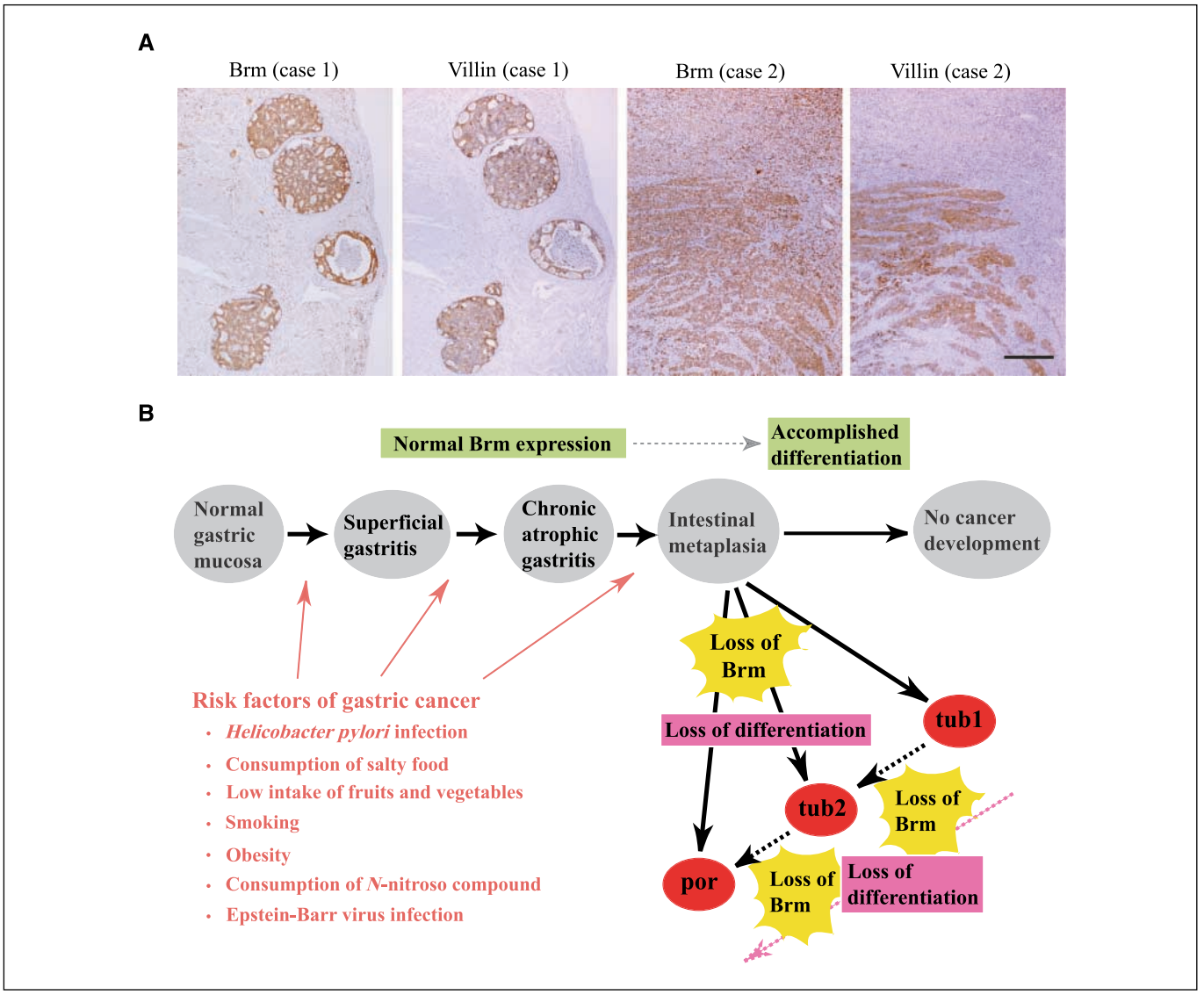

Figure 5. *A,* immunostaining of Brm and villin in sequential sections of two gastric cancer tissues: case 1, moderately differentiated tubular adenocarcinoma (tub2); case 2, poorly differentiated adenocarcinoma (por). *Scale bar,* 200 μm. *B,* schematic representation of the hypothetical roles of Brm in gastric cancer. The loss of Brm would occur during several stages of gastric carcinogenesis. In the major gastric cancer pathway from incomplete intestinal metaplasia to tub1, tub2, and por, the Brm deficiency facilitates a more anaplastic and hence more malignant gastric cancer.

was observed in cells transduced with either *Brm* or *BRG1*, as has been reported (25, 43). From these results, we conclude that Brm (Brm-type SWI/SNF complex) and not BRG1 plays a principal role in regulating *villin* expression.

We next analyzed the promoter region of the human *villin* gene using ChIP experiments. Using 5′-RACE analysis of the total RNA from NUGC-4 cells that express *villin* and *Brm* (Fig. 4*A*), we identified the transcription start site (TSS) and the noncoding exon 1 at ~4.6 kb upstream of the translation start ATG (Supplementary Fig. S7). Our 5′-RACE assays did not detect the reported TSS (44), which is 13 bp upstream of the 5′ end of our second exon. We did the ChIP analysis using the primer pairs detecting the newly identified first or second exon and observed a clear recruitment of Brm specifically around the first exon of the *villin* gene (Fig. 4*D*).

Because our analysis of the gastric cell lines predicted that the expression of villin would be low in Brm-deficient gastric tumors, the expression patterns of Brm and villin in our clinical tissue cohort were examined. In cancer lesions wherein Brm is positive, we almost always observed clear villin expression (Fig. 5*A, case 1*). In cancer lesions wherein Brm expression is severely decreased (i.e., evaluated as "1" in Table 3), villin expression is low or undetectable among 9 of 12 cases. We think faint villin staining in such regions would be explained by the difference in immunoreactivity of two antibodies; anti-villin antibody has much higher titer than anti-Brm antibody. Importantly, in tumor lesions that contained both Brm-positive and Brm-negative regions, the expression patterns of villin and Brm were very similar and were almost superimposable when observed in the adjacent sections (Fig. 5*A, case 2*). From these results, we concluded that Brm or Brm-type SWI/SNF plays a prime role in the regulation of *villin* expression in gastric tumors. As we observed three cases of exception where villin is moderately expressed in some Brm negative cancer lesions, we cannot exclude the possibility that *villin* expression could be retained in certain tumor lesions even after Brm expression is lost.

Discussion

The roles of Brm in the development of gastric cancers. The loss or severe reduction of Brm in a variety of malignancies has been reported (4, 18), and we have also shown previously using human tumor cell lines that Brm exhibits clear tumor suppressive effects (Fig. 2; ref. 24). Taking the findings of each of these reports into consideration, Brm would be expected to universally play some anti-neoplastic role. Yet, it is intriguing that a deficiency in Brm is much more frequent in stomach cancer than in any other tumor type and is also noteworthy that the BRG1 expression levels are mostly unaffected in the Brm-deficient gastric cancers, unlike other cancer types. These findings suggest that Brm has significant tumor suppressive effects, particularly against gastric oncogenesis.

It is of further note that a decrease in Brm expression is never observed in gastric intestinal metaplasia, a well-established premalignant lesion. This suggests that the involvement of this factor in gastric malignancy does not occur during the early stages of carcinogenesis (from chronic atrophic gastritis to intestinal metaplasia to gastric carcinoma; ref. 45). It should also be considered that gastric cancer is a multifactorial disease; *Helicobacter pylori* infection, the consumption of salty foods and *N*-nitroso compounds, low intake of fresh fruits and vegetables, smoking, obesity, and ERV infection are all considered to be among the risk factors (1). Analyses of *Brm* gene knockout mice have further indicated that the loss of Brm alone cannot trigger tumorigenesis (46). Considering all of these, it is likely that a deficiency in Brm causes significant effects in the later stages of gastric tumor development, after the accumulation of several known risk factors.

Differentiation of gastric cancer from the viewpoint of Brm, villin, and intestinal metaplasia. Using gastric cancer cell lines and primary malignant tissues, we have herein shown that Brm and not BRG1 is crucial for the expression of definitive intestinal differentiation marker villin, which emerges in the stomach along with intestinal metaplastic changes to the gastric mucosa. Intestinal metaplasia is a well-characterized precancerous condition of the stomach and is divided into complete (type I) and incomplete types (type II or type III; refs. 47, 48). In a previous retrospective study of 1525 patients, it was reported that complete intestinal metaplasia is associated with a low risk of stomach cancer, whereas the incomplete type (type III in particular) has a strong association with gastric carcinogenesis (49). In addition, our current finding that Brm decrease in major gastric cancer types tends to be more prominent in more undifferentiated lesions is quite suggestive (Table 3). Taking all of these data into consideration, we speculate if the intestinal metaplastic mucosa would lose Brm expression, the complete differentiation would be prevented, and it will facilitate the development of a more anaplastic and thence more malignant gastric cancer.

The current hypothetical roles of Brm in gastric tumorigenesis are highlighted in Fig. 5*B*. The loss (epigenetic suppression) of Brm would likely occur over multiple steps during gastric carcinogenesis, but never occurs in the nonmalignant gastric mucosa, i.e., normal mucosa, chronic atrophic gastritis, or intestinal metaplasia. In the major gastric cancer pathways from incomplete intestinal metaplasia to tubular adenocarcinoma (tub1, tub2) and poorly differentiated adenocarcinoma (por), the Brm deficiency facilitates a more undifferentiated phenotype via down-regulation of gastrointestinal marker genes, such as *villin*. It has not yet been determined whether the other minor gastric cancer types originate from intestinal metaplasia, but the loss of Brm must be intimately involved in the tumorigenesis of papillary adenocarcinomas (pap). In the case of signet-ring cell carcinoma (sig) and mucinous adenocarcinoma (muc), on the contrary, the corresponding carcinogenesis processes proceed in the presence of Brm expression. Tumor formations of sig and muc remain unresolved, but we surmise that their characteristic feature of extraordinarily abundant levels of mucus secretion develop as a result of altered differentiation.

Future prospects for the association between Brm and gastric cancer from a clinical standpoint. In the near future, we plan to conduct a prospective cohort study of gastric cancer patients focusing on Brm expression. The correlation between Brm deficiency and prognostic indicators of gastric cancer (5-year survival, lymph node metastasis, etc.) should be precisely elucidated to verify the clinical utility of evaluating gastric Brm levels. The loss of Brm expression seems to be a severe risk factor for gastric malignancy, because Brm is demonstrably reduced especially in poorly differentiated adenocarcinoma (por), a major type of gastric cancer with a very poor prognosis.

We further expect that assaying Brm expression will prove to be invaluable from a therapeutic standpoint, as a genetic defect in the *Brm* gene (i.e., mutation or deletion in the *Brm* locus) is rarely detectable. Epigenetically suppressed Brm can be restored for more than a week by the transient treatment with HDAC inhibitors (24), which are considered to be next-generation antineoplastic reagents (50). Therefore, it is anticipated that Brm will be a useful marker for not only gastric cancer prognoses but also for predicting the efficacy of tumor-suppressive HDAC inhibitors. The induction of endogenous Brm would promote the differentiation of gastric cancer, particularly from poorly differentiated adenocarcinoma (por) to tubular adenocarcinoma (tub1/tub2) and, thereby, could reduce the malignant potentials, such as metastatic and invasive properties.

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Frequent Loss of Brm Expression in Gastric Cancer Correlates with Histologic Features and Differentiation State

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