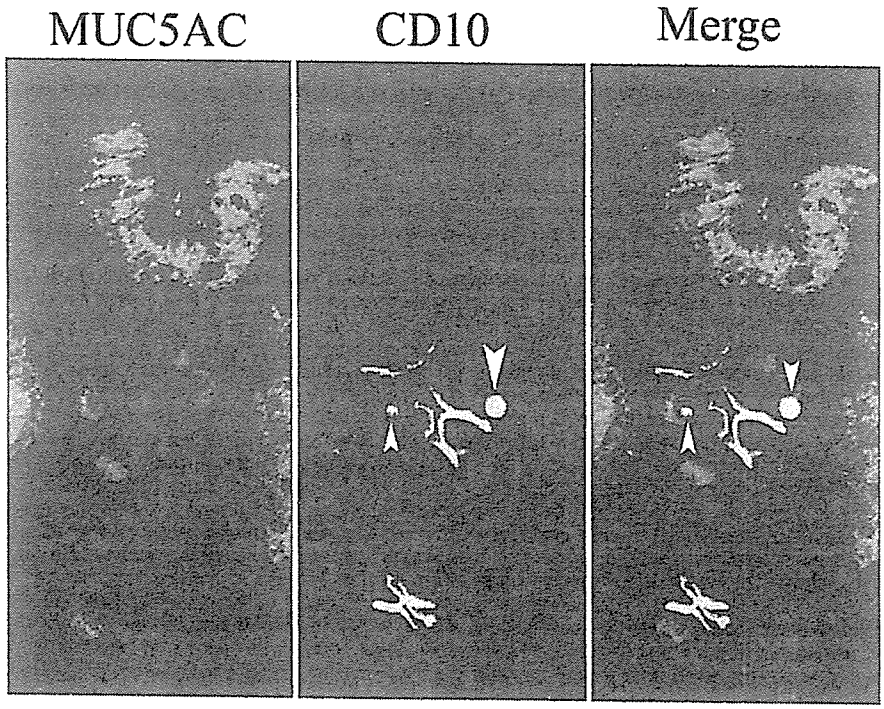


**Figure 5** Cellular localization of CD10 in GI-mixed IM glands. Frozen sections were stained by the double-immunofluorescence method using two mouse MAbs against MUC5AC and CD10 directly labeled with AlexaFluor 568 and 488, respectively. This figure was obtained by fluorescence microscopy. CD10 (green) was expressed in the microvilli of columnar cells without MUC5AC expression (red), while CD10 stained as inclusion body-like signals in MUC5AC-positive cells. Arrowheads indicate CD10-positive inclusion bodies (×60).

ure 6). Analogously, in the development of fetal mouse small intestine, villin appears on the brush border prior to CD10 (Landry et al. 1994; Montgomery et al. 1999). Villin is an actin-binding cytoskeletal protein essential for brush border formation in normal epithelial cells of the intestine, while CD10 is a brush border-associated neutral peptidase. Adapting these findings to human IM, the structural accomplishment of IMs such as villin expression might precede by functional maturations such as digestive enzyme, CD10 expression. Moreover, it may be reasonable to assume that villin-positive and CD10-negative cells in GI-mixed-type IM are functionally immature absorptive cells, and that the phenotype shift from GI-mixed-type IM to solely I-type IM is a kind of maturation. Furthermore, the expression of CD10 in the cytoplasm of columnar cells in GI-mixed IM (Figure 5) might support this idea, because it has been found in the surface enterocytes of familial microvillus inclusion disease due to the immaturity of CD10 (Groisman et al. 2002).

Co-expression ratios of MUC5AC to MUC2, villin, or CD10 varied greatly in terms of establishing a dominant MUC5AC type or a MUC2/villin/CD10 dominant type. Based on these findings, the cells in mixed-type IM glands seem to be about to free themselves from the gastric phenotype, finally becoming solely I-type IM cells. As villin, CD10, and MUC2 expressions progressively increase, MUC5AC expression reciprocally diminishes. Gastric-type cells are gradually reduced and finally replaced by intestinal-type cells, leading to solely I-type IM. These results indicate that GI-mixed-type IM cells are multi-phenotypic, suggesting that the phenotype shift from a GI-mixed type to a solely I-type IM should occur in each cell over time (Figure 7).

In conclusion, we demonstrated that intestinalization occurs in individual cells with MUC5AC expression in GI-mixed-type IM glands. The cells with an intestinal phenotype in GI-mixed type IM glands are morphologically and functionally less mature than those in I-type IM glands. As they are midway between gastric and intestinal phenotypic cells with varying degrees of differentiation and maturation, the GI-mixed-type IM glands consist of a heterogeneous population of cells. Based on observations in this study, we hypothesize that the cells in GI-mixed-type IM glands remain out of some regulations on intestinal differentiation and subsequent functional maturation toward becoming intestinal type cells. Furthermore, considering that stem cells are also present in GI-mixed-type IM, such unstable phenotypes might be induced at stem cells by either transcriptional factors or DNA methylation. To clarify and confirm these possibilities, further studies based on molecular biological techniques and applying our findings will have to be initiated starting with the identification of GI-mixed IM cells.

## Acknowledgments

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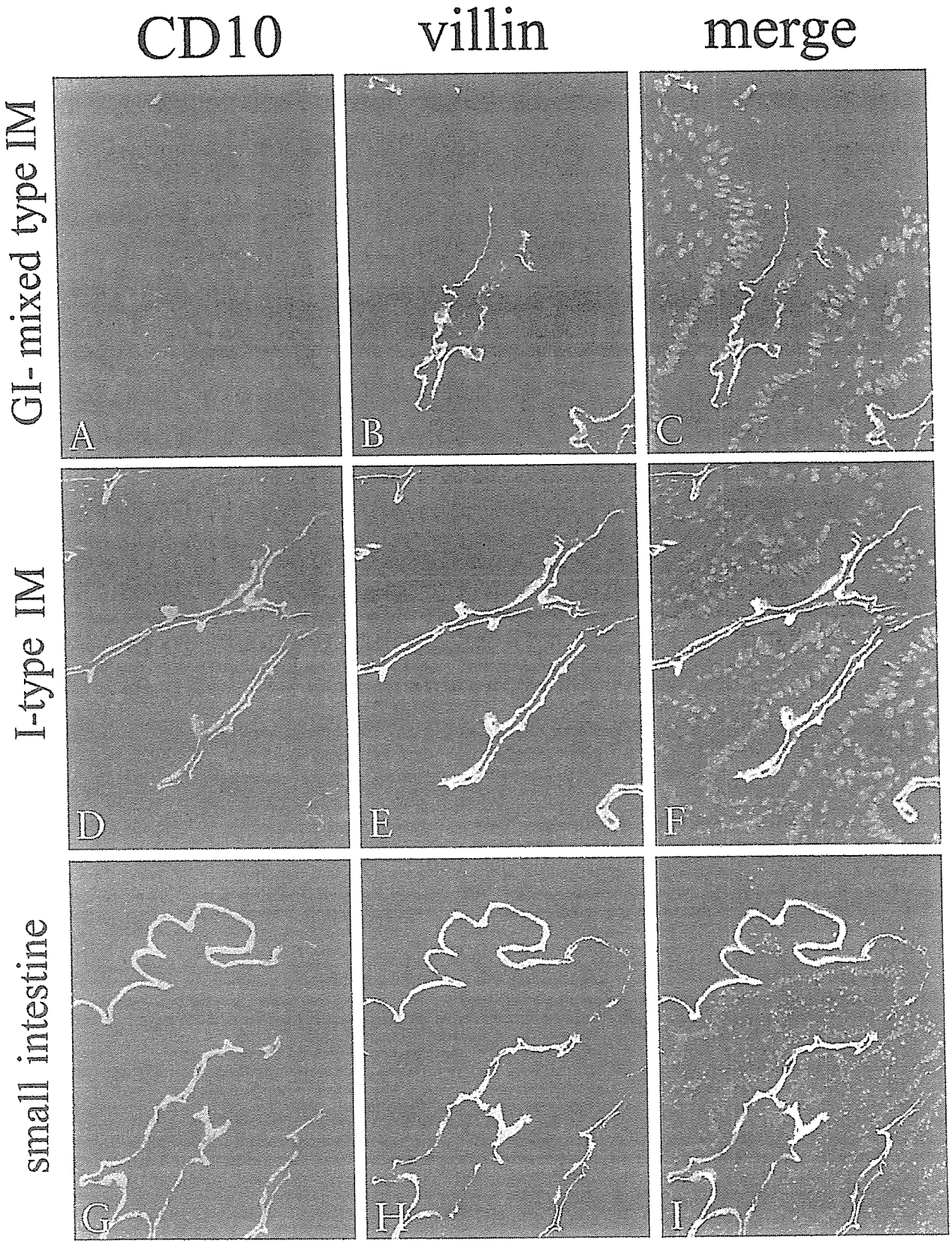


**Figure 6** Expression of villin and CD10 in GI-mixed IM glands. Frozen sections were stained by the double-immunofluorescent method using two mouse MAbs against villin and CD10 directly labeled with AlexaFluor 488 and 568, respectively. Nuclei were counter-stained by DAPI (blue) and observed by fluorescence microscopy. CD10 (red; **A,D,G**) and villin (green; **B,E,H**) were observed to be colocalized in the micro-villi of columnar cells in I-type IM glands and small intestine by composite images (yellow) (**F,I**). In contrast, compared with villin (**B**), CD10 expression (**A**) was faint in GI-mixed-type IM glands, and the colocalization was barely visible (**C**). (**A–I**, ×20).

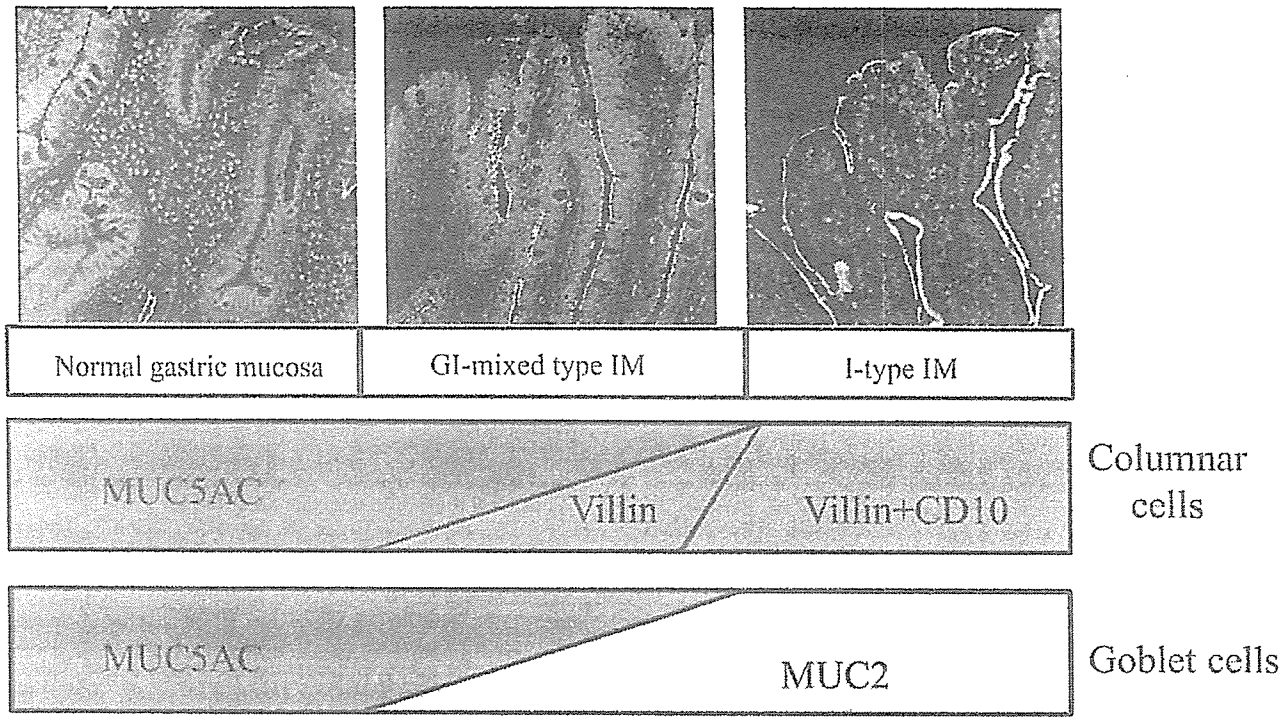


**Figure 7** Schematic representation of phenotypic shift of differentiation markers from GI-mixed-type IM to I-type IM glands. Gastric foveolar epithelial cells express only MUC5AC. IM begins from such foveolar epithelial cells, along two different cellular pathways. One begins with an aberrant expression of villin and subsequent CD10 on columnar epithelial cells, resulting in absorptive-like intestinal cells. The other starts from an ectopic expression of MUC2 and then accumulates in mucous vesicles, developing into goblet-like intestinal cells. As villin, CD10, and MUC2 expressions progressively increase, MUC5AC expression reciprocally diminishes. Gastric-type cells are gradually reduced and are finally replaced by intestinal-type cells, leading to solely I-type IM (upper panel, ×20).

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# Contrast Harmonic Sonographically Guided Radio Frequency Ablation for Spontaneous Ruptured Hepatocellular Carcinoma

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Intraperitoneal bleeding due to a ruptured tumor is a serious complication in patients with hepatocellular carcinoma (HCC). According to data compiled by the Liver Cancer Study Group of Japan,[1] ruptured HCC accounts for around 10% of deaths in these patients. Clinical features include the sudden onset of abdominal pain and distension and, if bleeding is massive, the presence of shock. Other causes of an acute abdominal emergency must be ruled out. Diagnostic imaging generally includes sonography, contrast computed tomography (CT), and angiography. In patients with ruptured HCC, prompt diagnosis and treatment is essential to avoid hepatocyte necrosis and secondary hepatic failure associated with shock and decreased hepatic perfusion due to bleeding.

The underlying liver disease varies in such patients with ruptured HCC. Chronic hepatitis, cirrhosis, or both may be present, and the severity of hepatic dysfunction as well as the size, number, and progression of the neoplastic lesions present varies from case to case. A common feature is the presence of a responsible lesion on or protruding from the surface of the liver. If hemostasis can be achieved early after HCC rupture, then overall prognosis depends on the patient's liver function and degree of tumor progression. Although there is a risk of intraperitoneal seeding, long-term survival is possible if the tumor can be completely resected by hepatectomy. One study has already reported a good 5-year survival rate after resection of ruptured and nonruptured HCC.[2] In another study, rather than performing emergency surgery, Marini et al[3] used transcatheter arterial embolization (TAE) to control bleeding; in those patients who could then undergo surgery, elective hepatectomy was associated with long-term survival. Treatment of ruptured HCC involves more than just hemostasis. Subsequent therapy is important, and, whenever possible, complete resection should be performed after bleeding has been controlled.

**Abbreviations**
CT, computed tomography; HCC, hepatocellular carcinoma; PEIT, percutaneous ethanol injection therapy; RFA, radio frequency ablation; S, segment; TAE, transcatheter arterial embolization

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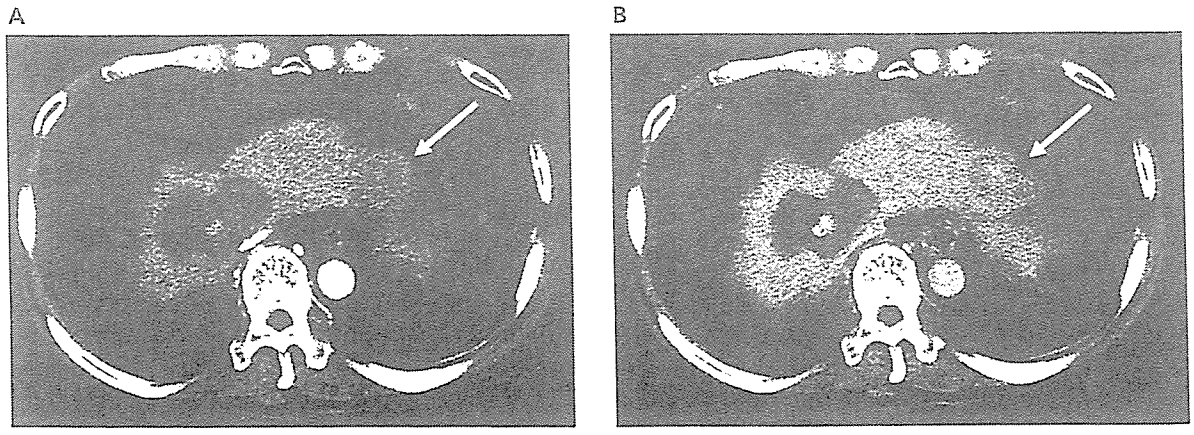
Nevertheless, in a series of 172 patients with ruptured HCC in Japan, Miyamoto et al[4] reported that subsequent hepatectomy was possible in only 12% of cases; in most cases, the presence of multiple lesions or underlying cirrhosis made surgery difficult. In patients in whom hepatectomy cannot be performed, relatively radical yet less invasive treatment with percutaneous radio frequency ablation (RFA) may lead to an improved prognosis.

Transcatheter arterial embolization is now widely used as first-line treatment to achieve safe and reliable hemostasis in ruptured HCC. However, extensive TAE may worsen liver function and lead to post-TAE hepatic failure. In addition, angiographic localization of the bleeding site in ruptured HCC is difficult and is successful in 20% of cases at most.[5] Accurate localization of the bleeding site allows for hemostasis with superselective TAE and local ablative therapy that can minimize injury to nontumor tissue and reduce the risk of posttreatment hepatic failure. In the case of ruptured HCC reported here, we identified the site of bleeding by contrast harmonic sonography and performed RFA under sonographic guidance to achieve hemostasis. This case shows the successful application of percutaneous ablative therapy guided by contrast harmonic sonography.

## Case Report

The patient was a 56-year-old man who had been followed for chronic hepatitis C at our outpatient clinic since 1992. In June 1999, hepatomas involving Couinaud segment 7 (S7; 18 mm in diameter) and S8 (25 mm) were found and were subsequently treated with TAE and percutaneous ethanol injection therapy (PEIT). In 2001, local recurrence as well as recurrence at other hepatic sites was noted, and RFA was performed. Eight months later, the patient had abdominal pain and distension and was hospitalized after consulting our clinic 2 days later. The only episode of note in the medical history was appendectomy at age 15 years; blood transfusion was required during that admission. The family history was notable in that both parents and an older brother had died of HCC. The patient also reported a 35-year history of alcohol consumption (≈0.54 L of sake, Japanese rice wine, per day). At admission, the patient was lucid and afebrile, and physical examination revealed pallor and tachycardia with no sign of shock (blood pressure, 120/60 mm Hg; pulse rate, 124 beats per minute, regular). Jaundice and ascites were apparent, but peripheral edema was not. The hemoglobin concentration was 10.6 g/dL. Liver function was severely impaired, with a total bilirubin level of 3.2 mg/dL, an albumin level of 2.6 g/dL, and prothrombin time of 36.2%. Serum aspartate aminotransferase and alanine aminotransferase levels were markedly elevated at 825 and 353 IU/L, respectively. α-Fetoprotein (106.0 ng/mL), lens culinaris agglutinin-reactive α-fetoprotein (12.9%), and des-γ-carboxy prothrombin (241 milli-arbitrary units/mL) levels were also elevated, indicating a recurrence of HCC. Abdominal

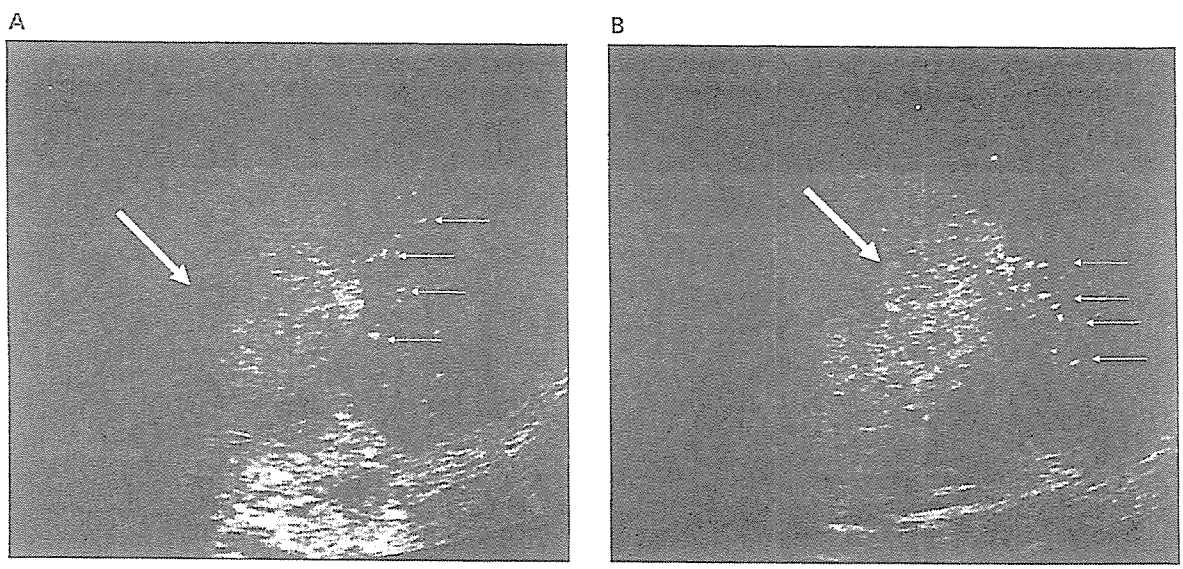
**Figure 1.** Abdominal contrast CT. **A**, An irregular low-density mass in S7 and S8 indicates scarring from previously treated HCC, but a new 3-cm tumor is also present on the S2 liver surface (arrow). During the arterial phase, no contrast enhancement is observed. **B**, During the equilibrium phase, the tumor has low density (arrow). A large amount of ascites is present, but no areas of high density suggesting blood clots and no leakage of contrast material are shown.



contrast CT showed a large volume of ascites present in the peritoneal cavity but no areas of high density suggestive of hematoma or leakage of contrast into the peritoneum (Figure 1). Several posttreatment nodules were evident in the right lobe of the liver, and a new 3-cm tumor was observed on the surface of S2. No enhancement occurred during the arterial phase, and the lesion was hypodense during the equilibrium phase. Although there was no sign of intraperitoneal hemorrhage, as abdominal paracentesis revealed grossly hemorrhagic fluid, we considered a diagnosis of ruptured HCC to be highly probable.

Although we initially considered TAE for hemostasis because the patient had severe hepatic dysfunction, TAE was decided against because of a potentially increased risk of hepatic failure. The patient was therefore treated conservatively. However, bleeding continued, shock developed, and the patient required transfusion of more than 1000 mL of blood to maintain blood pressure. On the second hospital day, the anemia progressed and bleeding became difficult to control. Because the tumor was exactly 3 cm in diameter, we thought it would be possible to achieve hemostasis together with complete tumor necrosis with minimal damage to the surrounding hepatic parenchyma if the lesion could be thermocoagulated with 1 application of RFA. Contrast sonography (Sonoline Elegra; Siemens AG, Erlangen, Germany) was used to localize the bleeding site. Because a vascular signal could not be detected with conventional power Doppler imaging of the S2 lesion, 2.5 g of the contrast agent Levovist (SH U 508A; Schering AG, Berlin, Germany) was administered by bolus intravenous injection, and sonography was performed with contrast harmonic (gray scale B-mode) imaging. During the early phase, real-time observation of leakage of microbubbles from the tumor surface confirmed the diagnosis of ruptured HCC (Figure 2). After obtaining informed consent, we performed RFA (Cool-Tip radio frequency system; Radionics, Burlington, MA) of the bleeding lesion under sonographic guidance on the same day (Figure 3). The tumor was pierced with a 3-cm electrode needle, and RFA was performed for 12 minutes. Immediately after RFA, contrast harmonic imaging was repeated, confirming no tumor blood flow and no leakage of the contrast agent into the peritoneum (Figure 4). After the procedure, blood pressure stabilized and anemia did not progress. No complications occurred. Liver function also improved, and the postprocedure course was initially good. However, hepatic failure progressed 1 month after the procedure, and the patient died on the 60th hospital day.

**Figure 2.** Abdominal contrast sonography. **A**, The 3-cm tumor is visible on the S2 liver surface (large arrow). During the early phase, the contrast agent flowed into the tumor, and leakage of microbubbles from the tumor surface into the peritoneum was observed (small arrows). The diagnosis of ruptured HCC was thereby confirmed. **B**, The HCC tumor (large arrow) shows uniform contrast enhancement.



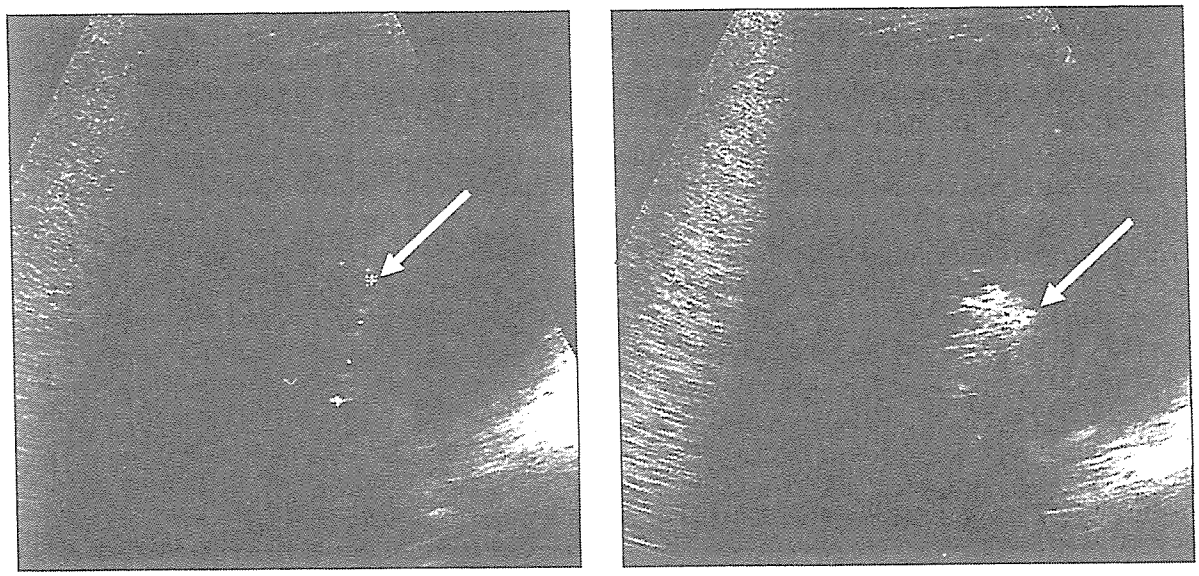
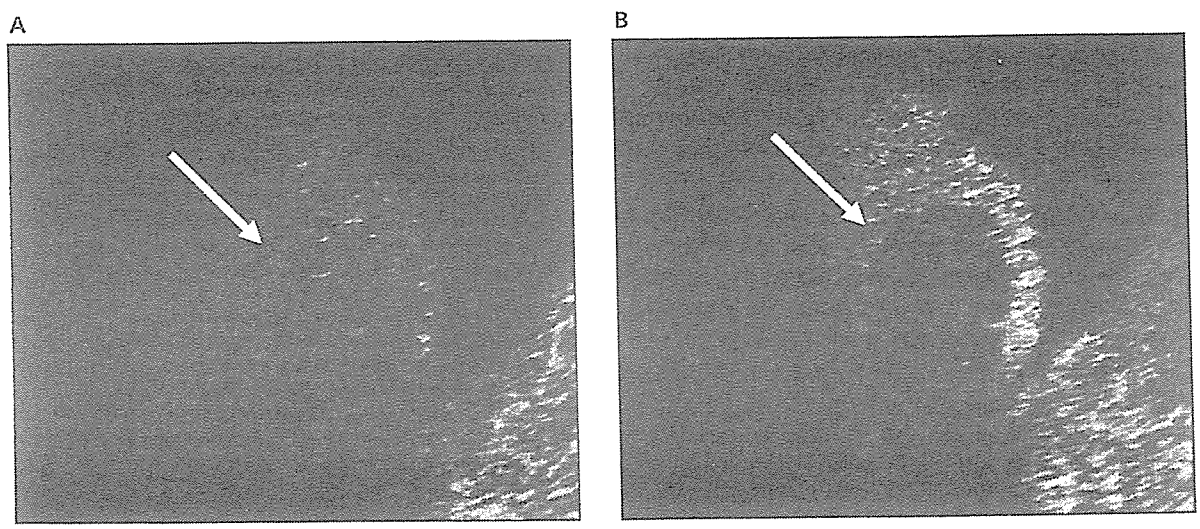



**Figure 3.** Sonographically guided RFA for ruptured HCC. **A**, The RFA electrode needle was inserted into the tumor along with the puncture guideline (arrow). **B**, After RFA, the entire tumor became strongly echoic (arrow).

## Discussion

In ruptured HCC, imaging findings suggestive of blood within the peritoneum include hyperdense areas within the ascites on CT scanning and floating hyperechoic areas (corresponding to blood clots) on sonography. Leakage of contrast medium on contrast CT helps confirm the diagnosis, but localizing the bleeding site is often difficult. Even on angiography, the diagnostic rate is less than 20% based on extravascular leakage of contrast medium.[1] In a study of color Doppler sonography performed to identify the site of bleeding in ruptured HCC, Ishida et al[6] reported that jet color flow imaging was useful in delineating the surface of the ruptured tumor. However, all the ruptured tumors were in the right hepatic lobe, so the usefulness of this procedure for tumors in the left hepatic lobe, which is subject to a cardiac apical motion artifact, could not be evaluated.

**Figure 4.** Contrast sonography after RFA. **A**, On contrast sonography after RFA, no blood flow into the tumor was observed during the early phase (arrow). **B**, During the late phase, the tumor appeared as a defect (arrow). There was no leakage of contrast into the peritoneum during either phase, thus confirming hemostasis.



In the case reported here, the ruptured tumor was in the left hepatic lobe. However, the blood flow signal from the tumor surface could not be identified on color flow imaging, perhaps because of the decreased sensitivity that was used to avoid a motion artifact from the cardiac apex. We were able to localize the site of bleeding by using contrast harmonic imaging. Compared with conventional color Doppler imaging, contrast harmonic imaging is highly sensitive in the detection of blood flow and can identify bleeding sites even when only slight bleeding is present. The disadvantage of contrast sonography is attenuation of contrast effects in deeper areas. In our patient, however, the ruptured tumor was on the liver surface, and imaging could be performed under conditions of optimal sensitivity in the presence of ascites. In addition, the use of an intravenous sonographic contrast agent is safer than iodine contrast material. Hence, contrast harmonic imaging is useful because it can be repeated to evaluate the effects of treatment immediately after hemostasis. A literature search revealed only 1 other report of ruptured HCC in which extravascular leakage of contrast material was observed in real time with the use of an intravenous sonographic contrast agent.[7] In that study, contrast color Doppler imaging was used, whereas in our study, we used contrast harmonic imaging. In comparison with conventional Doppler imaging, contrast harmonic imaging provides higher resolution because it is not affected by motion artifacts or blooming. Indeed, we were able to clearly observe extravascular leakage of each microbubble in our case. In addition, because contrast harmonic imaging is a gray scale B-mode technique, it is easier to perform percutaneous local ablative procedures under contrast guidance than with color Doppler imaging.

Very few reports have discussed control of bleeding in ruptured HCC with the use of percutaneous local ablation procedures such as PEIT and percutaneous microwave coagulation therapy. One reason is because accurate localization of the site of bleeding is difficult with conventional sonography. However, identification of the bleeding site with contrast harmonic sonography permits a percutaneous approach to the tumor, allowing hemostasis to be achieved with local ablative therapy. Radio frequency ablation is a novel procedure that allows thermocoagulation of a 3-cm lesion during a single procedure. For lesions 3 cm or less in diameter, RFA yields a high local cure rate, a less than 5% local recurrence rate, and a low incidence of posttreatment complications, including death.[8] We could only find 3 other case reports in which RFA was used, as in our patient, to control bleeding due to ruptured HCC.[9-11] Two of these cases were refractory to initial hemostatic treatment with TAE, and the remaining case was treated with RFA alone, although the site of bleeding was not identified. In each of these cases, hemostasis was achieved with a single procedure, without serious complications. One of the limitations of RFA is a cooling effect near large vessels and potential injury to bile ducts.[12] Because of this cooling effect, hemostasis with RFA may be insufficient in cases of extensive bleeding. However, contrast harmonic imaging is repeated immediately after the procedure, so even if hemostasis is incomplete, another method to control the bleeding such as PEIT or percutaneous microwave coagulo-necrotic therapy can be selected. Moreover, for ruptured HCC on the hepatic surface, the risk of bile duct injury is low. In addition, in the presence of intraperitoneal bleeding, an ample space forms between the liver surface and the skin, abdominal wall, diaphragm, and bowel. Therefore, the risk of complications due to thermal injury is low; as a result, RFA can be considered a safe procedure.

By the time our patient arrived at the hospital, secondary hepatocyte necrosis due to hemorrhage had already occurred. The development of delayed hepatic failure and subsequent limited survival may have resulted from hemostasis being performed long after the onset of tumor rupture. Ruptured HCC is generally associated with a poor prognosis, but prompt control of bleeding may permit radical treatment, prevent progression of hepatic failure, and improve the prognosis. Radio frequency ablation is useful for both hemostasis and radical cure. Furthermore, when compared with hepatectomy, RFA is minimally invasive and thus minimizes loss of the surrounding hepatic parenchyma.

In conclusion, the combination of contrast harmonic sonography and RFA enabled bedside diagnosis, hemostasis, and radical treatment of ruptured HCC. This case suggests that the procedure might become quite valuable and well worth trying, at least as a palliative treatment of ruptured HCC. Further studies are needed to elucidate indications for such treatment.

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# Inhibitory effect of etodolac, a selective cyclooxygenase-2 inhibitor, on stomach carcinogenesis in *Helicobacter pylori*-infected Mongolian gerbils

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**Abstract**

The effect of the selective COX-2 inhibitor, etodolac, on *Helicobacter pylori* (*Hp*)-associated stomach carcinogenesis was investigated in Mongolian gerbils (MGs). *Hp*-infected MGs were fed for 23 weeks with drinking water containing 10 ppm *N*-methyl-*N*-nitrosourea. They were then switched to distilled water and placed on a diet containing 5–30 mg/kg/day etodolac for 30 weeks. We found that etodolac dose-dependently inhibited the development of gastric cancer, and no cancer was detected at a dose of 30 mg/kg/day. Etodolac did not affect the extent of inflammatory cell infiltration or oxidative DNA damage, but it significantly inhibited mucosal cell proliferation and dose-dependently repressed the development of intestinal metaplasia in the stomachs of *Hp*-infected MGs. These results suggest that COX-2 is a key molecule in inflammation-mediated stomach carcinogenesis and that chemoprevention of stomach cancer should be possible by controlling COX-2 expression or activity.


*Keywords:* Stomach cancer; COX-2; *Helicobacter pylori*; Mongolian gerbils; Chemoprevention; Etodolac; Carcinogenesis

Despite a recent decline in its incidence, gastric cancer remains one of the most common malignancies in the world. Its pathogenesis is known to be closely associated with several environmental factors, including a high intake of salted foods and nitrates, and insufficient intake of fresh fruits and vegetables [1–6]. Clinicopathological and histological studies suggest a correlation between chronic atrophic gastritis (CAG) and the development of gastric cancer [1,7]. Moreover, since the discovery of *Helicobacter pylori* (*Hp*) in 1983, considerable evidence has accumulated for its involvement in CAG. *Hp* colonizes the stomach mucosa and triggers a series of inflammatory reactions [8,9], and it is now considered to be the most important cause and a potent driving force for subsequent progression of CAG.

Chronic atrophic gastritis is the first step in the series of events leading to carcinogenesis of the stomach, a process referred to as the CAG-metaplasia-dysplasia-cancer sequence [1,7]. By following up 4655 healthy asymptomatic males for a mean period of 7.7 years, we recently found that the risk of developing gastric cancer increases in a step-wise manner with the progression

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of CAG, and the cancer incidence rate reached a maximal level of 871 per 100,000 person-years in individuals with metaplastic gastritis [10]. Because gastric cancer did not develop in *Hp*-free subjects during the study period, it appears that *Hp*-associated gastritis is central in the Japanese carcinogenic pathway.

Meanwhile, elevated expression of cyclooxygenase 2 (COX-2), a prostaglandin-synthesizing enzyme, is observed in a wide variety of human malignancies, including gastric cancer [11–14]. Various in vitro and in vivo studies strongly suggest that COX-2 is involved in a major early oncogenic event in various human malignancies. In addition, epidemiological and preclinical animal studies indicate that regular intake of either nonselective or selective COX-2 inhibitors reduces the risk of several forms of human cancer [15–17]. Thus, it is conceivable that targeted inhibition of abnormally or improperly elevated COX-2 will be a highly effective strategy for preventing these epithelial malignancies.

The current model for human stomach carcinogenesis emphasizes the importance of *Hp* infection. *Hp*-induced chronic gastritis increases the level of COX-2 expression in the stomach mucosa [18–20]. Enhanced expression of COX-2 is also observed in intestinal metaplasia, dysplasia, and gastric adenoma, which are considered precancerous lesions [11,21]. Thus, it is likely that the development of cancer can be reduced by regulating COX-2 expression or activity. The selective COX-2 inhibitor, Celecoxib, has been reported to prevent *N'*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced chemical carcinogenesis in the rat [22]. Furthermore, while we were preparing this manuscript, Nam et al. [23] reported that another selective COX-2 inhibitor, nimesulide, represses the development of gastric cancer in *Hp*-infected C57/BL6 mice. In this mouse model, however, *Hp*-associated gastritis is mild and cancer development does not follow the characteristic series of events seen in humans [9,24]. The response of Mongolian gerbils (MGs) to infection with *Hp*, in contrast, is quite similar to that in humans: as in humans, *Hp* causes severe and persistent infection in the stomach of MGs; induces a similar series of pathological events, including the development of chronic active gastritis, peptic ulcers, intestinal metaplasia; and results in the same histological types of gastric cancer. These pathological events are not observed in any other animal model [25–27]. Thus, MGs are considered to be the best experimental model for human stomach carcinogenesis. Therefore, in the present study, we investigated the effect of a selective COX-2 inhibitor, etodolac, on *Hp*-associated stomach carcinogenesis in MGs.

## Materials and methods

*Chemicals. N*-Methyl-*N*-nitrosourea (MNU; Wako Pure Chemical Industries, Tokyo, Japan) was freshly prepared at 10 ppm in distilled water twice a week. The MGs were fed the solution ad libitum as drinking water from light-shielded bottles. The stock diet was made up by mixing various amounts of etodolac (kindly provided by Nippon Shinyaku, Kyoto, Japan) with CRF-1 (Oriental Yeast, Tokyo, Japan) so that daily intake of etodolac would be 5, 10, or 30 mg/kg/day.

*Bacteria. Hp* samples (Type I Strain ATCC 43504; American Type Culture Collection, Rockville, MD) containing approximately $3.0 \times 10^8$ colony forming units per milliliter were used as the inoculum. After a 24-h fast, samples (1 ml) were delivered intragastrically (i.g.) using an oral catheter. Vehicle alone (Brucella broth) was administered i.g. as a control.

*Animals.* Specific pathogen-free 6-week-old male MGs (*Meriones unguiculatus*; MGS/Sea; Seac Yoshitomi, Fukuoka, Japan) were housed in steel cages with hardwood chip bedding in an air-conditioned biohazard room with a 12-h light/12-h dark cycle. The animals were fed γ-irradiated (30 kGy) stock diet.

*Experimental design.* The experimental design was approved by the Animal Research Committee of Wakayama Medical University. As shown in Fig. 1, a total of 154 gerbils were divided into five groups (A–E). Animals were inoculated with *Hp* (groups A–D; $n = 28$, 35, 35, and 40, respectively) or vehicle (Brucella broth) alone (group E; $n = 16$). After 1 week, groups A–D were given MNU in the drinking water at a concentration of 10 ppm. MNU was not administered to group E. After 23 weeks, all groups were switched to autoclaved distilled water as drinking water. In groups B–D, the animals were given the stock etodolac diet from week 24 until week 53, whereas the animals in groups A and E received the control diet. The daily administered dosage of etodolac was 0 mg/kg/day in groups A and E, 5 mg/kg/day in group B, 10 mg/kg/day in group C, and 30 mg/kg/day in group D. On the 53rd experimental week, the animals were fasted for 24 h, placed under deep ether anesthesia, and subjected to laparotomy with excision of the stomach. One small segment of this stomach tissue was stored at −80 °C immediately after resection for isolation of total RNA. The remainder was used for histological examination.

*Tissue preparation and histological examination.* The excised stomachs were fixed in 10% neutral-buffered formalin and embedded in paraffin. Tissues sections (5 μm) were stained with haematoxylin–eosin (H&E) and were analyzed by immunohistochemistry with anti-*Hp* serum (Dako, Glostrup, Denmark), anti-COX-2 serum (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-proliferating cell nuclear antigen (PCNA) serum (Dako). Histological features of mucosal inflammation and intestinal metaplasia were evaluated for each specimen under a light microscope. The degree of inflammatory cell infil-

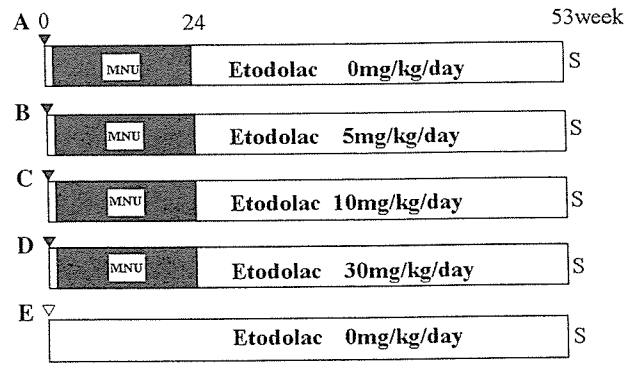


Fig. 1. Protocol of the animal experiment. At the beginning of the experiment, animals were inoculated i.g. with *Hp* (groups A–D; ▼) or vehicle (Brucella broth; group E; ▽). After 1 week and until 23 weeks, the animals were given drinking water containing no (group E; open bars) or 10 ppm MNU (groups A–D; filled bars). All groups were then switched to distilled water and given a diet containing no drug (groups A and E) or 5 (group B), 10 (group C), or 30 (group D) mg/kg/day etodolac. The animals were sacrificed (S) on week 53.

tration and the area of intestinal metaplasia were scored as follows: 0, normal; 1, mild; 2, moderate; 3, marked. For the evaluation of mucosal cell proliferation, the proportion of PCNA-positive cells per 1000 mucosal cells was assessed in the antrum and corpus as described previously [28].

*Isolation of total RNA and quantitative reverse transcription-polymerase chain reaction.* Total RNA was isolated using Trizol reagent (Life Technologies, Gaithersburg, MD) from the frozen stomach segments, and cDNA synthesis was carried out using 1 μg of total RNA and a First Strand cDNA Synthesis Kit for reverse transcription-polymerase chain reaction (RT-PCR) (AMV) (Roche, Indianapolis, IN). PCR was performed with a LightCycler (Roche Diagnostics, Mannheim, Germany), with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as a reference. Primers for COX-2 and GAPDH were designed based on their cDNA sequences (GenBank Accession Nos. AB044784 and AB040445, respectively). PCR was carried out with an initial denaturation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s, and 72 °C for 16 s. LightCycler software, version 3.5 (Roche Diagnostics) was used to determine the amount of mRNA from each sample.

*Serum levels of anti-Hp antibodies and 8-hydroxy-2′-deoxyguanosine (8-OHdG).* Blood samples from each animal were centrifuged and separated sera were stored at −80 °C until use. The anti-*Hp* IgG level was measured in the serum using an enzyme-linked immunosorbent assay (ELISA) (Seac Yoshitomi) with peroxidase-conjugated anti-mouse immunoglobulin as the secondary antibody. Optical density at 450 nm ($OD_{450}$) was measured spectrophotometrically. A cut-off value of 0.1 was used as an indicator of Hp infection [25]. These sera were also centrifuged at 10,000*g* for 30 min at 4 °C through centrifugal filter devices (Microcon YM-10, Millipore, Bedford, MA) and used for the measurement of 8-OHdG by ELISA (high sensitive 8-OHdG check; Japan Institute for the Control of Aging, Shizuoka, Japan).

*Statistical analyses.* An unpaired *t* test or a Mann–Whitney *U* test was applied to determine the significance of differences between two groups. Survival curves of the animals were calculated by the Kaplan–Meier method and the differences were evaluated using a log-rank test. The incidence of cancer was assessed using Fisher's exact probability method. *P* values <0.05 were considered to be statistically significant.

## Results

In the present study, we investigated whether long-term treatment with etodolac, a selective COX-2 inhibitor, can be used to prevent *Hp*-associated stomach carcinogenesis. The animals were separated into five groups: groups A–D were *Hp-infected* and were divided according to the dose of drug administration, and group E (control group) was neither infected with *Hp* nor treated with etodolac (Fig. 1). During the 53-week experimental period, the body weight gain of group C (10 mg/kg/day etodolac) was significantly less than group E (control), but there was no significant difference in the food intake or body weight gain among the other experimental groups of the animals. Also, a significant difference was not observed in the appearance or survival rates among the various groups.

In all of the *Hp*-infected animals (groups A–D), marked infiltration of inflammatory cells was observed in the lamina propria and submucosa. This infiltration was predominantly lymphocytes, although some macrophages and neutrophils were also observed. The histological examination also revealed hyperplasia of the epithelia together with erosions, lymphoid follicle formation, and intestinal metaplasia. These results are consistent with our previous findings [29]. COX-2 expression, which was not observed in the stomach of non-infected animals, was also induced by *Hp* infection. Immunohistochemistry with a specific anti-COX-2 antibody showed that a considerable number of mesenchymal cells and infiltrating mononuclear cells became COX-2-positive in the *Hp*-infected stomach (not shown).

Etodolac treatment did not have significant influence on the number of COX-2-expressing mesenchymal or inflammatory cells in the stomach, but the intensity of the staining was significantly reduced. The mucosal level of COX-2 mRNA expression tended to be dose-dependently reduced by etodolac treatment. However, the difference in the COX-2 mRNA level between groups with and without etodolac treatment was not significant (Fig. 2). This is probably due to the fact that RNA from COX-2 expressing cells represents a relatively small proportion of the total mucosal RNA.

The number of inflammatory cells infiltrating the *Hp*-infected stomach did not appear to be influenced by etodolac treatment because there was no significant difference in the inflammatory scores among the etodolac-treated groups (Fig. 3A). Infection with *Hp* remarkably elevated the serum level of 8-OHdG, a biomarker of oxidative DNA damage, but it was not significantly reduced by etodolac treatment, which suggests that etodolac did not reduce inflammation-mediated mucosal cell damage (not shown). In contrast, we found that the etodolac treatment dose-dependently reduced the serum

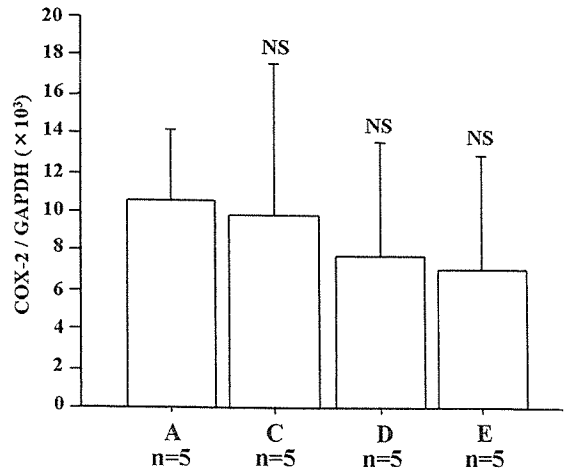


Fig. 2. Effect of etodolac on the mucosal level of COX-2 mRNA expression in *Hp*-infected MG stomach. Total RNA was isolated from *Hp*-infected MG stomach mucosa and used to generate cDNA. PCR was performed using specific primers for COX-2 and GAPDH, and the level of COX-2 mRNA in each sample was determined using LightCycler software. NS, not significant vs. group A.

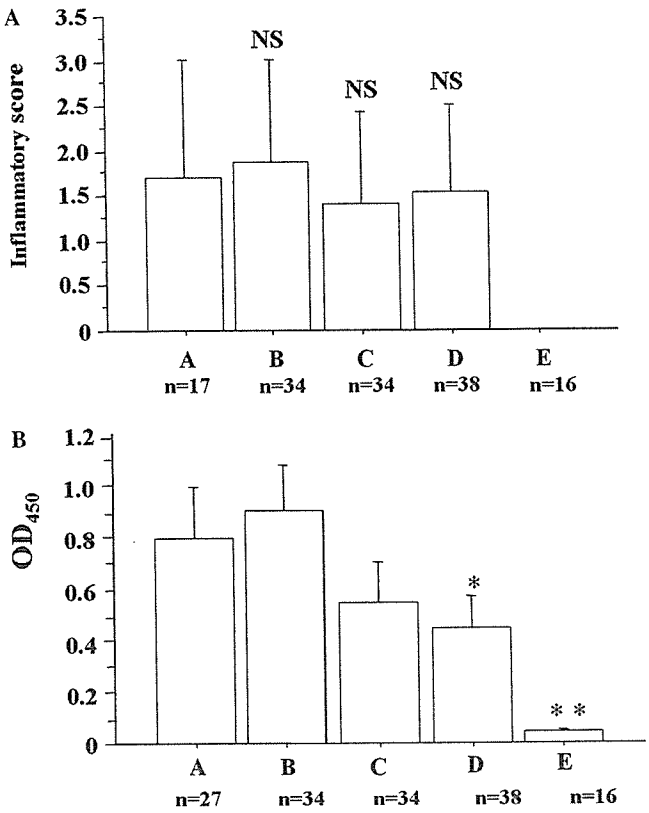


Fig. 3. Effect of etodolac on: (A) the number of inflammatory cells infiltrating the stomach mucosa and (B) the serum level of anti-*Hp* antibodies in *Hp*-infected MGs. (A) The degree of inflammatory cell infiltration was scored as follows: 0, normal; 1, mild; 2, moderate; 3, marked. NS, not significant vs. group A. (B) The anti-*Hp* IgG levels in serum were measured by ELISA. An $OD_{450} > 0.1$ was used to confirm infection by *Hp*. $^{*}p < 0.05$ vs. group B; $^{**}p < 0.01$ vs. groups A–D.

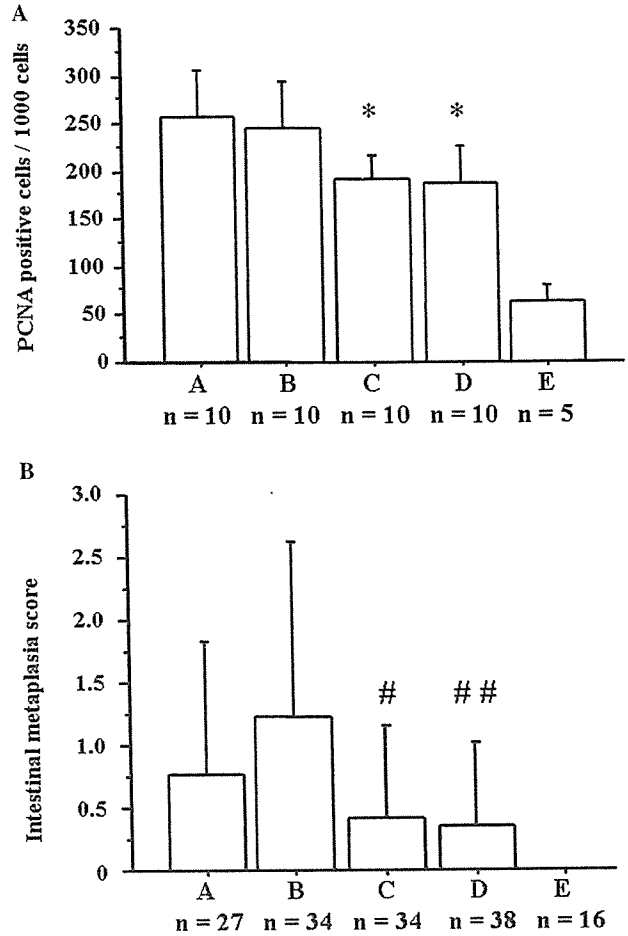


Fig. 4. Effect of etodolac on: (A) the proliferation of mucosal cells and (B) the development of intestinal metaplasia in the stomachs of *Hp*-infected MGs. (A) Anti-PCNA immunohistochemistry was used to evaluate mucosal cell proliferation. The proportion of PCNA-positive cells per 1000 mucosal cells was determined in each experiment. $^{*}p < 0.01$ vs. groups A and B. (B) The extent of intestinal metaplasia in each experimental group was scored as described as follows: 0, normal; 1, mild; 2, moderate; 3, marked. $^{\#}p < 0.05$ vs. group B; $^{\#\#}p < 0.01$ vs. group B.

level of anti-*Hp* antibodies, with a statistically significant reduction at 30 mg/kg/day (group D) (Fig. 3B).

Infection with *Hp* greatly enhanced mucosal cell proliferation as revealed by anti-PCNA immunohistochemistry (Fig. 4A). Etodolac treatment resulted in a step-wise and significant reduction in the proliferation of *Hp*-infected mucosal cells ($p < 0.01$). In addition, etodolac repressed the development of intestinal metaplasia, an end result of persistent gastritis (Fig. 4B). These metaplastic changes were significantly reduced in the groups C and D which were administered higher doses of etodolac.

Finally, we examined the incidence and development of cancer in the infected animals at the end of the experimental period. Cancers were mostly observed in the pyloric mucosa adjacent to the fundic region. As shown in Fig. 5, a majority of them were well-differentiated adenocarcinomas, although we also observed the development of signet-ring cell carcinoma. The incidence of cancer in the experimental groups is shown in Table 1. As a result of long-lasting infection with *Hp*, 14.8% of the animals in group A developed cancer. The cancer incidence in the group treated with a low dose of etodolac (group B) was higher than the control group, but the difference was not significant. The incidence of cancer decreased in a step-wise manner with the dose of etodolac, and the development of the cancer was completely inhibited at a dose of 30 mg/kg/day (group D).

## Discussion

In the present study, etodolac, a selective inhibitor of COX-2, dose-dependently inhibited the development of gastric cancer in the mucosa of *Hp*-infected MGs. The effect of etodolac was so strong that no cancer developed in MGs treated with 30 mg/kg/day. This is the first demonstration that a selective COX-2 inhibitor can prevent stomach carcinogenesis in the CAG-metaplasia-cancer sequence.

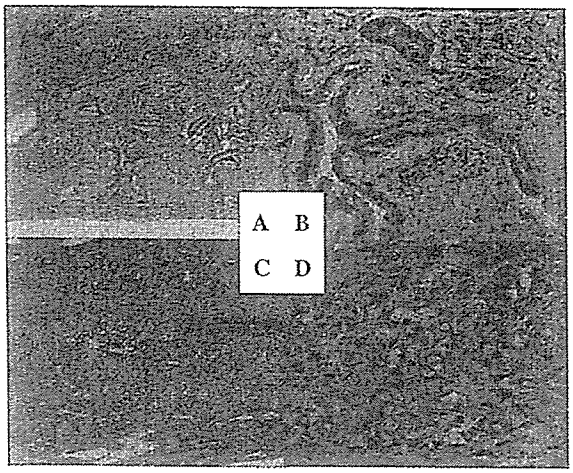


Fig. 5. Typical adenocarcinoma in the pyloric mucosa of *Hp*-infected MGs. Shown are a typical well-differentiated adenocarcinoma (A,B) and a signet-ring cell carcinoma (C,D) stained with H&E. Images were obtained at 100× (A,C) and 400× (B,D).

The basic mechanism for the observed inhibitory effect of etodolac remains unclear, but our current findings agree with epidemiological studies showing that aspirin and other non-steroidal anti-inflammatory drugs, which are non-selective COX inhibitors, reduce the risk for gastric cancer [30–32]. We expect that etodolac acts by inhibiting the inducible form of COX-2, a key enzyme in prostaglandin biosynthesis. COX-2 is readily induced by various stimuli, including proinflammatory cytokines, growth factors, and tumor promoters. *Hp* has been shown to directly upregulate COX-2 mRNA expression in gastric mucosal cells in vitro [33]. Chronic inflammation in other organs, for example, in ulcerative colitis or hepatitis C virus-associated chronic hepatitis, is thought to play a central role in the accumulation of genetic events leading to transformation and cancer by increasing the number of target cells or by promoting the proliferation of initiated cells [34–36]. In these cases, the induction of COX-2 appears to participate in the establishment of the carcinogenic state. In the current studies, we found that etodolac inhibits mucosal cell proliferation and the development of intestinal metaplasia, a precancerous lesion caused by accumulation of genetic events in the stomach epithelia. This strongly suggests that COX-2 also participates in inflammation-mediated carcinogenesis in the *Hp*-infected stomach.

Measurement of the serum 8-OHdG level showed that etodolac did *not* cause significant differences in the extent of oxidative DNA damage. In addition, etodolac has a stronger inhibitory effect on the development of cancer than on the development of intestinal metaplasia. This suggests that the anti-carcinogenic effect of etodolac is mediated by the suppression of tumor promotion rather than initiation, and it is consistent with studies showing that the COX-2 pathway is involved in tumor promotion in several other organs [37]. The effect of etodolac on gastritis was unclear because treatment with etodolac did not result in significant difference in the inflammatory score.

Interestingly, the titers of anti-*Hp* antibodies were significantly lower in etodolac-treated animals. This is consistent with the recent hypothesis that COX-2 inhibitors reduce B cell antibody production, in part, by attenuating their differentiation to plasma cells [38]. A high antibody titer is thought to reflect severe inflammation in *Hp*-infected stomach mucosa, and its long-term persistence appears to lead to the progression of atrophic changes and intestinal metaplasia [39,40]. Indeed, in previous studies, the enhancement of carcinogenesis by *Hp* has been observed mainly in animals with a high antibody titer [29,41]. This is not surprising because a T helper-2 humoral immune response is thought to be important in the development of cancer [29]. Therefore, reduction of the humoral immune response may contribute to the anti-carcinogenic activity of etodolac.

A variety of previous studies have provided evidence that the effect of selective COX-2 inhibitor is mediated not only by selectively inhibiting COX-2 but also by modifying COX-2-independent mechanisms. For example, both non-selective and COX-2 selective inhibitors have been shown to suppress the activation of NF-κB by *Hp*. This may subsequently reduce transcription of the COX-2 gene [42]. A relatively high dose of etodolac is needed for the anti-carcinogenic effect, indicating that it may be mediated by COX-2-independent mechanisms. Further studies are required to determine how this COX-2 inhibitor prevents stomach carcinogenesis.

Table 1
Effect of etodolac on development of gastric cancer in male Mongolian gerbils

| Group | Treatment | Number of tumor-bearing Mongolian gerbils (%) | No. of Mongolian gerbils | No. of cancer | | |
|---|---|---|---|---|---|---|
| | | | | Well | Poor | Sig |
| A | *Hp* + MNU + etodolac (0 mg/kg/day) | 4 (14.8)* | 27 | 3 | 0 | 1 |
| B | *Hp* + MNU + etodolac (5 mg/kg/day) | 8 (23.5)* | 34 | 8 | 0 | 0 |
| C | *Hp* + MNU + etodolac (10 mg/kg/day) | 3 (8.8) | 34 | 3 | 0 | 1 |
| D | *Hp* + MNU + etodolac (30 mg/kg/day) | 0 (0.0) | 39 | 0 | 0 | 0 |
| E | Br + etodolac (0 mg/kg/day) | 0 (0.0) | 16 | 0 | 0 | 0 |

*Hp*, *H. pylori* (i.g.); Br, Brucella broth (i.g.); Well, well-differentiated adenocarcinoma; Poor, poorly differentiated a denocarcinoma; Sig, signet-ring cell carcinoma.

* Significantly different from group D ($p < 0.05$) by Fisher's exact test.

In conclusion, the present study clearly demonstrates that etodolac potently inhibits inflammation-mediated carcinogenesis in the *Hp*-infected stomach. This effect is due to the inhibition of cell proliferation, alteration of the humoral immune response, and slowing of the CAG-metaplasia-cancer sequence. In addition, the potent anti-carcinogenic effect of etodolac strongly suggests that COX-2 plays a key role in *Hp*-associated stomach carcinogenesis, which is a main route for the development of cancer in high-risk areas throughout the world. Furthermore, it may be possible to prevent stomach cancer by chemotherapy aimed at reducing COX-2 expression. In addition to eradication of *Hp*, selective COX-2 inhibitors should provide a potent strategy for cancer prevention. However, the long-term use of other selective COX-2 inhibitors is known to be associated with an elevated risk of cardiovascular events [43–46], and etodolac is expensive and less cost-effective than therapy aimed at eradicating *Hp*. These factors must be considered before etodolac is used clinically for the chemoprevention of gastric cancer.

Our previous study revealed that subjects with extensive metaplastic gastritis have the highest risk for the development of gastric cancer (the annual cancer incidence, 0.87%). These individuals represent less than 1% of the middle-aged Japanese population [10], and they cannot be treated with therapy to eradicate *Hp* because the infection is no longer present [10]. In this small population, inhibition of COX-2 may be useful for the prevention of gastric cancer.

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# The *Brm* gene suppressed at the post-transcriptional level in various human cell lines is inducible by transient HDAC inhibitor treatment, which exhibits antioncogenic potential

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**The mammalian SWI/SNF chromatin remodeling complex is composed of more than 10 protein subunits, and plays important roles in epigenetic regulation. Each complex includes a single BRG1 or Brm molecule as the catalytic subunit. We previously reported that loss of Brm, but not BRG1, causes transcriptional gene silencing of murine leukemia virus-based retrovirus vectors. To understand the biological function and biogenesis of Brm protein, we examined seven cell lines derived from various human tumors that do not produce Brm protein. We show here that these Brm-deficient cell lines transcribe the *Brm* genes efficiently as detected by nuclear run-on transcription assay, whereas *Brm* mRNA and *Brm* hnRNA were undetectable by reverse transcription–polymerase chain reaction analysis. These results indicate that expression of *Brm* is strongly and promptly suppressed at the post-transcriptional level, through processing and transport of the primary transcript or through stability of mature *Brm* mRNA. This suppression was attenuated by transient treatment of these cell lines with HDAC inhibitors probably through indirect mechanism. Importantly, all of the treated cells showed prolonged induction of *Brm* expression after the removal of HDAC inhibitors, and acquired the ability to maintain retroviral gene expression. These results indicate that these Brm-deficient human tumor cell lines carry a functional *Brm* gene. Treatment with HDAC inhibitors or introduction of exogenous *Brm* into Brm-deficient cell lines significantly reduced the oncogenic potential as assessed by colony-forming activity in soft agar or invasion into collagen gel, indicating that, like *BRG1*, *Brm* is involved in tumor suppression.**

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## Introduction

The SWI/SNF complex contributes to the regulation of gene expression by altering the chromatin structure, and plays many important roles in epigenetic regulation in many organisms (Narlikar *et al.*, 2002). In mammals, this complex is composed of more than 10 subunits, and each of the complex contains a single molecule of either Brm or BRG1, but not both (Wang *et al.*, 1996). These two proteins are the catalytic subunits and have DNA-dependent ATPase activity that drives remodeling of nucleosomes (Laurent *et al.*, 1993). We previously reported that Brm and BRG1 have clear differences in their biological activities; Brm is essential for maintenance of murine leukemia virus (MuLV)-based retroviral gene expression, whereas BRG1 is not (Mizutani *et al.*, 2002). Therefore, cell lines that do not express detectable levels of Brm protein (designated hereafter as Brm-deficient cell lines) undergo very rapid retroviral gene silencing that occurs stochastically and discontinuously.

The SWI/SNF complex interacts with various proteins through many specific interfaces of its different subunits. These interacting proteins include products of proto-oncogenes such as c-*fos*, c-*jun* (Ito *et al.*, 2001), and c-*myc* (Cheng *et al.*, 1999), and tumor suppressor proteins such as Rb (Dunaief *et al.*, 1994; Trouche *et al.*, 1997; Strobeck *et al.*, 2000), p53 (Lee *et al.*, 2002), and β-catenin (Barker *et al.*, 2001). We previously reported that the heterodimer of c-Fos and c-Jun requires functional SWI/SNF complex for transactivation through AP-1 DNA binding sites (Ito *et al.*, 2001). Therefore, this complex would be involved in multiple processes associated with formation or suppression of tumors.

Genetic and molecular evidence indicate that some subunits of the SWI/SNF complex act as tumor suppressors in human and mice. The Ini1 subunit is deleted in malignant rhabdoid tumors, which occur commonly in pediatric brains and kidneys in the first 2 years of human life (Versteege *et al.*, 1998; DeCristofaro *et al.*, 1999). In addition, loss of heterozygosity of *Ini1* gene causes rhabdoid tumors in heterozygous (+/−) knockout mice (Klochendler-Yeivin *et al.*, 2000; Roberts *et al.*, 2000; Guidi *et al.*, 2001). Therefore, *Ini1* gene is now categorized as a typical tumor suppressor gene. The expression of BRG1 subunit is sometimes deleted in non-small cell lung carcinomas (Reisman *et al.*, 2003) and other cancers (Wong *et al.*, 2000; DeCristofaro *et al.*, 2001). Besides, experiments on *BRG1* knockout mice revealed that heterozygous (+/−) mice were prone to developing tumors (Bultman *et al.*, 2000), suggesting that *BRG1* is a tumor suppressor gene.

Brm, which has very similar structure to BRG1, has been also reported to be deficient in several human cell lines derived from various human tumors (Dunaief *et al.*, 1994; Mizutani *et al.*, 2002; Iba *et al.*, 2003; Reisman *et al.*, 2003). However, Brm was previously shown to be dispensable in mice, and Brm homozygotes were not prone to developing tumors (Reyes *et al.*, 1998). Therefore, biological meanings of frequent loss of Brm expression accompanied with tumor formation were not clear at present. Interestingly, expression of Brm has not been detected in several human tumor cell lines (Reisman *et al.*, 2002), and some cell lines such as SW13 were reported not to produce BRG1 protein either (Dunaief *et al.*, 1994). In our previous report, we showed that this cell line comprises two subtypes: SW13(vim−), which expresses neither Brm nor BRG1, and SW13(vim+), which produces both proteins (Yamamichi-Nishina *et al.*, 2003). We also found that in both SW13(vim-) and SW13(vim+) cells, the *BRG1* and *Brm* genes were transcribed efficiently, but that in SW13(vim-) cells, mRNA expression and protein production of *BRG1* and *Brm* were tightly suppressed, indicating that the regulation is at the post-transcriptional level.

In the present work, we selected several Brm-deficient cell lines derived from a wide variety of human tumors, and studied the molecular mechanisms underlying the loss of Brm protein production. Our results indicate that Brm deficiency is not caused by mutation or deletion in the *Brm* locus, but caused by post-transcriptional regulation of the *Brm* gene in all the Brm-deficient cell lines tested here, and that this unique regulation is independent of expression status of BRG1 protein or *BRG1* mRNA. We also observed that HDAC inhibitors attenuate suppression of Brm expression in these cells, and induction of either endogenous or exogeneous *Brm* reduced the oncogenic potential of these Brm-deficient cell lines.

## Results

### *The* Brm *gene is transcribed constitutively in human tumor cell lines deficient in Brm expression*

We screened expression of Brm, BRG1 and Ini1 proteins and mRNAs in many cell lines derived from a wide variety of human tumors, and some of them are summarized in Table 1. Including those previously reported, seven Brm–deficient cell lines (SW13(vim-), C33A, PA-1, NCC-IT, NCI-H522, A427, and G401), five BRG1-deficient cell lines (SW13(vim-), NCI-H522, A427, H1299, and A549), and two Ini1-deficient cell lines (G401 and A204) were identified by Western blotting analysis. As shown in Table 1, some cell lines, such as G401 and A427, lacked expression of two proteins simultaneously among these three proteins. *Brm* mRNA was not detected in all the seven Brm-deficient cell lines, when reverse transcription-polymerase chain reaction (RT–PCR) was performed with two independent pairs of primers (Figure 1a). On

**Table 1** Expression of *Brm*, *BRG1* and *Ini1* in several human tumor cell lines

| *Cell line* | *Origin* | *Brm* | | *BRG1* | | *Ini1* | |
|---|---|---|---|---|---|---|---|
| | | *R* | *P* | *R* | *P* | *R* | *P* |
| SW13 (vim−) | Adrenocortical carcinoma | − | − | − | − | + | + |
| C33A | Cervical carcinoma | − | − | + | + | + | + |
| PA-1 | Embryonic carcinoma | − | − | + | + | + | + |
| NCC-IT | Embryonic carcinoma | − | − | + | + | + | + |
| NCI-H522 | Non-small-cell lung carcinoma | − | − | + | − | + | + |
| A427 | Non-small-cell lung carcinoma | − | − | + | − | + | + |
| H1299 | Non-small-cell lung carcinoma | + | + | + | − | + | + |
| A549 | Non-small-cell lung carcinoma | + | + | − | − | + | + |
| G401 | Rhabdoid tumor | − | − | + | + | − | − |
| A204 | Rhabdomyosarcoma | + | + | + | + | + | − |
| MDA-MB435 | Breast ductal carcinoma | + | + | + | + | + | + |
| SW620 | Colorectal adenocarcinoma | + | + | + | + | + | + |
| HeLa-S3 | Cervical carcinoma | + | + | + | + | + | + |

R = expression of mRNA analysed by RT–PCR; P = protein expression analysed by Westem blotting

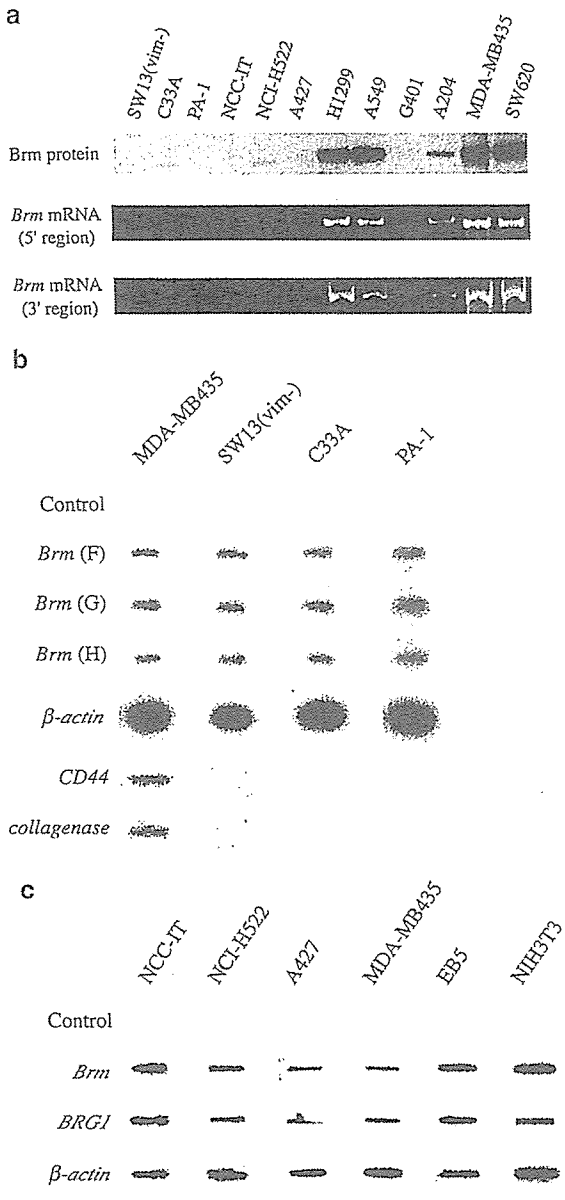


**Figure 1** (**a**) Expression of Brm protein detected by Western blotting and *Brm* mRNA analysed by RT–PCR (using two pairs of primers). (**b**) Nuclear run-on transcription assay with nuclear extracts of one Brm-expressing cell (MDA-MB435) and three Brm-deficient cells (SW13(vim-), C33A, and PA-1). *Brm*(F), *Brm*(G), and *Brm*(H) were DNA probes originated from the 5′-, center, and 3′-regions of the *Brm* cDNA, respectively. *Brm*(F) and *Brm*(H), respectively, cover the 5- and 3′-region amplified by RT–PCR shown in (**a**). DNA probes for β-actin and empty vector plasmid (control) were used for positive and negative controls. To certify the accuracy of run-on analysis, probes for *CD44* and *collangease*, which are under the control of transcription factor AP-1 that requires the SWI/SNF complex for its activity (Ito *et al*., 2001), were added. (**c**) Run-on transcription assay with nuclear extracts of four Brm-deficient cells (NCC-IT, NCI-H522, A427, and EB5), and two Brm-expressing cells (MDA-MB435 and NIH3T3). DNA probes for *BRG1* and *Brm* were derived from the corresponding full-length cDNA

the contrary, in four BRG1-deficient cell lines (NCI-H522, A427, H1299, and A549) and one Ini1-deficient cell line (A204), *BRG1* and *Ini1* mRNA was clearly detected (data not shown). All these observations except for NCI-H522 can be explained by the previous reports that these cell lines have frame-shift mutations (Wong *et al*., 2000; Betz *et al*., 2002).

To examine whether the *Brm* gene is largely defected or arranged in the seven Brm-deficient cell lines, we isolated genomic DNA and performed the PCR analysis using two pairs of primers, which cover fourth exon and 34th exon. The expected PCR products were clearly detected in all the Brm-deficient cell lines as well as MDA-MB435 and HeLa-S3 cells, which were used for control cell lines competent for the SWI/SNF complex (data not shown). Next, to elucidate the suppressive regulation of the *Brm* gene, a series of nuclear run-on transcription assays was performed. At first, we performed a set of run-on transcription assays with nuclear extracts of three Brm-deficient cell lines, SW13(vim-), C33A and PA-1 (Figure 1b). We used three independent DNA probes from the 5′-, central, and 3′-regions of the *Brm* cDNA, and found that these Brm-deficient cell lines transcribe the *Brm* gene as efficiently as MDA-MB435 cells. In addition, the relative levels of transcription of the 5′-, central, and 3′-regions of the *Brm* gene were similar in these cell lines, regardless of the status of mRNA and protein production (Figure 1a). These results suggest that both initiation and elongation of the *Brm* transcription in the Brm-deficient cell lines proceed as efficiently as those in Brm-expressing cells. When *CD44* and *collagenase* cDNA probes were used for the nuclear run-on assay, active transcription was detectable in nuclei isolated from MDA-MB435, whereas no signal was detected in those from SW13(vim-) (Figure 1b). These results are consistent with the observation that SW13(vim-) cells never express both of two genes at the transcriptional level (Ito *et al*., 2001; Yamamichi-Nishina *et al*., 2003), confirming that the run-on transcription assay faithfully reflects transcriptional activity in these cells.

We performed an additional set of run-on transcription assays with nuclear extracts of three other Brm-deficient human cell lines, NCC-IT, NCI-H522, and A427 (Figure 1c); and found that these three Brm-deficient cell lines also transcribe the *Brm* gene as efficiently as MDA-MB435 cells. We also checked the transcription of a mouse embryonic stem cell line EB5, which do not express *Brm* mRNA (data not shown), as has been reported for mouse embryonic cells in early development (LeGouy *et al*., 1998). The results showed that EB5 cells transcribe *Brm* gene as efficiently as mouse NIH3T3 cells that express Brm, indicating that regulation of *Brm* gene at the post-transcriptional level is not restricted to the tumor cell lines. The results of two independent sets of run-on assays indicate that post-transcriptional regulation of the *Brm* gene is a common property of the Brm-deficient cell lines. Among them, some are BRG1 positive and others are BRG1 negative (Table 1), indicating that post-transcriptional suppression of *Brm* is not affected by the status of BRG1 protein expression.