Cell-Type-Specific Repression of the *Maspin* Gene Is Disrupted Frequently by Demethylation at the Promoter Region in Gastric Intestinal Metaplasia and Cancer Cells

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Maspin, a serine protease inhibitor, was originally reported as a tumor suppressor gene in breast and prostatic cancers. We examined maspin expression and/or the allele-specific methylation status in four gastric cancer cell lines, as well as normal, metaplastic, and cancerous epithelia obtained from 50 gastric cancer patients. Three gastric cancer cell lines exhibiting maspin overexpression showed hypomethylation at either both alleles or a haploid allele. Only one cell line (GCIY) was maspin-negative but maspin expression was reactivated after treatment with a demethylating agent, 5-aza-2'-deoxycytidine. Dense and diffuse immunoreactivity for maspin was observed in 40 (80%) of 50 gastric cancers and all gastric normal epithelia (GNE) with intestinal metaplasia (IM), but not in GNE without IM. We further analyzed the allele-specific methylation status in 10 of 50 cases subjected to immunohistochemistry by the crypt isolation technique followed by a bisulfite genome sequencing method. The *maspin* gene promoter region of all GNE without IM was hypermethylated on both alleles whereas those with IM frequently represented the haploid type of hypomethylation status. In six of seven gastric cancers in which crypt isolation was possible, demethylation frequently occurred and extended to both alleles. Maspin mRNA was amplified from GNE with IM and cancerous crypts but not from GNE without IM. These results suggest that demethylation at the *maspin* gene promoter disrupts the cell-type-specific gene repression in both GNE and gastric cancer. (Am J Pathol 2003, 163:1911–1919)

Maspin, a mammary serine protease inhibitor, was originally identified in normal mammary epithelium by subtractive hybridization.¹ A *maspin*-transfected mammary

cancer cell line had reduced capacity for tumorigenesis and metastasis in nude mice.¹ *Maspin* has been shown to have tumor suppressive activity attributable to inhibition of breast cancer cell motility, invasion, and metastasis.^{2–7} Loss of maspin protein expression has been observed frequently and is associated with poor prognoses in breast, prostatic, and oral cancers.^{8–11} Recent studies have suggested that maspin interacts with the p53 tumor suppressor pathway¹² and may function as an inhibitor of angiogenesis¹³ *in vitro* and *in vivo*. These observations indicate that *maspin* acts as a tumor suppressor gene. However, it has been reported that *maspin* is overexpressed in pancreatic¹⁴ and ovarian cancers,¹⁵ whereas their normal tissues are *maspin*-negative. Precancerous lesions also express maspin protein and its up-regulation is highly correlated with malignant behavior.¹⁵ In these tumors, *maspin* seemed to behave as an oncogene rather than a tumor suppressor gene. Thus, paradoxical maspin expression has been described in various cancer cell types.

In gastric cancer, there have been two conflicting reports. Maass and colleagues¹⁴ failed to detect maspin mRNA in six gastric cancer cell lines by Northern blot analysis. Later, an immunohistochemical study detected frequent maspin overexpression in tumor tissues and intestinal metaplasia (IM) but not in gastric normal epithelium (GNE) without IM.¹⁶ Maspin expression and its functional significance in gastric epithelium and cancer cells have not fully been elucidated.

To clarify the significance of *maspin* gene expression in gastric carcinogenesis, its regulation mechanisms have to be examined in GNE and cancer cells. Recently, Futscher and colleagues¹⁷ showed that the *maspin* expression of normal cells is regulated by epigenetic modifications in a cell-type-specific manner. The maspin-positive cells (mammary and prostatic epithelia, and skin and oral keratinocytes) showed no methylation at the CpG islands of the *maspin* gene promoter region.¹⁷ By contrast,

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maspin-negative cells (skin fibroblast, lymphocytes, heart, liver, and bone marrow) showed extensive methylation.¹⁷ Futscher and colleagues¹⁷ and Costello and Vertino¹⁸ provided a new insight, demonstrating that cell-type-specific gene regulation was controlled by epigenetic modifications. Moreover, aberrant methylation of *maspin* gene CpG islands is associated with silencing of the gene expression in breast cancer cells. Treatment with a demethylation agent [5-aza-2'-deoxycytidine (5-Aza-dC)] reactivated maspin expression in these cells.¹⁹ These data suggested that epigenetic modification of the *maspin* gene might play an important role in not only the establishment and maintenance of normal cells in a cell-type-specific manner but also tumor progression of breast cancers.

Epigenetic modifications involving several tumor suppressor genes were examined in gastric precancerous epithelium and cancer cells. Aberrant methylations in tumor-related genes (*p16*, *p15*, *p14*, *E-cadherin*, and *hMLH1*) accumulated in the sequence from precancerous lesions (gastric adenoma and epithelium with IM) to cancers.^{20–22} Epigenetic changes have been recognized as an important mechanism underlying gastric carcinoma progression,^{23–30} whereas the methylation status of the *maspin* gene has never been examined.

We analyzed cytosine methylation of the *maspin* gene promoter region and its mRNA and protein expression in GNE, with or without IM, and cancer cells. To eliminate stromal cell contamination, we used the crypt isolation technique,^{31,32} and the methylation status was determined allele specifically by the bisulfite genome-sequencing method.³³

Materials and Methods

Cell Lines and Tissue Samples

Four gastric cancer cell lines (MKN7, MKN28, MKN74, and GCIY) were obtained from Riken Cell Bank (Tsukuba, Japan). All cell lines were maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum under the recommended conditions. The tumor and matching normal tissue samples subjected to crypt isolation were obtained surgically from 10 patients with gastric cancer. For immunohistochemical staining, 40 additional patients with gastric cancer were examined. Permission for the study was obtained from the Institutional Review Board (Iwate Medical University School of Medicine, Morioka, Japan) and written consent was obtained from all patients before surgery.

Crypt Isolation and Extraction of DNA and RNA

Just after surgical excision, tissue specimens were obtained from the cancerous lesion, and unaffected areas of the corpus and pyloric parts of the stomach, and cut into 2-mm squares. The crypts were isolated as previously described;^{31,32} briefly, the tissue was incubated at 37°C for 30 minutes in calcium- and magnesium-free Hanks' balanced salt solution containing 30 mmol/L of

ethylenediaminetetraacetic acid. The crypts were then stirred in calcium- and magnesium-free Hanks' balanced salt solution. The isolated crypts obtained from the non-cancerous lesion were stained with Alcian blue (pH 2.5) to identify the goblet cells. Subsequently, the isolated crypts were divided into two groups: intestinal metaplastic and nonmetaplastic epithelia. DNA and RNA were isolated for the bisulfite modification and real-time quantitative polymerase chain reaction (RQ-PCR), respectively. Genomic DNA was isolated with a Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN) and total RNA was isolated using Trizol reagent (Invitrogen). mRNA was reverse-transcribed with the ThermoScript RT-PCR system and oligo (dT) (Invitrogen) to produce cDNA.

Bisulfite Modification and PCR Amplification

Bisulfite-modified DNA was examined for the methylation status of 19 CpG dinucleotides within the *maspin* gene promoter region.^{19,34} Genomic DNA was digested by *Pst*I, then subjected to bisulfite modification as described previously.³³ Briefly, 1 μ g of genomic DNA was denatured with NaOH and modified by sodium bisulfite. Samples were then purified using Wizard DNA purification resin (Promega Corp., Madison, WI), again treated with NaOH, precipitated with ethanol, and resuspended in water. Modified DNA was PCR-amplified under conditions described previously.¹⁷ The *maspin* gene promoter was amplified from the bisulfite-modified DNA by two rounds of PCR using nested primers specific to the bisulfite-modified sequence of the *maspin* gene CpG island. The first round primers were: primer U2 (nucleotides –284 to –255), 5'-AAA AGA ATG GAG ATT AGA GTA TTT TTT GTG-3'; primer D2 (nucleotides 157 to 184), 5'-CCT AAA ATC ACA ATT ATC CTA AAA AAT A-3'.¹⁷ The second round primers were: primer U3 (nucleotides –238 to –212) 5'-GAA ATT TGT AGT GTT ATT ATT ATA-3'; primer D3 (nucleotides 107 to 133) 5'-AAA AAC ACA AAA ACC TAA ATA TAA AAA-3'.¹⁷ Both rounds of PCR were performed under the same PCR conditions, with 1% of the first-round PCR product serving as the template in the second-round PCR. PCR amplification was performed under the following conditions: 94°C for 4 minutes followed by five cycles of 94°C for 1 minute, 56°C for 2 minutes, 72°C for 3 minutes, then 35 cycles of 94°C for 30 seconds, 56°C for 2 minutes, 72°C for 1.5 minutes, and ending with a final extension of 72°C for 6 minutes.

Subcloning and Sequencing

Amplified PCR products were analyzed by electrophoresis on a 2% agarose gel. PCR products were purified with a QIAquick Gel Extraction Kit (Qiagen). The PCR fragments were ligated to pGEM-T Easy Vectors (Promega) and transformed into DH5 α competent cells (Toyobo, Tokyo, Japan). Ten or 30 subcloned colonies were chosen randomly in GNE and cancer cell lines, or primary gastric cancers, respectively. Plasmid DNA was purified by a PI-200 DNA automatic isolation system (Kurabo, Osaka, Japan). Cycle sequencing used a primer of the

T7 promoter and a BigDye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems; ABI, Foster City, CA). The product was analyzed with an ABI PRISM 3100 DNA Sequencer (ABI).

5-Aza-dC Treatment

Cells were seeded at a density of 5×10^5 cells/10-cm plate on day 1. Twenty-four hours later, 5-Aza-dC (Sigma, St. Louis, MO) was added to a final concentration of 2 $\mu\text{mol/L}$ or 10 $\mu\text{mol/L}$. Three days after 5-Aza-dC treatment, the cells were harvested for RQ-PCR and Western blotting.

Western Blotting

All gastric cancer cell lines were cultured to 70 to 80% confluence on a 10-cm Petri dish, added to 1 ml of cold phosphate-buffered saline (PBS) and removed from the dishes by cell scraping. The cell suspension was centrifuged at 7500 rpm for 1 minute. After removal of the supernatant, the cell pellet was dissolved in 1.0% Nonidet P-40 lysis buffer [50 mmol/L HEPES (pH 7.5)/1 mmol/L ethylenediaminetetraacetic acid/150 mmol/L NaCl/2.5 mmol/L EGTA/1.0% Nonidet P-40] and rotated at 4°C for 30 minutes. Insoluble material was spun down (20 minutes, 14,000 rpm) and the clear supernatants were collected. The protein concentration of the lysates was measured using a Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Cell lysates containing equal amounts of protein were mixed with 6 $\times$ concentrated loading dye, heated for 4 minutes at 95°C, and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel (Bio-Rad). The proteins were then transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, Buckinghamshire, UK) by electroblotting. The membrane was washed twice in 0.05% Tween-20 PBS (2 $\times$ 10 minutes), then blocked by 5% blocking reagent (Amersham Biosciences) in 0.05% Tween-20 PBS for 1 hour at room temperature. The membrane was washed twice in 0.05% Tween-20 PBS (2 $\times$ 10 minutes). Primary monoclonal antibody for maspin (anti-human maspin antibody; Pharmingen International, San Diego, CA) was diluted 1:1000 in 0.05% Tween-20 PBS. The membrane was incubated for 1 hour at room temperature and washed as already described. For the second antibody, anti-mouse IgG (Amersham Biosciences) was diluted 1:10,000 in blocking buffer. The membrane was incubated for 45 minutes at room temperature and washed. Maspin protein was detected by ECL Plus (Amersham Biosciences).

RQ-PCR

For RQ-PCR assay, primers and a fluorogenic probe were designed with Primer Express software (ABI): maspin F (nucleotides 646 to 665; 5'-CGA CCA GAC CAA AAT CCT TG-3'), maspin R (nucleotides 778 to 796; 5'-GAA CGT GGC CTC CAT GTT C-3'), probe (nucleotides 745 to 772; 5'-FAM-CAA CAA GAC AGA CAC CAA

ACC AGT GCA G-TAMURA-3'). For RQ-PCR assay, an ABI PRISM 7700 Sequence Detector (ABI) was used. The reaction mix contained 50 ng of cDNA, 200 nmol/L of each primer, 5 $\mu\text{mol/L}$ of probe, and 25 μl of TaqMan Universal PCR Master Mix (ABI), in a final volume of 50 μl . The cDNA was subjected to 50 cycles of a two-step PCR consisting of a 15-second denaturation step at 95°C and a 1-minute combined annealing/extension step at 60°C. Plasmids were diluted in a precise series, ranging from 5 pg to 0.005 fg (2×10^6 to 2 copies). For normalization of each target in the samples, the copy number of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The normalized values of maspin mRNA were expressed as the ratio of maspin copy number per 10^3 copy number of GAPDH.

Immunohistochemical Staining

Four- μ slices were cut from formalin-fixed, paraffin-embedded samples and stained with hematoxylin and eosin. Serial sections were stained immunohistochemically. Antigen was retrieved by incubating the microwave-based antigen retrieval method with 10 mmol/L of citrate buffer (pH 6.0) for 15 minutes. After deparaffinization and antigen recovery, the sections were washed three times in PBS (3 $\times$ 5 minutes) and immersed in 0.3% hydrogen peroxide in methanol for 30 minutes to block the endogenous peroxidase activity. After three washes with PBS (3 $\times$ 5 minutes), the sections were incubated with 5% bovine serum albumin for 10 minutes to block nonspecific reactions. Then the sections were incubated with anti-human maspin antibody (dilution, 1:50), overnight at 4°C. Secondary antibody and peroxidase labeling were performed with a Simple Stain MAX-PO Kit (Nichirei, Tokyo, Japan), colorization was produced by diaminobenzidine substrate (DAKO), and counterstaining was performed with Mayer's hematoxylin.

Results

Maspin Expression and Methylation Status in Gastric Cancer Cell Lines

Three gastric cancer cell lines (MKN7, MKN28, and MKN74) strongly expressed maspin protein; only GCIY was undetectable (Figure 1A). Maspin expression of GCIY was reactivated after treatment with the demethylating agent, 5-Aza-dC (2 $\mu\text{mol/L}$ and 10 $\mu\text{mol/L}$; tumor cell viability did not differ between the two concentrations) (Figure 1B). The same result for maspin mRNA was also confirmed in GCIY after treatment with 5-Aza-dC (Table 1). These results indicated that maspin expression is regulated by epigenetic modification in gastric cancer cell lines; therefore, we evaluated the methylation status of the *maspin* gene promoter using the bisulfite genome-sequencing method.

We found a single nucleotide polymorphism (SNP) site in the sequenced region (A/C at -156; the number indicates the location of the site with reference to the start site of the major transcription; Figure 2A). We determined the

Figure 1. Western blot analyses of maspin expression before (A) and after (B) treatment with 5-Aza-dC (2 μ mol/L) in gastric cancer cell lines. Equal loading was confirmed by incubation with an anti-actin antibody (C-2, Santa Cruz). **A:** Maspin protein expression was observed in three cancer cell lines. Only GCIY was undetectable. **B:** Reactivation of the maspin protein was observed in GCIY by the treatment with 5-Aza-dC.

allele-specific methylation status using SNP. The promoter region was completely unmethylated in both alleles in a maspin-positive cell line (MKN7) (Figure 2B). All sequenced subclones of MKN28 were completely demethylated, but SNP revealed homozygosity. MKN74, a maspin-positive cell line, was methylated at 19 of 19 CpG sites in one-half of the clones (Figure 2B). Unfortunately, the SNP revealed homozygosity, and we could not determine the allele-specific methylation status. However, one allele may be completely demethylated because one-half of the sequenced clones were completely demethylated (Figure 2B). A maspin-negative cell line (GCIY) showed hypermethylation. One allele was methylated at 18 of 19 CpG sites, and another allele was methylated at six sites (Figure 2B).

Immunohistochemistry for Maspin Expression in Gastric Cancerous and Noncancerous Tissue Samples

The expression of maspin product was assessed immunohistochemically in 50 specimens obtained surgically from gastric cancer patients. Two sections in which the tumor cells infiltrated most deeply were stained in each case. Sections of GNE obtained from the corpus and pyloric parts of the stomach were also examined in each case.

Table 1. Results of RQ-PCR Assay for Measuring Maspin mRNA in Gastric Cancer Cell Lines

Cell line	Copy number of maspin mRNA/ 10^3 GAPDH	
	5-Aza-dC (–)	5-Aza-dC (+)
MKN7	40.2	50.0
MKN28	7.80	11.8
MKN74	9.00	5.70
GCIY	0	0.21

Maspin expression was observed in 40 (80%) of 50 tumor specimens (Table 2). Their immunoreactivities were diffusely positive in 38 (95%) of 40 cases (Table 2 and Figure 3, E and F), whereas the relative staining intensities varied. Subcellular localization of maspin protein was observed in the cytoplasm (Figure 3, E and F). Cells with nuclear localization were extremely rare. There was no significant correlation between immunoreactivities of tumor cells and histological subtypes (Table 2).

All cases contained GNE with IM. Dense immunoreactivity was observed selectively in GNE with IM (Figure 3, A and D). Forty-six cases (92%) of 50 had GNE without IM but those showed no diffuse and dense immunoreactivity (Figure 3, A and B). Fourteen (30.5%) of the 46 patients showed focal and weak maspin expression in the foveolar epithelium (Figure 3C). Subcellular localization in the epithelium with IM was confined to the cytoplasm (Figure 3D).

Crypt Isolation and Maspin Analyses on Methylation Status and mRNA Expression

We studied the methylation status of the *maspin* gene promoter in more detail for 10 of 50 patients whose tissue was assessed immunohistochemically. For precise examination, we eliminated stromal cell contamination by isolating crypts. Crypts isolated from normal mucosa were stained with Alcian blue (pH 2.5) to distinguish those with IM from those without IM. Normal epithelia were obtained from the corpus and pylorus of the stomach. DNA was extracted from more than 200 crypts in each sample and prepared for the bisulfite genome-sequencing method.

The results of the assessment of the methylation status of *maspin* gene promoter regions are shown in Figure 4. In all patients, the *maspin* gene promoter region of GNE without IM was frequently methylated at almost all CpG sites, whereas those with IM were frequently demethylated (Figure 4, A and B). Six of 10 patients exhibited heterozygosity at the SNP, and we determined the allele-specific methylation status in these patients. We found that methylation occurred in one allele at 17 to 19 CpG sites and that another allele was hypermethylated (Figure 4B, P42). Haploid demethylation might have occurred in the remaining four patients because one-half of the sequenced clones were completely methylated (Figure 4B, P43).

We failed to isolate cancerous crypts in 3 of 10 examined patients because they had diffuse-type gastric cancer. Demethylation frequently occurred in six of seven gastric cancers (Figure 4C; P42, P50, P41, P46, P49, and P43), but the remaining one exhibited hypermethylation (Figure 4C, P44), and we confirmed negative immunoreactivity for maspin protein in that patient (Figure 2G). We were able to determine the allele-specific methylation status in four of six patients using the SNP site (Figure 4C; P42, P50, P41, and P46) both alleles were frequently demethylated in three of the four patients (Figure 4C; P42, P50, and P41). One patient (P46) exhibited frequent demethylation on one allele, although another allele was methylated at 13 of 19 CpG sites. The other two patients were homozygous but approximately one-half of the se-

Table 2. Immunohistochemistry for Maspin-Product in 50 Gastric Cancer Patients

Histology	Number of cases	Maspin staining		
		Negative (%)	Positive (%)	
			Focal	Diffuse
GNE without IM	46	32 (69.5)	14 (30.5)	0 (0)
GNE with IM	50	0 (0)	0 (0)	50 (100)
Gastric cancer	50	10 (20.0)	2 (4.0)	38 (76.0)
Intestinal type	38	7 (18.4)	1 (2.6)	30 (79.0)
Diffuse type	12	3 (25.0)	1 (8.3)	8 (66.7)

quenced clones showed demethylation (Figure 4C, P49 and P43). Small numbers of clones exhibiting different methylation patterns from major subclones (Figure 4C; P41, P43, P46, and P49) were identified in the cancerous crypts. These clones revealed hypermethylation; therefore, they might have been amplified from contaminating stromal cells.

We examined maspin mRNA expression in cancerous and noncancerous crypts using RQ-PCR, and we were able to amplify maspin mRNA from GNE with IM and cancerous crypts but not from GNE without IM (Figure 5). The quantitative results of maspin mRNA did not differ between normal epithelium with IM and cancerous crypts (Table 3 and Figure 5).

Discussion

The present study demonstrated that maspin protein production was high in gastric cancer cells as well as GNE with IM, but not in GNE without IM. The mechanism of regulation of maspin expression primarily depended on epigenetic modification in normal gastric cells and gastric cancer cells. Our allele-specific methylation analyses showed that demethylation on the haploid allele was sufficient to produce abundant maspin protein *in vitro* and *in vivo*. Moreover, the extent of demethylation of the *maspin* gene promoter was greater in gastric cancers. These data suggest that demethylation at the *maspin* gene promoter region disrupts the cell-type-specific gene repression of the establishment and/or maintenance of differentiated GNE and gastric cancer.

The aberrant expression of maspin protein in precancerous lesions has been described for the pancreas and ovary,^{14,15} but the methylation status of the lesions has not been examined. Overexpression of maspin protein frequently occurred in both types of cancer cells, and lack of immunoreactivity was confirmed in the corre-

Figure 2. Diagram of the *maspin* gene promoter region analyzed (**A**) and methylation status in four gastric cancer cell lines (**B**). **A:** The vertical lines mark the locations of CpG dinucleotide sites. The bent arrow indicates the transcription start site. The locations of nested PCR primers (U2/D2 and U3/D3) are indicated by arrows. SNP site (-156) is indicated by arrow-head. *PstI* indicates the position of the restriction site. **B:** Location and methylation status of 19 CpG sites in the *maspin* gene promoter. Each row of circles represents the methylation pattern obtained from individual clones of the *maspin* gene promoter. Each circle represents a CpG dinucleotide site. The filled circles are methylation-positive and the open circles are methylation-negative. The number at the top indicates the position of each CpG site from the major transcription start site. The allele status of each clone at the SNP site is indicated on the right.

Figure 3. Immunohistochemistry for maspin protein. **A:** A low-power view of a GNE. Immunoreactivity for maspin is observed in GNE with IM but not in GNE without IM. **B–D:** High-power views of GNE without (**B** and **C**) and with (**D**) IM. GNE without IM are almost negative (**B**) but focal and faint immunoreactivity is observed in the foveolar epithelia (**C**). Dense immunoreactivity is observed in GNE with IM (**D**). **E–G:** High-power views of gastric cancers. **E:** Maspin-positive intestinal-type gastric cancer. **F:** Maspin-positive diffuse-type gastric cancer. **G:** Maspin-negative intestinal-type gastric cancer. Scale bars: 200 μm (**A**); 50 μm (**B–D**); 100 μm (**E–G**).

sponding normal cells.^{14,15} This immunophenotype analogous to that observed in the present study led us to propose the following explanation for the paradoxical expression of maspin among cancers. Maspin plays an important role in the establishment and/or maintenance in a cell-specific manner (breast and prostate).¹⁷ In tumors arising from these organs, *maspin* had tumor-suppressive

activity, such as inhibition of cell motility, invasion, and metastasis.^{2–7} Unlike the situation in these tumors, maspin is expressed aberrantly in pancreatic and ovarian cancers as well as in gastric cancers. In these tissues, maspin is usually repressed in a cell-type-restricted manner. The *maspin* gene will be expressed aberrantly when differentiated normal epithelial cells transform to meta-

Figure 4. Features of isolated crypts and methylation status of GNE without (**A**) and with (**B**) IM, and gastric cancer (**C**). **A:** The crypts are negative for Alcian blue staining. **B:** The goblet cells of IM are stained with Alcian blue. **C:** Irregularly shaped crypts with multiple branching are observed in cancerous crypts. **A–C:** Location and methylation status of 19 CpG sites in the *maspin* gene promoter. Each **row of circles** represents the methylation pattern obtained from individual clones of the *maspin* gene promoter. Each **circle** represents a CpG dinucleotide site. The **filled circles** are methylation-positive and the **open circles** are methylation-negative. The number at the **top** indicates the position of each CpG site from the major transcription start site. The allele status of each clone at the SNP site and number of clones exhibiting each methylation status are indicated on the **right**. Scale bars, 100 μ m.

plastic or dysplastic cells. Tumor cells derived from these tissues were disrupted in a cell-type-restricted manner. Our present *in vivo* study is the first to have demonstrated that demethylation at the *maspin* gene promoter may play an important role in disruption of the maspin regulation mechanism and contribute to transformation from normal differentiated cells to metaplastic cells.

The functional significance of aberrant maspin expression in IM and gastric cancer cells has remained unclear. Although some morphological and genetic studies have suggested the precancerous nature of IM,^{35–43} this issue remains controversial. We examined maspin expression in the gastric epithelia of autopsied patients without gastric cancer immunohistochemically, and demonstrated

Figure 5. Results of real-time quantitative PCR (A) and agarose gel electrophoresis (B) for maspin mRNA expression. **A:** A standard curve for the RQ-PCR. The **red dots** are quantitative results for 7 gastric cancers and the **blue dots** are those for 10 GNE with IM. **B:** PCR products are detectable in GNE with IM (IM) but not in GNE without IM (NIM). SM, size marker.

that most cases of IM strongly expressed maspin protein (data not shown). Our study did not suggest that the aberrant expression of maspin was related directly to tumor development. However, the high incidence of aberrant maspin expression in gastric cancers suggests that most gastric cancers arise from GNE with IM. Our observations are consistent with those from the immunohistochemical study by Son and colleagues.¹⁶

Targeted overexpression of *maspin* in mice disrupts mammary gland development and inhibits the development of lobular-alveolar structures during pregnancy.⁴⁴ Architectural and differential abnormalities can occur in gastric mucosa, and these abnormalities may facilitate the development of gastric cancer. Recent epigenetic studies have examined tumor-related genes and also suggested that silencing of the genes because of hypermethylation at CpG islands tended to be accumulated along the multistep pathway of gastric carcinogenesis from IM to gastric cancer.^{20–22} Only a few studies have examined the relationship between aberrant hypomethylation and overexpression of specific genes in other types

of carcinoma.^{45,46} In gastric cancer, there has been no report concerning aberrant expression because of hypomethylation at its CpG sites. Further functional studies are needed to clarify the contribution of inappropriate maspin expression to gastric carcinogenesis. The loss of control of epigenetic changes may be related to the formation of IM and/or cancers of the stomach.

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Table 3. Results of RQ-PCR Assay for Measuring Maspin mRNA in GNE and Cancers

Samples	Number of cases	Copy number of maspin mRNA/10 ³ GAPDH*
GNE without IM	10	0
GNE with IM	10	12.2 ± 12.5
Gastric cancer	7	13.9 ± 14.4

*Values indicate mean ± standard deviation.

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