

# Epigenetic status and aberrant expression of the *maspin* gene in human hepato-biliary tract carcinomas

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We examined expression of *maspin* and the epigenetic status of its gene in 40 primary hepato-biliary tract carcinomas and 11 cell lines originating from hepato-pancreatico-biliary tract carcinomas. Aberrant *maspin* expression was frequently observed immunohistochemically in biliary tract carcinomas (22/25, 88%) but not in hepatocellular carcinomas (HCCs) (0/15, 0%). Aberrant *maspin* expression by five pancreatico-biliary tract carcinoma cell lines was closely associated with demethylation at the *maspin* promoter. Five of six HCC cell lines were *maspin*-negative and exhibited extensive hypomethylation and hypoacetylation at the *maspin* promoter. Treatment with 5-aza-2'-deoxycytidine did not activate *maspin* expression in these five *maspin*-negative HCC cell lines, whereas treatment with Trichostatin A (TSA) activated *maspin* expression in two of them. Treatment with TSA increased histone acetylation in some HCC cell lines. These results suggest that aberrant *maspin* expression in biliary tract carcinomas is closely associated with demethylation at the promoter region, but that some HCC cell lines additionally require histone acetylation. In addition, the fact that *maspin*-negative HCC cell lines remain after treatment with TSA suggests the existence of other repressive factors controlling *maspin* expression.

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*Maspin* is a member of the serine protease inhibitor family, which is related to the *SERPIN* family. The *maspin* gene was originally identified in normal mammary epithelial cells. A *maspin*-transfected mammary cancer cell line was found to have reduced capacity for tumorigenesis and metastasis in nude mice.<sup>1</sup> Exogenous *maspin* protein can inhibit certain invasive and malignant characteristics of human breast carcinoma cell populations.<sup>1–3</sup> Loss of *maspin* protein expression has frequently been observed, and is associated with poor prognosis, in breast, prostatic and oral cancers.<sup>4–8</sup> However, it has been reported that *maspin* is overexpressed in pancreatic and ovarian cancers,

whereas their normal tissues are *maspin*-negative.<sup>9,10</sup> Since *maspin* has a metastasis suppressor function in human breast cells, the gain of expression observed in these other tumor types is paradoxical.

Although the precise cellular and biochemical activities of *maspin* are currently unknown, we recently demonstrated that aberrant *maspin* expression was frequently observed in intestinal metaplasia of gastric epithelium and gastric cancers,<sup>11</sup> and in undifferentiated carcinomas of the thyroid gland.<sup>12</sup> This aberrant pattern of expression (where tumor cells are positive for *maspin* but their normal counterparts are negative) was closely associated with demethylation status at the 5' regulatory region of the *maspin* gene.<sup>11–13</sup> The concept that DNA methylation might play a role in the establishment and/or maintenance of tissue-specific gene expression was first proposed by Holliday<sup>14</sup> and Riggs.<sup>15</sup> Futscher *et al*<sup>16</sup> demonstrated that this concept applies to cell-type-specific expression of the *mas-*

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*pin* gene in normal human tissues, supporting the hypothesis that disruption of cell-type-specific repression by demethylation at *maspin* CpG islands contributes to revealing metaplastic or dedifferentiated morphological phenotypes in normal or tumor cells.

To test our hypothesis, we initially used an immunohistochemical method to screen various human tumors and/or normal tissues for aberrant expression of the *maspin* gene in relation to metaplastic or dedifferentiated phenotypes. We found that aberrant *maspin* expression was frequently observed in biliary tract carcinomas, including cholangiocellular carcinomas (CCCs), common bile duct carcinomas (CBDCs) and gallbladder carcinomas (GBCs), whereas hepatocellular carcinomas (HCCs) were completely negative for *maspin* expression. In the series, biliary tract tumors demonstrated the expected relationship between DNA methylation and protein expression of the *maspin* gene, whereas the relationship was disrupted in HCCs. The present study describes in detail the epigenetic status and protein expression of the *maspin* gene in hepato-biliary tract carcinomas.

## Materials and methods

### Hepato-Biliary Tract Tumors and Cell Lines

Tumor specimens were obtained from 40 patients with hepato-biliary tract carcinomas, consisting of 15 HCCs, six CCCs, eight CBDCs and 11 GBCs, and were subjected to immunohistochemistry for *maspin*. All specimens were from autopsy cases at the Department of Pathology, Iwate Medical University School of Medicine (Morioka, Japan), between 1992 and 2002. The cases were 29 males and 11 females, in the age range 48–82 years (mean 68 years). To study *maspin* protein expression in normal tissues (hepatocytes, intrahepatic bile duct, common bile duct and gallbladder), 10 autopsied patients (mean age 68 years, age range 45–89 years) who had died of noncancerous disease were also examined. Additionally, specimens obtained from three autopsy cases (66, 68 and 75 years old) who died of noncancerous disease were subjected to genomic DNA extraction to determine the methylation status at the *maspin* promoter of the normal liver and epithelium of the common bile duct and gallbladder. Permission for this study was obtained from the Institutional Review Board (Iwate Medical University School of Medicine, Morioka, Japan).

Six human HCC cell lines (HepG2, Hep3B, HT17, HuH-7, Li-7 and PLC/RPF/5), three human CBDC cell lines (HuH-28, HuCCT1 and TFK-1), a human GBC cell line (OCUG-1) and a human PC cell line (PK-8) were obtained from the Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). These cells were maintained under the recommended conditions.

### Immunohistochemistry

Four-micrometer slices were cut from formalin-fixed paraffin-embedded samples and were stained with hematoxylin and eosin. Serial sections were stained immunohistochemically. A microwave-based antigen-retrieval method with 10 mM citrate buffer (pH 6.0) for 15 min was used. After deparaffinization and antigen recovery, the sections were immersed in 0.3% hydrogen peroxide in methanol for 30 min to block endogenous peroxidase activity. After three washes with PBS for 5 min, the sections were incubated with 5% bovine serum albumin for 10 min to block nonspecific reactions. The sections were then incubated overnight at 4°C with anti-human *maspin* antibody (diluted 1:50; clone G167-70, Pharmingen, San Diego, CA, USA). Incubation with secondary antibody and peroxidase labeling were performed with a Simple Stain MAX-PO Kit (Nichirei, Tokyo, Japan). Color was produced with DAB substrate (Dako, Glostrup, Denmark), and counterstaining was performed with Mayer's hematoxylin. The extent of immunohistochemical reactivity for *maspin* was evaluated according to the incidence of *maspin*-positive cells and the relative density of *maspin*-positive cells. The incidence of *maspin*-positive cells was graded as follows: negative, no immunoreactive cells were seen; <20, 20–80 and >80%, the indicated proportion of immunoreactive cells. Relative density of *maspin*-positive cells was graded as follows: negative, no immunoreactivity; faint, moderate and strong, the indicated staining intensity.

### Bisulfite-Modified Genomic DNA Sequencing

The methylation status of 19 CpG dinucleotides within the *maspin* gene promoter region was examined in bisulfite-modified genomic DNA, as previously described.<sup>17</sup> Briefly, genomic DNA was digested with *Pst* I and then subjected to bisulfite modification as described elsewhere.<sup>16</sup> The *maspin* gene promoter was amplified from bisulfite-modified DNA by using nested primers specific to the bisulfite-modified sequence of the *maspin* gene CpG islands. PCR primers and conditions were as previously described.<sup>16</sup> PCR products were purified with a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany), ligated to pGEM-T Easy Vectors (Promega Corp., Madison, WI, USA) and transformed into DH5 $\alpha$ -competent cells (Toyobo, Tokyo, Japan). Ten subcloned colonies were chosen randomly. Plasmid DNA was purified with a PI-200 automatic DNA 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, USA). The product was analyzed with an ABI PRISM 3100 DNA Sequencer (ABI).

### 5-Aza-dC Treatment

Cells were seeded at a density of  $5 \times 10^5$  cells/10 cm plate on day 1. After 24 h, 5-aza-2'-deoxycytidine (5-Aza-dC; Sigma, St Louis, MO, USA) was added to a final concentration of 10  $\mu$ M. At 3 days (72 h) after 5-Aza-dC treatment, the cells were harvested for Western blotting and real-time quantitative-PCR (RQ-PCR) assay.

### Trichostatin A Treatment

Treatment with a histone deacetylase (HDAC) inhibitor (Trichostatin A (TSA); Sigma, St Louis, MO, USA) was performed according to the method used by Maass *et al*<sup>18</sup> to examine maspin expression in breast cancer cell lines. Cells were seeded at a density of  $1 \times 10^6$  cells/10 cm plate on day 1. After 24 h, TSA was added to a final concentration of 1  $\mu$ M. At 2 days (48 h) after TSA treatment, the cells were subjected to chromatin immunoprecipitation (ChIP) assay, Western blotting and RQ-PCR assay.

### Western Blotting

The cell pellet was dissolved in 1.0% NP-40 lysis buffer [50 mM HEPES (pH 7.5)/1 mM EDTA/150 mM NaCl/2.5 mM EGTA/1.0% NP-40] and rotated at 4°C for 30 min. Insoluble material was spun down (20 min, 14 000 rpm) and the clear supernatants were collected. The protein concentration of the lysates was measured with a Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Cell lysates containing equal amounts of protein were mixed with 6 $\times$  concentrated loading dye, heated for 4 min at 95°C and subjected to SDS-PAGE on a 10% polyacrylamide gel (Bio-Rad). The proteins were then transferred to a PVDF membrane (Amersham Biosciences, Buckinghamshire, UK) by electroblotting. The same primary monoclonal antibody for maspin used in immunohistochemistry was diluted 1:500 in PBST. The membrane was incubated for 1 h at room temperature and washed. For the secondary antibody, anti-mouse IgG (Amersham Biosciences) was diluted 1:10000 in blocking buffer. The membrane was incubated for 45 min at room temperature and washed. Maspin protein was detected with ECL Plus (Amersham Biosciences). Equal loading was confirmed by incubation with an antiactin antibody (C-2; Santa Cruz Biotechnology, Santa Cruz, CA, USA).

### RQ-PCR for Maspin mRNA

Total RNA was isolated with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA). mRNA was reverse-transcribed with a ThermoScript™ RT-PCR system and oligo(dT) (Invitrogen) to produce cDNA. For the RQ-PCR assay, primers and a fluorogenic probe were designed with Primer Express software

(ABI): maspin F (5'-CGA CCA GAC CAA AAT CCT TG-3'), maspin R (5'-GAA CGT GGC CTC CAT GTT C-3'), probe (5'-FAM-CAA CAA GAC AGA CAC CAA ACC AGT GCA G-TAMURA-3'). An ABI PRISM 7700 Sequence Detector (ABI) was used for RQ-PCR. The reaction mix contained 50 ng of cDNA, 200 nmol/l of each primer, 5  $\mu$ M of probe and 25  $\mu$ l of TaqMan Universal PCR Master Mix (ABI) in a final volume of 50  $\mu$ l. The cDNA was subjected to 50 cycles of a two-step PCR consisting of 15 s denaturation at 95°C and 1 min combined annealing/extension at 60°C. Plasmids were diluted in a precise series, ranging from 50 pg to 0.005 fg ( $2 \times 10^7$  to two 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  $10^4$  copy number of GAPDH, as previously described.<sup>11</sup>

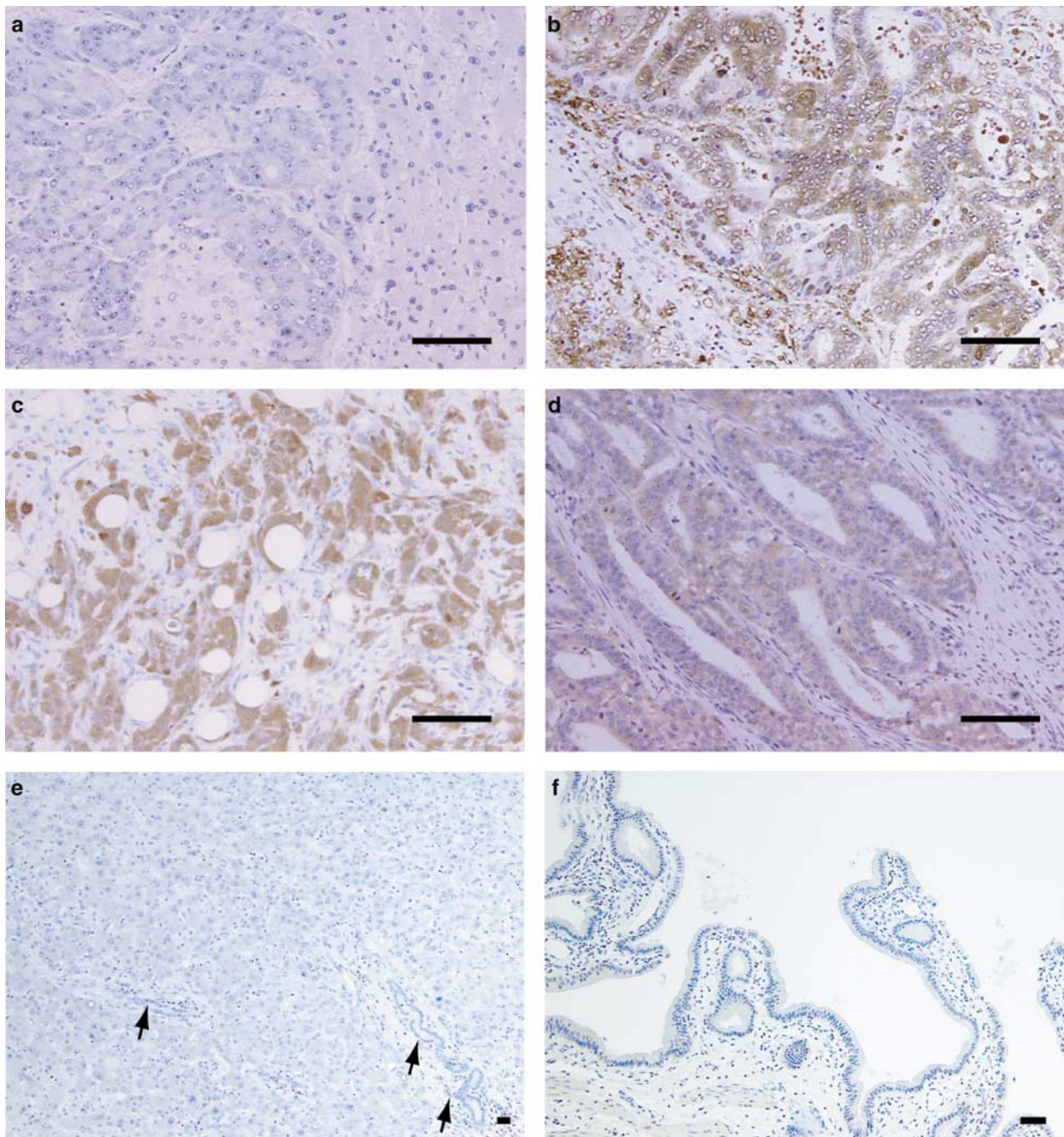
### ChIP Assay

The ChIP assay with acetyl-histone H3 and H4, and dimethyl histone H3 (Lys9) was performed according to the manufacturer's instructions (Upstate Biotech, Lake Placid, NY, USA) with slight modifications. Cells were rinsed in 1 $\times$  HBSS with 0.1% EDTA and treated with 1% formaldehyde for 10 min at 37°C to form DNA-protein crosslinks. The cells were rinsed in ice-cold 1 $\times$  HBSS containing 0.1% EDTA and protease inhibitors (1 mM PMSF/1  $\mu$ g/ml aprotinin/1  $\mu$ g/ml pepstatin A), scraped and collected by centrifugation at 4°C. Cells were then resuspended in PIPES buffer (5 mM PIPES, pH 8.0/85 mM KCl/0.5% NP40) containing protease inhibitors and incubated for 10 min on ice. Cells were then collected by centrifugation and resuspended in SDS lysis buffer containing protease inhibitors and incubated on ice for 10 min. The DNA-protein complexes were sonicated to lengths between 200 and 1000 bp as determined by gel electrophoresis. Samples were centrifuged at 14 000 rpm at 4°C to spin out cell debris, and then the supernatant was diluted 10-fold with ChIPs dilution buffer containing protease inhibitors. One-tenth of the sample was set aside for input control, and the remaining sample was then precleared with Salmon Sperm DNA/Protein A Agarose (Upstate Biotech, Lake Placid, NY, USA). Following preclearing, the samples were split into thirds, with two of the three samples being treated with each antibody, while the third was left as a minus antibody (-Ab) control. All samples were rotated overnight at 4°C. The chromatin-antibody complexes were collected with Salmon Sperm DNA/Protein A Agarose and then sequentially washed with the manufacturer's low salt high salt and LiCl buffers, then twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA). The chromatin-antibody complexes were eluted and the DNA-protein crosslinks

were reversed with 400 mM NaCl at 65°C for 6 h for all samples, including the input DNA control. All samples were treated with proteinase K, and each antibody-enriched fraction of genomic DNA was recovered by phenol/chloroform extraction and ethanol precipitations.

The RQ-PCR assay was used to analyze ChIP DNA with the ABI Prism 7700 sequence detector (ABI).

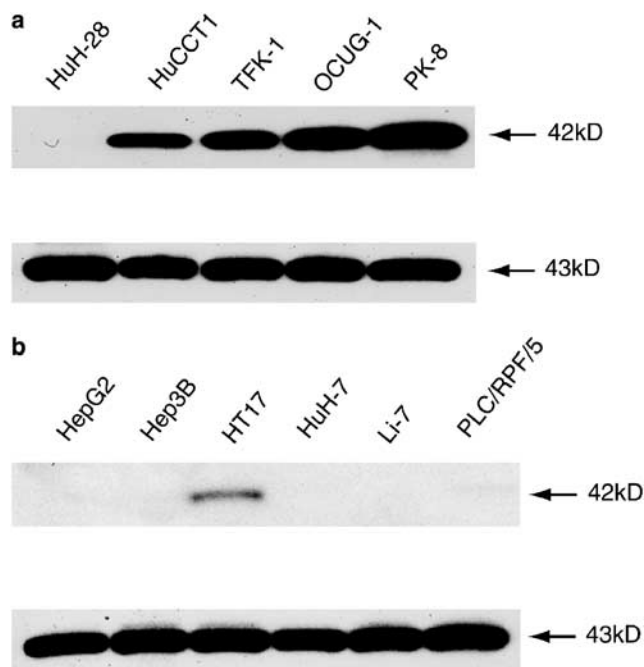
Primers and fluorogenic probes for the *maspin* and *GAPDH* promoters were designed with Primer Express software (ABI): *maspin* CF (5'-CCA CCA ACG TGT CTG AGA AAT T-3'), *maspin* CR (5'-TCT TGG CAG CTT GTC CAC A-3'), *maspin* Cprobe (5'-FAM-CAC AGC CCC TTC CTG CCC GAA C-TAMURA-3'), *GAPDH* CF (5'-CTA GTG TCC TGC TGC CCA C-3'), *GAPDH* CR (5'-AGG TCT TGA GGC



**Figure 1** Immunohistochemistry for maspin in primary hepato-biliary tract carcinomas; (a) hepatocellular; (b) cholangiocellular; (c) common bile duct; (d) gallbladder carcinomas; (e) normal liver; (f) normal gallbladder; (all scale bars = 200  $\mu$ m). Immunoreactivity for maspin is observed in the cholangiocellular, common bile duct and gallbladder carcinomas, but not in the hepatocellular carcinoma. Normal hepatocytes, biliary ducts (arrows), and gallbladder epithelium were completely negative for maspin.

**Table 1** Immunohistochemistry for maspin in 40 patients with hepato-biliary tract carcinomas

Carcinoma type (number of cases)	Incidence of maspin-positive cells Number of cases (%)				Relative density of maspin-positive cells Number of cases (%)			
	Negative	<20%	20–80%	>80%	Negative	Faint	Moderate	Strong
Hepatocellular (15)	15 (100)	0	0	0	15 (100)	0	0	0
Cholangiocellular (6)	0	0	1 (17)	5 (83)	0	2 (34)	0	4 (66)
Common bile duct (8)	0	0	3 (38)	5 (62)	0	1 (13)	1 (13)	6 (74)
Gallbladder (11)	3 (28)	0	4 (36)	4 (36)	3 (28)	1 (9)	2 (18)	5 (45)



**Figure 2** Western blotting analyses of maspin expression in pancreatico-biliary tract (a) and hepatocellular (b) carcinoma cell lines. Equal loading was confirmed by incubation with an anti-actin antibody (43 kD, C-2; Santa Cruz Biotechnology). (a) Maspin protein expression was observed frequently in pancreatico-biliary tract carcinoma cell lines, and was only undetectable in the HuH-28 common bile duct carcinoma cell line. (b) A relatively weaker signal for maspin was observed in only one HCC cell line (HT17); the other HCC cell lines were negative.

CTG AGC TAC-3'), *GAPDH* Cprobe (5'-FAM-AGC GGT TTT ACG GGC-TAMURA-3'). PCR conditions and reaction mixtures were the same as for the quantification of *maspin* mRNA. A standard curve method was used for the quantitative evaluation of ChIP DNAs. Plasmids into which *maspin* or *GAPDH* PCR products had been inserted were diluted in a precise series, ranging from 5 pg to 0.005 fg ( $2 \times 10^6$  to two copies). For normalization of each target in the samples, the copy number of the input control was used. The normalized values of *maspin* ChIPs DNA were expressed as the ratio of *maspin* copy numbers of the acetyl-histone H3-, acetyl-histone

**Table 2** Results of RQ-PCR assay for *maspin* mRNA expression with or without 5-Aza-dC or TSA treatment in biliary tract and hepatocellular carcinoma cell lines

Cell lines	Without treatment	With treatment (copy number of <i>maspin</i> /10 <sup>4</sup> <i>GAPDH</i> )	
		5-Aza-dC	TSA
<i>CBDC</i>			
HuH-28	0		
HuCCT1	100		
TFK-1	72.7		
<i>GBC</i>			
OCUG-1	107		
<i>PC</i>			
PK-8	230		
<i>HCC</i>			
HepG2	0	0	0.283
Hep3B	0	0	1.71
HT17	3.91	14.3	26.3
HuH-7	0	0	0
Li-7	0	0	0
PLC/RPF/5	0	0.653	0.833

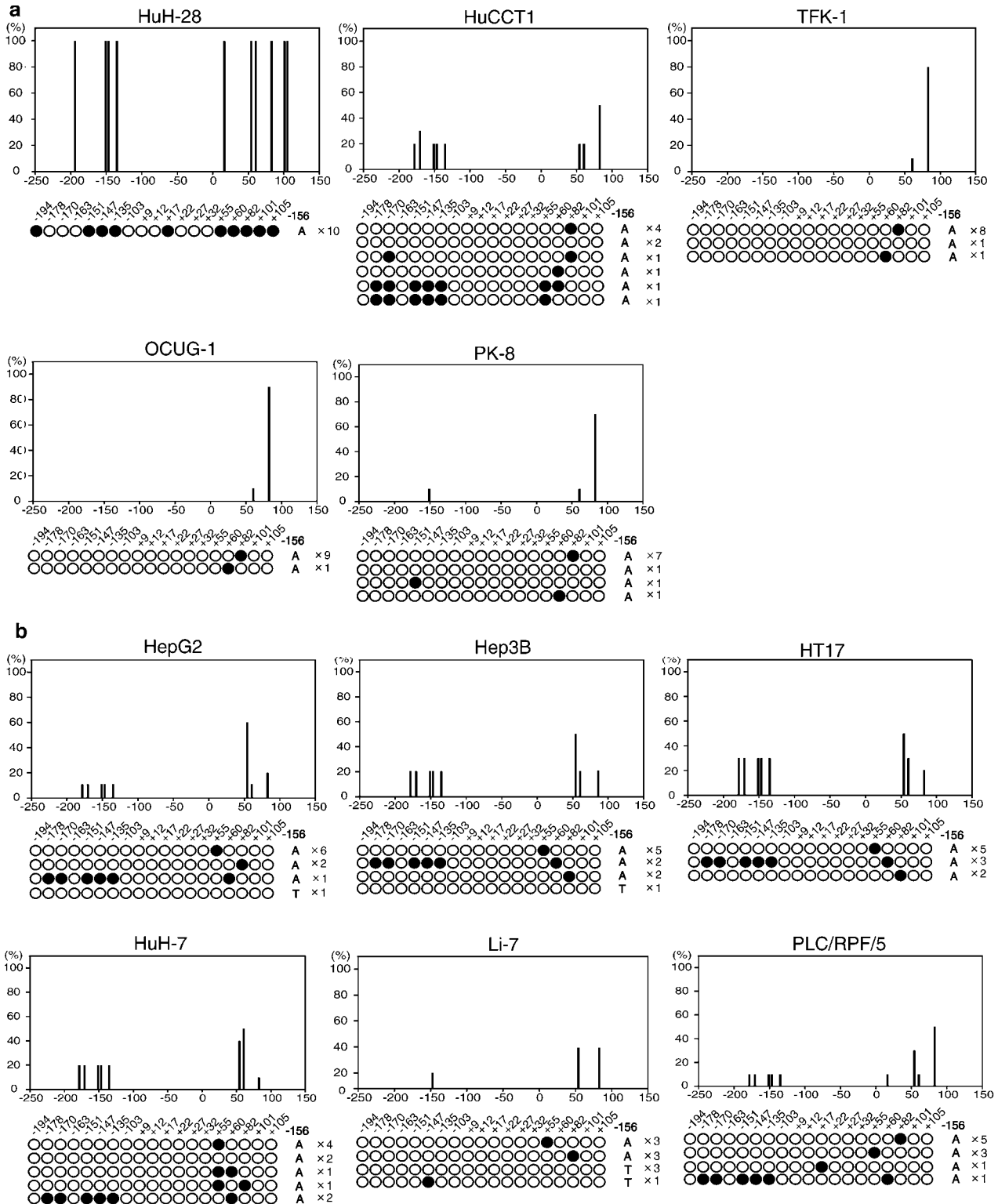
Blank, not carried out.

H4- or dimethyl histone H3 (Lys9)-enriched fractions to each input.

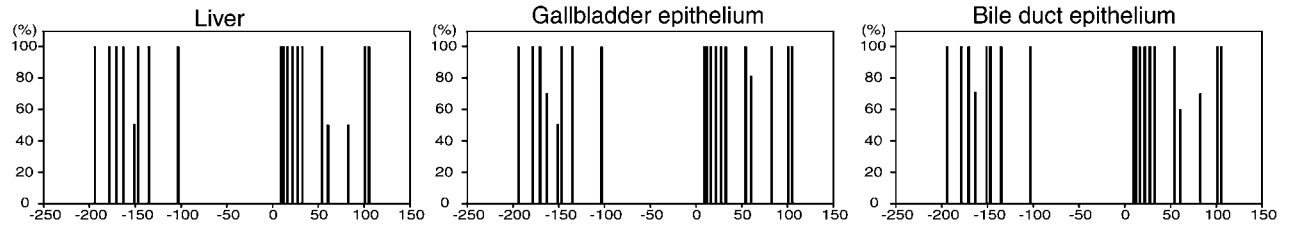
## Results

### Immunohistochemistry for Maspin in Primary Hepato-Biliary Tract Carcinomas

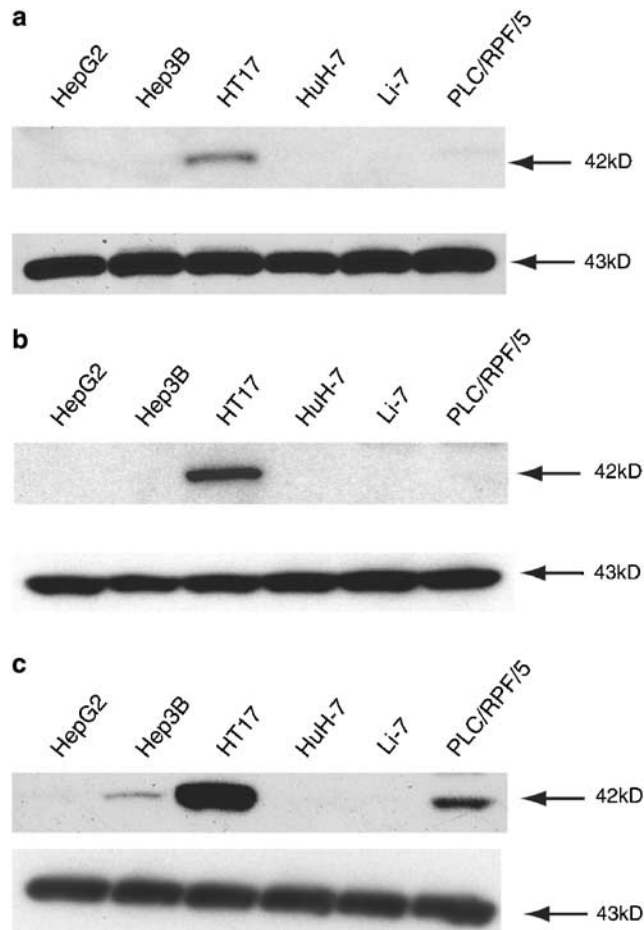
We immunohistochemically screened maspin expression in 40 hepato-biliary tract carcinomas. Immunoreactivity for maspin was completely negative in all HCCs (0/15, 0%) and their corresponding background hepatocytes, whereas maspin expression was highly positive in biliary tract carcinomas (Figure 1 and Table 1). No immunoreactivity for maspin was also observed in normal hepatocytes, intrahepatic bile duct common bile duct and gallbladder from autopsy cases of noncancerous disease (Figure 1). Immunoreactivity and the incidence of maspin-positive cells are summarized in Table 1. The high incidence of maspin overexpression



**Figure 3** Summary of 5-methylcytosine levels obtained by bisulfite-modified genomic DNA sequencing of the *maspin* promoter in pancreatico-biliary tract (a) and hepatocellular (b) carcinoma cell lines. In all, 10 cloned PCR products were sequenced to determine the percentage methylation of the 19 CpG sites in the region analyzed. The y-axis represents percentage cytosine methylation and the x-axis shows the nucleotide position relative to the transcription start site. Each row of circles represents the methylation pattern obtained from individual clones of the *maspin* gene promoter, with the number of the clone at the right. Each circle represents a CpG dinucleotide site. The filled circles are methylation-positive and the open circles are methylation-negative. The number above the circle indicates the position of each CpG site from the major transcription start site. The allele status of each clone at the SNP site (–156 from the major transcription start site) is indicated on the right.



**Figure 4** Summary of 5-methylcytosine levels obtained by bisulfite-modified genomic DNA sequencing of the *maspin* promoter in the normal liver and epithelia of the gallbladder and common bile duct. Ten cloned PCR products were sequenced for each autopsy case, and the percentage methylation of the 19 CpG sites in the region was analyzed. The percentage shown is the average of the three autopsy cases.



**Figure 5** Western blotting analyses of maspin expression before (a) and after treatment with 5-Aza-dC (b) or TSA (c) in hepatocellular carcinoma cell lines. Equal loading was confirmed by incubation with an antiactin antibody (C-2, Santa Cruz Biotechnology). (a) Maspin protein expression was observed in only one cell line (HT17). (b) A relative signal increase was observed in HT17 after treatment with 5-Aza-dC. (c) Gain of maspin expression was observed in three HCC cell lines (Hep3B, HT17, and PLC/RPF/5) after treatment with TSA.

in biliary tract carcinomas led us to speculate that cell-type-specific maspin repression was frequently disrupted in these carcinomas, whereas the repressive character was preserved in HCCs.

### *Maspin* mRNA and Protein Expression in Hepato-Biliary Tract Carcinoma Cell Lines

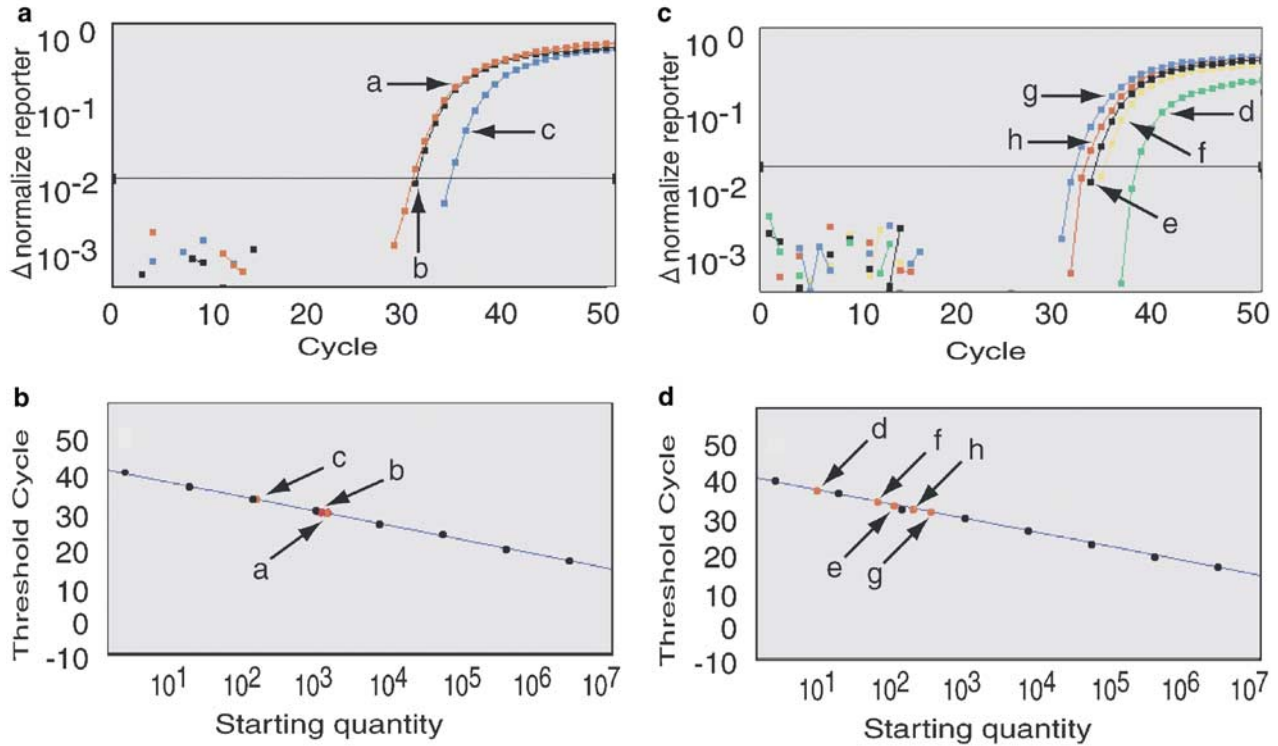
Overexpression of maspin protein was observed in two (HuCC1 and TFK-1) of three CBDC cell lines, the GBC cell line (OCUG-1) and the PC cell line (PK-8) (Figure 2a). Only one (HT17) of the six HCC cell lines exhibited a *maspin* signal, and this was relatively weak (Figure 2b). One of the CBDC cell lines (HuH-28) and 5 of the HCC cell lines (HepG2, Hep3B, HuH-7, Li-7 and PLC/RPF/5) were negative (Figure 2). *Maspin* mRNA expression correlated well with the Western blotting results (Table 2). Thus, *maspin* mRNA and protein expression by human hepato-biliary tract carcinoma cell lines was compatible with the immunostaining results in the primary tumors.

### Methylation Status at the *Maspin* Promoter in Hepato-Biliary Tract Carcinoma Cell Lines

The *maspin* promoter of the maspin-negative CBDC cell line (HuH-28) was relatively hypermethylated, but those of the maspin-positive cell lines originating from pancreatico-biliary tract carcinomas were hypomethylated (Figure 3a). Surprisingly, all HCC cell lines exhibited extensive hypomethylation at the *maspin* promoter (Figure 3b). Further, bisulfite-modified genomic DNA sequencing showed extensive hypermethylation in normal tissues of the liver and of the epithelia of the gallbladder and the common bile duct (Figure 4). In previous studies, demethylation status at the *maspin* promoter and aberrant *maspin* gene expression were well correlated, whereas in the HCC cell lines the sequencing results are inconsistent with mRNA/protein expression status.

### Treatment with TSA and 5-Aza-dC, and ChIP Assay

To test whether cytosine methylation or histone acetylation in the *maspin* promoter is functionally linked to its silencing in HCC cell lines, we treated the cells with 5-Aza-dC, a demethylating drug, and TSA, a histone acetylating drug.



**Figure 6** Quantitative chromatin immunoprecipitation assay for *maspin* promoter in a common bile duct carcinoma cell line (HuCCT1) (a and b) and an HCC cell line (Hep3B) (c and d) with acetyl-histone H3 (a and f) and H4 (b, d and g) antibodies. Gene promoter-specific RQ-PCR was carried out on DNA from the immunoprecipitated chromatin (a, b, d, f and g) as well as input DNA (c, e and h). (a) Representative amplification plots of HuCCT1 (a, b and c). Fluorescent signals of H3 (a) or H4 (b) are observed at earlier cycles than that of input. The degree of histone acetylation of a common bile duct carcinoma cell line (HuCCT1) is markedly higher than that in a hepatocellular carcinoma cell line (Hep3B) (see Figure 1c). As it has been confirmed that the acetylation level of HuCCT1 is higher, we did not examine TSA treatment. (c) Representative amplification plots of Hep3B before (d and e) and after (f, g and h) treatment with TSA. No amplification plot was observed for H3 before treatment with TSA. We judged that the histone H3 acetylation status of the hepatocellular carcinoma cell line, Hep3B, was absent or extremely low. Increasing histone acetylation was observed in H3 (f) and H4 (d–g) before and after treatment with TSA. (b and d), Standard curve for the RQ-PCR.

**Table 3** Results of quantitative ChIP assay of the *maspin* promoter acetyl H3 and H4, and methyl H3 Lys9 using real-time PCR in the biliary tract and hepatocellular carcinoma cell lines

Cell lines	Normalized copy number of <i>maspin</i> promoter acetyl H3 and H4 (copy numbers of H3 or H4/input)		Normalized copy number of <i>maspin</i> promoter methyl H3 Lys9 (copy numbers of methyl H3 Lys9/input)	
	No treatment		Treatment with TSA	
	H3	H4	H3	H4
<b>CBDC</b>				
HuH-28	0	0	0.529 ± 0.0417	1.62 ± 0.349
HuCCT1	111 ± 15.1	83.7 ± 6.98		
TFK-1	30.9 ± 1.04	46.2 ± 1.89		
<b>GBC</b>				
OCUG-1	39.2 ± 1.77	60.1 ± 3.69		
PC				
PK-8	119 ± 7.64	102 ± 2.88		
<b>HCC</b>				
HepG2	0	0	0	4.83 ± 0.293
Hep3B	0	1.02 ± 0.307	3.92 ± 0.994	17.0 ± 2.398
HT17	3.97 ± 0.871	8.15 ± 0.798	32.9 ± 2.33	33.4 ± 3.28
HuH-7	0	0	0	6.70 ± 0.554
Li-7	0	0	0	0
PLC/RPF/5	0	1.98 ± 0.293	10.3 ± 0.351	25.1 ± 1.20

Each copy number represents the mean value of three independent experiments with standard deviation. Blank, not carried out.

**Table 4** Summary of maspin mRNA/protein expression, DNA methylation, histone acetylation statuses in hepatocellular carcinoma cell lines

Cell lines	maspin mRNA/protein expression			DNA methylation	Gain of histone acetylation by treatment with TSA
	No treatment	5-Aza-dC treatment	TSA treatment		
HepG2	-/-	-/-	+/-	Hypomethylation	Gain
Hep3B	-/-	-/-	+/+	Hypomethylation	Gain
HT17	+/+	+/+	+/+	Hypomethylation	Gain
HuH-7	-/-	-/-	-/-	Hypomethylation	Gain
Li-7	-/-	-/-	-/-	Hypomethylation	No change
PLC/RPF/5	-/-	+/-	+/+	Hypomethylation	Gain

After treatment with 5-Aza-dC, gain of maspin expression was observed only in one cell line (HT17), and the others did not exhibit maspin expression even after 5-Aza-dC treatment (Figure 5b). The response of each cell line was compatible with the result of bisulfite-modified genomic DNA sequencing. We also examined the effects of 5-Aza-dC treatment at 2 and 50  $\mu$ M, but the responses at 2  $\mu$ M were almost identical and those at 50  $\mu$ M were not evaluable because of strong cytotoxicity (data not shown).

In three (Hep3B, HT17 and PLC/RPF/5) of the six HCC cell lines, maspin expression was activated by treatment with TSA (Figure 5c). The RQ-PCR assay for *maspin* mRNA showed identical results (Table 2). To assess whether the chromatin structure at the *maspin* promoter participates in the regulation of this gene, we performed ChIP experiments with antibodies directed against acetylated histones H3 and H4 or dimethyl histone H3 (Lys9), and amplified the *maspin* promoter of the hepato-biliary carcinoma cell lines (Figure 6). Three independent experiments were performed in each cell line. The acetylation status of these nucleosome constituents was tightly correlated with cytosine methylation status and gene expression in biliary tract carcinoma cell lines (Table 3). On the other hand, maspin-negative HCC cell lines exhibiting a hypomethylated promoter showed a chromatin structure with hypoacetylated histones (Table 3). After treatment with TSA, increased histone acetylation was observed in three (Hep3B, HT17 and PLC/RPF/5) of the six HCC cell lines, although the extent of histone acetylation in the HCC cell lines was less than that in the pancreatico-biliary tract carcinomas. We failed to demonstrate any differences in the degree of histone methylation (dimethyl H3K9) between maspin-negative cell lines in comparison with positive cell lines, using the same primer for the ChIP assay with acetyl-histone H3 and H4 antibodies (Table 3). Table 4 gives a summary of maspin mRNA/protein expression, DNA methylation and histone acetylation status in the hepatocellular carcinoma cell lines.

## Discussion

The high incidence of aberrant maspin expression in biliary tract carcinomas suggests that they share the same genetic pathway with pancreatic carcinomas (PCs).<sup>19,20</sup> In previous reports, gain of maspin expression was correlated well with demethylation at the *maspin* promoter region.<sup>11–13,16,19,21</sup> Although the methylation status of biliary tract carcinoma cell lines was consistent with this pattern, most maspin-negative HCC cell lines surprisingly exhibited not only hypomethylated but also hypoacetylated CpG islands. In other types of tumors, hypomethylation at the *maspin* promoter is usually concomitant with hyperacetylation status.<sup>22</sup> The unexpected epigenetic status of HCC cell lines suggested the existence of other repressive factors controlling maspin expression.

Zou *et al*<sup>23</sup> identified two closely spaced *p53* consensus binding sites in the *maspin* promoter region, and found that wild-type *p53* is a positive transcription factor in human prostatic cancer cell lines. Several immunohistochemical studies have suggested inverse correlations between mutant *p53* and maspin expression.<sup>7,24,25</sup> Recently, Oshiro *et al*<sup>19</sup> demonstrated that mutations of the *p53* gene and aberrant cytosine methylation contribute to silencing of maspin expression in breast cancer cell lines. We screened *p53* gene mutations in our series of cell lines. Four of the six HCC cell lines and three of the five pancreatico-biliary tract carcinoma cell lines had *p53* gene mutations (data not shown). There was no correlation between the presence of *p53* gene mutations and maspin expression. If mutant *p53* negatively controls maspin expression in primary biliary tract carcinomas, the incidence of aberrant maspin expression detected by immunohistochemistry would be lower, since the incidence of *p53* gene mutations is over 50% and equal to that of HCCs.<sup>26,27</sup> Thus, loss of *p53* function as a result of mutation might be insufficient to repress aberrant maspin expression in pancreatico-biliary tract carcinomas, whereas it contributes to silencing of maspin expression in tumors where maspin acts as a tumor suppressor, such as breast cancers.<sup>21</sup>

The question remains as to why some HCC cell lines with hypomethylation at the *maspin* promoter do not gain aberrant maspin expression after treatment with TSA. Histone H3 lysine 9 (H3K9) methylation is another epigenetic factor for silencing gene expression.<sup>28,29</sup> Histone methylation does not always correlate with DNA methylation. We failed to demonstrate the gain of H3K9 methylation in HCC cell lines in comparison with maspin-positive cell lines in experiments using the same primer for ChIP assay with acetyl-histone H3 and H4 antibodies. A clear explanation for maspin repression in HCC has not yet been provided, since in this study we did not systematically examine both methylation and acetylation status at the *maspin* promoter. Although gain of maspin expression in some HCC cell lines may be caused by histone acetylation, further studies are needed to define the mechanism of repression of the *maspin* gene in HCC cell lines.

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