Expression of metastasis-associated protein 1 (MTA1) gene correlates with the degree of invasion and metastasis in hepatocellular carcinoma (HCC). Expression of MTA1 is induced by hepatitis B virus X protein (HBx); however, little is known about the transcriptional regulation of MTA1 gene expression. Here, we report that the 5′-flanking region of the human MTA1 promoter contains two CpG islands. Transient expression of HBx in Chang liver cells increased the methylation of the CpG island1 from 18 to 49% when measured by bisulfite-modified direct sequencing. Chromatin immunoprecipitation showed that HBx recruited DNA methyltransferase 3a (DNMT3a) and DNMT3b to the CpG island1. In silico analysis of CpG island1 predicted the existence of putative p53-binding sequences. p53 was pulled down by a DNA probe encoding the p53-binding sequences but not by the methylated DNA probe. The mouse MTA1 promoter also contains a CpG island encoding a p53-binding sequence of which p53 binding was decreased in the presence of HBx, and the expression of MTA1 and DNMT3 was increased in the liver of HBx-transgenic mice. Comparison of MTA1 and DNMT3a expression in the human normal liver and HCC specimens produced a significant correlation coefficient ($r = 0.5686, P = 0.0001$) for DNMT3a, and a marginally significant coefficient ($r = 0.3162, P = 0.0103$) for DNMT3b. These data show that HBx induces methylation of CpG island in the MTA1 promoter, which interferes with DNA binding of p53 in the specific DNA region. This result may explain the molecular mechanism responsible for the induction of MTA1 gene expression by HBx.

Oncogenesis (2012) 1, e25; doi:10.1038/oncsis.2012.26; published online 3 September 2012

**Subject Category:** virus-induced oncogenesis

**Keywords:** MTA1; p53; HBx; DNA methylation

**INTRODUCTION**

Chronic infection by hepatitis B virus (HBV) is a major cause of the development and progression of hepatocellular carcinoma (HCC). Among the viral proteins encoded in the 3.2kb HBV genome, HBV X (HBx) protein is implicated in hepatocellular carcinogenesis because it leads to modulation of transcriptional expression of many cellular genes involved in oncogenesis, proliferation, inflammation, and immune responses. Recently published data support the idea that HBx has critical roles in the invasive and metastatic properties of intrahepatic and extrahepatic metastases of HCC. The balance between adhesion and de-adhesion in cells is modulated by HBx, which may facilitate integrin-mediated cell migration in primary tumor sites. HBx increases the invasion potential by upregulating the expression of the membrane-type matrix metalloproteinases and miR-143, which contributes to tumor metastasis.

The metastasis-associated protein 1 (MTA1) is a strong candidate for a metastasis-promoting gene in liver cancer. The MTA1 expression level is significantly higher in HCC hepatocytes than in nonmalignant hepatocytes. We reported previously that the expression of the MTA1 gene is a key transcriptional target of HBx protein, which contributes to angiogenesis in liver cancer by activating hypoxia-inducible factor 1α and vascular endothelial growth factor. It was shown recently that HBx recruits p65 to the nuclear factor-kB consensus motif on the relaxed MTA1 gene chromatin. HBx controls the expression of the MTA1 gene by suppressing miR-661 and subsequently activates inducible nitric oxide synthase in liver cancer cells. However, the mechanisms responsible for the increased expression of MTA1 in HCC remain largely unknown.

Epigenetic modifications are defined as heritable changes in gene expression occurring without alteration of underlying DNA sequences and comprise many layers of complexity, including DNA methylation, histone modifications, and chromatin remodeling. DNA methylation is a covalent modification of the cytosine ring at the 5′ position of a CpG dinucleotide and is performed by DNA methyltransferases (DNMTs). In HCC, the epigenetic inactivation caused by DNA hypermethylation has been established for several tumor suppressors and adhesion molecules such as p16INK4A, p14ARF, and cadherins. For example, hypermethylation around the E-cadherin promoter region is associated with reduced E-cadherin level in HCC. Methylation-induced silencing of the M-cadherin gene has significance as a prognostic indicator of poor survival in HCC patients.
The promoter of tissue factor pathway inhibitor-2, a Kunitz-type serine protease inhibitor that represses cellular invasion, is highly methylated and is repressed in human HCC. However, the epigenetic regulation of the MTA1 gene promoter has not been reported.

HBx protein is involved in epigenetic modifications during hepatocarcinogenesis by increasing the recruitment of DNMTs and methyl-binding proteins to the promoters of target genes. HBx induces the transcriptional activation of DNMT1, and methyl-binding proteins to the promoters of target genes. HBx also induces hypermethylation of the promoters of genes such as insulin-like growth factor-binding protein-3 (IGFBP-3), p16NK4A, retinoic acid receptor-β (RAR-β), ankyrin repeat-containing, SH3 domain-containing, and proline-rich region-containing protein 1 (ASPP1), and ASPP2 by recruiting DNMT1, DNMT3a1, and DNMT3a2. Here, we report on an important epigenetic regulation of the MTA1 promoter that is mediated by HBx during hepatocarcinogenesis. A CpG island and its methylation patterns of the human MTA1 promoter were characterized in HBx-expressing cell lines and in tissues obtained from patients with HBV-associated HCC. We also found that the HBx-induced methylation of the MTA1 promoter was linked to a decrease in the tumor-suppressor function of p53, which further supports the oncogenic and metastatic potential of HBx in the development and progression of HBV-associated HCC.

RESULTS

HBx increases methylation of the MTA1 promoter

Although HBx is associated with high expression of MTA1 in HCC, the mechanism underlying this regulation of MTA1 expression is largely unknown. To identify the molecular details of HBx-induced MTA1 gene expression, we cloned a potential MTA1 promoter containing the putative human MTA1 promoter with the HBx expression vector dose-dependently increased the reporter activity (Figure 1a), suggesting that the 5' upstream sequence functions as a promoter of MTA1. An analysis using the CpGplot program identified two potential CpG islands that span positions from −583 to −459 and −212 to +160 on the MTA1 promoter (http://www.ebi.ac.uk/emboss/cpgplot) (Figure 1b). To study the methylation status of the regions, we performed bisulfite sequencing. The methylation level of CpG island 1 was about 35%, whereas that of CpG island 2 was about 0.1% in Chang liver cells, white blood cells, and normal liver tissues obtained from healthy donors (Figure 1c). Surprisingly, exogenously introduced HBx significantly increased methylation level of the CpG island 1 (span positions −548 to −475) in WRL-68 and Chang liver cells. Doxycycline increased the methylation level of CpG island 1 in Chang X-34 cells whose expression of the HBx gene is under the control of an inducible doxycycline promoter (Figures 2a and b). The increases in methylation were accompanied by increases in the expression level of MTA1 (Figure 2c). Expression of HBx was slightly detectable without doxycycline treatment in Chang X-34, which probably represented background expression of HBx, and a relatively high basal expression level of MTA1 and methylation status of CpG island 1. By contrast, the methylation level of CpG island 2 was not changed in the presence of HBx (data not shown).

p53 does not bind to the methylated promoter of the MTA1 gene

We found that CpG island 1 in the MTA1 promoter contains a p53-binding site that is associated with transcriptional repression of MTA1 (Figure 3a). We hypothesized that the HBx-induced methylation of the CpG island interferes with the p53 binding and that this could rescue transcription of MTA1. To prove that p53 binds to this region, we performed chromatin immunoprecipitation (ChIP) analysis with primers that amplify the CpG island 1 and the region near the transcription start site. Introduction of exogenous HBx decreased the binding of p53 to CpG island 1 (ChIP1) and increased acetylation level of the lysin 9 repeat-containing, SH3 domain-containing, and proline-rich region-containing protein 1 (ASPP1), and ASPP2 by recruiting DNMT1, DNMT3a1, and DNMT3a2. Here, we report on an important epigenetic regulation of the MTA1 promoter that is mediated by HBx during hepatocarcinogenesis. A CpG island and its methylation patterns of the human MTA1 promoter were characterized in HBx-expressing cell lines and in tissues obtained from patients with HBV-associated HCC. We also found that the HBx-induced methylation of the MTA1 promoter was linked to a decrease in the tumor-suppressor function of p53, which further supports the oncogenic and metastatic potential of HBx in the development and progression of HBV-associated HCC.
in histone 3 at near the transcription start site (ChIP2; Figure 3b). It has been reported that p53 does not bind to methylated DNA promoters, resulting in the upregulation of genes such as survivin. We performed DNA pull-down experiments using nuclear extracts containing p53. A clear binding of p53 was observed with the unmethylated probe, but the binding was reduced with the methylated probe, indicating that p53 binds mainly to the unmethylated MTA1 promoter (Figure 3c). The involvement of p53 in HBx-induced MTA1 was demonstrated further in p53-knocked down WRL-68 cells. Repression of p53 expression by si-RNA markedly increased the protein level of MTA1 (2.9-fold, lane 3). However, coexpression of HBx did not increase MTA1 further (2.6-fold, lane 4), suggesting that MTA1 expression is fully activated by p53 knockdown. HBX recruits DNMT3a and DNMT3b to the CpG island of the human MTA1 promoter.

To identify the catalytic enzymes associated with HBx-induced DNA methylation, we examined whether DNMT3a and DNMT3b

Figure 2. HBx increases methylation of the human MTA1 promoter CpG island1. (a) Representative sequencing traces from bisulfite-modified direct sequencing. ▼ denotes methylated or unmethylated cytosines. Seven of unmethylated cytosines were converted to uracil in empty vector (EV)-transfected WRL-68 cells, but two in p3XFLAG7.1-HBx-transfected cells. (b) p3XFLAG7.1-HBx or EV was transfected into the indicated cells or treated with vehicle or 2 μg/ml of doxycycline (Doxy) for 24 h. Results from bisulfite sequencing analysis of the MTA1 promoter CpG island1 were shown. Filled squares, methylated; and open squares, unmethylated. Numbers at the bottom indicate the proportion of methylated CpG sites relative to total CpG sites examined. (c) Indicated amount of p3XFLAG7.1-HBx or EV was transfected into cells, or treated with doxycycline. Whole-cell lysates were prepared and protein levels were evaluated by western blotting.
are recruited in the CpG island of the MTA1 promoter. When HBx was exogenously introduced in Chang cells, expression level of DNMT3a was enhanced as previously shown (data not shown). ChIP analysis showed that both DNMT3a and DNMT3b bound to CpG island 1 and that the binding increased in the presence of HBx. Interestingly, HBx itself bound to the same region of the promoter (Figure 4a). Further, DNMT3a and DNMT3b knock down by si-RNA reduced expression levels of MTA1 (Figure 4b). To confirm the involvement of DNMT3a and DNMT3b in the methylation of CpG island 1, we monitored the DNA methylation status of CpG island1 after DNMT3a and DNMT3b were knocked down by si-RNA. HBx did not significantly alter the methylation status of CpG island1 when expression of DNMT3a and DNMT3b was repressed (Figure 4c). This result demonstrates that HBx increases the transcription of MTA1 by releasing p53 from CpG island1 by increasing promoter methylation through recruitment of DNMT3a and DNMT3b (Figure 4d).
The expression level of DNMT3 increases in the liver of HBx-expressing transgenic mice.

Previously, we showed that expression level of MTA1 was elevated markedly in the livers from HBx-transgenic mice compared with wild-type mice. Therefore, we next examined whether the expression levels of DNMT3a and DNMT3b are associated with the increased expression of MTA1 in the HBx transgenic mouse livers. In a similar pattern with the MTA1 expression, the levels of DNMT3a and DNMT3b were increased markedly in HBx-transgenic livers, suggesting a potential link between the induction of MTA1 and DNMT3 expression (Figures 5a and b). The level of p53 protein was significantly decreased in 9 month-old HBx TG compared with wild-type control mice. This result may be relevant with the previous observation that HBx downregulates p53 promoter activity. Interestingly, a p53-binding sequence was found in one of the two potential CpG islands in the mouse MTA1 promoter (Figure 5c). When HBx was exogenously introduced, DNA binding of p53 in the CpG island1 was reduced, whereas DNA bindings of DNMT3a and DNMT3b were enhanced greatly (Figure 5d). Increases in expression levels of DNMT3a and DNMT3b were also observed in the mouse NIH3T3 cells in the presence of HBx (Figure 5e).

Expression level of MTA1 is correlated with the MTA1-promoter methylation in HCC specimens

Finally, we performed qRT–PCR analysis to assess the expression levels of MTA1, DNMT3a, and DNMT3b in 30 HBV-associated HCC specimens after microdissection of HCC tissues and the adjacent nontumorous tissues. The expression level of MTA1 did not differ significantly between the HCC tissues and the adjacent nontumorous region; however, the expression levels in both groups were elevated considerably compared with the level in the normal human livers ($P = 0.001$) (Figure 6a). The expression level of DNMT3a in the HCC and nontumorous tissues varied considerably in the groups, and the mean values were higher than normal, but the differences were not significant. The level of DNMT3b was significantly higher in the HCC samples than in the nontumorous tissues ($P = 0.004$), but the expression levels of DNMT3b in both the HCC and nontumorous tissues did not differ significantly compared with the level in the normal human livers. Pearson correlation analysis of MTA1 and DNMT3 expression in the human normal livers and the HCC specimens showed a strong correlation ($r = 0.5686$, $P = 0.0001$) for DNMT3a and a marginally higher correlation ($r = 0.3162$, $P = 0.0103$) for DNMT3b, which may indicate the involvement...
of DNMT3a and DNMT3b in the regulation of MTA1 expression (Figure 6b).

Bisulfite sequencing was used to analyze the CpG island 1 methylation status of four normal human livers and five HCC tissues with higher MTA1 expression. The methylation level was significantly higher \((P = 0.0088)\) in the HCC tissues. MTA1 mRNA expression and the MTA1 promoter methylation level correlated significantly \((r = 0.7201, P = 0.0287)\), indicating a close association between the DNA methylation status of the MTA1 promoter and MTA1 expression level (Figure 6c). The results obtained from the HBx-transgenic mice and clinicopathological studies strongly support our hypothesis that the HBx-induced methylation of the MTA1 promoter upregulates the transcription of MTA1, which may contribute to the development of HBV-associated HCC.

**DISCUSSION**

Because of the strong connection between MTA1 gene expression and progression of HBV-associated HCC, identifying the molecular mechanism responsible for the effect of HBx on the transcriptional activation of the MTA1 gene is an important task. In the present study, we demonstrated the existence of an epigenetic mechanism for MTA1 gene regulation during hepatocarcinogenesis, which was mediated by HBx protein. We observed that HBx induced the methylation of human MTA1 gene promoter CpG island1, which contains the specific DNA-binding sequences for p53 (Figure 2b). The MTA1 gene promoter also contains a CpG island2 region; however, this region did not respond to HBx-mediated methylation, probably because of the presence of DNA-binding sites for transcription factors that confer resistance to de novo methylation in cancer cells.

Epigenetic inactivation caused by hypermethylation of a promoter is well established for genes involved in the initiation and progression of HCCs.\(^{13}\) The levels of DNMT1, DNMT3a, and DNMT3b were increased significantly in HCC tissues compared with nonneoplastic liver tissues (Figure 6a).\(^{27,28}\) In many cases, the aberrant DNA methylation is associated with gene silencing. For example, the expression levels of tumor-suppressor genes, such as \(p16^{INK4A}\), correlates inversely with the expression of DNMT3a.\(^{13}\) However, we observed a significant positive correlation between the mRNA expression levels of MTA1, DNMT3a, and DNMT3b in the present study (Figure 6b). Similar to our observation, DNA methylation-mediated derepression was reported for several genes with oncogenic potential. The methylation of the hTERT promoter at the CCCTC-binding factor-binding site inhibits the DNA binding of CCCTC-binding factor, which increases hTERT expression, especially in human tumors.\(^{29}\) Methylation of the survivin gene promoter inhibits the binding of p53 and causes derepression of survivin gene expression.\(^{25}\) Interestingly, the observation that HBx recruited DNMTs to the MTA1 promoter in
our investigation (Figures 4a and 5d) raises the possibility that the function of HBx is linked to specific targeting of promoters of both tumour suppressor genes and genes with oncogenic potential. Indeed, HBx induces hypermethylation of the IGFBP-3 promoter by recruiting DNMT1, DNMT3A1, and DNMT3A2, which suppress IGFBP-3 expression.18 By contrast, HBx suppresses the expression of p16Ink4a, RAR-β2, ASPP1, and ASPP2 in HCC tissues by upregulating or recruiting DNMT1 and DNMT3A.19–21 HBx induces the transcriptional activation of DNMT1, which causes subsequent DNA hypermethylation of the promoter of E-cadherin.17 Therefore, HBx may be one of the most potent and efficient epigenetic regulators that control cellular gene expression and may have beneficial effects for viral survival and proliferation through immortalization of host cells.

In this study, we found that DNA methyltransferase-induced derepression of the MTA1 gene was closely associated with the function of p53. This observation may be related to the previous observation that the loss of p53 function increases invasion and metastasis in several in vivo models of HCC.30,31 However, mRNA level of p53 was significantly higher in the non-tumorous and tumor tissues compared with the p53 level in the normal human livers (Supplementary Figure). However, the mRNA level of p53 may not represent the functional p53, as inactivation of p53 by mutations is frequently found in tumors associated with HBV infection.22 Further, the negative cross-talk between HBx and p53 protein has been addressed in the context of HBV-associated hepatocarcinogenesis.23 HBx binds to the wild-type p53 protein, inhibits sequence-specific DNA binding, and sequesters p53 in the cytoplasm, thereby preventing its nuclear entry.32,33 Here we show a new type of cross-talk between HBx and p53, in which HBx-mediated methylation of DNA inhibits specific DNA binding of p53 (Figures 3 and 5), and p53 is then unable to interact with its cognate binding sites if a methylated cytosine is present. The survivin promoter contains a region containing a p53-binding element, and methylation of the region inhibits the binding of p53.34 In an independent study, HBx increased the expression of survivin, suggesting that the survivin promoter may be a target of HBx-mediated methylation.35 We reported recently that poly(ADP-ribose) polymerase 1 (PARP-1)-mediated poly(ADP-ribose)ylation (PARylation) of p53 is necessary for the transcriptional repression of DNA methylation.36,37 Our data together with those of the other groups may suggest that PARP-1-mediated PARylation on certain cellular proteins has a role in maintaining the methylation pattern of genomic DNA. Whether the PARylation of p53 is linked to HBx-mediated DNA methylation remains as an interesting question.

Several recent studies have shown that MTA1 can be a molecular target for antimitastatic therapy. Downregulation of MTA1 by RNA interference successfully suppressed the in vitro growth and experimental metastasis of mouse B16F10 melanoma cells in vivo.38 Silencing MTA1 significantly suppressed the invasion and angiogenic activity of several cancer cell lines and delayed tumor formation and development in mouse xenografts.39,40 In this study, we found that the expression of MTA1 was of significant importance in both pre-cancerous and cancerous regions in the adjacent noncancerous regions than in the normal liver (Figure 6a). This result suggests that MTA1 also has a role in pre-cancerous changes in HBV-associated HCC. Epigenetic modification has emerged recently as an attractive therapeutic strategy against HBV-associated HCC. DNMT inhibitor demethylation agents, such as 5-aza-2′-deoxyocytidine, and histone deacetylase inhibitors, such as SAHA, trichostatin, and valproic acid, have been tested preclinically against HCC and have shown promising results.41 Interestingly, 5-aza-2′deoxyocytidine can increase the sensitivity of hepatoma cell lines to 5-fluorouracil, which represses the transcriptional expression of MTA1, as demonstrated in our recent study39 and by another study.42 In the present study, we showed that MTA1 expression was upregulated by DNA methylation, which may provide a rationale for an epigenetic combination therapy to HBV-associated HCC in patients with an increased MTA1 expression level.

In summary, the present study highlights the important role of DNA methylation in MTA1 expression in HBx-positive HCC. Our findings shed light on the basic molecular mechanisms involved in HBx-induced HCC progression and support a role for DNMTs as a potential target for antimitastatic therapy.

**MATERIALS AND METHODS**

**Cells and cell culture**

Human normal liver cell lines, Chang and WRL-68, a human colon cancer cell line, HCT 116, and a mouse embryonic fibroblast cell line, NIH3T3, were obtained from American-type culture collection. Chang X-34 cells, in which HBx gene expression is under the control of a doxycycline-inducible promoter, were described previously.43 Cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum at 37°C in a 5% CO2/95% air incubator.

**Plasmids, si-RNA, western blotting, and transient transfection**

The eukaryotic expression vectors for p3XFLAG-C12-HBx was described previously.24 The human MTA1 promoter (from –860 to +216) was cloned from human BAC genomic clone RP6D737A112145D (RP2P, Berlin, Germany) by PCR amplification and subsequent insertion into ScaI and Nhel site of the pGKL-2 Basic vector (Promega, Madison, WI, USA). The si-RNA duplexes targeting si-DNMT3a (5′-CAA CAUGCAUUCAUGAUAAU-3′ and 5′-UUCAUGAAGUAAUGUUGU-3′) and si-DNMT3b (5′-ACGCACGCG GACGACUUCAU-3′ and 5′-UCAUGUCCUGACGUGGUGCUU-3′) and control si-GFP (green fluorescent protein), were synthesized and purified by ST Pharm (Seoul, Korea).43 Transient transfection, reporter gene analysis, and western blotting were performed as described previously.24

**Bisulfite-modified direct sequencing**

Genomic DNA was extracted using the Wizard Genomic DNA purification kit (Promega) and treated with sodium bisulfite according to the protocol of EpI-Tect Bisulfite Kit (Qiagen, Valencia, CA, USA). The modified DNA was PCR amplified using the following primers: 5′-GAAAGGTATTTTGTATGAG TGGT-3′ (sense) and 5′-CCCTATACCCACAAATAATC-3′ (antisense) for the region 1 (CpG island of human MTA1 promoter); 5′-AGGGTGATAG GGGAGTTATTAG-3′ (sense) and 5′-AACCATTTTAATATATACAC-3′ (antisense) for the region 2 (CpG island2 of human MTA1 promoter). The resulting PCR products were cloned using a pGEM-T-easy vector system (Promega) and subjected to sequencing analysis.

**Chromatin immunoprecipitation (ChIP)**

ChIP assay was performed basically as previously described.44 Briefly, cells were fixed for 15 min in 0.7% formaldehyde, placed in lysis buffer (50 mmol/l HEPES [hydroxyethyl piperazineethanesulfonic acid]-KOH (pH 7.5), 140 mmol/l NaCl, 1 mmol/l EDTA (pH 8), 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate and fresh protease inhibitor cocktail), and then chromatin was sheared into 500–200 bp fragments by sonication. Equal amounts of sample were immunoprecipitated with 2 μg of anti-p53 (DO-1), anti-DNMT3a, anti-DNMT3b (Santa Cruz Biotech, Santa Cruz, CA, USA), anti-p53 (PAb421) (Calbiochem, La Jolla, CA, USA), anti-FLAG (Sigma, St Louis, MO, USA), or anti-ACh3K9 (Abcam, Cambridge, MA, USA). Bound target DNA fragments were analyzed for the presence of sequences –718 to –348 (ChIP1), +89 to +366 (ChIP2) of human MTA1 promoter and –2323 to –2017 (mChIP1) of mouse MTA1 promoter by PCR amplification.

**DNA pull-down assay**

Promoter templates biotinylated at the 5′ end of the upper strand were produced by PCR using primers, 5′-GCCCAACACAGGCGGCCGC-3′ (sense) and biotin-5′-GCCCAACACCTCCTTGCGGCG-3′ (antisense). Nuclear extracts were prepared as described previously.25 Nuclear extracts (100 μg) from HCT 116 cells were incubated with the biotinylated DNA probe (10 nM) in a binding buffer (20 μM HEPES, 150 μM NaCl, 1 μM EDTA, 0.01% NP-40, and fresh protease inhibitor cocktail) containing 1 μg/ml herring sperm DNA and 20 μl streptavidin Sepharose High Performance (GE Healthcare, Piscataway, NJ, USA) at 4°C for 4 h. The protein–DNA complexes
were washed three times with the binding buffer, and then subjected to SDS–PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) followed by western blotting with anti-p53 antibodies (Santa Cruz Biotech). Methylated PCR products were prepared in vitro using SssI Cpg DNA methyltransferase (New England Biolabs, Cambridge, MA, USA).

HCC Samples and qRT–PCR

Six normal liver samples and 30 patients with HBV-associated HCC were described previously. All samples were collected anonymously according to the Institutional Review Board guidelines, and the histopathological features of the examined HCCs are described previously. To prevent total RNA extraction from formalin-fixed paraffin-embedded tissues, each tissue section was stained with hematoxylin, and nontumor or cancer regions were microdissected with a laser microdissection system or cut directly with a needle from sections. RNA isolation and qRT–PCR was performed as previously described previously. The following primers and probes were used for qRT–PCR: DNMT3a: 5'-AGCTACATCAAGAAGCCGACG-3' (forward primer), 5'-CACAGCATTATCAAGCAGA-3' (reverse primer), and Universal ProbeLibrary probe: No. 75, DTMT3b: 5'-CCGAGAAAATGCTTCGACAG-3' (forward primer), 5'-TCTCGGCCACAGACAAACA-3' (reverse primer), and Universal ProbeLibrary probe: No. 64, MTA1: 5'-GGGAGAATCAGCAGCTGTG-3' (forward primer), 5'-ACACCTGGTGTCCACCGTCT-3' (reverse primer), and Universal ProbeLibrary probe: No. 54 (Roche Diagnostics, Mannheim, Germany). Five HCC specimens showing high expression of MTA1 in tumor were selected for bisulfite sequencing.

Statistics

Experimental values are expressed as the mean ± s.d. of three independent experiments. The significance of any difference was determined by Student’s t-test and expressed as a probability value. For clinicalopathological studies, the independent data (normal vs non-tumor region) and the matched data (non-tumor vs cancer) of gene expression was analyzed using Wilcox signed rank test and Mann–Whitney test, respectively. Mean differences were considered significant at P < 0.05. The relationship between the expression of DNMTs and MTA1 was analyzed by calculating Pearson correlation coefficient.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by grants from the NRF (2009-0080757), and the SRC/ERC (R11-2007-017-01001-0).

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