

# Epigenetic activation of E-cadherin is a candidate therapeutic target in human hepatocellular carcinoma

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**Abstract.** E-cadherin is a key cell adhesion molecule implicated in tumor suppression that is frequently altered in hepatocellular carcinoma (HCC), particularly in hepatitis B virus-related tumors. Here, we report that the epigenetic drugs 5-azacytidine and trichostatin A up-regulated E-cadherin expression in HCC cells. The depletion of DNMT1 restored E-cadherin expression via demethylation, whereas the depletion of DNMT3A or DNMT3B did not. Activated E-cadherin suppressed HCC cell colony formation. However, E-cadherin expression was repressed by HBx transfection due to the DNA methylation induced by the elevation of DNMT1 in the HCC cell lines. The present study indicates that E-cadherin expression is regulated by epigenetic agents in HCC cells, which suggests a schema for restoring E-cadherin by targeting its epigenetic mechanism.

## Introduction

E-cadherin (CDH1) is a well-known suppressor of invasion/metastasis and an important Ca<sup>2+</sup>-dependent adhesion molecule that mediates cell-cell contact and is important for tissue morphogenesis and cell polarity (1). Owing to its critical function in intercellular adhesion, E-cadherin has been assumed to act as a tumor suppressor negatively regulating several critical steps of invasion and metastasis (2). E-cadherin expression is frequently suppressed or reduced in carcinoma tissues of the breast and liver and in many carcinoma cell lines derived from the colon, stomach and prostate (3). The loss of E-cadherin function induced by promoter methylation was associated with the metastasis and invasion of tumors. Studies using animal models and human hepatocellular carcinoma (HCC)

tissues have shown that hypermethylation is associated with decreased E-cadherin expression but also with microvascular invasion and recurrence of HCC (4,5). Transcriptional or post-transcriptional down-regulation may be the mechanism of underexpression of E-cadherin in HCC (6). The decrease or loss of E-cadherin expression is observed in HCC as well, particularly in poorly differentiated cancers (7,8). E-cadherin plays a role in cancer progression, and its therapeutic restoration as a strategy to suppress metastasis has recently been considered (9). The presence of the HBx protein, which is one of the crucial factors in HCC, was found to be involved in this pathway and may be associated with the hypermethylation of the E-cadherin promoter (10).

Over the past few years, many epigenetic drugs have been discovered and found to effectively reverse DNA methylation and histone modification aberrations that occur in cancer (11). 5-Azacytidine (5'-aza) and 5-aza-2'-deoxycytidine lead to the inhibition of DNA methylation and induce gene expression via the blocking of DNA methyltransferases (DNMTs). Trichostatin A (TSA), one of the effective HDAC inhibitors, re-establishes normal histone acetylation patterns and reactivates silenced tumor suppressor genes. These discoveries have led to the possibility of 'epigenetic therapy' as a treatment option, and epigenetic agents are defined as a legitimate set of targets for therapeutic approaches to cancer. In the present study, we investigated E-cadherin-up-regulating drugs, proposing a schema for restoring E-cadherin by targeting its epigenetic mechanism.

## Materials and methods

**Cell culture and 5'-aza/TSA treatment.** The human HCC cell line SMMC-7721 and human hepatocellular pericarcinoma cell line QSG-7701 (Cell Bank Shanghai, P.R. China) were maintained by serial passage in RPMI-1640 (Gibco, USA) containing 10% heat-inactivated newborn bovine serum, and incubated at 37°C in an atmosphere containing 5% CO<sub>2</sub>. Cells were cultured in medium containing 120 ng/ml of TSA (Sigma, USA) or DMSO for 24 h. For 5'-aza (Sigma) treatment, cells were plated and treated with 0, 10, 50 and 100 µmol/l 5'-aza for up to 2 days.

**Transfection of DNMT1 siRNA, DNMT3A siRNA and DNMT3B siRNA into the HCC cell line SMMC-7721.** SMMC-7721 cells

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Table I. Primers and annealing temperature of genes analyzed by RT-PCR.

Gene	Primers (5'-3')	Temperature (°C)	Amplicon size (bp)
<i>E-cadherin</i>	F: GGTGGGTGACTACAAAATCAATCT R: TTCTCCGCCTCCTTCTTCATCATA	58	310
<i>DNMT1</i>	F: CCGAGTTGGTGTATGGTGTGTAC R: AGGTTGATGTCTGCGTGGTAGC	61	324
<i>DNMT3A</i>	F: TATTGATGAGCGCACAAGAGAGC R: GGGTGTTCAGGGTAACATTGAG	65	110
<i>DNMT3B</i>	F: GACTTGGTGATTGGCGGAA R: GGCCCTGTGAGCAGCAGA	64	270
<i>HBx</i>	F: TTCTTCGTCTGCCGTTCC R: TCGGTCGTTGACATTGCT	54	201
<i>ACTB</i>	F: AAAGACCTGTACGCCAACAC R: GTCATACTCCTGCTTGCTGAT	61	220

were transfected with DNMT1 siRNA (pMT1), DNMT3A siRNA (pMT3A) and DNMT3B siRNA (pMT3B) constructs, and their scramble sequences as control, respectively, using Transfectamine™ 2000 transfection reagent (Invitrogen, USA) as in our previous studies (12,13). Cells were grown and selectively cultured in 0.4 mg/ml Geneticin (Life Technologies, USA) for 2 months after the initial transfection. SMMC-7721 cells transfected with pMT1, pMT3A and pMT3B were labeled as 7721-pMT1, 7721-pMT3A and 7721-pMT3B; those transfected with DNMT scramble sequence were labeled as 7721-sMT1, 7721-sMT3A and 7721-sMT3B.

**Semi-quantitative reverse transcription-PCR (RT-PCR)-detected expression of genes.** The expression of the tumor suppressor gene E-cadherin and of DNMTs was analyzed by semi-quantitative RT-PCR. Total RNA was purified with TRIzol (Invitrogen). First-strand complementary DNA (cDNA) was synthesized from 2 µg of total RNA using Oligo(dT)<sub>18</sub> primer and M-MLV reverse transcriptase (Invitrogen). β-actin was used as an internal control. Each PCR was repeated with at least three different cDNA preparations and three independent PCRs for each cDNA with β-actin co-amplification. The primer sequence of each gene and the PCR conditions are listed in Table I.

**Antibody and Western blotting.** The protein concentration of each extract was quantitated using the BCA assay (Pierce, USA). Total protein (2-40 µl) was electrophoresed on 7-15% SDS-polyacrylamide gel and transferred to polyvinylidene fluoride membranes (PVDF; Amersham) electrophoretically. Western blotting was performed with the mouse monoclonal anti-E-cadherin or the mouse monoclonal anti-β-actin (Sigma) antibodies and detected by Super Signal chemiluminescence substrate (Pierce). β-actin protein levels were used as a control for equal protein loading.

**Methyl-specific PCR (MSP) for promoters of E-cadherin.** Genomic DNA was obtained from cell lines and modified with

sodium bisulfite as described previously (14). Sodium bisulfite-treated genomic DNA from 7721-sMT1 and 7721-pMT1 were specifically amplified by methylated and unmethylated primers of E-cadherin as reported previously (15).

**Colony formation assay.** Cells (1×10<sup>3</sup>) were evenly plated onto 6-well plates in medium containing 10% FBS and incubated at 37°C in 5% CO<sub>2</sub>. After 14 days of incubation, colony growth on the plates was assayed by the visual counting of the colonies. Cells were then fixed in methanol and stained using crystal violet solution to evaluate foci formation. Experiments were performed in triplicate during two independent repetitions.

**Transfection with HBx construct.** Cells were transfected with 4 µg of the pcDNA4/TO-HBx construct (a gift from Professor X.Y. Guan, University of Hong Kong) and the control pcDNA4/TO using Lipofectamine™ 2000 transfection reagent (Invitrogen) for 36 h.

## Results

**Treatment with 5'-aza/TSA up-regulates E-cadherin expression.** After cells were treated with 5'-aza or TSA, semi-quantitative RT-PCR was performed on E-cadherin expression (Fig. 1). Both 5'-aza and TSA treatments up-regulated E-cadherin expression. 5'-aza restored E-cadherin in a dose-dependent manner. TSA-regulated E-cadherin expression did not occur through DNMTs.

**Depletion of DNMT1 induced E-cadherin expression via demethylation of the promoter.** In order to determine which DNMTs play a major role in reducing the expression of E-cadherin, we detected the expression of E-cadherin in 7721-pMT1, 7721-pMT3A and 7721-pMT3B cells. RT-PCR results showed that the knockdown of DNMT1 restored E-cadherin expression, whereas the knockdown of DNMT3A or DNMT3B did not (Fig. 2A). In DNMT1-depleted HCC cells, E-cadherin expression was upregulated at the protein level and coincided

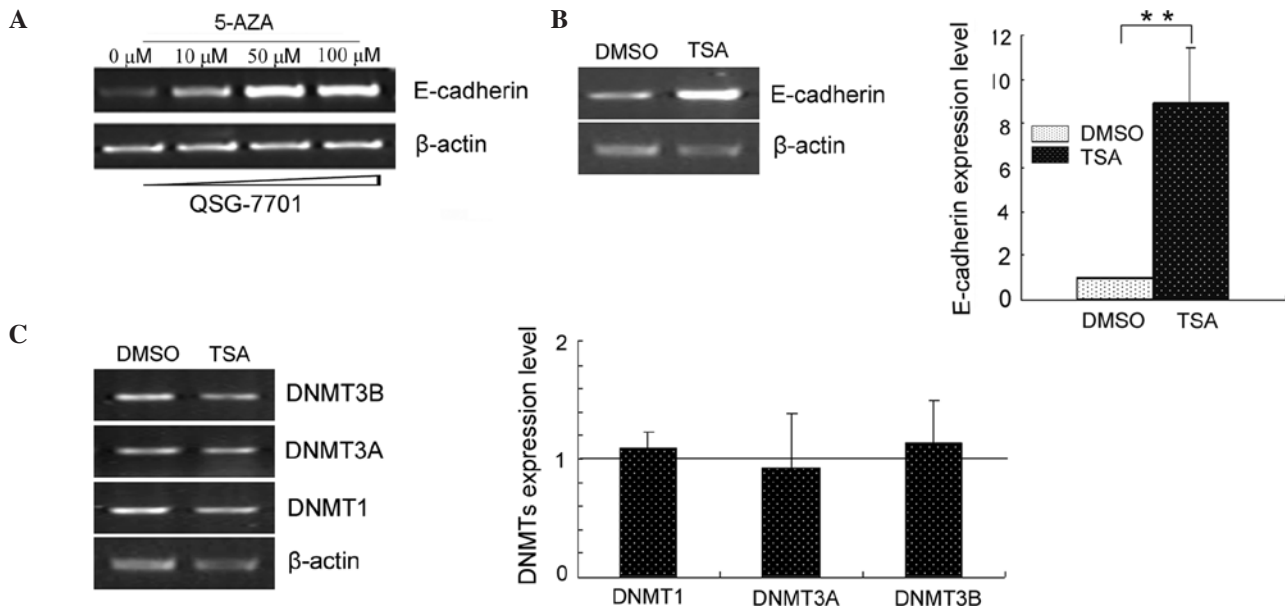


Figure 1. 5'-aza and TSA restored E-cadherin expression in the HCC cell lines. (A) The restoration of mRNAs encoding E-cadherin was measured by RT-PCR after treatment with 10, 50 and 100  $\mu$ mol/l 5'-aza for up to 2 days in QSG-7701 cells. (B) Left: TSA restored E-cadherin expression detected by RT-PCR in the SMMC-7721 cell line after treatment with 120 ng/ml of TSA or DMSO (control) for 24 h. Right: pattern of gel electrophoresis analyzed by Gel-Pro Analyzer 3.0 software to quantitatively evaluate the expression of E-cadherin to  $\beta$ -actin ratios. (C) Left: DNMT expression levels in the SMMC-7721 cell line after treatment with TSA as measured by RT-PCR. Right: quantitative evaluation of the expression of DNMT to  $\beta$ -actin ratios.

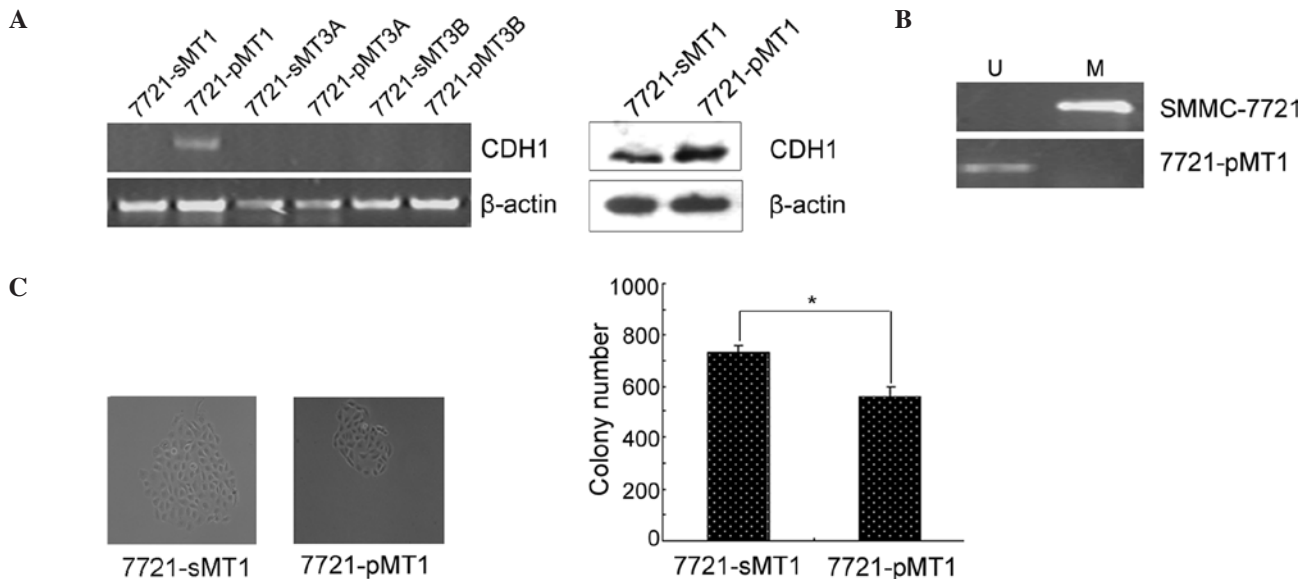


Figure 2. The depletion of DNMT1 induced E-cadherin expression via demethylation of the promoter. (A) Left: E-cadherin expression levels in the 7721-pMT1, 7721-pMT3A and 7721-pMT3B cells, and their scramble sequence control measured using RT-PCR.  $\beta$ -actin was used as an internal control. Right: Western blot analysis of E-cadherin expression levels in DNMT1 knockdown HCC and control cells. (B) The methylation status of the E-cadherin promoter was analyzed using MSP in the 7721-pMT1 and control cells. (C) Colony formation assays were performed on the 7721-pMT1 and control cell lines. The rate of colony formation was significantly lower in the 7721-pMT1 cells compared to the control cells.

with the transcriptional level. These results indicated that the E-cadherin gene may be one of the direct targets of DNMT1. We next investigated whether the up-regulated expression of E-cadherin induced by DNMT1 RNAi would be reflected in the promoter methylation status of the genes. Therefore, we determined the methylation status of the promoter using MSP as shown in Fig. 2B. The results showed that the restoration

of E-cadherin was significantly associated with its promoter demethylation in the 7721-pMT1 cell line. Subsequently, colony formation assays were performed using the 7721-pMT1 and control cell lines. Compared to the random control, siRNA-treated HCC cells with knocked down DNMT1 expression exhibited a significantly decreased colony size in the colony formation assays (Fig. 2C).

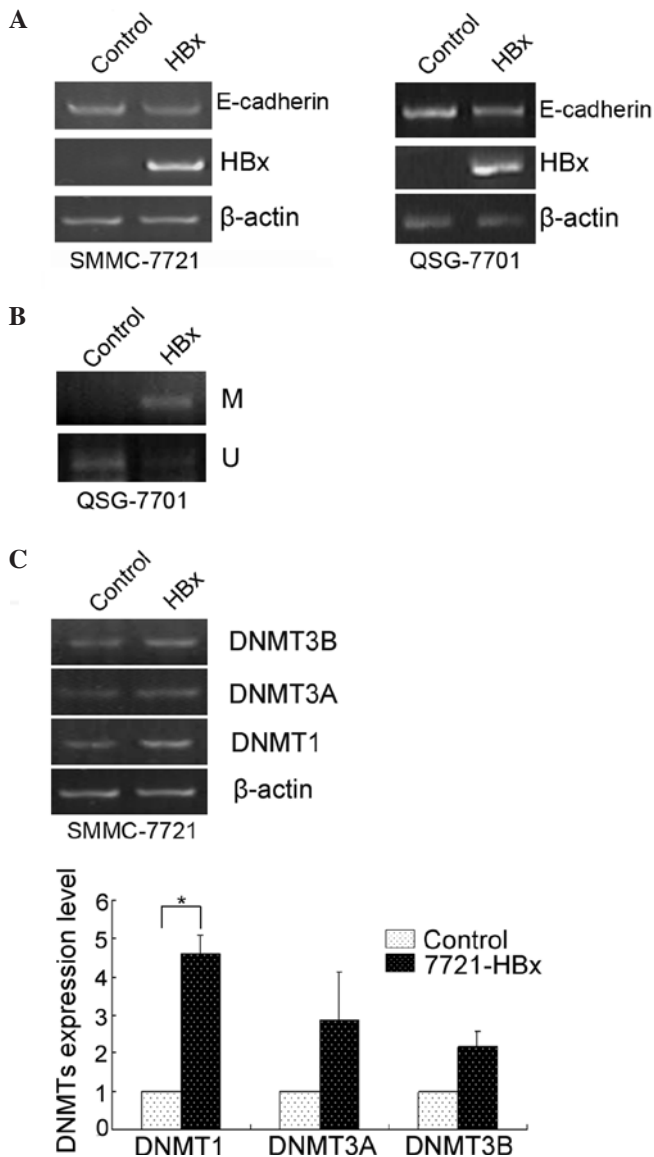


Figure 3. HBx suppressed E-cadherin expression through DNA methylation via DNMT1. (A) Reduced expression of mRNAs encoding E-cadherin measured using RT-PCR after transfection with the HBx construct for 36 h in HCC cells. (B) The methylation status of the E-cadherin promoter was analyzed using MSP in QSG-7701 cells transfected with HBx and control cells. (C) Top: DNMT expression in HCC cells measured using RT-PCR after transfection with the HBx construct for 36 h.  $\beta$ -actin was used as an internal control. Bottom: histogram representing the results of the RT-PCR. Error bars, SE. \*Statistically significant difference ( $p < 0.05$ ).

HBx leads to the promoter hypermethylation of the E-cadherin gene by activating DNA methyltransferase-1. Previous immunohistochemical studies of E-cadherin expression in HBV-related HCC have demonstrated the significant down-regulation of E-cadherin expression in tumor tissues compared with adjacent non-tumor tissues (16). Although the pathogenesis of HBV-related HCC has not been fully described, evidence suggests that HBx plays a crucial role in the pathogenesis of HCC (17). Therefore, we first investigated whether HBx represses E-cadherin expression in cultured human liver cells. For this purpose, we transfected the transiently pcDNA4/TO-HBx construct into QSG-7701 and SMMC-7721 cells. As a result, the

E-cadherin mRNA level was reduced by HBx (Fig. 3A), suggesting that HBx represses E-cadherin expression. The repression of E-cadherin by HBx was significantly associated with its promoter methylation (Fig. 3B) and increased DNMT1 (Fig. 3C).

## Discussion

It has been suggested that genetic alterations such as loss of heterozygosity and mutations in tumor suppressor genes accumulate during multistep hepatocarcinogenesis (18,19). Recently, epigenetic alterations including histone deacetylation and DNA methylation in promoter areas were also hypothesized to play crucial roles in the development of HCC. DNA methylation inhibitors including 5'-aza induce gene expression. 5'-aza was the first epigenetic drug proposed for use in cancer therapeutics (20). TSA alone or in combination with 5'-aza is capable of reactivating the transcription of tumor suppressor genes that are silenced by methylation-mediated mechanisms in human cancer cells (21,22). A number of genes involved in cell cycle- or apoptosis-regulation were also up-regulated in hepatoma cell lines, as previously reported (23).

Hypermethylation of CpG regions of the E-cadherin promoter represents the most common cause for its inactivation and has been observed in many malignancies, including HCC (24-27). Reactivation of E-cadherin, proposed as a target of epigenetic therapy for tumors (28), may be effective in HCC. In the present study, we investigated for the first time the epigenetic activation of E-cadherin by treatment with 5'-aza in an HCC cell line, and found it to be dependent on the administered dose of 5'-aza. Several studies have suggested that 5'-aza restores the expression of silenced genes by selective degradation or the partial influence of DNMT1 (30,31). In our study, we found that the depletion of DNMT1 restored E-cadherin gene expression by demethylating the promoter and suppressed HCC cell colony formation.

As HBV is the main factor leading to HCC in Chinese populations (29), HBx, an important gene associated with HBV, was transfected into HCC cells to evaluate the potential cause of inactivated E-cadherin in HCC samples from Chinese patients. It was observed that some of the tumor-associated genes, including IGFBP3, were epigenetically silenced in HCC cell lines infected with the recombinant HBx (32,33). This preliminary observation led to further *in vivo* and *in vitro* analyses of this characteristic abnormality of HBV-associated HCC. Few studies have focused on the mechanisms of the promoter methylation of host genes in association with HBV infection. Here, we showed that HBx suppressed expression of the E-cadherin gene by activating DNMT1. Our study regarding the epigenetic modulation of E-cadherin by HBx may suggest a mechanism for the epigenetic silencing of tumor suppressor genes in HBV-related HCC.

The findings presented in the present study demonstrate that diverse epigenetic agents restore E-cadherin expression. In addition, results obtained from studies involving HBx-transfected HCC cell lines suggest that the inhibition of DNMT1 may be considered a strategy by which silenced E-cadherin in HBV-related HCC may be inactivated.



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