Dual infection of hepatitis B virus (HBV) and hepatitis C virus (HCV) is closely associated with an increased risk of hepatocellular carcinoma; however, the underlying mechanism is poorly understood. In the present study, we found that HBV X protein (HBx) and HCV core protein work together to inhibit E-cadherin expression in human hepatoma cells. For this effect, they additively increased both the level and activity of enzymes, DNA methyltransferase 1, 3a, and 3b to induce promoter hypermethylation of E-cadherin in a p53-dependent fashion. Their additive effect on E-cadherin levels was reproduced in an in vitro HBV/HCV dual infection system using Huh7D-NTCP cells. As a result, HBV and HCV additively upregulated mesenchymal marker such as N-cadherin, Snail, Twist and Vimentin but cooperatively downregulated epithelial markers such as E-cadherin, Slug and Plakoglobin. In addition, the coinfected cells exhibited faster cell migration and higher invasion ability, as compared with monoinfection, which are hallmarks of epithelial-mesenchymal transition required for the initiation of metastasis in cancer progression.

1. Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are taxonomically unrelated viruses belonging to two different families, *Hepadnaviridae* and *Flaviviridae*, respectively [1]. The two viruses have several common properties, including transmission routes, hepatotropism, and the potential to establish chronic hepatitis, which often results in hepatocellular carcinoma (HCC) [2]. A substantial number of patients are coinfected with both viruses because they can be co-transmitted through common transmission routes, such as sexual contact and sharing needles [3]. In addition, coinfection occurs as a consequence of superinfection, i.e., new infection of HBV in a patient with preexisting chronic infection by HCV, or vice versa [4]. The estimated prevalence of coinfection is 10–20% in HBV-positive patients, and 2–10% in HCV positive patients, with wide variations depending on the geographical region [5, 6].

Coinfection with HBV and HCV is usually associated with a higher risk of advanced hepatic disease, including liver cirrhosis and HCC, as compared with monoinfection [4, 7], while the molecular mechanism remains poorly understood. Both HBV and HCV encode a multifunctional protein termed HBx and HCV core protein, respectively, which have been strongly implicated in HCC pathogenesis due to their roles in cell growth, signal transduction, transcriptional activation, transformation and immune modulation [8, 9]. They also epigenetically silence tumor suppressor genes, including p14, p16 and E-cadherin, via DNA methylation [10, 11]. For this purpose, HBx and HCV core protein upregulate levels of DNA methyltransferase (DNMT) 1, 3a, and 3b. Therefore, it is possible to assume that HBx and HCV core protein cooperate to repress tumor suppressor genes via DNA methylation and contribute to a higher risk of HCC development in patients with dual infection of HBV and HCV. In the present study, we first investigated whether HBx and HCV core protein cooperate to downregulate E-cadherin levels in human hepatoma cells. Second, we investigated whether the two viral proteins additively activate DNMTs to repress E-cadherin expression via DNA methylation. Third, we examined the effect of HBx and HCV core protein on E-cadherin expression during HBV and HCV coinfection. Finally, we attempted to prove that the decreased E-cadherin expression actually contributes to a higher risk of HCC development, focusing on the epithelial-mesenchymal transition (EMT) of coinfected cells.
2. Materials and methods

2.1. Plasmids

Plasmids pCMV-3 × HA1-HBx [12] and pCMV-3 × HA1-core protein [13] encode the full-length HBx (genotype C) and HCV core protein (genotype 1b), respectively, downstream of three copies of the influenza virus hemagglutinin (HA) epitope. The E-cad-luc, containing the promoter region of E-cadherin in pGL2-Basic (Promega, Cat No. E1641) was described before [14]. The HBV replicon (1.2-mer WT), containing 1.2 units of the HBV genome (genotype D) [15], and pJFH-1, containing HCV cDNA from a Japanese patient with fulminant hepatitis [16], were described previously. The p53 expression plasmid, pCMV p53-WT, was kindly provided by Dr. C.-W. Lee (Sungkyunkwan University). Scrambled (SC) shRNA (Cat No. sc-37007) and p53 shRNA plasmids (sc-29435-SH and sc-44218-SH) were purchased from Santa Cruz Biotechnology. Plasmids RC210241 (Cat No. 003049) encoding the Myc-DDK-tagged human Na⁺-taurocholate cotransporting polypeptide (NTCP) and pCH110 (Cat No. 27-4508-01) encoding the Escherichia coli β-galactosidase (β-gal) gene under the control of the SV40 promoter were purchased from OriGene and Amersham, respectively.

2.2. Cell transfection

The human hepatoma cell lines, HepG2 (Cat No. 88065) and Hep3B (Cat No. 88064), were purchased from the Korean Cell Line Bank (KCLB). Huh7D, a derivative of another human liver cancer cell line, Huh7, was kindly provided by Dr. S.M. Feinstone (US FDA). HepG2 cells express wild-type p53, whereas Huh7 cells have Y220C-mutated p53 [17]. In contrast, Hep3B cells with a homozygous deletion in the p53 gene do not express p53 [17]. Huh7D-NTCP cells were established by transfection with RC210241, followed by selection with 500 μg ml⁻¹ G418 sulfate (Sigma, Cat No. A1720). For transient expression, 4 × 10⁵ cells per 60-mm diameter plate were transfected with 2 μg of appropriate plasmid(s), using the TurboFect™ transfection reagent (Thermo Scientific, Cat No. R0532). Cells were treated with 5-aza-2′-dC (Sigma, Cat No. A3656), if necessary, to inhibit DNMTs for 24 h before harvesting.

2.3. Preparation of HBV and HCV stocks and virus infection

For 1.2-mer HBV replicon system, Huh7D-NTCP cells were transiently transfected with 1.2-mer-WT for 48 h, as described above. HBV titers in cultured medium were determined by immunoprecipitation (IP)-coupled real-time PCR, as previously described [18]. For HCV stocks, 10 μg of JFH-1 RNA prepared from the plasmid pJFH-1, containing HCV cDNA from a Japanese patient with fulminant hepatitis behind a T7 promoter [16] by in vitro transcription (MEGAscript; Ambion Cat No. AM1333) was delivered to Huh7.5 cells by electroporation, as previously described [19]. Huh7D-NTCP cells in 6-well plates were either mock-infected or infected with HBV and/or HCV at 10.0 multiplicity of infection (MOI) in 500 μl serum-free Dulbecco’s modified Eagle’s medium (DMEM; WELGENE, Cat No. LM001-05). After incubation for 1 h, cells were washed 3

Figure 1. HBx and HCV core protein cooperate to repress E-cadherin expression in p53-positive human hepatoma cells. (A, C) HepG2 and Hep3B cells were transiently transfected with HBx and/or HCV core expression plasmid along with 0.2 μg of E-cad-luc for 48 h, followed by the luciferase assay (n = 4). (B, D) HepG2 and Hep3B cells were transiently transfected with HBx and/or HCV core expression plasmid for 48 h, followed by western blotting. The HA-tagged HBx and HCV core protein were detected together using an anti-HA antibody. Uncropped full-length pictures of western blotting membranes are presented in the Supplementary Figure 1.
times with PBS and then incubated for an additional 47 h in DMEM containing 3% FBS, 4% polyethylene glycol 8000 (PEG 8000), and 2% dimethyl sulfoxide (DMSO).

### 2.4. Determination of HBV and HCV levels

The HBV titers were determined, as described previously [18]. Briefly, HBV genomic DNA was purified from the precipitated HBV particle-antibody complexes using the QIAamp DNA mini kit (Qiagen, Cat No. 51306). For quantitative PCR (qPCR) of HBV, HBV DNA was amplified using the SYBR premix Ex Taq II (Takara Bio, Cat No. RR82LR) and a primer pair, HBV 379F (5’-GTG TCT GCG GCG TTT TAT CA-3’) and HBV 476R (5’-GAC AAA GGG GCA ACA TAC CTT-3’), in a Rotor Gene Q PCR machine (Qiagen). For quantitation of HCV virions, HCV RNA extracted from the culture supernatant using the QIAamp Viral RNA mini kit (Qiagen, Cat No. 52904) was reverse transcribed with a reverse primer, HCV 290R (5-AGT ACC ACA AGG CCT TTC G-3), using the AccuPower RT PreMix kit (Bioneer, Cat No. K-2041). Quantitative real-time RT-PCR (qRT-PCR) of HCV RNA levels was performed using HCV 130S and HCV 290R primers and SYBR green PCR master mix (Takara, Cat No. RR82LR) using Rota-Gene Q (Qiagen), as previously described [20].

### 2.5. Methylation-specific PCR (MSP)

Total genomic DNA was purified from cells using QIAamp DNA Mini Kit (Qiagen, Cat No. 51306), according to the manufacturer’s instructions. Bisulfite modification of the genomic DNA (1 μg) was performed using Epitext Bisulfite kit (Qiagen, Cat No. 59104). The modified DNA (100 ng) was subjected to MSP using a methylated primer pair and an unmethylated primer pair of E-cadherin, as previously described [21].

### 2.6. DNMT activity assay

Approximately 4 × 10⁵ cells per 60-mm diameter plate were transiently transfected with the indicated amount of HBx expression plasmid and HCV core protein expression plasmid for 48 h. DNMT activity in the cell lysates was measured using EpiQuick DNMT Activity/Inhibition Assay Ultra Kit (Epigentek, Cat No. P-3009-96), following the manufacturer’s instructions.

### 2.7. Western blot analysis

Cells were lysed in buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, and 1% NP-40) supplemented with a cocktail of protease inhibitors (Roche). Cell extracts were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (GE Healthcare Life Science). Membranes were then incubated with antibodies to DNMT1 (Cat No. ab19905, 1:500 dilution), HCV core protein (Cat No. ab58713, 1:500 dilution) (Abcam), to p53 (Cat No. sc-126, 1:1,000 dilution), DNMT3a (Cat No. sc-373905, 1:500 dilution), DNMT3b (Cat No. sc-81252, 1:500 dilution), E-cadherin (Cat No. sc-71009, 1:500 dilution), HA (Cat No. sc-7392, 1:500 dilution), N-cadherin (Cat No. sc-59987, 1:500 dilution),

Figure 2. HBx and HCV core protein cooperate to repress E-cadherin expression by upregulating p53 levels in human hepatoma cells. (A) Levels of p53 and E-cadherin in HepG2 and Hep3B cells were measured by western blotting. (B, D) HepG2 and Hep3B cells were transiently transfected with the indicated plasmids, as described in Figure 1A and C, followed by the luciferase assay (n = 4). (C, E) HepG2 and Hep3B cells were transiently transfected with HBx and/or HCV core expression plasmid along with p53 expression plasmid, scrambled (SC) shRNA or p53 shRNA plasmid for 48 h, followed by western blotting. Uncropped full-length pictures of western blotting membranes are presented in the Supplementary Figure 2.
γ-tubulin (Cat No. sc-17787, 1:500 dilution), Slug (Cat No. sc-15391X, 1:500 dilution), Snail (Cat No. sc-10433X, 1:500 dilution), Twist (Cat No. sc-15393X, 1:500 dilution), Vimentin (Cat No. sc-6260, 1:500 dilution) (Santa Cruz Biotechnology), to HBx (Millipore, Cat No. MAB8419, 1:500 dilution) and to Plakoglobin (BD Biosciences, Cat No. 610253, 1:500 dilution). The ECL kit (Advansta, Cat No. K-12043-D20) was employed to visualize the protein bands with the ChemiDoc XRS imaging system (Bio-Rad).

2.8. Luciferase reporter assay

Approximately $2 \times 10^5$ HepG2 or Hep3B cells per well in 6-well plate were transiently transfected with 0.2 μg of a reporter plasmid under the indicated conditions. To control for transfection efficiency, 0.1 μg of pCH110 was included as an internal control. At 48 h after transfection, a luciferase assay was performed using the Luciferase Reporter 1000 Assay System (Promega, Cat No. E4550). The values were normalized to the β-gal activity measured in the corresponding cell extracts.

2.9. Immunofluorescence analysis

Cells that were grown on coverslips under the indicated conditions were fixed in 4% formaldehyde at 20 °C for 15 min and permeabilized in methanol at −20 °C for 10 min. The coverslips were then incubated at 20 °C for 3 h with anti-HCV core protein polyclonal (Abcam, Cat No. ab58713, 1:200) and anti-HBx monoclonal (Santa Cruz, Cat No. sc-57760, 1:500 dilution) antibodies. The cells were then incubated with anti-rabbit IgG-rhodamine (Invitrogen, Cat No. 31670, 1:200 dilution) and anti-mouse IgG-FITC (Sigma-Aldrich, Cat No. F0257-1ML, 1:100 dilution) antibodies at 20 °C for 1 h. The slides were prepared with UltraCruz mounting medium (Santa Cruz Biotechnology, Cat No. sc-24941) were visualized using an Eclipse fluorescence microscope (Nikon).

2.10. Wound-healing assay

For the measurement of cell migration rate, the scratch wound-healing assay was performed, as described before [22]. Briefly, $1 \times 10^6$ cells were seeded and cultured to create a confluent monolayer in 60-mm dish. Cells were either mock-infected or infected with HBV and/or HCV at 10.0 MOI each for 48 h and then gently scratched with a pipette tip to produce a wound. The closure rate of the wound was measured after incubation for 24 h to determine wound healing rate.

2.11. Cell invasion assay

Cell invasion analysis was performed with an EZCell™ Cell Invasion Assay Kit (BioVision, Cat No. K912-100) according to the manuals supplied by the manufacturer. Briefly, $5 \times 10^3$ Huh7D-NTCP cells infected with HBV and/or HCV for 48 h were seeded in 50 μl serum-free media in
wells of the top chamber, which were coated with a basement membrane solution, and were incubated for an additional 24 h. Migrated cells were incubated with a mixture of cell dissociation solution and cell invasion dye (100 μl) for 1 h, followed by fluorometric quantification at Em/Ex = 530/590 nm.

2.12. Statistical analysis

The values indicate means ± standard deviations from at least three independent experiments. The difference between the means of the treatment group and the control was assessed with the paired two-tailed t-test; the difference was considered to be statistically significant if \( P < 0.05. \)

3. Results

3.1. HBx and HCV core protein cooperate to inhibit E-cadherin expression in a p53-dependent manner

Consistent with previous studies [14, 23], transient expression of HBx or HCV core protein significantly decreased the E-cadherin promoter activity (Figure 1A), resulting in downregulation of E-cadherin levels in HepG2 cells (Figure 1B). The effects of HBx and HCV core protein on the promoter activity and protein level of E-cadherin were additive in HepG2 cells (Figure 1A and B), indicating that they cooperate to inhibit E-cadherin expression in human hepatoma cells. In contrast, the effects of HBx and HCV core protein on the promoter activity and protein level of
E-cadherin were negligible or insignificant in Hep3B cells, in which p53 was absent (Figure 1C and D). In addition, HBx and HCV core protein individually increased p53 levels in HepG2 cells and the effect was additive in cells expressing both proteins (Figure 1B). Accordingly, the p53 levels were inversely proportional to the E-cadherin levels in HepG2 cells (Figure 1B), suggesting that HBx and HCV core protein inhibit E-cadherin expression by upregulating p53 levels.

We attempted to prove that upregulation of p53 levels is necessary and sufficient for the inhibition of E-cadherin expression by HBx and HCV core protein in human hepatoma cells. Levels of E-cadherin was much higher in Hep3B cells than in HepG2 cells (Figure 2A). In addition, ectopic p53 expression significantly decreased the E-cadherin promoter activity and dose-dependently lowered E-cadherin levels in HepG2 (Figure 2B and C) and Hep3B cells (Figure 2D and E) in the absence of HBx and HCV core protein. In contrast, p53 knockdown downregulated E-cadherin levels in HepG2 cells in the absence of HBx and HCV core protein (Figure 2C). In addition, p53 knockdown almost completely abolished the combinatorial effects of HBx and HCV core protein on the E-cadherin promoter activity and protein level in HepG2 cells (Figure 2B and C). Moreover, HBx and HCV core protein individually or in combination upregulated ectopic p53 levels, lowered the E-cadherin promoter activity, and downregulated E-cadherin levels in Hep3B cells (Figure 2D and E). Based on these observations, we concluded that HBx and HCV core protein cooperate to repress E-cadherin expression via elevation of p53 levels in human hepatoma cells.

3.2. HBx and HCV core protein cooperate to repress E-cadherin expression via DNA methylation

Consistent with previous reports [13, 14], both HBx and HCV core protein elevated protein levels of DNMT1, 3a, and 3b and their enzyme activities in HepG2 cells (Figure 3A and B), resulting in promoter hypermethylation of E-cadherin gene in HepG2 cells (Figure 3C). However, these effects were negligible or insignificant in Hep3B cells (Figure 3A–C). These results enabled us to hypothesize that HBx and HCV core protein collaborate to inhibit E-cadherin expression via DNA methylation in a p53-dependent manner. Indeed, HBx and HCV core protein cooperated to upregulate protein levels of DNMT1, 3a, and 3b and their enzyme activities (Figure 3A and B), resulting in higher frequencies of DNA methylation of the E-cadherin promoter in HepG2 cells (Figure 3C), while none of these effects were clear in Hep3B cells (Figure 3A–C). In addition, the ability of HBx and HCV core protein to activate DNMTs and thereby inhibit E-cadherin expression via DNA methylation was directly proportional to their potential to upregulate p53 levels (Figure 3A–C). Moreover, treatment with 5-Aza-2′-dC, a universal DNMT inhibitor, almost completely abrogated the potential of HBx and HCV core protein to downregulate E-cadherin levels in HepG2 cells, as in Hep3B cells (Figure 3D). Based on these observations, we conclude that HBx and HCV core protein cooperate to activate cellular DNMTs and inhibit E-cadherin expression via DNA methylation in a p53-dependent manner.

3.3. HBx and HCV core protein cooperate to repress E-cadherin expression via DNA methylation during HBV/HCV coinfection

The combinatorial effect of HBx and HCV core protein on the E-cadherin expression was investigated by an in vitro HBV/HCV coinfection using Huh7D cells stably expressing NTCP, which can support replication of HBV [24] and HCV [25]. Replication of HBV and HCV in Huh7D-NTCP cells was evidenced through detection of intracellular viral proteins, including HBx and HCV core protein (Figure 4A and C), and extracellular HBV and HCV particles (Figure 4A). According to the IFA, 88% of the coinfected cells expressed both HBx and HCV core protein (Figure 4A). Coinfection with HBV and HCV, as compared to monoinfection, exerted stronger effects on the protein level and promoter activity of E-cadherin, which were almost completely abolished by treatment with 5-Aza-2′-dC (Figure 4C and D). These results suggest that HBx and HCV core protein cooperate to repress E-cadherin expression via DNA methylation during HBV/HCV coinfection in a p53-dependent fashion, as demonstrated with overexpression of HBx and HCV core protein (Figure 3).

Finally, we investigated whether HBx and HCV core protein cooperate to induce EMT of the coinfected cells as a consequence of E-cadherin downregulation. Monoinfection with either HBV or HCV upregulated mesenchymal marker such as N-cadherin, Snail, Twist and Vimentin but downregulated epithelial markers such as E-cadherin, Slug and Plakoglobin in Huh7D-NTCP cells (Figure 4E). Coinfection with HBV and HCV, as compared to monoinfection, exerted stronger effects on the protein levels of both mesenchymal and epithelial markers in Huh7D-NTCP cells (Figure 4E). According to the results of the wound-healing assay, the Huh7D-NTCP cells monoinfected with HBV or HCV exhibited a relatively fast migratory behavior, colonizing 42.5% and 42.8% of the wounded surface, respectively, in 24 h, whereas in the mock-infected cells at this time point, 69.9% of the wounded surface remained uncovered (Figure 4F). Coinfection with HBV and HCV induced faster cell migration, colonizing 59.3% of the wounded surface in 24 h. Coinfection with HBV and HCV, as compared to monoinfection, also exhibited significantly higher effects on the cell invasion of Huh7D-NTCP cells (Figure 4G). Based on these observations, we concluded that HBx and HCV core protein cooperate to induce EMT of the coinfected cells by downregulating E-cadherin levels.

4. Discussion

Epidemiological studies have demonstrated a close correlation between coinfection with HBV and HCV, as compared to monoinfection, and a high risk of HCC development [4, 7, 26, 27]. Accumulating evidence suggests that HBx and HCV core protein as the representative viral oncoprotein of HBV and HCV, respectively, play critical roles in the development of virus-associated HCC [28, 29]. These multifunctional proteins have been implicated in HCC development owing to their abilities in the modulation of diverse signaling pathways in the cytoplasm and alteration of gene expression in the nucleus, and dysregulation of immune responses, apoptotic cell death, and lipid metabolism [30, 31, 32, 33]. Therefore, it is possible to assume that the additive or synergistic effects of HBx and HCV core protein contributes, at least in part, to a high risk of HCC development in patients coinfected with both viruses. The present study provides, to our knowledge, the first example of such additive action of HBx and HCV core protein—cooperative repression of E-cadherin expression via DNA methylation, which can be correlated with a higher risk of HCC development.

Previous reports have demonstrated that both HBx and HCV core protein activates DNMTs to repress E-cadherin expression via DNA methylation to induce EMT in human hepatocytes [13, 14]. The present study showed that HBx and HCV core protein cooperate to elevate the protein levels of DNMT1, 3a, and 3b that are responsible for de novo and maintenance DNA methylation [17] to increase DNMT activity, resulting in a decrease in E-cadherin expression via promoter hypermethylation (Figure 3). The present study also provides several lines of evidence that these effects result from cooperative activation of p53 by HBx and HCV core protein. It is likely that HBx and HCV core protein activate p53 to inhibit E-cadherin expression via DNA methylation. The roles of p53 in the regulation of protein levels and enzyme activities of DNMTs are controversial. Radiation or etoposide, both of which can stabilize and activate p53, induces a 5-fold increase in DNMT1 levels [34]. In addition, p53 activates DNMT1 and 3a via direct interaction to inhibit expression of survivin and p21, respectively, via DNA methylation [35, 36]. The present study also showed that HBx and HCV core protein upregulate both the protein levels and enzyme activities of DNMTs via activation of p53 (Figure 3). However, an opposite role for p53 in the regulation of
DNMTs also has also been reported. p53 negatively regulates DNMT1 expression through direct DNA binding [34] or by forming a complex with Sp1 and chromatin modifiers on the DNMT1 promoter [37]. More detailed studies are required to unveil the mechanism HBx and HCV core protein cooperate to activate cellular DNA methylation systems via upregulation of p53 levels.

Inactivation of the E-cadherin gene is one of the most common genetic alterations that is associated with progression to malignant HCC [38]. DNA methylation is likely to be the prominent cause for E-cadherin inactivation in HCC [38, 39]. Epidemiological studies have shown relatively higher frequencies of DNA methylation in the E-cadherin promoter from HCCs with HBV or HCV monoinfection compared to those with no such infection [39, 40]. Later studies have further demonstrated that both HBx and HCV core protein induce promoter hypermethylation of the E-cadherin gene in human hepatocytes, resulting in inhibition of its expression [9, 14, 23, 39]. For this effect, HBx and HCV core protein individually upregulate levels of DNMT1, 3a, and 3b in human hepatoma cells [13, 14, 23]. Treatment with 5-Aza-2′-dC effectively reactivates E-cadherin expression and inhibits EMT in HBx- or HCV core protein-expressing cells [13, 14, 23]. Most of these studies were performed using cells overexpressing viral proteins, which may cause non-natural effects or artifacts due to the extremely high levels of viral proteins in the transfected cells. In the present study, the combination effect of HBx and HCV core protein could be exactly reproduced in an in vitro HBV and HCV coinfection system, which may properly reflect the natural course of coinfection. The present coinfection experiments, however, cannot exclude the possible involvement of other viral proteins, such as NS3 and NS4A of HCV and HBs proteins of HBV, which are also known to be implicated in the development of HCC [28, 29]. More detailed studies are required to evaluate their relative contributions to the development of HCC during coinfection with HBV and HCV.

**Declarations**

**Author contribution statement**

Hyunyoung Yoon: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Kyung Lib Jang, Ph.D.: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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**Data availability statement**

Data included in article/ supp. material/ referenced in article.

**Declaration of interests statement**

The authors declare no conflict of interest.

**Additional information**

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