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Hepatitis B Virus X Protein and Hepatitis C Virus Core Protein Cooperate to Inhibit P16 Expression Via DNA Methylation

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1. Abstract

Coinfection with hepatitis B virus (HBV) and hepatitis C virus (HCV) is often associated with a higher risk of hepatocellular carcinoma (HCC). Here, we found that HBV X protein (HBx) and HCV core protein cooperate to inhibit p16 expression via DNA methylation in human hepatoma cells. For this purpose, they additively upregulated both protein levels and enzyme activities of DNA methyltransferases (DNMTs) 1, 3a and 3b and induced promoter hypermethylation of p16. As a result, HBx and HCV core protein in combination activated the Rb-E2F pathway to stimulate cell cycle progression from G_1 to S phase, resulting in an increase in cell proliferation. The potential of HBx and HCV core protein to cooperatively induce these effects was reproduced in an *in vitro* HBV and HCV coinfection system but was almost completely abolished when p16 levels were restored by either 5-Aza-2'dC treatment or p16 overexpression, providing an insight on the coinfection-associated higher risk of HCC development.

2. Introduction

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are phylogenetically unrelated viruses belong to two different families, *Hepadnaviridae* and *Flaviviridae*, respectively [1]. The two viruses however have several common properties, including transmission routes, hepatotropism, and the potential to establish chronic hepatitis, which often leads to hepatocellular carcinoma (HCC) in human [2]. A substantial number of patients are coinfected with both viruses mainly because they share transmission routes, such as sexual contact, sharing needles, and vertical transmission [3]. In addition, coinfection occurs as a consequence of superinfection, i.e., new infection of one virus in a patient with preexisting chronic infection by the other virus [3,4]. The estimated prevalence of coinfection is 10-20% in patients with chronic HBV infection and 2-10% in patients with chronic HCV infection, showing wide variations depending on the geographical region [5,6].

Coinfection with HBV and HCV is usually associated with a higher risk of advanced liver disease, including liver cirrhosis and HCC, as compared with monoinfection [4,7-9]; however, the molecular mechanism remains poorly defined. Both HBV and HCV encode a multifunctional protein termed HBx and core protein, respectively, which have been implicated in HCC pathogenesis by virtue of their roles in cell growth, signal transduction, reactive oxygen species (ROS) formation, lipid metabolism, transcription activation, transformation and immune modulation [9,10]. They also epigenetically silence several tumor suppressor genes, including p14, p16 and E-cadherin, via DNA methylation [11,12]. For this purpose, HBx and HCV core protein individually upregulate levels of DNA methyltransferases (DNMTs) 1, 3a, and 3b [8,13,14]. Therefore, it is possible to assume that HBx and HCV core protein cooperate to silence tumor suppressor genes via DNA methylation to contribute to a higher risk of HCC development in patients coinfected with HBV and HCV. In the present study, we first examined whether HBx and HCV core protein cooperate to activate DNMTs in human hepatoma cells using both coexpression and *in vitro* coinfection systems. In addition, we attempted to prove that the activated DNMTs correlates with a high risk of HCC development in coinfected patients, focusing on the cooperative inhibition of p16 expression via DNA methylation and its effects on the Rb-E2F pathway and cell proliferation.

3. Materials and Methods

3.1. Plasmids

Plasmids pCMV-3 × HA1-HBx [15] and pCMV-3 × HA1-core [12] encode the full-length HBx (genotype C) and HCV core (genotype 1b), respectively, downstream of three copies of the influenza virus hemagglutinin (HA) epitope. The HBV replicon (1.2-mer WT) containing 1.2 units of the HBV genome (genotype D) [16] and pJFH-1, containing HCV cDNA from a Japanese patient with fulminant hepatitis behind a T7 promoter [17] were described previously. The pCMV-3 × HA1-p16, encoding the full-length HA-tagged p16, was described previously [13]. The E2F1-luc and pHA-Ub were gifts from C.-W. Lee (Sungkyunkwan University, Korea) and Y. Xiong (University of North Carolina at Chapel Hill), respectively. Plasmids RC210241 encoding the human Na^+-taurocholate cotransporting polypeptider (NTCP) and pCH110 encoding the *Escherichia coli* β*-galactosidase* (β*-Gal)* gene were purchased from OriGene and Pharmacia, respectively.

3.2. Cell Lines and Cell Transfection

A human hepatoma cell line HepG2 (KCLB No. 58065) was obtained from the Korean Cell Line Bank. Huh7.5 and Huh7D cells were kindly provided by C. M. Rice (Rockefeller University) and D. A. Feigelstock (US FDA), respectively. For transient expression, 4×10^5 cells per 60-mm diameter plate were transfected with 2 μg of appropriate plasmid(s), using the TurboFect transfection reagent (Thermo Scientific) according to the manufacturer's instructions. Cells were treated with 5 μM 5-Aza-2'dC (Sigma), if necessary, to inhibit DNA methylation for 24 h before harvesting.

3.3. Preparation of HBV Stocks from A 1.2-Mer Replicon System

Cells were transiently transfected with 1.2-mer-WT for 48 h, as described above. Virus titers in cultured medium were determined by immunoprecipitation (IP)-coupled real-time PCR. Briefly, HBV particles were immunoprecipitated with an anti-HBs antibody (Santa Cruz Biotechnology) from the culture supernatant using a Classic Magnetic IP/Co-IP assay kit (Pierce), according to the manufacturer's specifications. HBV genomic DNA was purified from the precipitated HBV particle-antibody complexes using the QIAamp DNA mini kit (Qiagen). Quantitative real-time PCR assay of HBV was carried out, as described previously [54].

3.4. Preparation of HCV Stocks from the JFH1-Based HCV Infection System

The plasmid pJFH-1 was linearized at the 3' end of the HCV cDNA by *Xba*I digestion. The linearized DNA was then used as a template for *in vitro* transcription (MEGAscript; Ambion). *In vitro* HCV infection was performed in Huh7.5 cells, in which the virus can replicate efficiently due to a defect in the innate antiviral signaling pathway of these cells [18]. Ten micrograms of JFH-1 RNA were delivered to Huh7.5 cells, by electroporation, and virus stocks were prepared, as previously described [19]. HCV titers were determined by real-time RT-PCR, as previously described [20].

3.5. Virus Infection

For virus infection, Huh7D cells in 6-well plates were first transfected with 1 μg of NTCP expression plasmid for 24 h and then either mock-infected or infected with HBV and/or HCV at a multiplicity of infection (MOI) of 1.0 in 500 μl serum-free DMEM (WelGENE). After incubation for 1h, cells were washed 3 times with PBS and then incubated for an additional 23 h in DMEM containing 5% FBS. To monitor virus multiplication, both intracellular viral proteins and extracellular virus particles were detected by western blotting and real-time PCR, respectively.

3.6. Methylation-Specific PCR (MSP)

Genomic DNA (1 μg) denatured in 50 μl 0.2 M NaOH was modified by treatment with 30 μl 10 mM hydroquinone (Sigma) and 520 μl 3 M sodium bisulfite (pH 5.0; Sigma) at 50 °C for 16 h. MSP was performed under the conditions described by Herman *et al.* [21].

3.7. DNMT Activity Assay

Approximately 4×10^5 cells per 60-mm diameter plate were transiently transfected with the indicated plasmids for 48 h. DNMT activity in the cell lysates was measured using EpiQuick DNMT Activity/Inhibition Assay Ultra Kit (Epigentek), following the manufacturer's instructions.

3.8. 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 protease inhibitors (Roche). Cell extracts were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (Amersham). Membranes were then incubated with antibodies to p16 (Abcam), to DNMT1, DNMT3a, DNMT3b, E2F1, HBs, and HCV E2 (Santa Cruz Biotechnology), to HA (Roche), to phosphorylated Rb (Cell signaling), to Rb (Oncogene), to HBx (Millipore), to HCV core protein (Thermo Scientific) and to γ-tubulin (Sigma) and subsequently with an appropriate horseradish peroxidase-conjugated secondary antibody: anti-mouse or anti-rabbit IgG (H+L)-HRP (Bio-Rad). The ECL kit (Advansta) was used to visualize the protein bands with the ChemiDoc XRS imaging system (Bio-Rad).

3.9. Luciferase Reporter Assay

Approximately 2×10^5 cells per well in 6-well plate were transfected with 0.2 μg of E2F1-luc along with the indicated plasmids in Figure 2B and 4C. To control for transfection efficiency, 0.1 μg of pCH110 was cotransfected as an internal control. At 48 h after transfection, a luciferase assay was performed using the Luciferase Reporter 1000 Assay System (Promega). The obtained values were normalized to the β-Gal activity measured in the corresponding cell extracts.

3.10. Cell Viability Analysis

For the determination of cell viability, the MTT assay was performed

as previously described [22]. Briefly, 2×10^4 cells per well in 96-well plates were treated with 10 μM MTT (Sigma) for 4 h at 37°C. The formazan compounds derived from MTT by mitochondrial reductases present in the living cells were then dissolved in dimethyl sulfoxide, and quantified by measuring absorbance at 550 nm.

3.11. BrdU Incorporation Assay

For determination of DNA synthesis rate, the amount of BrdU incorporated into DNA was measured by a colorimetric immunoassay (Roche). Briefly, 2×10^4 cells per well in 96-well plates were incubated for 48 h under the indicated conditions and treated with 10 μM BrdU for an additional 24 h. Fixed cells were reacted with anti-BrdU-peroxidase for 2 h, and the color that developed after addition of trimethyl benzidine was measured at 490 nm and 405 nm.

3.12. Statistical Analysis

The values indicate means ± standard deviations (SD) from at least three independent experiments. A two-tailed Student's *t*-test was used for all statistical analyses. A P value of < 0.05 was considered statistically significant.

4. Results

4.1. HBx and HCV core protein cooperate to inhibit p16 expression via DNA methylation

Initially, we investigated whether HBx and HCV core protein activate a host DNA methylation system to inhibit p16 expression via DNA methylation. HBx and HCV core protein separately upregulated DNMT1, 3a and 3b levels and elevated DNMT activity, resulting in downregulation of p16 levels via DNA methylation in HepG2 cells (Figure 1A and B, lanes 2 and 3), which is consistent with previous findings [13,14]. The individual potentials of HBx and HCV core protein to induce these effects were similar. Their combination effects on DNA methylation and protein levels of p16 as well as protein levels and enzyme activities of DNMTs were additive, as demonstrated in cells expressing both HBx and HCV core protein (Figure 1A and B, lane 4), indicating that HBx and HCV core protein cooperate to induce these effects. As demonstrated with individual expression studies [13,14], treatment with a universal DNMT inhibitor, 5-Aza-2'dC, almost completely removed the combination effects of HBx and HCV core protein on the expression of p16 (Figure 1C), confirming that they cooperate to inhibit p16 expression via DNA methylation in human hepatoma cells.

4.2. HBx and HCV core protein cooperate to activate the Rb-E2F pathway by inhibiting p16 expression

As a potent inhibitor of G_1 cyclin-dependent kinases 4 and 6, p16 inactivates the Rb-E2F pathway to arrest the cell cycle progression from the G_1 to S phase [23]. Consistently, ectopic p16 expression reduced Rb phosphorylation without affecting total Rb protein levels in HepG2 cells, resulting in a decrease of E2F1 levels in a dose-dependent manner (Figure 2C, lanes 3 and 5). Having established the role of p16 in HeG2 cells, we investigated whether the p16 downregulation in the presence of HBx and/or HCV core protein results in activation of the Rb-E2F pathway. Consistent with previous reports [13,14], HBx and HCV core protein individually induced Rb phosphorylation without affecting total Rb levels, resulting in an increase of E2F1 protein levels and its transcriptional activity in HepG2 cells (Figure 2A and B, lanes 2 and 3). In addition, coexpression of HBx and HCV core protein led to additive activation of the Rb-E2F pathway, presumably due to their combination effect on p16 levels (Figure 2A and B, lane 4). Indeed, restoration of p16 levels in cells coexpressing HBx and HCV core protein by either p16 overexpression or 5-Aza-2'dC treatment almost completely abolished the potential of HBx and HCV core protein in combination to activate the Rb-E2F pathway (Figure 2C and D). Based on these observations, we conclude that HBx and HCV core protein cooperate to activate the Rb-E2F pathway by additively inhibiting p16 expression via DNA methylation.

4.3. HBx and HCV core protein cooperate to stimulate cell growth by inhibiting p16 expression

In agreement with its role as a negative regulator of the Rb-E2F pathway (Figure 2C), ectopic p16 expression significantly decreased DNA synthesis rate and thereby inhibited cell growth in HepG2 cells, as demonstrated with BrdU incorporation and MTT assays, respectively (Figure 3C and D). Therefore, it was investigated whether HBx and HCV core protein individually and in combination stimulate cell growth by downregulating p16 levels via DNA methylation. Consistent with their potential to activate the Rb-E2F pathway (Figure 2A and B), HBx and HCV core protein individually stimulated DNA synthesis and cell growth in HepG2 cells, as demonstrated with the BrdU incorporation and MTT assays, respectively (Figure 3A and B). As expected from the combination effect on the Rb-E2F pathway (Figure 2A), HBx and HCV core protein additively stimulated both DNA synthesis and cell growth in HepG2 cells (Figure 3A and B). In addition, restoration of p16 levels by either 5-Aza-2'dC treatment or ectopic p16 expression almost completely removed the combination effects of HBx and HCV core protein on the DNA synthesis and cell growth (Figure 3C and D), which is consistent with their effects on the Rb-E2F pathway (Figure 2C and D). Therefore, we conclude that HBx and HCV core protein cooperate to stimulate cell growth by downregulating p16 levels via DNA methylation.

4.4. HBV and HCV cooperate to stimulate cell growth by inhibiting p16 expression via DNA methylation during coinfection

It was attempted to prove that HBx and HCV core protein cooperate to stimulate cell growth via downregulation of p16 levels in cells coinfected with HBV and HCV. For this purpose, we employed a human hepatoma cell line, termed Huh7D, which is permissive for infection with both HBV and HCV [24,25]. Prior to HBV infection, NTCP as an HBV receptor [26] was introduced into Huh7D cells to facilitate HBV entry into cells. HBV replication in Huh7D cells was evidenced by the detection of viral proteins, such as HBV sur-

face antigen (HBs) and HBx (Figure 4A), and HBV particles (data not shown). In addition, infection with the JFH-1 strain of HCV in Huh7D cells was proved by the detection of viral proteins, such as HCV core protein and E2 (Figure 4A), and HCV particles (data not shown). Monoinfection with either HBV or HCV upregulated DNMT1, 3a and 3b levels, resulting in downregulation of p16 levels via DNA methylation (Figure 4A). Coinfection with HBV and HCV, as compared to monoinfection, exhibited stronger effects on the DNMT levels, p16 promoter methylation, and p16 protein levels (Figure 4A), suggesting that HBx and HCV core protein cooperate to inhibit p16 expression via DNA methylation in cells coinfected with HBV and HCV.

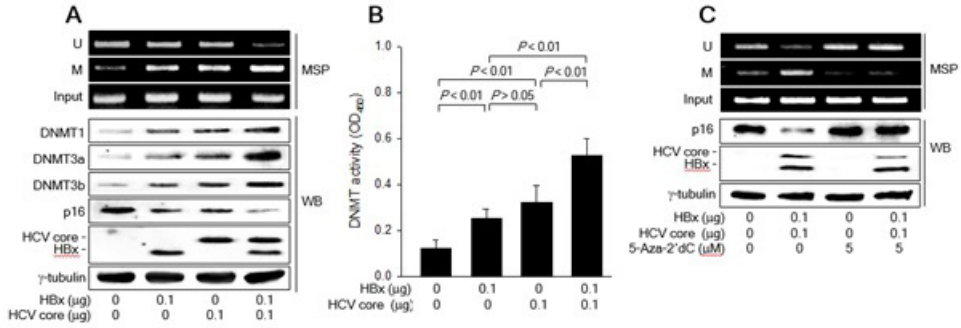

Figure 1: HBx and HCV core protein cooperate to inhibit p16 expression via DNA methylation. HepG2 cells were transiently transfected with the indicated amount of HBx expression plasmid and HCV core expression plasmid for 48 h. (A, C) The methylation status of p16 promoter was analyzed by MSP (upper panel). Protein levels were determined by western blotting (lower panel). HBx and HCV core protein were detected together using an anti-HA antibody. For lanes 3 and 4 in (C), cells were treated with 5 μM 5-Aza-2'dC for 24 h before harvesting. (B) DNMT activity in the cell extracts was determined (n = 6).

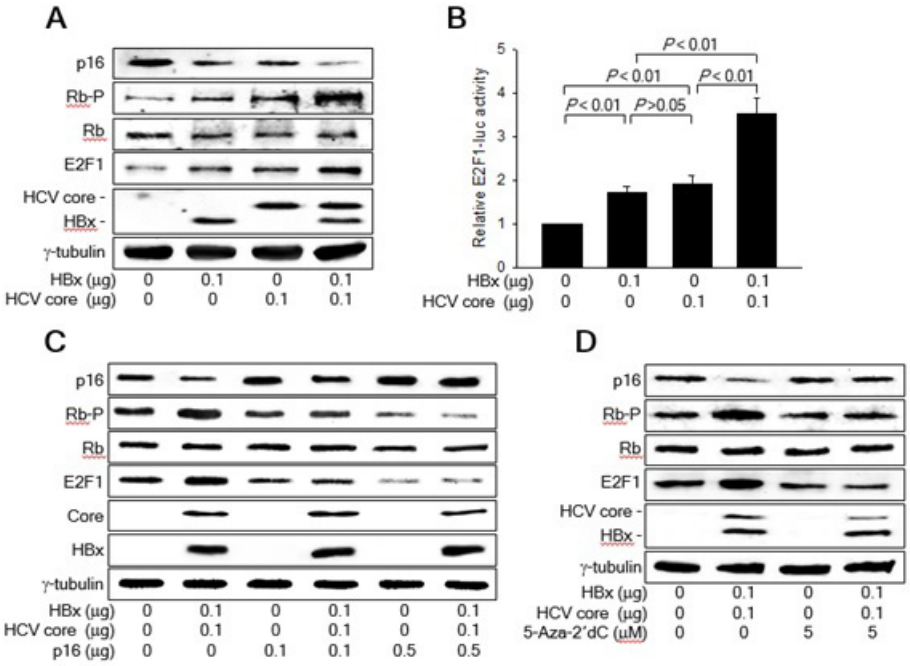

Figure 2: HBx and HCV core protein cooperate to activate the Rb-E2F pathway by inhibiting p16 expression. (A, C, D) HepG2 cells were transiently transfected with the indicated amount of HBx expression plasmid and HCV core expression plasmid for 48 h. An increasing amount of p16 expression plasmid was included in (C). Protein levels were determined by western blotting. (B) HepG2 cells were transiently transfected with 0.1 μg of E2F1-luc along with the indicated plasmid for 48 h, followed by luciferase assay (n = 4).

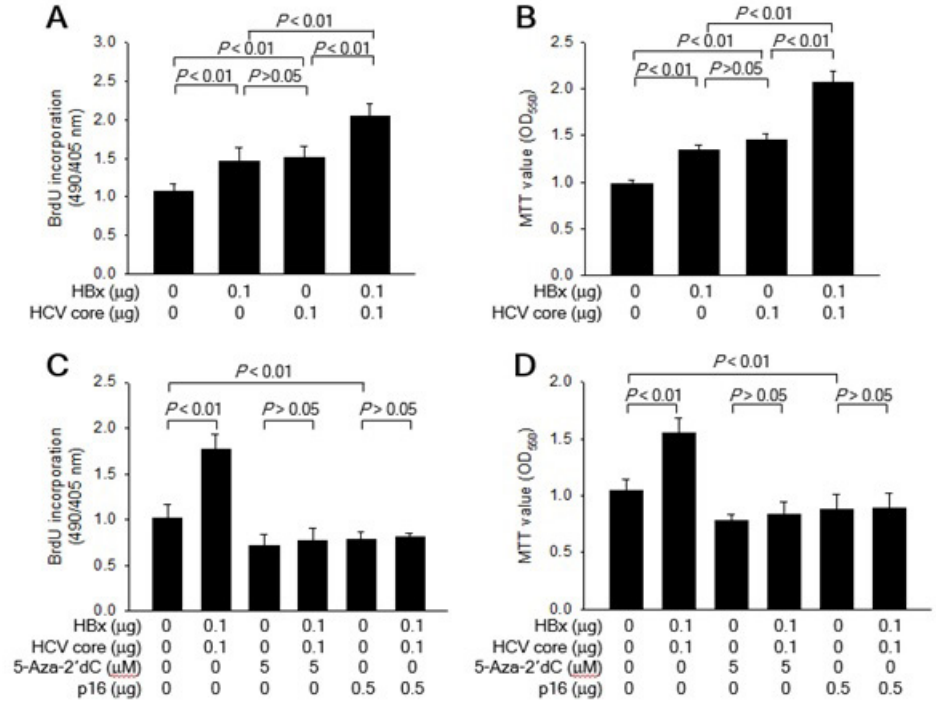

Figure 3: HBx and HCV core protein cooperate to stimulate cell growth by inhibiting p16 expression. Approximately 2 × 10^5 cells per well in 6-well plates were transfected with the indicated plasmids for 48 h. For columns 3 and 4 in (C, D), cells were treated with 5 μM 5-Aza-2'dC for 24 h before harvesting. Cells were subjected to BrdU incorporation (A, C) and MTT assays (B, D) (n = 4).

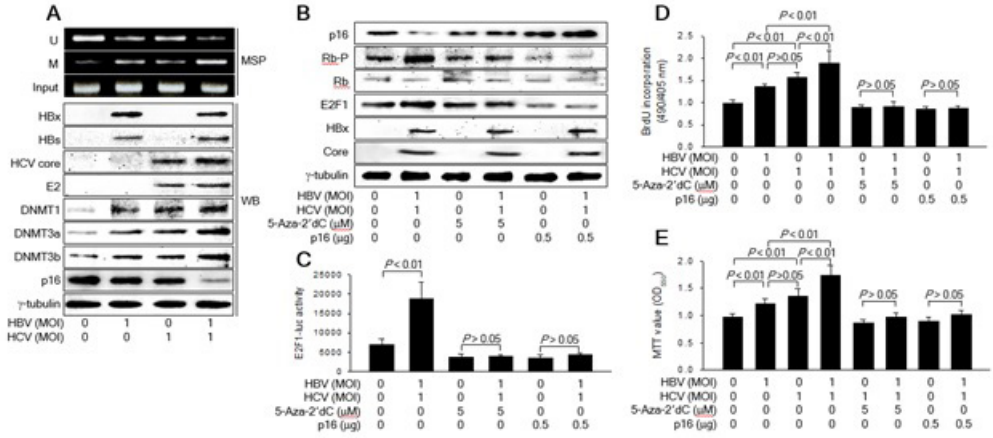

Figure 4: HBV and HCV cooperate to stimulate cell growth by inhibiting p16 expression via DNA methylation during coinfection. (A) Huh7D cells were transfected with an NTCP expression plasmid along with the indicated plasmid for 24 h and either mock-infected or infected with HBV and/or HCV at an MOI of 1.0 for an additional 24 h. (B) Huh7D cells were transiently transfected with 0.1 μg of E2F1-luc along with an empty vector or p16 expression plasmid for 24 h and infected with HBV and HCV as in (A), followed by luciferase assay (n = 3). (C, D, E) Huh7D cells were transiently transfected with either an empty vector or p16 expression plasmid for 24 h and infected with HBV and HCV as in (A). Levels of the indicated proteins were determined by western blotting (C). Cells were subjected to BrdU incorporation (D) and MTT assays (E) (n = 3).

As shown in HepG2 cells (Figure 2C, 3C and 3D), ectopic p16 expression lowered both E2F1 levels (Figure 4B) and its transcription factor activity (Figure 4C) by inactivating the Rb-E2F pathway, decreased the DNA synthesis rate (Figure 4D) and inhibited cell growth (Figure 4E), Accordingly, p16 downregulation in Huh7D cells coinfected with HBV and HCV not only activated the Rb-E2F pathway (Figure 4B and C) but also increased both DNA synthesis and cell growth rates (Figure 4D and E). The biological significance of p16 downregulation in these processes was confirmed by restoration of p16 levels through either treatment with 5-Aza-2'dC or ectopic p16 expression, which almost completely removed the effects of coinfection on the Rb-E2F pathway (Figure 4B and C), DNA synthesis (Figure 4D), and cell growth (Figure 4E). These results suggest that HBx and HCV core protein cooperate to stimulate the growth of

cells coinfected with HBV and HCV by downregulating p16 levels via DNA methylation.

5. Discussion

Several epidemiological studies have demonstrated a close correlation between HBV and HCV coinfection, as compared to monoinfection, and a high risk of HCC development [4,7,8]. Accumulating evidence suggests that HBx and HCV core protein as the representative viral oncoproteins of HBV and HCV, respectively, play critical roles in the development of virus-associated HCC [9,10]. The multifunctional protein HBx of HBV has been implicated in HCC development owing to its roles in the modulation of diverse signaling pathways, transcriptional activation of cellular genes and dysregulation of immune responses, apoptosis, and lipid metabolism [9,10]. In addition to its role as a capsid protein, HCV core protein also has been strongly implicated in HCC development because of its roles in the alteration of diverse signaling pathways, transcriptional activation, and modulation of immune responses, apoptosis, and lipid metabolism [9,10]. Considering the accumulating evidence on their roles in hepatocellular carcinogenesis, it is possible to assume that the additive or synergistic effects of HBx and HCV core protein contributes, at least in part, to the higher risk of HCC development in patients coinfected with both viruses. The present study provides, to our knowledge, the first example of such an additive action of HBx and HCV core protein-cooperative repression of p16 expression via DNA methylation, which can be correlated with an increased risk of HCC development.

Inactivation of the *p16* gene is one of the most common genetic alterations that is associated with progression to malignant HCC [27,28]. DNA methylation appears to be the prominent cause for p16 inactivation in HCC [29,30]. Higher frequencies of DNA methylation have been detected in the p16 promoter from HCCs with HBV or HCV infection [31,32]. Later studies have further demonstrated that HBx and HCV core protein induce promoter hypermethylation of the p16 gene in human hepatocytes to inhibit its expression [13,14]. For this effect, HBx and HCV core protein individually upregulate DNMT1, DNMT3a, and DNMT3b in human hepatoma cells [13,14]. Treatment with a universal DNMT inhibitor, 5-Aza-2'dC, can effectively reactivate p16 expression in HBx- or HCV core-expressing cells to inhibit cell proliferation [13,14]. Most of these studies, however, 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 reproduced in an *in vitro* HBV and HCV coinfection system, which may more 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 NS5A of HCV and HBs proteins of HBV, which are also known to be implicated in the development of HCC [9,10]. The present study may provide the first insight into the increased risk of HCC development in patients coinfected with HBV and HCV. In addition, the p16 inactivation via DNA methylation may serve a potential target for the development of therapeutic schemes to treat HCC patients associated with coinfection with HBV and HCV.

6. Acknowledgement

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