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Hepatitis B X protein upregulates decoy receptor 3 expression via the PI3K/NF-κB pathway

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ABSTRACT

Chronic hepatitis B (CHB) is associated with the development of hepatocellular carcinoma (HCC). Decoy receptor 3 (DcR3) is a tumor necrosis factor receptor that promotes tumor cell survival by inhibiting apoptosis and interfering with immune surveillance. Previous studies showed that DcR3 was overexpressed in HCC cells and that short hairpin RNA (shDcR3) sensitizes TRAIL-resistant HCC cells. However, the expression of DcR3 during hepatitis B virus (HBV) infection has not been investigated. Here, we demonstrated that DcR3 was overexpressed in CHB patients and that DcR3 upregulation was positively correlated with the HBV DNA load and liver injury (determined by histological activity index, serum alanine aminotransferase level, and aspartate aminotransferase level). We found that hepatitis B virus X protein (HBx) upregulated DcR3 expression in a dose-dependent manner, but this increase was blocked by NF-κB inhibitors. HBx also induced the activation of NF-κB, and the NF-κB subunits p65 and p50 upregulated DcR3 by directly binding to the DcR3 promoters. Inhibition of PI3K significantly downregulated DcR3 and inhibited the binding of NF-κB to the DcR3 promoters. Our results demonstrate that the HBx induced DcR3 expression via the PI3K/NF-κB pathway; this process may contribute to the development of HBV-mediated HCC.

1. Introduction

The hepatitis B virus (HBV) infects > 300 million people worldwide, and patients with HBV infections are prone to develop cirrhosis, liver failure, and hepatocellular carcinoma (HCC) [1,2]. HBV is a small, double-stranded, circular DNA virus, which contains four overlapping open reading frames (S, C, P, and X) that encode several viral proteins, including HBe, pre−S1, preS2, pol, core, S, and X [3]. Of these, HBx is believed to be one of the most critical determinants of HCC development, as this protein interferes with numerous host proteins [4–6].

Decoy receptor 3 (DcR3), a member of the tumor necrosis factor receptor (TNFR) superfamily, is a soluble decoy receptor. Since DcR3 lacks a transmembrane domain, this protein can be secreted. DcR3 inhibits cell apoptosis and interferes with immune surveillance by competitively binding with related ligands such as the Fas Ligand (FasL), the lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells (LIGHT), and the TNF-like ligand 1A (TL1A) [7–9]. DcR3 is overexpressed in various inflammatory diseases, including bacterial infections [10], rheumatoid arthritis [11], acute ulcerative colitis [12], and tumors arising from the lung, colon, glia, and gastrointestinal tract [7,13,14].

Recent studies have shown that, in addition to the effects of DcR3 on the progression and immune suppression of tumor cells, this protein also functions as an effector molecule, modulating pathological and physiological activity [CITE]. The DcR3.Fc fusion protein modulates monocyte differentiation and improves monocyte adhesion by upregulating adhesion-associated proteins, cross-linking heparan sulfate proteoglycan to increase ICAM-1 and VCAM-1 in endothelial cells [15,16]. DcR3 also modulates the differentiation and apoptosis of dendritic cells and downregulates MHC-II in tumor-associated macrophages [17,18]. Previous studies showed that Epstein-Barr viral infection upregulated DcR3 via the DcR3 latent membrane protein 1, and increased nasopharyngeal carcinoma cell migration and invasion [19]. However, the regulatory mechanism of DcR3 in HBV infection remains mostly unknown.

DcR3 is overexpressed in HCC cells, and DcR3 knockdown sensitizes

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tumor necrosis factor (TNF) – related apoptosis – inducing ligand (TRAIL) -resistant HCC cells [20]. Recent studies have shown that DcR3 is significantly upregulated in patients with chronic hepatitis B (CHB) [21], indicating that DcR3 may play an essential role in HBV-related HCC. In this study, we first quantified the expression levels of DcR3 in the serum and livers of CHB patients. We then explored the molecular mechanisms underlying HBV-induced DcR3 expression.

2. Materials and methods

2.1. Patients

Blood samples from 132 CHB patients were collected at Songjiang Central Hospital (Shanghai, China) between January 2014 and January 2015. The diagnosis and treatment of all patients complied with previously described criteria [22]. Patients had been treated with antiviral or immunosuppressive agents within six months before blood and liver sampling. Other causes of liver disease, including hepatitis C, hepatitis D, human immunodeficiency virus, autoimmune liver disease, and alcoholic liver disease, were excluded. We selected 70 age- and sex-matched healthy individuals as healthy controls (HCs). Paraffin-embedded liver sections were collected from 34 patients with CHB. We also obtained ten healthy liver specimens, collected from age- and sex-matched donors whose livers were used for transplantation. The study was approved by the Research Ethics Committee of SongJiang Central Hospital (Shanghai, China), and written informed consent was obtained from all study participants. The clinical characteristics of the enrolled populations are listed in Table 1.

2.2. Cells and reagents

The human hepatoma cell line (Huh7 and HepG2) and HepG2.2.15 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Shanghai, China) supplemented with 10% fetal bovine serum (Gibco, NY, USA). The standard hepatocyte cell lines (Chang liver and HL-7702) were grown in RPMI 1640 (Shanghai, China) with 10% fetal bovine serum (Gibco, NY, USA). All cells were cultured at 37 °C in a 5% CO2 incubator.

2.3. Real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, USA) and reverse transcribed into cDNA using a ReverTra Ace qPCR RT Kit (Toyobo, FSQ-101). cDNA was quantified with real-time PCR, using an SYBR RT-PCR kit (QPK-212; Toyobo, Osaka, Japan) and a LightCycler 7500 (Applied Biosystems, Foster, CA, USA). We used specific primers to amplify DcR3 and GAPDH: GAPDH-F (5′-TGACCA CAGTCCATGCCATC-3′), GAPDH-R (5′-GACGGACACATTGGGGG TAG-3′), DcR3-F (5′-TCTCAGCCAGCAGCTCCA-3′), and DcR3-R (5′-CACACTCCTCAGCTCCTGGTAC-3′). The relative expression level of each gene was normalized to Gapdh, and calculated using the eq. $2^{-\Delta\Delta Ct}$, where ΔCt = Ct (target)-Ct (Gapdh), and $\Delta\Delta Ct = \Delta Ct$ of the experimental group-ΔCt of the control group. All reactions were performed at least three times.

Table 1
Clinical characteristics of the studied subjects.

Parameters	HCs	CHB
Case	70	132
Gender (M/F)	41/29	74/58
Age, y (range)	35 (20–63)	38 (21–66)
ALT	27 (8–42)	227 (56–437)
AST	26 (8–39)	233 (46–423)
DNA (log copies/ml)	ND	6.4 (4.0–8.9)

Data are expressed as median and range.
ND, not determined.

2.4. Western blotting

Proteins from lysed tissues or cells were fractionated using a sodium dodecyl sulfate (SDS)-polyacrylamide gel and then transferred to a nitrocellulose membrane. After being blocked in 5% milk in phosphate buffered solution (PBS)for one hour at room temperature, the membranes were incubated with specific primary antibodies at 4 °C overnight. After washing with PBS three times, the membranes were incubated with the secondary antibody for one hour at room temperature. After a final wash, signals were detected using ECL reagent (ThermoFisher Scientific, Rockford, USA).

2.5. Immunohistochemistry

Liver tissues were fixed in 4% paraformaldehyde for 12 or 24 h, embedded in paraffin and cut into 4–5 μm slides. After deparaffinization, hydration, and blocking with 1.5% blocking serum, the slides were incubated with anti-DcR3 Ab (1:200; ab57956) at 4 °C overnight. The peroxidase-coupled secondary antibody was then applied for 30 min at room temperature. Antibody binding was detected with a diaminobenzidine solution. The slides were then counterstained with hematoxylin; brown-yellow staining was considered a positive signal. Stained slides were observed using a light microscope (Leica, Wetzlar, Germany).

2.6. Plasmids and transfection

PcDNA3.1-HBV plasmids, each containing 1.3 copies of the full-length HBV genomic sequence, were constructed in our lab. All other plasmids were constructed by Heyuan Biotechnology (Shanghai, China). For the overexpression assay, HepG2 cells were plated on six-well plates at a density of 5×10^4 cells/well. After 12–24 h, cells were transfected with either the overexpression plasmids or the control vector using Lipofectamine 2000 (Invitrogen, Carlsbad, USA). After an additional 12 or 24 h, the cells were lysed or incubated for further study. Cells used for the luciferase assay were plated for 12–24 h, and then transfected with a mixture of 0.6 μg expression plasmids, 0.18 μg promoter reporter plasmids, and 0.02 μg pRL-TK plasmids using Lipofectamine 2000. At five hours post transfection, the medium was replaced with fresh medium supplemented with 1% FBS. Cells were then cultured for an additional 24 h.

2.7. Luciferase reporter assay

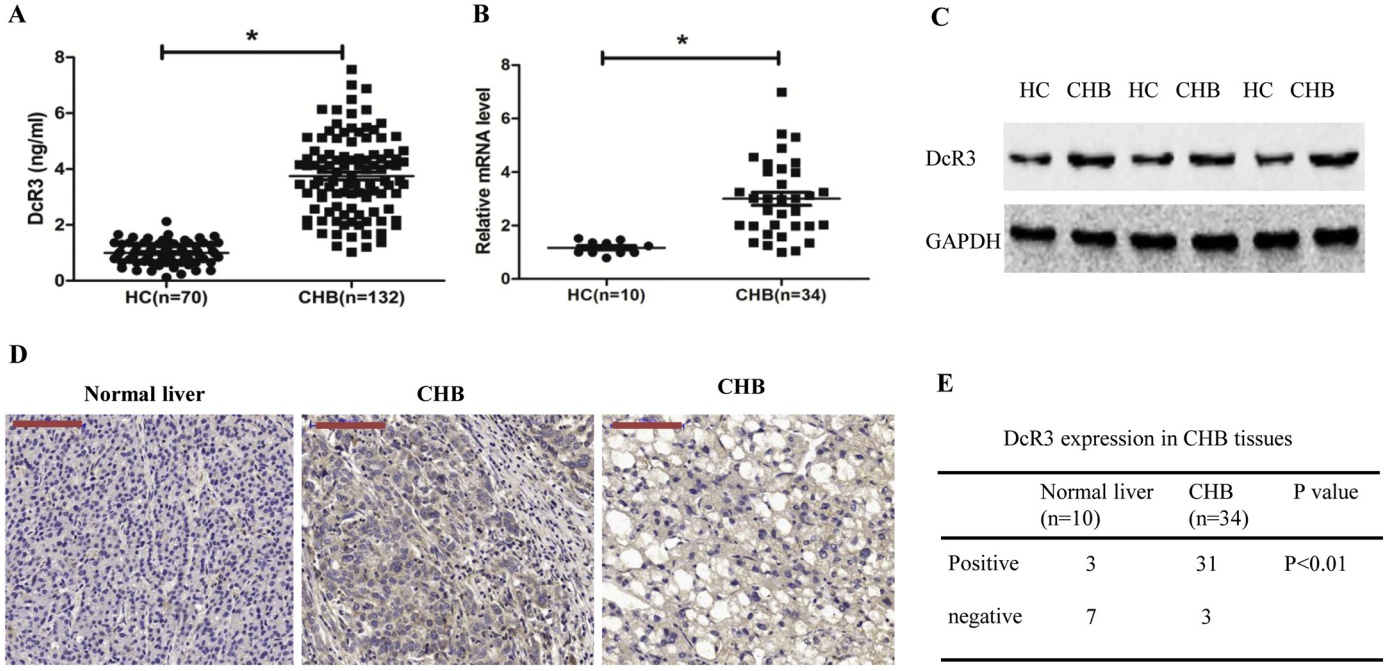
Luciferase activity was measured with a Dual Luciferase Assay (Promega, USA). At 48 h post-co-transfection with the promoter luciferase reporter plasmid and the control plasmid, cells were lysed with reporter lysis buffer, and the luciferase activity levels in the lysates were measured with a Modulus TD-20/20 Luminometer (Turner Biosystems, USA). Luciferase activity was normalized against Renilla. The promoter-reporter plasmids and mutant plasmids were designed and constructed by GenePharma Co., Ltd. (Shanghai, China).

2.8. ELISA

DcR3 levels in the serum or culture supernatants were determined using ELISA kits (R&D Systems, Abingdon, UK) following the manufacturer's instructions.

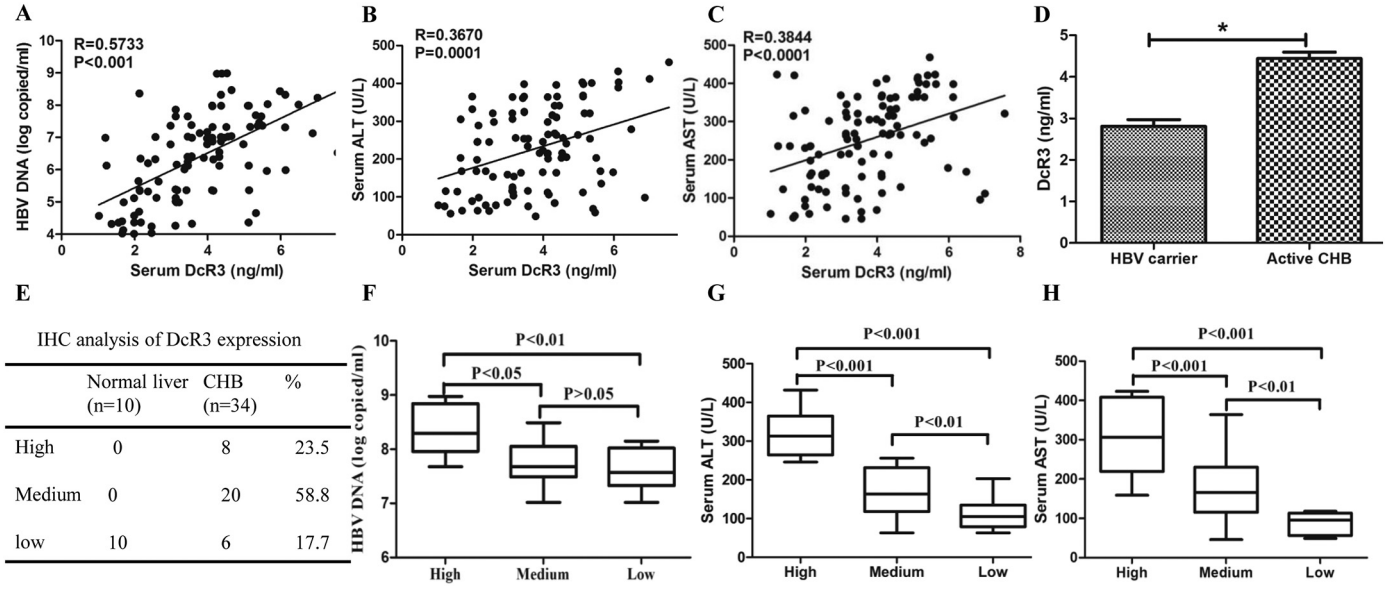
2.9. EMSA

EMSAs were performed using a LightShift Chemiluminescent EMSA Kit (Thermo, Massachusetts, USA) following the manufacturer's instructions. For the DNA binding reaction, 4 μg of nuclear extract was



	Normal liver (n=10)	CHB (n=34)	P value
Positive	3	31	P<0.01
negative	7	3	

Fig. 1. Serum and hepatic expression of DcR3 are upregulated in CHB patients. A, ELISA analysis of serum DcR3 levels in CHB patients and HCs. B, Real-time PCR analysis of hepatic DcR3 mRNA levels in CHB patients and HCs. C, Western blot analysis of hepatic DcR3 protein levels in three different CHB patients and three different HCs. D, Immunohistochemical analysis of hepatic DcR3 protein levels in two different CHB patients and one HCs. E, Statistical analysis of the results of the immunohistochemistry. *$p < 0.01$.



	Normal liver (n=10)	CHB (n=34)	%
High	0	8	23.5
Medium	0	20	58.8
low	10	6	17.7

Fig. 2. Increased DcR3 expression is associated with viral loads and liver injury. A, B and C, Correlation analysis of serum DcR3 levels with HBV DNA (A) and serum ALT (B) and AST (C). D, ELISA analysis of serum DcR3 levels in patients with active CHB and patients who are CHB carriers. E, Statistical analysis of DcR3 expression in CHB and HCs liver tissues. F, G and H, Distribution of serum HBV DNA (F), ALT (G) and AST (H) in CHB patients with low, medium and high DcR3 expression. Statistical significance and correlation coefficients are indicated. *$p < 0.01$.

incubated for 20 min at room temperature in a reaction buffer containing 40 fmol of biotin-labeled probes, 2 μl binding buffer, and 50 ng poly (I:C). To construct the competitor EMSA, a 100-fold volume of unlabeled oligonucleotides were added before the labeled probe. Samples were separated on a 6% polyacrylamide gel for 30 min, and a chemiluminescent detection method was used to identify the samples. Biotin end-labeled DNA probes were synthesized and generated by annealing complementary oligonucleotides.The p65 probe sequence was 5′-AGTTGAGGGGACTTTCCCAGGC- 3′.

2.10. Chromatin immunoprecipitation assay

Formaldehyde was used to cross-link the HepG2 cells for 10 min at 37 °C. After washing with PBS, cells were resuspended in a 300 μl lysis buffer containing 50 mMTris (pH 8.1), 10 mMEDTA, 1% SDS, and 1 mMPMSF. The cells were then sonicated into fragments. Herring sperm DNA and a G-Sepharose protein slurry (Sigma-Aldrich) were used to preclear the supernatants at 4 °C overnight.

The precleared supernatants were immunoprecipitated with anti-p50, p65, p52, c-Rel, RelB, or an isotype control for 2 h. We then added

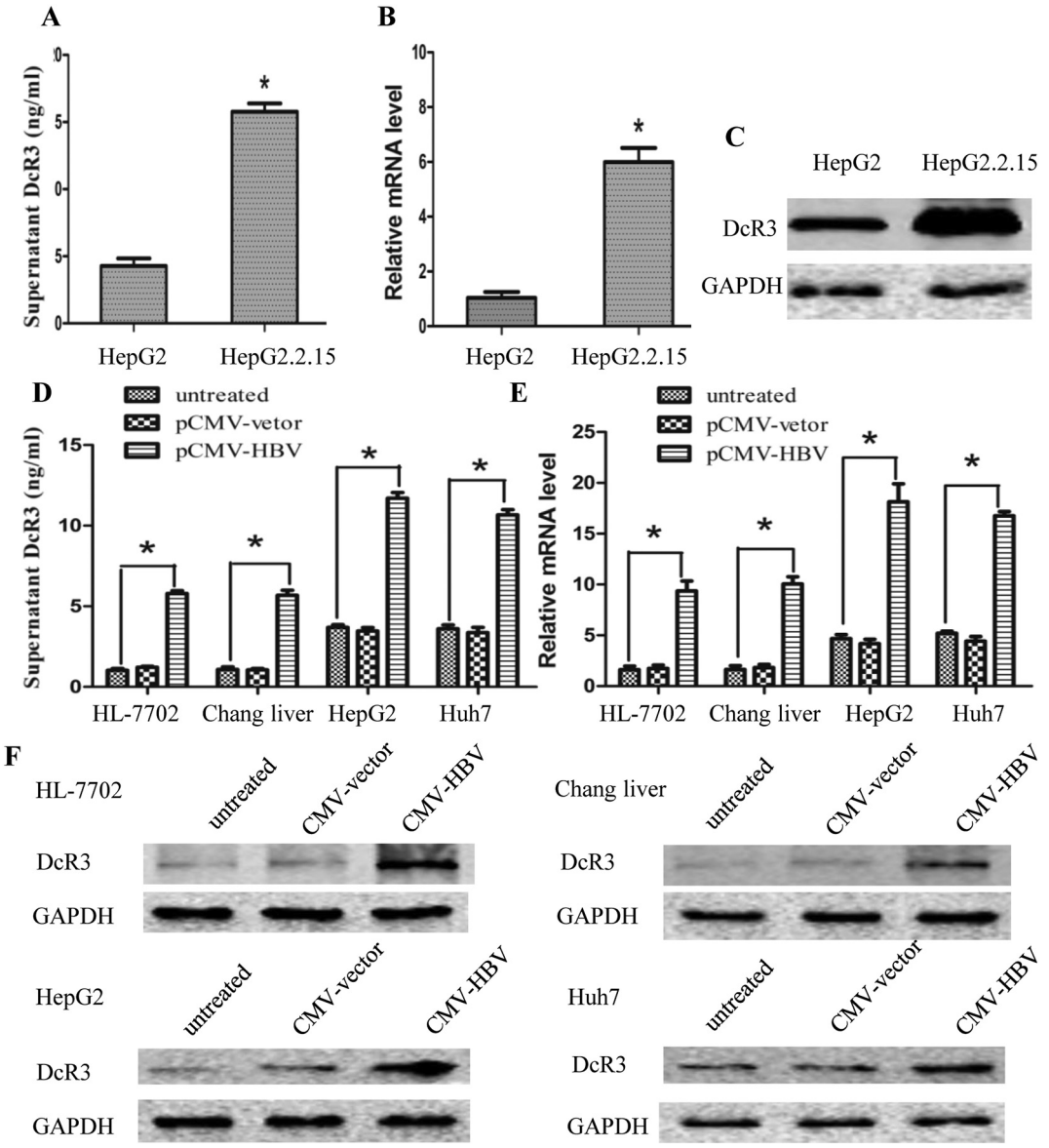


Fig. 3. HBV induces DcR3 expression in hepatocytes. A, ELISA analysis of supernatant DcR3 levels in HepG2 and HepG2.2.15 cells. B and C, Real-time PCR and western blot analysis of cellular DcR3 mRNA levels and protein levels in HepG2 and HepG2.2.15 cells. D, E and F, After transfected with the pcDNA3.1-flag-HBV which contained 1.3 copies of full-length HBV genome into four hepatocytes, supernatant DcR3 levels were detected by ELISA (D), cellular DcR3 mRNA levels were detected by real-time PCR (E) and cellular DcR3 protein levels were measured by western blot (F). Each experiment performed in triplicates and each value represents the mean ± SEM of 3 independent experiments. *$p < 0.01$ compared with control.

1% SDS and a 1.1 M $NaHCO_3$ solution to retrieve the DNA-protein complexes at 65 °C for 6 h. After purifying the extracted DNA using a PCR purification kit (Qiagen, USA), DcR3 was amplified using real-time PCR with DcR3 promoter-specific primers (forward: 5′-AGTTGGCAGA GGCCCC; reverse: 5′-ACCCACCTGGTACCATCCC).

2.11. Nuclear and cytoplasmic protein extraction

A NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific, Rockford, USA) was used to extract the nuclear and cytoplasmic proteins following the manufacturer's instructions. Cells were suspended in CER I for 10 min, followed by the addition of ice-cold CER II. After vortexing and centrifugation, the cytoplasmic supernatant was removed. The nuclear pellet was suspended in ice-cold NER. After vortexing and centrifugation, the supernatant (nuclear extract) was transferred to a new tube.

2.12. Immunofluorescence staining and confocal microscopy

HepG2 cells were fixed with 4% formaldehyde for 30 min at room temperature and then permeabilized with 0.1% Triton X-100 for 5 min. To block non-specific binding, 1% bovine serum albumin was added for 5 min. Cells were incubated with anti-p65 antibody and secondary antibody and then incubated with DAPI for 5 min. Finally, cells were examined under a confocal laser scanning microscope (TCSSP2; Leica, Wetzlar, Germany).

2.13. Statistical analyses

Data are presented as means ± SEM. We used Student's *t*-tests to determine the significance of differences between the two groups, and ANOVAs were used to determine the significance of differences among two or more groups. Correlations were evaluated using Spearman's rank correlation. We considered $p < 0.05$ statistically significant. All

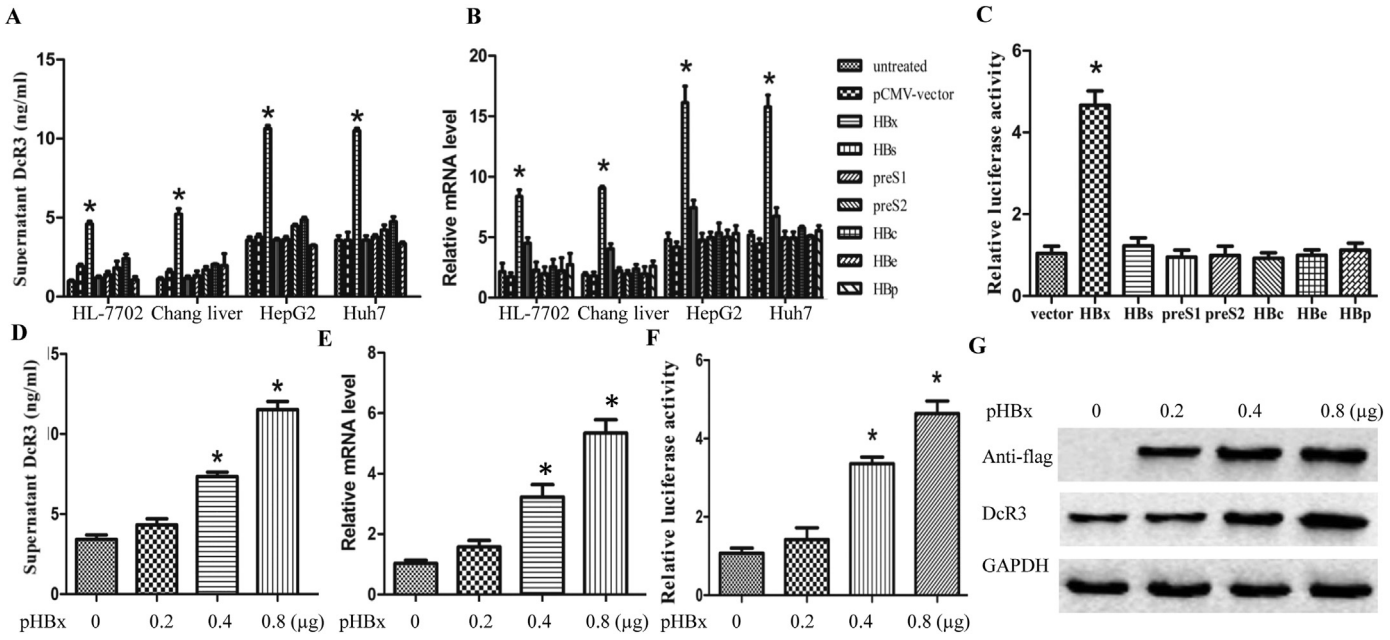


Fig. 4. HBx induces DcR3 expression and transactives its promoters activity. A and B, After transfected with seven HBV viral protein plasmids (Pre−S1, S2, HBc, HBe, HBx or HBp) into four different hepatocytes, respectively, supernatant DcR3 levels were detected by ELISA (A), cellular DcR3 mRNA levels were detected by real-time PCR (B). C, After co-transfected with DcR3 promoter luciferase reporter plasmids and the seven viral protein plasmids into HepG2 cells, relative luciferase activities were detected by standard procedures. D, E and F, HepG2 cells were transfected with HBx expression plasmids in a dose dependent manner, and then supernatant DcR3 levels were detected by ELISA (D), cellular DcR3 mRNA levels were detected by real-time PCR (E), and relative luciferase activities were detected by standard procedures (F). G, HBx protein was detected by western blot. Each experiment performed in triplicates and each value represents the mean ± SEM of 3 independent experiments. *$p < 0.01$ compared with control.

statistical analyses were performed in SPSS (SPSS Science, Chicago, IL, USA).

3. Results

3.1. DcR3 is significantly upregulated in the serum and livers of CHB patients

To explore the association between DcR3 and HBV infection, we first measured the expression levels of DcR3 in the serum samples from 132 CHB patients and 70 HCs using ELISAs. We found that DcR3 levels in the serum from CHB patients were significantly higher than those from the HCs (Fig. 1A). Real-time PCRs and western blots were used to quantify hepatic DcR3 expression. DcR3 mRNA and protein expression levels were significantly higher in CHB patients, as compared to the HCs (Fig. 1B, C). Furthermore, immunohistochemical staining of liver tissue sections showed that DcR3 was mainly expressed in the bile duct epithelial cells, infiltrating lymphocytes as well as hepatocytes in CHB liver tissues. In contrast, little or no DcR3 expression was observed in normal liver tissues (Fig. 1D, E).

3.2. DcR3 upregulation is associated with increased viral load and liver injury

To investigate whether DcR3 upregulation is correlated with viral replication and liver injury, we measured levels of DcR3, HBV DNA, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) in the serum of the CHB patients. Spearman's correlation analysis showed that the DcR3 expression level in the serum was positively correlated with serum levels of HBV DNA, ALT, and AST (Fig. 2A–C).

To investigate if active viral replication is correlated with DcR3 upregulation, we compared serum DcR3 expression levels between patients with active CHB and HBV carriers. We found that patients with active CHB had higher levels of DcR3 expression (Fig. 2D). Following the methods used to detect HER-2 IHB expression in breast cancer patients [23], we categorized CHB patients as high, medium, or low based on our immunohistochemical staining of DcR3. We then compared levels of HBV DNA, ALT, and AST among CHB patients with high, medium, and low levels of DcR3 expression. Patients with high DcR3 expression levels also had high levels of HBV DNA, ALT, and AST (Fig. 2E–H).

3.3. The HBx protein induces DcR3 expression and transactivates DcR3 promoters

To understand whether HBV infection upregulates DcR3, we measured the expression of DcR3 in HepG2 and HepG2.2.15 cells, which had been transfected with 1.3 copies of the full-length HBV genome. DcR3 mRNA and protein expression levels were much higher in the HepG2.2.15 cells than in the HepG2 cells (Fig. 3A–C), suggesting that HBV infection might trigger DcR3 expression. To verify that HBV directly induces DcR3 expression, we transfected pcDNA3.1-flag-HBV, which contained 1.3 copies of the full-length HBV genome in four hepatocytes. This transfection significantly increased DcR3 mRNA and protein expression levels in the four hepatocytes (Fig. 3D–F).

To understand how HBV induces DcR3 expression, we constructed seven different types of plasmid, each encoding one HBV viral factor (Pre−S1, S2, HBc, HBe, HBx, and HBp). Each of these plasmids was transfected into four different types of hepatocytes. ELISAs were used to detect the DcR3 secreted into the supernatant, and real-time PCR was used to detect cellular DcR3 mRNA expression. Both protein and mRNA DcR3 expression increased only in cells transfected with the HBx-encoding plasmid (Fig. 4A, B). We then transfected HepG2 cells with different concentrations of the HBx expression plasmid and found that DcR3 expression increased both in the supernatant and within the cell as the concentration of the pHBx plasmid increased (Fig. 4D, E). HBx protein expression levels were verified using western blots (Fig. 4G). Next, we co-transfected DcR3 promoter luciferase reporter plasmids and each of the seven viral protein plasmids into HepG2 cells. We found that DcR3 transcription was increased markedly by HBx in a dose-

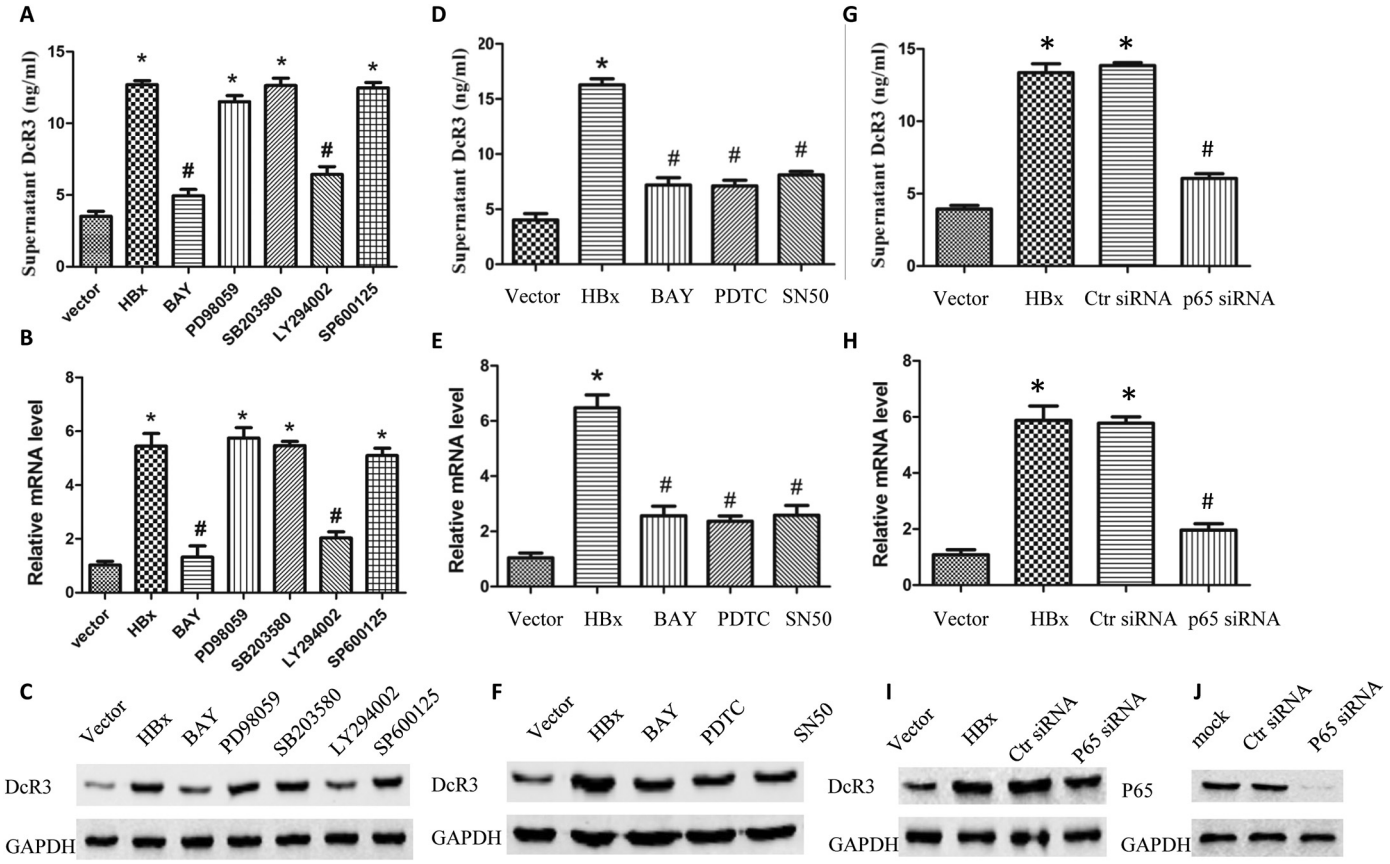


Fig. 5. NF-κB activity is required for HBx- induced DcR3 expression. A, B and C, Before transfected with pHBx, HepG2 cells were pretreated with DMSO (0.1%), BAY (20 μg/ml), PD98059(20 μM), LY294002 (25 μM), SB202190 (5 μM) or SP600125 (20 μM) for 30 min, and then supernatant DcR3 levels were detected by ELISA (A), cellular DcR3 mRNA levels were detected by real-time PCR (B), and cellular DcR3 protein levels were detected by western blot (C). D, E and F, HepG2 cells were pretreated with three different NF-κB inhibitors before transfection with pHBx, and supernatant DcR3 levels were detected by ELISA (D), cellular DcR3 mRNA levels were detected by real-time PCR (E), and cellular DcR3 protein levels were detected by western blot (F). G, H and I, Before transfected with pHBx, HepG2 cells were transfected with p65 siRNA for 12 h, and supernatant DcR3 levels were detected by ELISA (G), cellular DcR3 mRNA levels were detected by real-time PCR (H), and cellular DcR3 protein levels were detected by western blot (I). J, P65 protein was detected by western blot. Each experiment performed in triplicates and each value represents the mean ± SEM of 3 independent experiments. *$p < 0.01$ compared with vectorl. # $p < 0.01$ compared with pHBx-transfected cells.

dependent manner (Fig. 4C, F). Together, these results indicate that, during HBV infection, HBx is a critical factor for the upregulation of DcR3.

3.4. NF-κB activity is required for HBx-induced DcR3 expression

To determine how HBx induces DcR3 expression, we used various signal pathways inhibitors to inhibit the activation of certain signaling pathways. We pretreated HepG2 cells with BAY or LY294002, which inhibit the activation of NF-κB or PI3K, respectively. This pretreatment significantly reduced HBx-induced DcR3 expression. However, pretreatment of cells with PD98059, SB203580, or SP600125, which inhibit the activation of ERK1/2, P38 MAPKs, and JNK, respectively, did not significantly affect HBx-induced DcR3 expression (Fig. 5A–C). To determine whether HBx stimulates DcR3 expression via the NF-κB signaling pathway, we used NF-κB siRNA and three different NF-κB inhibitors (BAY, PDTC, and SN50) to inhibit NF-κB activation before pHBx transfection. NF-κB knockdown was verified with western blots. We found that HBx-induced DcR3 protein and mRNA upregulation was significantly inhibited after the inhibition of DcR3 (Fig. 5D–I). Luciferase activity was also inhibited considerably after the knockdown of NF-κB (Fig. 6A, B). These results suggested that NF-κB activity is required for HBx-induced DcR3 expression.

To investigate the mechanisms underlying NF-κB-induced DcR3 expression, we used bioinformatics analyses to identify the putative NF-κB binding sites located in the DcR3 promoter. We identified two sections; one located from nt-1531 to nt-1522 (NF-κB1) and one situated from nt-710 to nt-701 (NF-κB2).To explore the roles of these binding sites in the regulation of HBx-induced DcR3 expression, we mutated these sites (Fig. 6C). HBx-induced DcR3 promoter activity was significantly reduced after the mutation of NF-κB2, while the mutation of NF-κB1 did not substantially affect HBx-induced DcR3 promoter activity. To determine the role of the NF-κB binding site in the DcR3 promoter, we performed a series of 5′ deletions in the DcR3 promoter. We found that the suppression of nt-1602 to nt-1522 did not affect HBx-induced DcR3 luciferase activity. However, the suppression of nt-1522 to nt-701 reduced HBx-induced DcR3 luciferase activity (Fig. 6D). These results suggest that HBx upregulates DcR3 transcription by activating NF-κB.

3.5. The NF-κB subunit p65/p50 binds to the DcR3 promoter

We next investigated whether HBx-induced DcR3 transcription due to the direct binding of NF-κB to the DcR3 promoter. EMSAs were performed, using the biotin-labeled NF-kB consensus oligonucleotides in the DcR3 promoter as a probe. When transfected with the HBx expression plasmid, the DNA binding activity of NF-κB increased notably, as compared to NF-κB activity after transfection with the control plasmid (Fig. 7A).NF-κB binding specificity was verified by incubating the p65 antibody with either the unlabeled probe or the mutated probe.

In addition to p65 and p50, c-Rel, Rel, and p52 are also subunits of NF-κB. To confirm the direct binding between NF-κB and the DcR3

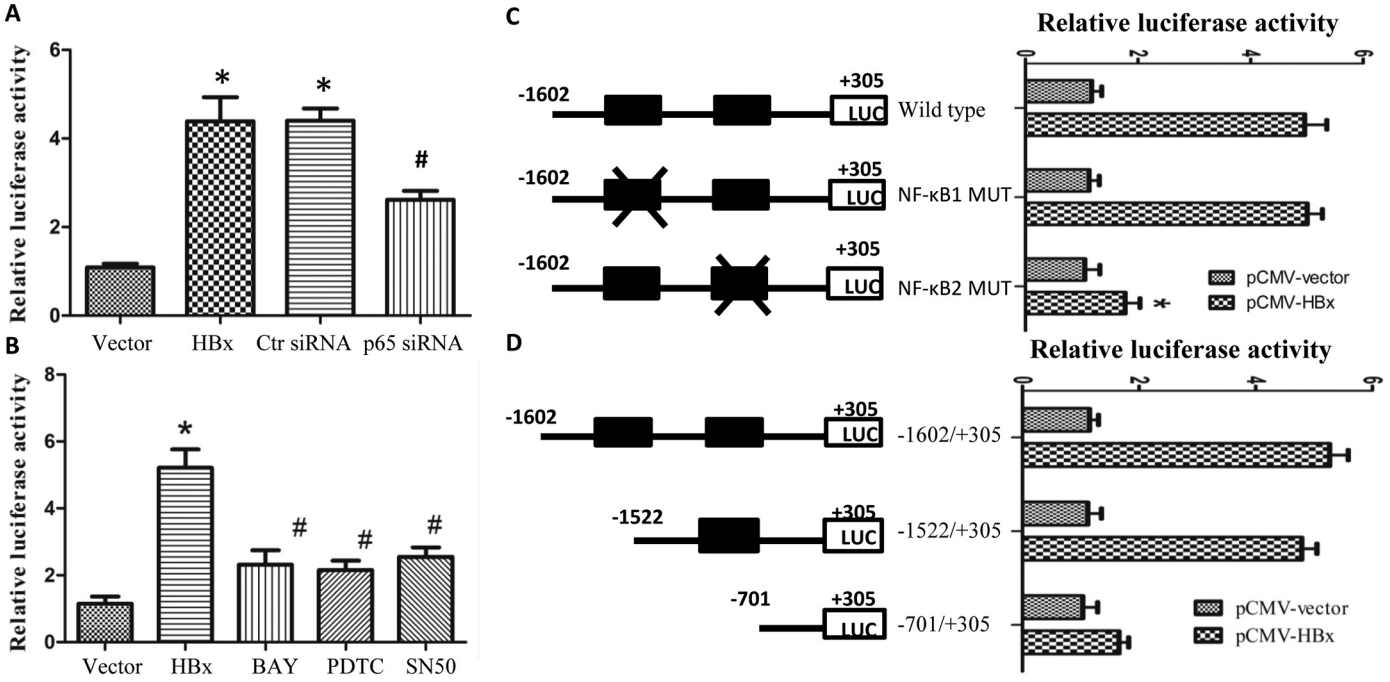


Fig. 6. NF-κB sites in the DcR3 promoters are involved in HBx-induced DcR3 expression. A and B, HepG2 cells were pretreated with p65 siRNA for 12 h or NF-κB inhibitors for 30 min before transfection with the reporter vector and pHBx, and luciferase activity was measured. C and D, HepG2 cells were cotransfected with pHBx and mutated or truncated DcR3 promoter, respectively, and the relative luciferase activity was determined. The left panel represents the constructs of the reporter gene. The right panel represents the luciferase activity in each of the transfected samples. Each experiment performed in triplicates and each value represents the mean ± SEM of 3 independent experiments. *$p < 0.01$ compared with vector. # $p < 0.01$ compared with pHBx-transfected cells.

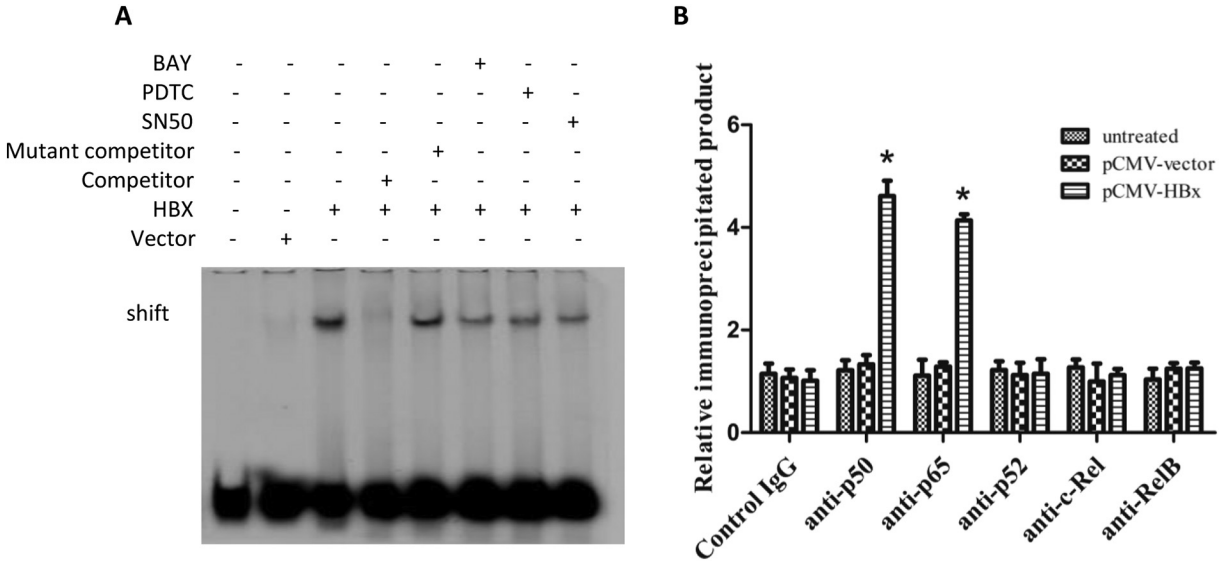


Fig. 7. NF-κB subunits p65/p50 bind to the DcR3 promoter directly. A, After transfected with pHBx or control plasmid for 48 h in the absence or presence of three NF-κB inhibitors, nuclear protein were extracted from HepG2 cells and subjected to EMSA. B, p65, c-Rel, RelB, p50, and p52 were immunoprecipitated with DNA fragments prepared from untreated, vector-transfected or pHBx-transfected HepG2 cells using the CHIP assay. Each experiment performed in triplicates and each value represents the mean ± SEM of 3 independent experiments. *$p < 0.01$ compared with untreated group.

promoter, we performed a chromatin immunoprecipitation (CHIP) assay using five subunits of the NF-κB family. Chromatin fragments were prepared from HepG2 cells transfected with either the control plasmid or the HBx expression plasmid. The chromatin fragments were then immunoprecipitated against each of these subunits with Abs. The binding between NF-κB p65/p50 and the DcR3 promoter was much stronger in cells transfected with the HBx expression plasmid than in cells transfected with the control plasmid (Fig. 7B). However, the DcR3 promoter could not be immunoprecipitated with c-Rel, RelB, or p52. These results indicate that HBx increases the binding of p65/p50 to the DcR3promoter.

3.6. The NF-κB p65/p50 subunit upregulates DcR3

To investigate the effects of NF-κB on DcR3expression, we transfected HepG2 cells with the p65-overexpression plasmid or the p50-overexpression plasmid. We then measured DcR3 expression. Transfection with either p65 or p50 upregulated DcR3 expression. Co-

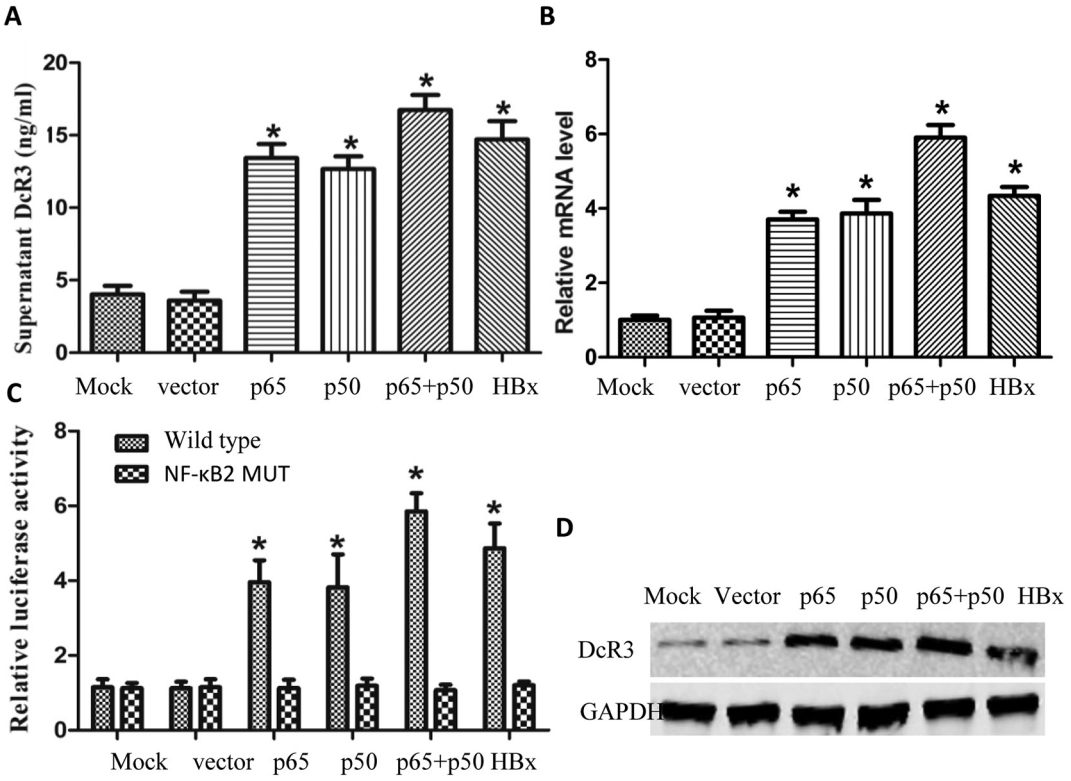


Fig. 8. The NF-κB subunits p65/p50 increase the expressing of DcR3. A, B and D, After transfected with p65 and/or p50 expression plasmids for 24 h, supernatant DcR3 levels were measured with an ELISA kit (A), cellular DcR3 RNA levels were detected by real time PCR (B) and cellular DcR3 protein levels were determined by western blot (D). C, After transfected with wild type or NF-κB2 MUT plasmids and p65 or p50 expression plasmids for 24 h, the luciferase activity of DcR3 was measured. Each experiment performed in triplicates and each value represents the mean ± SEM of 3 independent experiments. *$p < 0.01$ compared with untreated group.

transfection with both p65 and p50 strongly increased both protein and mRNA DcR3 expression levels, as compared to transfection with the control plasmid (Fig. 8A, B and D). Furthermore, when co-transfected with p65 or p50 plasmids and DcR3 promoter reporter plasmids, the luciferase activity of DcR3 was higher compared to co-transfection with control plasmids, and the luciferase activity was highest when cells were transfected with both p65 and p50. However, the increased luciferase activity of DcR3 was abolished by mutating the NF-κB2 binding site in the DcR3 promoter (Fig. 8C).

3.7. HBx induces the activation of NF-κB

Our results suggest that the activation of NF-κB is required for HBx-induced DcR3 expression. Therefore, we investigated whether HBx activated NF-κB. The protein expression levels of phosphor(p)-p65 in HepG2 cells, as measured with western blots, increased significantly after transfection with the HBx expression plasmid (Fig. 9A). P65 was localized more into nucleus under a confocal microscope when transfection with HBx expression plasmid, and both the nucleus p65 and p50 was significantly increased after nuclear and cytoplasmic separation (Fig. 9B, C). The expression of HBx was verified using western blots (Fig. 9D). The results showed that p65 and p50 protein expression in the nucleus was significantly upregulated after transfection with the HBx expression plasmid. These results indicate that HBx induces the activation of NF-κB.

3.8. HBx-induced DcR3 expression is mediated by the PI3K-NF-κB signaling pathway

The increase in HBx-induced DcR3 expression was reversed not only by CAMP (an inhibitor of NF-κB), but also by LY294002 (an inhibitor of PI3K) (Fig. 4), suggesting that the PI3K signaling pathway might be involved in HBx-induced DcR3 expression. We, therefore, investigated the possibility that HBx induced DcR3 expression via PI3K activation. The protein expression levels of phosphor (p) -PI3K in HepG2 cells, as measured with western blots, increased significantly after transfection with HBx expression plasmid (Fig. 10A). Then we transfected siRNA against PI3K into HepG2 cells before transfection with the HBx expression plasmid. DcR3 expression was detected with western blots and real-time PCR. We found that PI3K knockdown significantly inhibited HBx-induced DcR3 protein and mRNA expression (Fig. 10B, C and D).

To determine whether HBx-induced DcR3 expression is dependent on the PI3K-p65/p50 pathway, HepG2 cells were co-transfected with DcR3 promoter reporter plasmids containing NF-κB binding sites and HBx overexpression plasmids after transfection siRNA against PI3K for 12 h. We found that PI3K knockdown significantly inhibited the luciferase activity induced by HBx (Fig. 10E). The EMSA indicated that the binding of NF-κB to the CD36 promoter was also significantly inhibited after the knockdown of PI3K. (Fig. 10F). Thus, our data suggest that the PI3K-p65/p50 pathway might mediate the induction of DcR3 by HBx.

4. Discussion

DcR3, a soluble TNFR, is overexpressed in several diseases, including inflammatory diseases, autoimmune diseases, and cancer. By binding to LIGHT and TL1A, DcR3 inhibited cell apoptosis and interfered with immune surveillance; immune surveillance increases the survival rates of infected cells [24,25]. Lou et al. demonstrated that DcR3 is overexpressed in CHB patients and that DcR3 expression was correlated with the HBV DNA load [21]. This suggested that HBV might affect DcR3 expression. Here, DcR3 was upregulated in both the serum and the livers of CHB patients, as compared with the healthy controls.

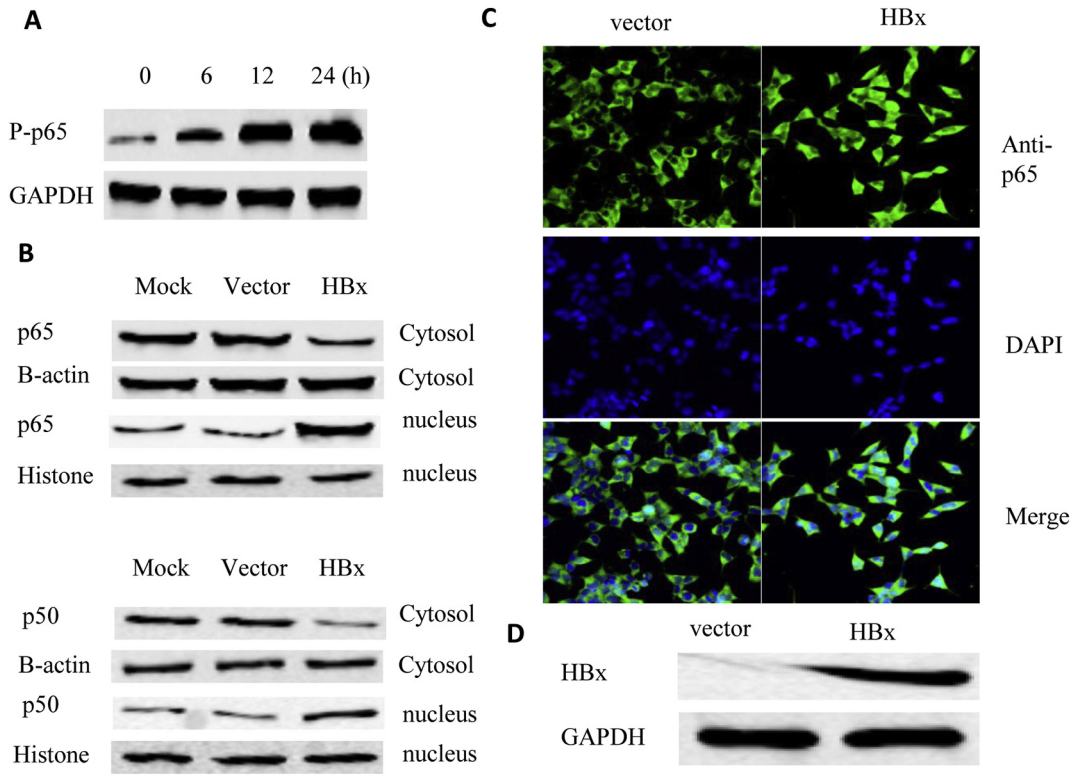


Fig. 9. HBx induces the activation of NF-κB. A, HepG2 cells were transfected with pHBx for the indicated time, and the phosphorylated (p) – 65 was detected by western blot. B, After transfected with pHBx into HepG2 cells for 24 h, the cytosolic and nuclear p65 and p50 proteins were extracted and determined by western blot. C, The cells were fixed, strained with anti-p65 antibody and immunofluorescence was determined by laser-scanning confocal microscopy after being transfected with pHBx for 24 h. D, HBx protein was detected by western blot. Each experiment performed in triplicates and each value represents the mean ± SEM of 3 independent experiments. *$p < 0.01$ compared with untreated group.

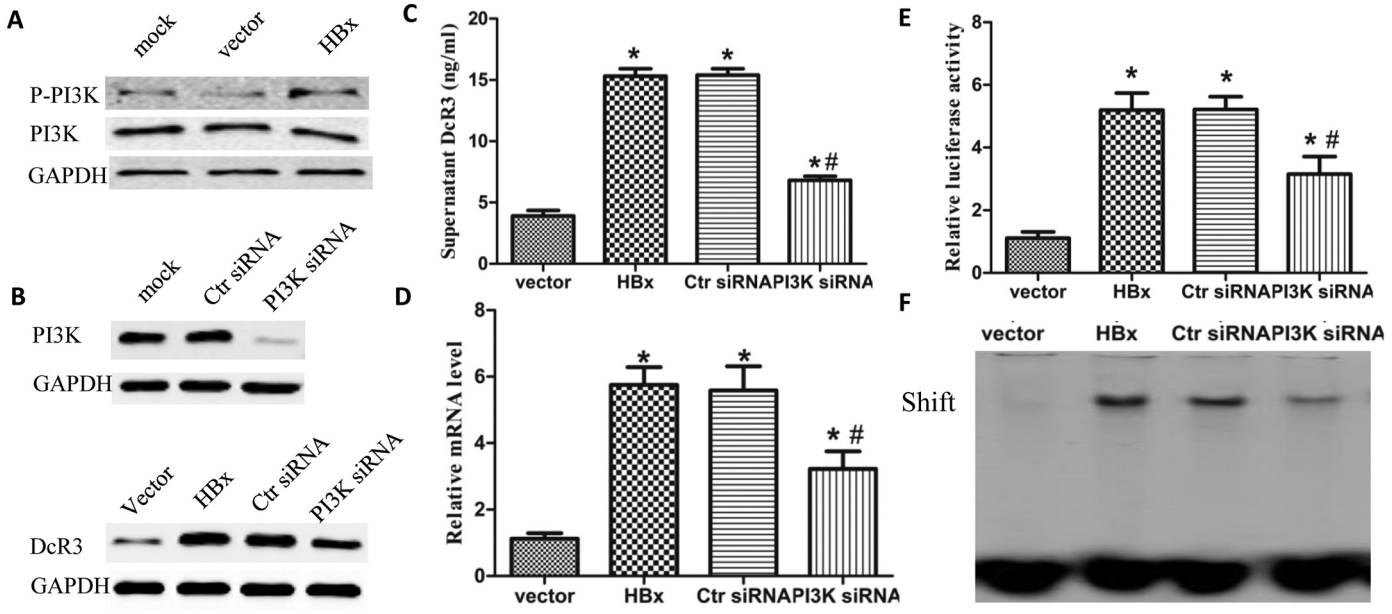


Fig. 10. PI3K-NF-κB signaling pathway mediates HBx-induced DcR3 expression.
A, HepG2 cells were transfected with pHBx for 24 h, and the phosphorylated (p) – PI3K and PI3K were detected by western blot. B–F, After pretreated with control siRNA or PI3K siRNA for 12 h, the HepG2 cells were transfected with pHBx for next 24 h. Cellular DcR3 protein were detected by western blot (B), supernatant DcR3 protein were detected by ELISA (C), cellular DcR3 RNA levels were detected by real time PCR (D), luciferase activity was determined by standard method (E), the binding of NF-κB to the DcR3 promoter was measured by EMSA (F). Each experiment performed in triplicates and each value represents the mean ± SEM of 3 independent experiments. *$p < 0.01$ compared with untreated group.

Both serum and hepatic DcR3 expression levels were positivity correlated with the HBV DNA load and liver injury markers such as ALT and AST. Besides, patients with active CHB or with higher HAI scores (as determined by liver biopsy) had higher serum and hepatic DcR3 expression levels. Our results suggest that DcR3 is closely associated with HBV infection and liver damage.

DcR3 is upregulated in response to various infectious diseases. DcR3 was upregulated in the skin lesions of psoriasis patients with Kaposi's sarcoma-associated herpesvirus, as well as in human umbilical vein endothelial cells infected with Kaposi's sarcoma-associated herpesvirus in vitro [26,27]. In Epstein Barr Virus (EBV)-associated nasopharyngeal carcinoma (NPC) cases, DcR3 was upregulated by latent EBV membrane proteins via the NF-κB signaling pathway [19]. Here, DcR3 was upregulated in patients with HBV infections. Furthermore, our immunohistochemistry results indicate that DcR3 was expressed not only in bile duct epithelial cells and infiltrating lymphocytes, but also in the hepatocytes. This is consistent with a previous study, which showed that cultured hepatoma cells are capable of producing DcR3 [28]. To verify the HBV-induced upregulation of DcR3 directly, we constructed plasmids carrying the full-genome of HBV and transfected these plasmids into hepatocytes. We found that both protein and mRNA DcR3 expression was upregulated, suggesting that HBV directly induced DcR3. Indeed, we specifically showed that the HBV X protein induced DcR3 transcription.

DcR3 is expressed in many types of cells and is regulated by various factors. In human renal cell carcinoma, DcR3 upregulation is mediated by the PI3K/AKT signaling pathway [29], while in gastric cancer cells, DcR3 is regulated by GSK-3β via the inactivation of the transcription factor NFAT [30]. Epstein-Barr virus, a ubiquitous herpesvirus that often infects B-lymphocytes, regulates DcR3 expression by binding to the promoter of the transcriptional factor RTA [31]. Besides, the EBV latent membrane protein-1 induced DcR3 expression by activating the NF-κB signaling pathway [19].

To identify the pathway involved in the HBx-induced upregulation of DcR3, we used different signal pathway inhibitors. We found that inhibition of NF-κB and PI3K suppressed the upregulation of DcR3 induced by HBx. To further determine the direct effects of NF-κB on HBx-induced DcR3 expression, we used siRNAs to knockdown NF-κB. We found that NF-κB knockdown suppressed both HBx-induced DcR3 expression and HBx-induced DcR3 transcription. Mutation of the NF-κB binding sites on the DcR3 promotor also suppressed HBx-induced DcR3 transcription. These results demonstrate that NF-κB was essential for the regulation of HBx-induced DcR3 expression and transcription.

NF-κB, which is a five-member transcription complex comprising the subunits p65, cRel, RelB, p50, and p52, is a member of the Rel family of transcription factors [32]. NF-κB subunits, through homo- or heterodimerization, become transcription factors; these transcription factors bind to the promoters of many genes, including those involved in the immune, inflammatory, and apoptosis responses, and regulate the expression of those genes [33–35]. Here, the EMSA showed that NF-κB bound to the DcR3 promoter and affected HBx-induced DcR3 expression. The CHIP assay also demonstrated that the p60/p50 NF-κB heterodimer bound to the DcR3 promoter. Notably, transfection of HepG2 cells with the p50 and p65 overexpression plasmids stimulated DcR3 expression. These results suggest that the activation of NF-κB, as well as the binding of the NF-κB p60/p50 heterodimer to the CD36 promoter, are essential for HBx-induced DcR3 expression.

The PI3K/Akt pathway upregulates NF-kB; in AsPC-1 cells, the PI3K/Akt/NF-κB pathway regulated insulin-like growth factor-1 (IGF-1)-induced DcR3 expression [29,36]. Pretreatment of HepG2 cells with LY294002, an inhibitor that specifically inhibits the activation of PI3K, significantly reduced HBx-induced DcR3 expression, like the effects of treatment with the NF-κB inhibitor (Fig. 4). To determine whether the PI3K pathway was involved in HBx-induced DcR3 expression, the siRNA targeting PI3K was used to knockdown PI3K expression. ELISAs and luciferase assays showed that the knockdown of PI3K with RNAi significantly decreased HBx-induced DcR3 expression. The EMSA showed that the inhibition of PI3K blocked the binding of NF-κB to the DcR3 promoter. These results indicate that both HBx-induced DcR3 expression and NF-κB activation are mediated by the PI3K pathway.

In summary, we showed that DcR3 was overexpressed in CHB patients, and that DcR3 upregulation was positively correlated with HBV DNA load and liver injury. We also demonstrated that, in hepatocytes, DcR3 expression was induced by the HBV X protein via the activation of the PI3K/ NF-κB pathway.

Declaration of Competing Interest

The authors have declared that no conflict of interest exists.

Acknowledgments

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