The hepatitis B virus X protein downregulates NF-κB signaling pathways through decreasing the Notch signaling pathway in HBx-transformed L02 cells

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Abstract. Hepatitis B virus X protein (HBx) is implicated in the pathogenesis of hepatocellular carcinoma, which has been found to be associated with Notch and NF-κB signaling. This study aimed to investigate the crosstalk between Notch and NF-κB pathways in HBx-related hepatocellular carcinoma. An HBx-transformed non-tumor hepatic cell line L02 (L02/HBx) was previously established. Immunofluorescence assays were performed to visualize HBx and the Notch intracellular domain (NICD) in cell nuclei. Co-immunoprecipitation assays were used to investigate physical interactions between HBx and components of the Notch signaling pathway (NICD and JAG1), NF-κB signaling pathway (p65 and p50) or IκBα. L02/HBx cells were treated with the Notch signal inhibitor DAPT or Notch1 siRNA to inhibit the Notch1 pathway. qRT-PCR was used to quantify the expression of the p65, p50 and IκBα genes. Protein expression changes in cytoplasm and nuclei after treatment with DAPT or Notch1 siRNA were analyzed by western blotting and EMSA assays. We found that HBx directly regulated Notch1 signaling, which cross-talked with the NF-κB pathway. Downregulation of Notch1 decreased the binding of NF-κB p65 to its target gene promoter, reduced NF-κB expression and enhanced IκBα expression. The results suggest that HBx functions through the Notch signaling pathway; Notch contributes to hepatocarcinogenesis partially by regulating the NF-κB pathway. Our findings provide new insights into the role of Notch and NF-κB signaling in the progression of hepatocellular carcinoma related to HBx.

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Abbreviations: ACTB, β-actin; ADAM, a disintegrin and metalloproteinase domain; BCL, B-cell CLL/lymphoma; coIP, co-immunoprecipitation; CSL, CBF1/RBP-Jκ/suppressor of hairless/LAG1; DAPI, 4',6-diamidino-2-phenylindole; DAPT, N-[N-(3,5-difluorophenacetyl)-1-alanyl]-S-phenylglycine t-butyl ester; DLL, δ-like protein; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assay; FITC, fluorescein isothiocyanate; HBV, hepatitis B virus; HBx, hepatitis B virus X protein; HCC, hepatocellular carcinoma; IκB, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor; JAG, jagged; MMLV, Moloney murine leukemia virus; NF-κB, nuclear factor κB; NICD, Notch receptor's intracellular domain; qRT-PCR, quantitative real-time reverse transcription PCR; RHD, Rel homology domain

Key words: Notch, NF-κB, hepatitis B virus X protein, crosstalk, hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of death from cancer (1). Many studies have shown that hepatitis B virus (HBV) infection is a risk factor for the development of HCC (2,3). The multifunctional oncoprotein HBx, encoded by the smallest open reading frame of the HBV and its interaction with signal transduction pathways are involved in the initiation of hepatocarcinogenesis (3). In our previous study we reported that the Notch signaling pathway is activated in HBx-transformed L02 cells and is involved in the malignant transformation of normal hepatic cells (4). However, the role of Notch signaling in the development of HCC has not been fully elucidated and its relationship with HBx still requires further exploration.

The evolutionarily conserved Notch signaling pathway mediates cell-to-cell communication and is involved in mediating binary cell fate decisions. There are four mammalian Notch receptors (Notch1-4) and two groups of ligands, jagged (JAG1 and JAG2) and δ-like protein (DLL1, 3 and 4) (5). Upon binding to Notch, the receptor is cleaved by ADAM10 or ADAM17 (formerly, a disintegrin and metalloproteinase domain 10 and 17) (6,7). There is then an intramembranous cleavage by the γ-secretase protease complex, resulting in the release of the Notch receptor's intracellular domain (NICD) (8). The NICD subsequently translocates to the nucleus, binds to the transcription factor CSL (i.e., CBF1/RBP-Jκ/suppressor

of hairless/LAG1), which then activates the transcription of a group of downstream genes (9) including pro-oncogenes (p21 and c-Myc), cyclin D1, cyclin A and the subunits of nuclear factor κB (NF-κB) (10-14). Our previous research indicated that Notch signaling might be one of the downstream targets through which HBx functions as an oncoprotein (4).

In addition, HBx interacts with many other signal pathways in the initiation of hepatocarcinogenesis, including AKT/PKB, ERK1/2, SAPK, NF-κB signal transduction pathway (15). Many researchers have also shown that HBx upregulates the activity of the NF-κB transcription factor (16-18). The

NF-κB pathway comprises a family of transcription factors, namely p50, p52, p65 (RelA), RelB and c-Rel (19), which homo- or heterodimerize to form transcriptional regulatory complexes. All five of these transcription factors contain Rel homology domains that participate in dimerization and DNA binding. The IκB (i.e., nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor) family, including IκBα, IκBβ, IκBε, Bcl-3, p100 (p52 precursor) and p105 (p50 precursor), are the inhibitors of NF-κB pathway, each with different roles (20). Activation of NF-κB typically involves the phosphorylation of IκB by the IκB kinase (IKK) complex, which results in IκB degradation (20). This releases NF-κB and allows it to translocate freely to the nucleus (20).

Accumulating evidence indicates that the Notch and NF-κB pathway are closely related and that they contribute to the pathogenesis of malignancies. The inhibitor of κB kinase 2 (Ikk2), a component of the canonical NF-κB signaling pathway, synergizes with basal Notch signaling to upregulate transcription of primary Notch target genes, resulting in suppression of anti-inflammatory protein expression and promotion of pancreatic carcinogenesis in mice (21). In MDA-MB-231 triple-negative breast cancer cells, genistein inhibited the growth of cells by inhibiting NF-κB activity via the Notch-1 signaling pathway in a dose-dependent manner (22). Schwarzer *et al* (23) reported that Notch is an essential upstream regulator of alternative NF-κB signaling and confirmed crosstalk between both pathways in B cell-derived Hodgkin and Reed-Sternberg cells. However, little is known about the involvement of the Notch and NF-κB pathways in the pathogenesis of HBx-associated HCC. This requires further investigation.

In this study we investigated whether HBx directly binds with components of the Notch and NF-κB pathways and explored evidence of interactions between these two pathways.

Materials and methods

Cell culture. The human non-tumor hepatic cell line L02/HBx, which was derived from L02 cells via transfection with an HBx expression plasmid, was successfully established previously (24). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 250 μg/ml G418 (Invitrogen, Shanghai, China) and maintained in a humidified incubator at 37˚C under a 5% CO_2 atmosphere.

Immunofluorescence assays. Immunofluorescence assays were conducted to visualize HBx, NICD and cell nuclei in L02/HBx cells. L02/HBx cells were cultured on glass cover slips for 24 h and fixed with 4% paraformaldehyde. The fixed cells were incubated with anti-HBx and anti-NICD (both 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) for 12 h at 4˚C. They were then incubated in CY3-conjugated goat anti-mouse IgG and fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (both 1:100; Boster, China) for 1 h to stain the HBx and NICD proteins red and green, respectively. The nuclei were stained blue with 4',6-diamidino-2-phenylindole (DAPI; Boster). The stained cells were observed under a FluoView 1000 laser scanning confocal microscope (Olympus, Japan).

Co-immunoprecipitation (coIP) assays. CoIP was performed to investigate physical interactions between HBx and components of the Notch signaling pathway (NICD and JAG1), components of the NF-κB signaling pathway (p65 and p50), or IκBα (inhibitor of NF-κB pathway) in L02/HBx cells. L02/HBx cells were lysed with RIPA Lysis buffer and phenylmethylsulfonyl fluoride (KeyGEN Biotech, China) and the lysates pretreated with Protein G-Agarose (Santa Cruz) to remove non-specifically bound proteins. After centrifugation, one third of the supernatants were immediately boiled for western blot analysis using antibodies directed serially against NICD, JAG1, p65, or p50, or IκBα as a positive control. The remaining supernatants were incubated for 2 h at 4˚C with 1 μg of non-immune mouse IgG (for the negative control) or mouse anti-HBx (experimental group; Santa Cruz). Then, the mixtures were incubated from 1 h to overnight at 4˚C with 20 μl Protein G-Agarose beads (Santa Cruz). The immunocomplexes were extensively washed with phosphate-buffered saline and samples were boiled in electrophoresis sample buffer, then assayed via western blotting using antibodies directed against NICD, JAG1, p65, p50, or IκBα.

DAPT treatment. To repress normal activity of the Notch signaling pathway, cells were treated with the γ-secretase inhibitor DAPT. DAPT was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in 100% dimethyl sulfoxide (DMSO; Sigma) to make a stock solution of 10 mM, which was then diluted in culture medium to obtain the desired final concentration of 20 μM. DMSO was diluted in culture medium to a final percentage of 0.05% without DAPT. Untreated cells were incubated in the culture medium without any additives. Cells with or without DAPT were cultured for 48 h. Total RNA or protein was then extracted.

Notch1 small interfering RNA (siRNA) transfection. To block Notch signaling, in another experiment L02/HBx cells were transfected with Notch1 siRNA. L02/HBx cells were seeded in 6-well plates. The next day the cells (30-50% confluence) were treated with Notch1 siRNA (sense 5'-GGUGUCUUCCA GAUCCUGAdTdT-3'; antisense 3'-dTdTCCACAGAAGGU CUAGGACU-5') or control siRNA (which does not match any known mammalian GenBank sequences). Notch1 siRNA and control siRNA were purchased from RiboBio (Guangzhou, China). Cells were transiently transfected with Notch1 siRNA or control siRNA using Lipofectamine™ 2000 (Invitrogen). Media were replaced 6 h after transfection. Cells were allowed to grow for 48 h and harvested for further analysis.

Table I. Primer sequences for real-time polymerase chain reaction analysis.

Gene	Primer sequence	PCR product (bp)	GenBank accession no.
p65	f: 5'-GGGGACTACGACCTGAATG-3' r: 5'-GGGCACGATTGTCAAAGAT-3'	118	NM_021975.3
p50	f: 5'-CGCGGTGACAGGAGACGTGAA-3' r: 5'-TGAGAATGAAGGTGGATGATTGCTAATGT-3'	162	NM_003998.2
IκBα	f: 5' -TCCACTCCATCCTGAAGGCTACCAA-3' r: 5'-GACATCAGCACCCAAGGACACCAAA-3'	108	NM_020529.2
β-actin	f: 5'-GTTGCGTTACACCCTTTCTTG-3' r: 5'-GACTGCTGTCACCTTCACCGT-3'	157	NM_001101.3

f, forward; r, reverse.

Quantitative real-time PCR (QRT-PCR) analysis. To quantify the expression of the genes of interest, total RNA was isolated from cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized from 5 μg of total RNA using Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI, USA) and oligo$(dT)_{18}$ as the primer. Real-time quantitative PCR was carried out using SYBR Premix Ex Taq (DRR041A, Takara, Japan). Each real-time PCR (20.0 μl) contained 2.0 μl of cDNA, 10.0 μl of 2X SYBR Premix Ex Taq, 7.2 μl nuclease-free water and primers at a final concentration of 0.2 μM. Real-time reverse transcription-polymerase chain reactions (qRT-PCR) were performed in a Step One Real-Time PCR system (Applied Biosystems, USA). The expression of RNA was determined from the threshold cycle (Ct) and the relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method. All standards and samples were assayed in triplicate. The primer sequences used to amplify specific target genes (p65, p50, IκBα, β-actin) are listed in Table I.

Extraction of nuclear and cytoplasmic proteins. To quantify protein expression changes in the cytoplasm and nuclei of L02/HBx cells after treatment with the Notch signal inhibitor DAPT or Notch1 siRNA, L02/HBx cells were treated with DAPT or DMSO for 48 h, or with Notch1 siRNA or control siRNA for 48 h. Proteins were subsequently extracted in accordance with the instructions in a nuclear and cytoplasmic protein extraction kit (KeyGEN Biotech). The nuclear and cytoplasmic proteins were measured using the bicinchoninic acid method (Boster, Wuhan, China). The samples were stored at -80˚C and assayed via western blotting and electrophoretic mobility shift assay (EMSA).

Western blot analysis. Cells were lysed and the lysates were subjected to SDS/PAGE. The resolved proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). The blotted membranes were blocked and subsequently incubated with rabbit anti-JAG1 and rabbit anti-NICD (both 1:400; Santa Cruz), rabbit anti-p65 and rabbit anti-p50 (both 1:1,000; Proteintech Group, Chicago, IL, USA), rabbit anti-IκBα (1:750; Proteintech) and rabbit anti-actin (1:1,000; Proteintech) according to the manufacturer's instructions. After incubation with horseradish peroxidase-labeled secondary antibody (1:4,000; Santa Cruz), visualization was performed with an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA) and exposure to X-ray film (Kodak, Rochester, NY, USA). Immunoblotting with anti-actin antibody was used as an internal control to confirm equivalent protein loading. Western blot experiments were repeated ≥3 times. The relative intensity of each protein band was assessed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

EMSA for measuring NF-κB activity. To evaluate changes in NF-κB activation after cells were treated with the Notch signal inhibitor DAPT (as reflected by DNA-binding activity), cell nuclear extracts from L02/HBx cells, L02/HBx cells treated with DMSO (0.05%), or L02/HBx cells treated with DAPT (20 μM) were subjected to EMSA. In addition, a reaction system sample without cell nuclear extracts was used as a blank control and with untreated positive cell nuclear extracts (Pierce) as a positive control. EMSA was performed by incubating 10 μg of nuclear protein extract with biotin-labeled NF-κB oligonucleotide (Pierce). The mixture included 1 μg of poly (dI-dC) in a binding buffer. The DNA-protein complex formed was separated from free oligonucleotides on a 6.0% polyacrylamide gel using buffer (45 mM Tris, 45 mM boric acid pH 8.3 and 1 mM ethylenediaminetetraacetic acid) for electrophoretic transfer of binding reactions to a nylon membrane and then transferred DNA was UV cross-linked to a membrane at 254 nm and exposed to X-ray film. The relative intensity of each protein band was assessed using Quantity One software (Bio-Rad Laboratories).

Statistical analyses. SPSS version 17.0 software (SPSS for Windows, Inc. and Chicago, IL, USA) was used for all statistical analyses. All results are expressed as mean ± standard error of the mean. Statistical analysis was performed using standard one-way ANOVA or one-way ANOVA for repeated measures, followed by the least significant difference *post hoc* test. Bonferroni's correction was used to adjust for multiple

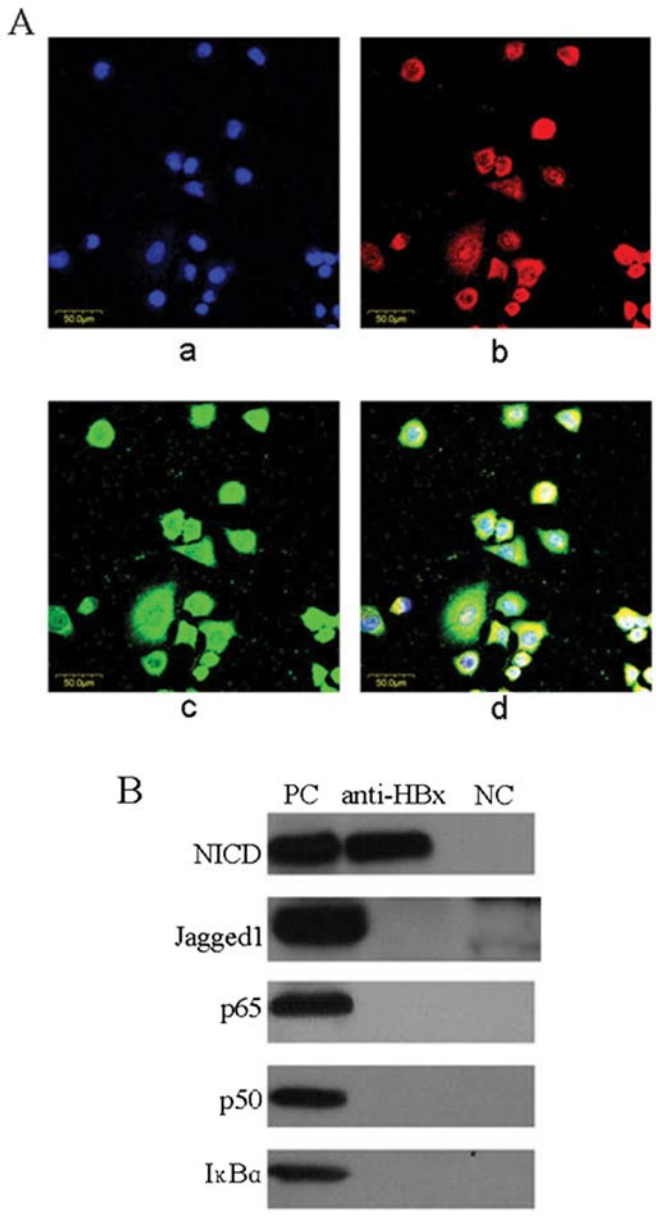

Figure 1. Co-localization and physical interactions between Notch1 or NF-κB HBx in L02/HBx cells. (A) Confocal micrograph of co-localization of NICD and HBx. Nuclei were stained with DAPI (blue) (a), HBx protein was stained with CY3 (red) (b) and the NICD protein was stained with FITC (green) (c). The yellow indicated overlapping areas of red and green (d) (magnification x100). (B) CoIP assay of interactions between NICD, JAG1, p65, p50, or IκBα and HBx. Data shown are representative of 3 independent experiments. Specific interaction was found between NICD and HBx and not between JAG1, p65, p50, or IκBα and HBx. PC, positive control; anti-HBx, coIP proteins treated with anti-HBx; NC, negative control; coIP proteins treated with non-immune mouse IgG.

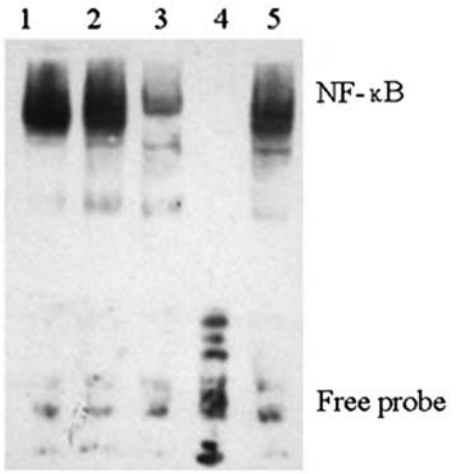

Figure 2. Effects of Notch1 inhibition on the DNA binding activity of NF-κB in nuclear extracts of L02/HBx cells. After treatment with DAPT, cell nuclear extracts were prepared and assayed for NF-κB activation by EMSA. Inhibition of Notch1 resulted in a reduction in NF-κB DNA binding activity when compared to control (untreated cells). The data shown are representative of 3 independent experiments. Lane 1, untreated cells; lane 2, cells treated with DMSO (0.05%); lane 3, cells treated with DAPT (20μM); lane 4, blank control; lane 5, positive control.

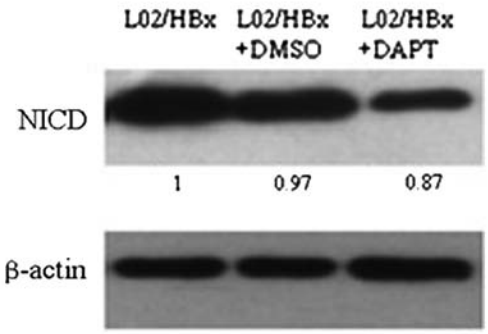

Figure 3. Notch signaling inhibition in L02/HBx cells. Inhibition of Notch signaling with indicated concentrations of DAPT was assessed by immunoblotting. Representative blots shown are from three independent experiments with identical results. The relative ratios of each band were normalized to actin and shown below.

comparisons. A 2-tailed Student's paired t-test was also used to compare the difference in values between 2 groups. $P<0.05$ was considered statistically significant.

Results

Co-localization and physical interactions of Notch1 or NF-κB with HBx. To investigate the potential association between Notch1 or NF-κB and HBx in the development of HCC, we performed immunofluorescence and coIP assays with HBx-transfected L02 cells (L02/HBx). In the L02/HBx cells, nuclei, HBx and NICD were stained blue, red and green, respectively (Fig. 1A, a-c). Yellow areas in dual-labeling experiments indicated overlapping of red and green fluorescent labels (Fig. 1A, d), suggesting the co-localization of NICD with HBx.

Then we investigated the possible physical interaction via the coIP assay. Compounds from L02/HBx cells were immunoprecipitated with anti-HBx or non-immune mouse IgG, which were then subjected to western blotting with anti-NICD, anti-JAG1, anti-p65, anti-p50 and anti-IκBα. NICD was observed to co-immunoprecipitate with HBx (Fig. 1B). No specific interaction was found between JAG1, p65, p50 or IκBα and HBx, or the protein immunoprecipitated with non-immune IgG, which indicates the specificity of the NICD-HBx interaction.

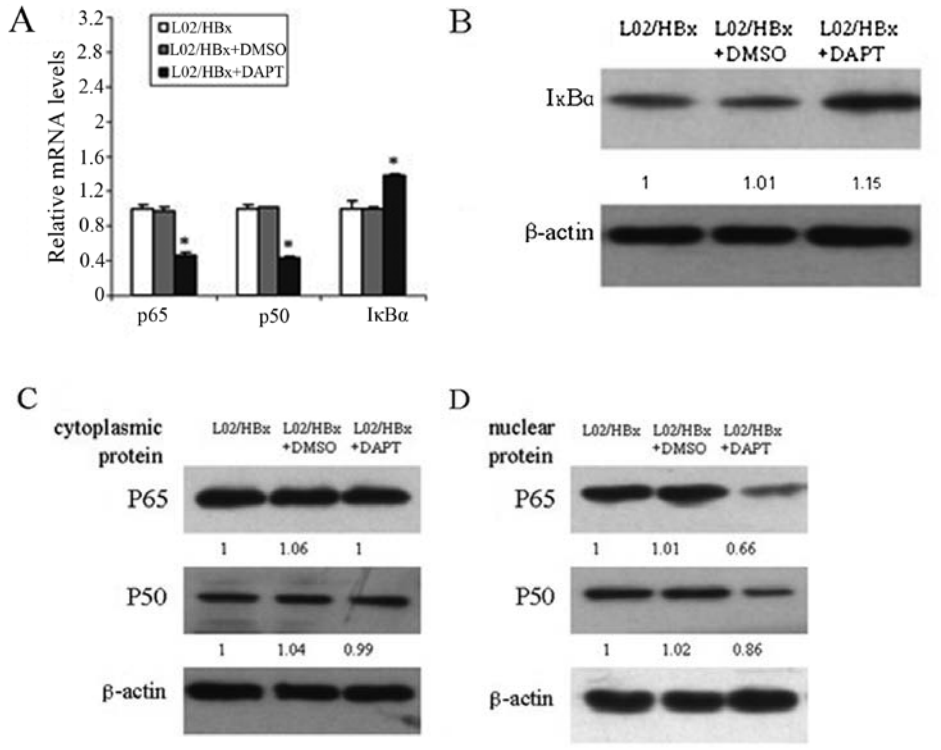

Figure 4. Downregulation of NICD by DAPT decreases activation of the NF-κB pathway in L02/HBx cells. (A) Significant decreases in p65 and p50 mRNA levels were observed (P<0.001); the IκBα mRNA level increased (P<0.001). (B) NICD protein levels were notably suppressed via DAPT treatment and the total protein level of IκBα was increased. (C) No significant changes were observed in cytoplasmic p65 and p50 protein levels and (D) the nuclear extract levels of p65 and p50 were decreased. β-actin was used as an internal control for equal loading of samples. Representative western blots shown are from three independent experiments with similar results. The relative ratios of each band were normalized to actin and are shown below each western blot band.

Downregulation of NICD decreases NF-κB DNA-binding activity. NF-κB (p50/p65) is a ubiquitous, constitutive and inducible heterodimer and the DNA binding activity of NF-κB traditionally refers to the p50/p65 (p50/RelA) heterodimer-mediated binding to DNA (25). In the present study, we inhibited Notch1 using DAPT in L02/HBx cells (Fig. 3) to examine the effect of Notch on NF-κB DNA binding activity. Nuclear proteins from untreated L02/HBx, DMSO-treated L02/HBx cells and DAPT-treated L02/HBx cells were subjected to an EMSA for NF-κB DNA-binding activity (Fig. 2). It was found that downregulation of NICD via DAPT significantly decreased the binding of NF-κB p65 to its target gene promoter. These results revealed that after translocating into the nucleus, NICD could function as a regulator to regulate the NF-κB DNA-binding activity, which provided evidence for a mechanistic crosstalk between Notch and NF-κB in HCC.

Inhibition of NICD by DAPT decreases the activation of NF-κB pathway. To investigate whether HBx acted through Notch signaling to activate the NF-κB pathway, we inhibited the Notch pathway by using the γ-secretase inhibitor DAPT, which blocks the processing of transmembrane (TM)-Notch1 to Notch1-IC and has been widely used for experimental studies of Notch signaling (4). We treated L02/HBx cells with DAPT (20 μM) for 48 h and then used western blotting to determine NICD protein expression. It turned out that NICD decreased significantly after DAPT treatment (Fig. 3). Moreover, our data also showed that inhibition of Notch1 decreased NF-κB DNA-binding activity in L02/HBx cells.

To further study the importance of activated Notch signaling for the NF-κB pathway, we used qRT-PCR and western blot analysis to observe changes in the NF-κB pathway after inhibition of Notch1 (Fig. 4). The mRNA levels of p65 and p50 were significantly decreased (P<0.001) and the IκBα mRNA level was increased (P<0.001; Fig. 4A).

The protein levels of NF-κB were then evaluated using western blot analysis and found that the total protein level of IκBα was increased in L02/HBx cells after treating with DAPT (Fig. 4B). No significant change was observed in p65 or p50 proteins in the cytoplasm (Fig. 4C) and the nuclear extract levels of p65 and p50 were decreased (Fig. 4D). These results indicated that inhibition of Notch1 suppressed the activation of the NF-κB pathway in L02/HBx cells by affecting the transcription of the components of NF-κB and inhibiting the nuclear transport of NF-κB dimers. This confirmed that in L02/HBx cells HBx induced Notch signaling, which is important for stimulating the NF-κB pathway.

Inhibition of Notch1 by specific siRNA also decreases the activation of NF-κB pathway. Notch1 siRNA-transfected L02/HBx cells showed significantly reduced Notch1 mRNA and protein expression (P<0.001; Fig. 5A and B). We then detected the expression of NF-κB pathway proteins in the Notch1 siRNA-transfected L02/HBx cells. In the cytoplasm, p50 and p65 proteins were not changed compared with either

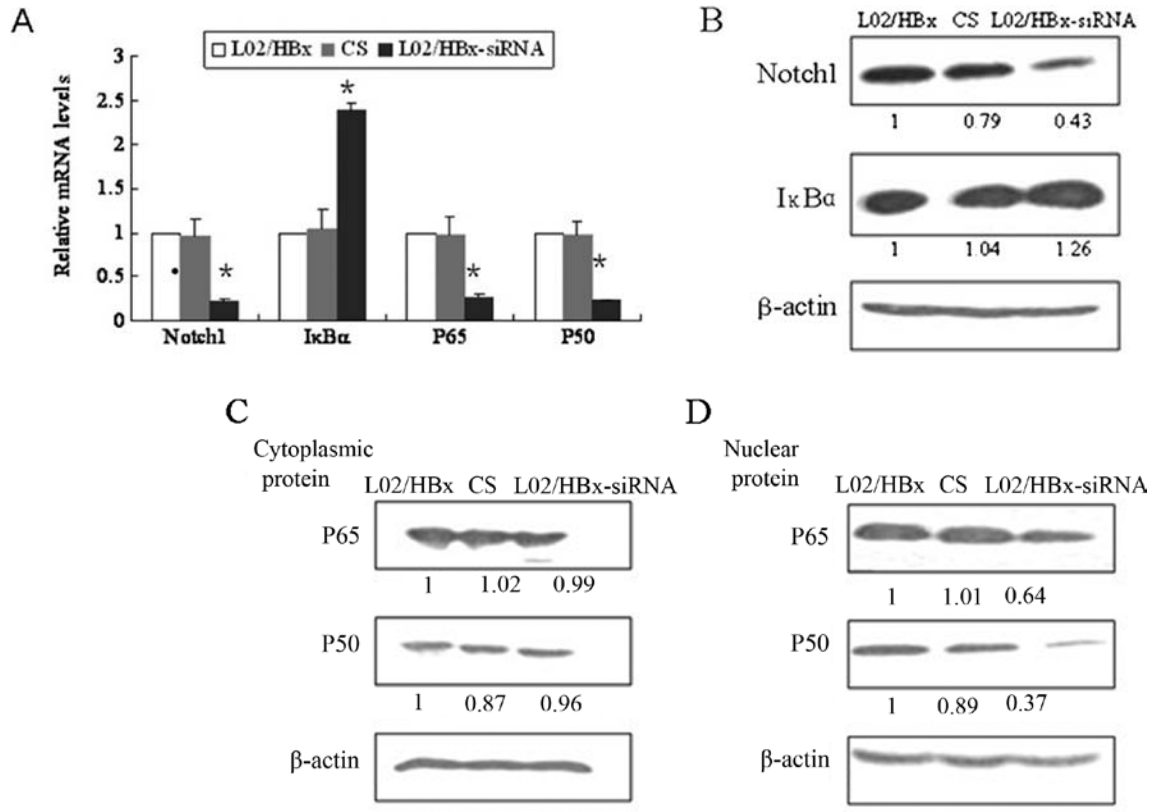

Figure 5. Inhibition of Notch1 decreases activation of the NF-κB pathway in Notch1 siRNA-transfected L02/HBx cells. CS, control siRNA group. (A) Notch1, p50 and p65 mRNA levels were significantly decreased in Notch1 siRNA-transfected cells, compared with non-transfected L02/HBx cells and control siRNA-transfected L02/HBx cells, but IκBα mRNA levels were remarkably increased. (B) Notch1 protein was reduced. IκBα was increased, consistent with the Q-PCR result. (C) In the cytoplasm there was no significant alteration in p50 and p65 proteins. (D) In the nucleus, both p50 and p65 decreased.

the blank or control groups (Fig. 5C). However, these proteins were decreased notably in the nucleus (Fig. 5D).

Consistent with the above results, Q-PCR also showed that p50 and p65 mRNA levels were significantly decreased ($P<0.001$; Fig. 5A). Moreover, the total protein level of IκBα was increased in the Notch siRNA-transfected L02/HBx cells (Fig. 5B) and results of the Q-PCR showed that the IκBα mRNA levels were also increased (Fig. 5A). Therefore, down-regulation of Notch1 suppressed the activation of p65 and p50, accompanied by an increase in IκBα. Altogether these results showed that the NF-κB pathway was regulated by the Notch signaling pathway.

Discussion

Stably HBx-expressing L02/HBx cells were previously established and the data indicated that HBx promoted growth and malignant transformation of the human non-tumor hepatic L02 line (24). To explore whether associations among Notch and NF-κB may be involved in the malignant transformation of hepatic cells induced by HBx, we investigated the interaction between HBx and Notch, or between HBx and NF-κB, in the present study. We found that NICD interacted with HBx directly, while the evidence that HBx acts directly through NF-κB was lacking. Thus, HBx affected Notch signaling by directly binding to the NICD upon its release from the cleaved Notch receptor, whereas HBx did not have an immediate interaction with NF-κB. We then continued to explore whether the Notch pathway receptor Notch1 and the NF-κB pathway are interrelated. We found that downregulation of Notch1 via the γ-secretase inhibitor DAPT decreased the ability of NF-κB to bind to DNA. RNA-mediated or DAPT inhibition of the Notch1 signaling pathway in HBx-transfected L02 cells reduced NF-κB expression and enhanced IκBα expression. These results showed that NF-κB was regulated through the Notch1 signaling pathway.

The development of HCC is a multifactor, multistep, complex process (26,27). Numerous reports have shown that hepatocarcinogenesis is associated with the HBx protein. Our previous research confirmed that HBx induced the malignant transformation cells of the human non-tumor hepatic cell line L02 (24). Moreover, HBx regulated a variety of cellular signaling pathways, including the Notch and NF-κB pathways, thereby contributing to the progression of HCC (28). In our previous study we also found that activated Notch signaling is required for HBx to promote the proliferation and survival of human hepatic cells (4). However, little is known about how HBx influences the Notch and NF-κB signaling pathways. Therefore, in the present study we continued to investigate the mechanisms by which HBx directly regulates Notch1 and NF-κB and how Notch1 affects the NF-κB signaling pathway.

The possibility of crosstalk between the HBx and Notch signaling pathways has become an important focus of research. One recent report showed that the protein and mRNA expressions of Notch1 and JAG1 were upregulated in HBx-stably transfected L02 cells (4), which was also consistent with the

report of Gao *et al* (29). But the underlying molecular mechanisms are still unknown. The present research revealed a direct association between HBx and the Notch signaling pathway and showed that NICD interacts with HBx in HBx-transformed L02 cells, while the ligand JAG1 did not co-precipitate with HBx. Here we could deduce that the malignant function of HBx is directly associated with the activity ofNotch1 signaling pathway, since HBx could immediately regulate the Notch1 signaling pathway and the key factor is NICD instead of other components of the Notch1 signaling pathway. Our study thus provides some novel clues to the mechanism by which HBx affects the Notch signaling pathway.

NF-κB is involved in the biological processes of cancers as an important modulator of genes that promote cell survival, proliferation, migration, inflammation, angiogenesis and metastasis (30), including the malignant transformation of hepatocytes (31). NF-κB (p50/p65) is a ubiquitous, constitutive and inducible heterodimer and the DNA binding activity of NF-κB traditionally refers to the p50/p65 (p50/RelA) heterodimer-mediated binding to DNA (25). IκBα interacts with and sequesters NF-κB in the cytosol (32). It can also export NF-κB from the nucleus to the cytoplasm (19) and directly inhibits the DNA-binding activity of NF-κB. Thus p50, p65 and IκBα play crucial roles in the NF-κB pathway. Recent studies have reported that HBx stimulates the phosphorylation of IκBα via the IκB kinase complex (28), thereby upregulating the activity of the NF-κB signaling pathway (29). In the present study our objective was to discover the mechanisms of HBx-induced activation of the NF-κB pathway. We found that HBx could not co-precipitate with the subunits p65 or p50 and the suppressor IκBα of NF-κB, suggesting that HBx does not activate NF-κB signaling by directly binding to p65, p50, or IκBα. Reports have described the regulation of NF-κB by Notch, the mechanisms of which are dependent on cell type. Notch1 upregulates the expression of the NF-κB subunits p50, p65, RelB and c-Rel in murine bone marrow hematopoietic precursors and therefore it can be concluded that Notch1 upregulates NF-κB activity (13). In satellite liver cells, Notch1 reversed the repression of IκBα expression and lowered NF-κB activity (33). Since numerous studies have reported crosstalk between Notch and the NF-κB pathway, we hypothesize that HBx may promote NF-κB signaling by activating the Notch pathway. Our studies attempted to reveal the molecular mechanism of the interaction between Notch1 and NF-κB in HBx-transformed L02 cells.

Our EMSA results showed that the NF-κB DNA-binding activity was decreased after Notch signaling was inhibited by DAPT. To learn more about how Notch1 influence NF-κB in HBx-related HCC, we used the γ-secretase inhibitor DAPT and Notch1 siRNA to block Notch signaling. We found that when Notch signaling was inhibited in HBx-transformed L02 cells, p50 and p65 mRNA levels were significantly decreased, while IκBα mRNA level was notably increased. This suggests that Notch activates the NF-κB pathway at the transcription level. At the same time, in the nucleus the proteins p50 and p65 were decreased, although their levels had not changed in the cytoplasm. The results imply that NICD stimulated the expression of the functional subunits and the nuclear transport of NF-κB dimers and inhibited the suppressor of NF-κB to active the NF-κB pathway. To our knowledge, several mechanisms potentially may explain the effect of Notch1 on NF-κB activity. These include transcriptional effects, physical binding between NF-κB and Notch1 and indirect effects on IκBα/β phosphorylation (13). There have been studies reported that Notch1 signaling promoted NF-κB translocation to the nucleus and DNA binding by increasing both phosphorylation of the IκBα/β complex and the expression of some NF-κB family members (34). However, the details on the molecular mechanism still required further confirmation, including *in vivo* experiments and in different kinds of HBx-expressing hepatic cells.

In conclusion, the results of this study showed that HBx could promote Notch signaling by binding to NICD, which then activates the NF-κB pathway. Therefore, crosstalk between the NF-κB and Notch1 pathways is partially responsible for the progression of HBx-induced HCC. However, further investigations are needed to delineate the exact mechanisms by which Notch1 effects IκBα/β phosphorylation and promotes NF-κB translocation into the nucleus.

Acknowledgements

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