Evaluation of Nuclear Factor-κB, Urokinase-Type Plasminogen Activator, and HBx and Their Clinicopathological Significance in Hepatocellular Carcinoma

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ABSTRACT

Purpose: Nuclear factor κB (NF-κB) signaling pathway is an important regulating pathway in human diseases and cancers. One of its downstream target genes is urokinase plasminogen activator (uPA), which is involved in cancer invasion and metastasis. The purpose of this study was to evaluate NF-κB activation, uPA up-regulation, and hepatitis B viral X protein (HBx) expression in human hepatocellular carcinoma (HCC) and to assess their clinicopathological significance.

Experimental Design: We evaluated NF-κB activation, expression of uPA, and presence of HBx in 32 human HCCs. Their clinicopathological significance was assessed by correlation with the clinicopathological features. Aberrant NF-κB signaling pathway and uPA up-regulation mediated by HBx were also analyzed in vitro.

Results: We found that NF-κB activation and uPA up-regulation were frequently (56% and 59%, respectively) observed in HCCs, and particularly in HBx-positive HCCs. NF-κB activation and uPA overexpression were closely associated with one another (P < 0.0001). Furthermore, both activation of NF-κB and up-regulation of uPA were significantly associated with a more aggressive tumor behavior in terms of venous invasion, direct liver invasion, and absence of tumor encapsulation. In vitro, NF-κB activation was induced by HBx transfection in HepG2 cells through inhibitor of nuclear factor-κB kinase β (IKKβ). HBx also up-regulated uPA and enhanced cell invasion synergistically with IKKβ.

Conclusions: The data indicate that NF-κB dysregulation and uPA overexpression may lead to a more aggressive tumor behavior in HCC. In addition, our data suggest that IKKβ plays a critical role in the HBx-activated NF-κB signaling pathway.

INTRODUCTION

Nuclear factor κB (NF-κB) transcription factors are crucial regulators of innate immune and inflammatory responses, cell proliferation, and apoptosis through activation of responsive genes (1, 2). In resting cells, NF-κB is present as a latent, inactive complex retained in the cytoplasm through association of inhibitory proteins, inhibitor of nuclear factor-κB (IκB; Ref. 3). After many extracellular stimulations, IκB proteins are phosphorylated by the IκB kinase (IKK) complex (4). IKK complex is composed of two catalytic subunits (IKKα and IKKβ) and regulatory subunits (IKKγ; 5). IKKβ and IKKγ are responsible for phosphorylating IκB in response to pro-inflammatory stimuli, whereas IKKα is necessary for inducible p100 processing in B-cell maturation and lymphoid organ development (6). The released NF-κB rapidly enters the nucleus and binds to specific κB-element-containing target genes (6, 7). It is believed that the products of certain NF-κB target genes inhibit apoptosis, regulate cell cycle progression, and govern invasion and metastasis (8–10). NF-κB activation has also been implicated in the development of hepatocellular carcinoma (HCC) related to hepatitis B virus (HBV) infection (11, 12).

HCC is one of the more common malignancies in the world and is the second most common fatal cancer in Southeast Asia and Hong Kong. Over 80% of the HCC worldwide are associated with chronic HBV infection (13). The pathogenesis of HBV-associated HCC still remains unclear, because of the involvement of complex and diverse molecular mechanisms. One of the protein products encoded by the HBV genome, the hepatitis B virus X protein (HBx), is closely related to the development of HCC (14). HBx is essential for viral replication and viral carcinogenesis (15). In addition, HBx has been shown to activate several transcription factors, in particular NF-κB. Previous studies have implicated that activation of NF-κB in HCC may play a critical role in cell survival and tumor development (11, 16–19). However, the possible role of NF-κB activation in tumor invasion and metastasis has not been examined.

HCC has high rates of intrahepatic invasion and metastasis. In tumor metastasis, degradation of the extracellular matrix by serine proteases is believed to be one of the important mechanisms (20). Urokinase-type plasminogen activator (uPA) is a serine protease that converts plasminogen into the active protease, plasmin. It is implicated in direct or indirect degradation of matrix proteins (21). Expression of uPA has been shown to be regulated by ras and NF-κB signaling pathways (22–24). In HCC, uPA overexpression has been reported previously (25–
but its involvement in NF-κB signaling pathway leading to tumor aggressiveness has not been defined in HCC.

In this study, we investigated the activation status of NF-κB and expression of uPA in human HCC. We found that NF-κB activation and uPA overexpression were closely related to one another, and both were associated with a more aggressive tumor behavior. Our results also suggest a key role for IKKα in regulating uPA expression and cell invasiveness, acting synergistically with HBx.

**MATERIALS AND METHODS**

**Patients and Samples.** Thirty-two patients who had primary HCC resected at Queen Mary Hospital, Hong Kong between October 1992 and August 1999 were randomly selected. Twenty-three were men and nine were women, with a mean age of 57 years (ranging from 36 to 74 years). Twenty-five (78.1%) of the 32 patients were positive for hepatitis B surface antigen (HBsAg), whereas only 2 (6%) patients were positive for hepatitis C virus antibody and they were HBsAg negative. The nontumorous liver samples were taken distal to the HCCs. Serum HBsAg and anti-HBs activity was taken as significant when the ratio of the tumor to that of the corresponding nontumorous liver was >2-fold.

**Antibodies and Plasmids.** Specific antibodies for NF-κB [anti-μ-Rel avian reticuloendotheliosis viral oncogene homolog A (RelA) (C-20) and anti-p50 (nuclear localization signal)], anti-Topo I (H-300), and anti-actin (H-196) were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA), the polyclonal rabbit anti-HBx was a gift from M. A. Feitelson, and antirabbit immunoglobulin horseradish peroxidase conjugate was from Amersham (Buckinghamshire, United Kingdom). The eukaryotic expression plasmid pcDNA3.1-HBX was constructed by inserting HBx gene (subtype ayw; accession number U95551; 1376–1840) containing flanked EcoRI and HindIII restriction sites into pcDNA3.1(–) vector (Invitrogen, Carlsbad, CA) under the control of cytomegalovirus promoter/enhancer. Expression vectors for IKKα wild type (WT) and IKKβ dominant negative (D/N) have been described previously (28). Reporter plasmids pNF-κB-Luc and pRL-cytomegalovirus were from Stratagene and Promega (Madison, WI), respectively.

**Cell Culture, Transfection, and Dual Luciferase Assay.** Human hepatoblastoma HepG2 cells, negative for HBV (and also HBx), were grown at 37°C, and 2 × 10⁵ cells were seeded onto a 35 mm culture plate a day before transfection. Cells were transiently transfected using FuGENE transfection reagent (Roche Molecular Biochemicals, Mannheim, Germany) with the respective constructs according to the manufacturer’s protocol. Cells were harvested 48 h after transfection, and the luciferase activity was determined using Dual Luciferase Assay System (Promega). Luciferase assay reagent II was added, and firefly luciferase was measured, followed by adding 100 μl of Stop and Glo reagent to measure the Renilla luciferase for normalization.

**Preparation of Cytoplasmic and Nuclear Extracts.** Cytoplastic and nuclear extracts were prepared as described previously (16) with some modifications. Briefly, frozen tissue blocks were homogenized, and cells were placed on ice for 15 min and then centrifuged at 1500 rpm for 5 min at 4°C. The supernatant was removed, and the cell pellet was resuspended and incubated on ice for 1 h. Cytoplastic protein was collected after centrifugation at 11,000 rpm for 5 min at 4°C. Nuclear pellets were resuspended and incubated on ice for 30 min. Nuclear extract was obtained by centrifugation at 11,000 rpm for 5 min at 4°C. The protein concentrations were determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA).

**Electrophoretic Mobility Shift Assay (EMSA).** The NF-κB DNA-binding activity of the HCC and corresponding nontumorous liver tissues was analyzed by EMSA as described previously (29). Five μg of nuclear extracts were incubated for 30 min at room temperature with 20 μl of binding buffer containing buffer C (10 mM HEPES [pH 7.9], 50 mM NaCl, 0.5 mM EDTA, 5 mM MgCl₂, 1 mM DTT, 10% glycerol), 1 μg of poly(deoxyinosinic-deoxycytidylic acid), 1 pmol [³²P] 5'-end-labeled probe (5 × 10⁶ cpm) containing NF-κB-binding site sequence (Santa Cruz Biotechnology). DNA-protein complexes were resolved in 5% polyacrylamide gels. Gels were dried and autoradiographed at ~80°C. For supershift EMSA, rabbit anti-RelA (anti-p65) and anti-p50 (at 1:50 dilution) were preincubated with nuclear extracts for 30 min at 4°C before the addition of labeled oligonucleotides. Increased NF-κB DNA-binding activity was taken as significant when the ratio of the tumor to that of the corresponding nontumorous liver was >2-fold.

**Western Blotting.** Protein (20 μg) extracted from each of the fresh frozen tumor and nontumorous liver tissues was resolved in 10% SDS-polyacrylamide gels and was transferred electrophoretically to Hybond-P membranes (Amersham). The membranes were blotted in Tris-buffered saline containing 0.1% Tween 20, 5% fat-free dry milk for 1 h followed by incubation with the appropriate primary antibodies (at 1:200 dilution) for 2 h at room temperature. The membranes were washed three times and then incubated with secondary antibody against rabbit immunoglobulin. Western blot analysis was carried out with enhanced chemiluminescence system (Amersham) according to the manufacturer’s recommendations. Overexpression of NF-κB was taken as significant when the ratio of RelA expression of the tumor to that of the corresponding nontumorous liver was >2-fold after normalization with β-actin or TopoI, respectively.

**RNA Extraction and cDNA Synthesis.** Total RNA was extracted from human HCCs, the nontumorous liver samples and HepG2 cells using Trizol reagent (Invitrogen, Tokyo) according to the manufacturer’s protocol. cDNA was synthesized from 1 μg of total RNA by using GeneAmp RNA PCR kit components (Roche).

**PCR.** The expression levels of uPA were determined by semi-quantitative reverse transcription-PCR using Perkin-Elmer 9700 thermocycler (ABI, Foster City, CA) and normalized with β-actin. The primer sets used for uPA were as follows: sense 5’-CAGAGACACTAAGCATCTCCAGCGG-3’ and antisense 5’-GAGGATTGATGAATACCTTAA-3; for β-actin, sense 5’-GAGGGCGGCCCAGGCAACA-3’ and antisense 5’-CTCCTTAATGTCACGCACGATTTC-3’. The primer sets used for HBx were as follows: sense 5’-AAAAATCTACTGTTGCTGTTGTAAC-3’ and antisense 5’-GGGCAGGACATTTTGT-3’. The PCR condition profile was 94°C for 30 s (55°C for HBx, 60°C for uPA, and 60°C for β-actin for 30 s) and 72°C for 45 s.

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The cycling profiles were 28 cycles for uPA, 40 cycles for HBx, and 22 cycles for β-actin. The PCR was terminated at the exponential phases without saturation. The final reverse transcription-PCR products were resolved on 1% agarose gel stained with ethidium bromide, and their signal intensities were measured by GelWorks 1D Intermediate Software (UVP Inc.). The sizes of the PCR products of HBx, uPA, and β-actin were 403-, 366-, and 190-bp, respectively. Negative controls, without adding reverse transcriptase, were included in all of the PCR reaction to prevent DNA contamination and ensure only RNA was being amplified in the PCR reaction. Overexpression of uPA was taken as significant when the ratio of uPA mRNA of the tumor to that of the corresponding nontumorous liver was >2-fold after normalization with β-actin.

**Immunostaining of HBx.** Immunohistochemical staining for HBx (at 1:500 dilution) was performed on formalin-fixed, paraffin-embedded sections using the labeled streptavidin-biotin method. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Nonspecific binding to the endogenous biotin was blocked by incubation of 10% normal swine serum (DAKO, Glostrup, Denmark). Negative controls were done by either incubating the tissues without primary antibody or incubating together with HBx synthetic peptide (a gift from M. A. Feitelson).

**Cell Invasion Assay.** Cell Invasion kit (Chemicon International, Temecula, CA) was used for the cell invasion assay according to the manufacturer’s protocol. Briefly, 2 × 10^5 cells were seeded onto a 35-mm culture plate, and the respective constructs were transfected 2 days before cell invasion assay. Warm, serum-free medium (300 μl) was added to the interior of the inserts and removed after rehydration of the extracellular matrix. Transiently transfected cells were trypsinized and added over the inner chamber of the insert in 300 μl of serum-free media. Five hundred μl of media containing 10% fetal bovine serum was added to the lower chamber. The plates were incubated for 48 h at 37°C. Then, the noninvasing cells and the extracellular matrix gel from the interior of the inserts were gently removed using a cotton-tipped swab. The invasive cells that migrated from the upper to the lower surface of the membrane were stained, and 30 fields of the invading cells were counted under the microscope at ×400 magnification and captured by photography. The experiment was performed thrice independently.

**Pathological Analysis.** The clinicopathological features of the patients included gender, age, tumor size, cellular differentiation, tumor encapsulation, presence of venous invasion without differentiation into portal or hepatic venules, direct invasion into the adjacent liver parenchyma, tumor microsatellite formation, tumor stage (pTNM stage), serum HBsAg and hepatitis C virus antibody status, and background liver disease in the nontumorous livers. They were analyzed as described previously (30).

**Statistical Analysis.** Statistical analysis was performed with SPSS 11.0 for Windows (SPSS Inc., Chicago, IL). Associations between quantitative variables were analyzed with the Fisher’s exact test. All Ps were 2-tailed and were considered significant when they were <0.05.

**Fig. 1** A, V-Rel avian reticuloendotheliosis viral oncogene homolog A (RelA) DNA-binding activity was assessed by electrophoretic mobility shift assay, and higher activity was found in HCC tissues (Lanes 1 and 3), as compared with the corresponding nontumorous livers (Lanes 2 and 4; cases 216 and 219, HBx-positive). T, tumor tissues; NT, nontumorous. B, nuclear RelA was detected by Western blotting and was overexpressed in HCC tissues (Lanes 1 and 3) but relatively weak expression was observed in the corresponding nontumorous livers (Lanes 2 and 4). C, nuclear RelA was overexpressed (Fig. 1B, Lanes 1 and 3), whereas cytoplasmic RelA was underexpressed in tumor tissues (Lanes 1 and 3). Two other HCC cases, cases 213 and 238 (HBx-negative), with neither RelA translocation nor increased RelA DNA-binding activity are shown for comparison (Lanes 5 to 8). D, specific polyclonal antibodies were added to binding reactions corresponding to p50 (Lane 2) and RelA (Lane 3). The addition of either antibody led to further retardation of the shifted bands highlighted by “*” and “#”. NF-κB complexes were composed of p50/p50 homo- and p50/RelA heterodimers. EMSA, electrophoretic mobility shift assay; HCC, hepatocellular carcinoma; HBx, hepatitis B viral X protein.
RESULTS

NF-κB Activation in HCCs. To investigate the DNA-binding activity of NF-κB, EMSA was performed with the nuclear extracts of the HCC and nontumorous liver tissues. A retarded band representing the NF-κB-bound oligonucleotides was observed (Fig. 1A). In the 32 HCC cases, 20 (62.5%) samples showed increased NF-κB DNA-binding activity in the tumor tissues compared with the corresponding nontumorous livers (Fig. 1A). To verify the identity of the retarded band, a supershift assay (Fig. 1D) was performed with specific antibodies against RelA (p65) and NF-κB1 (p50). The addition of either antibody led to further retardation of the shifted bands (Fig. 1D; supershift signals indicated by "+++" and "+++"). Thus, the NF-κB DNA-binding complexes contained both p50 and RelA.

In addition, consistent with the EMSA results, increased nuclear RelA expression was detected in 19 (59.4%) of 32 HCCs, as compared with the corresponding nontumorous livers (Fig. 1B; compare nuclear RelA in tumor tissues (T) to nontumorous livers (NT)). Specifically more RelA was found in the nuclear fractions of the HCC samples (Fig. 1B; compare RelA to Topol in the nuclear fractions), whereas RelA was much more abundant in the cytoplasmic fractions of the corresponding nontumorous livers (Fig. 1C; compare RelA to β-actin in the cytoplasmic fractions). Such nuclear translocation of RelA was observed in 17 (89.5%) of the 19 HCCs overexpressing nuclear RelA (Table 1).

uPA Up-Regulation in HCCs. To test the uPA expression in HCCs, semi-quantitative reverse transcription-PCR was performed, and its expression level was normalized with β-actin. Overexpression (>2-fold) of uPA mRNA was found in 19 (59.4%) of the 32 HCC samples as compared with the corresponding nontumorous livers (Fig. 2A; Table 1).

HBx Expression in HCCs. Twenty-three (71.8%) of the 32 HCCs showed HBx mRNA expression (Fig. 2B; Table 1) and positive HBx immunoreactivity (Fig. 2C). All of them were HBsAg positive. HBx was present submembranously and intracytoplasmically (Fig. 2C). No HBx immunoreactivity was found in all HBx mRNA-negative cases (Fig. 2C).

Correlation among NF-κB Activation, uPA Overexpression, and HBx Expression. NF-κB activation was closely associated with uPA mRNA overexpression. Overexpression of uPA mRNA was significantly associated with increased NF-κB DNA-binding activity (P < 0.0001) and with overexpression of nuclear RelA (P < 0.0001; Table 2). Increased NF-κB activation was also frequently found in those HCCs positive for HBx (P = 0.006; Fig. 1A; Table 2). In addition, there was higher RelA nuclear expression in HBx-positive HCCs (P = 0.015; Fig. 1B; Table 2). Interestingly,
up-regulation of uPA was significantly associated with HBx positivity \((P = 0.015; \text{Fig. 2B; Table 2})\). These results strongly support the notion that HBx induces activation of NF-\(\kappa\)B, which in turn stimulates the expression of uPA.

**Clinicopathological Correlation of NF-\(\kappa\)B Activation, uPA Overexpression, and HBx Expression.** For ease of correlation, increased NF-\(\kappa\)B DNA-binding activity and overexpression of nuclear RelA were combined together to represent NF-\(\kappa\)B activation, and a case was counted as having NF-\(\kappa\)B activation when there was simultaneous increased NF-\(\kappa\)B DNA-binding activity and overexpression of nuclear RelA. Eighteen (56.3\%) HCCs showed NF-\(\kappa\)B activation, which was significantly associated with direct liver invasion by the tumor \((P = 0.001)\), venous invasion \((P = 0.01)\), absence of tumor encapsulation \((P = 0.043)\) and positive HBsAg status \((P = 0.027; \text{Table 3})\). uPA overexpression was more frequently seen in HCCs with liver invasion \((P = 0.028)\), venous invasion \((P = 0.004)\), absence of tumor encapsulation \((P = 0.032)\), advanced tumor stages (stages III and IV; \(P = 0.005\)), tumor microsatellite formation \((P = 0.03)\) and presence of HBsAg \((P = 0.01)\). Significant correlation was also found between HBx expression and liver invasion \((P = 0.05)\) and positive HBsAg status \((P < 0.0001)\).

**Activation of NF-\(\kappa\)B by IKK and HBx in Vitro.** To further characterize the role of HBx in the activation of NF-\(\kappa\)B, \(\textit{in vitro}\) experiments were performed by transfecting the HBx-expressing plasmid into HepG2 cells. As shown in Fig. 2A, uPA mRNA expression was detected by semi-quantitative reverse transcription-PCR with a product size of 366 bp. \(\beta\)-Actin with a product size of 190 bp was used for normalization. Overexpression of uPA of >2-fold was detected in the tumor tissues, as compared with nontumorous livers (cases 216 and 219). Cases 213 and 238 were HBx negative and showed no aberrant uPA expression in the tumors. T, tumor tissues; NT, nontumorous; uPA, urokinase plasminogen activator. B, representative cases (cases 216 and 219) showing HBx mRNA expression by reverse transcription-PCR, with a band size of 402 bp. Cases 213 and 238 were negative cases for comparison. C, results of immunohistochemical staining of HBx in hepatocellular carcinoma samples, with positive submembranous and cytoplasmic HBx staining, respectively (C-1, case 216; C-2, case 219; magnification \(\times 200\)). No HBx immunoreactivity was found in HBx mRNA-negative case (C-3, case 238; magnification \(\times 200\)).

### Table 2 Correlation of expression in nucleus compartment, NF-\(\kappa\)B DNA binding activity, HBx expression and uPA expression

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\(^a\) NF-\(\kappa\)B, nuclear factor \(\kappa\)B; HBx, hepatitis B viral X protein; uPA, urokinase plasminogen activator.

\(^b\) Compared with the corresponding nontumorous livers.
NF-κB B activity was abolished in the HepG2 cells transfected with HBx and IKK WT. In contrast, if their actions are in parallel, the effects of IKK WT downstream of HBx in the same pathway, the expression of NF-κB by HBx significantly depends on IKK WT stimulated NF-κB activity by ~3-fold, whereas the NF-κB activity was abolished in the HepG2 cells transfected with IKK WT (~0.5-fold, P = 0.019; Fig. 3). To query for whether HBx activation of NF-κB is via IKK WT, we coexpressed HBx and IKK WT or IKK D/N in HepG2 cells. If IKK WT acts downstream of HBx in the same pathway, the expression of IKK WT should abolish the induction of NF-κB by HBx. On the contrary, if their actions are in parallel, the effects of IKK WT on HBx activation of NF-κB should at least be incomplete. Indeed, we observed that the activation of NF-κB by HBx was completely lost in the HepG2 cells cotransfected with HBx and IKK WT (~0.5-fold, P = 0.011; Fig. 3). In contrast, IKK WT further enhanced the HBx-mediated NF-κB activation by ~2- to 3-fold (P = 0.004; Fig. 3). Thus, IKK WT apparently cooperates with HBx in the activation of NF-κB, although activation of NF-κB by HBx may also be through other possibilities such as interaction with IkBα and hepatitis B virus X-associated protein, which have been investigated previously (32, 33). Collectively, our findings suggest that activation of NF-κB by HBx significantly depends on IKK WT.

**Up-Regulation of uPA by IKK WT and HBx.** We observed that uPA mRNA was not up-regulated in HBx-transfected cells (Fig. 4A), suggesting that transient expression of HBx alone is insufficient to activate uPA. Thus, HBx unlikely has a direct influence on the transcription of uPA gene. However, we found a 2.5-fold increase in the induction of uPA mRNA in the HepG2 cells transfected with IKK WT (P = 0.01; Fig. 4B), whereas uPA mRNA expression was reduced when IKK WT was transfected (~0.5-fold, P = 0.005; Fig. 4B). Hence, the transcription of uPA gene is activated by NF-κB through IKK WT. Interestingly, a remarkable induction of uPA mRNA expression was seen when HepG2 cells were cotransfected with HBx and IKK WT (~5-folds P = 0.005; Fig. 4B). Our interpretation of these data is that HBx may contribute to the induction of uPA by acting synergistically with other stimuli of NF-κB.

**Induction of Cell Invasiveness by IKK WT and HBx.** Above we have shown that the expression of uPA was tightly regulated by NF-κB activation, and further elevation of uPA was implicated by HBx cotransfected with IKK WT (Fig. 4). To investigate the association of NF-κB activation and invasiveness of HepG2 cells, cell invasion assay was conducted. With transfection of HBx alone, HepG2 cells invaded into the lower chamber modestly (Fig. 5A). Such induction of cell invasion by HBx was in accordance with previous findings (34, 35). The invasive ability of HepG2 cells was obviously increased by NF-κB activation through IKK WT signaling. This was evidenced by the marked induction of invasiveness of HepG2 cells with the introduction of IKK WT, whereas this phenomenon was abolished by the presence of IKK WT (Fig. 5B). Notably, cotrans-
Activation of NF-κB in Hepatocellular Carcinoma

In HCC, high rates of tumor recurrence after surgical resection because of intrahepatic metastasis contribute to the poor prognosis (36). Cancer cell invasion requires degradation of the surrounding extracellular matrix, and uPA is one of such mediators of degradation. In this present study, uPA was frequently overexpressed in HCC, and this finding is in agreement with those of the previous studies (25, 37). Indeed, uPA has also been regarded as a biomarker for aggressive HCC (38). Concerning the relationship between uPA overexpression and NF-κB activation, NF-κB has been shown to be essential for regulating uPA expression, because NF-κB-binding site is located at its promoter region (39). In our study, activation of NF-κB was closely associated with uPA overexpression. In addition, uPA overexpression was significantly associated with a more invasive tumor phenotype in terms of venous invasion, liver invasion, and absence of tumor encapsulation (same parameters for NF-κB activation), in addition to advanced tumor stages and tumor microsatellite formation. These results strongly support the notion that activation of NF-κB in HCC stimulates the expression of uPA and leads to a more aggressive tumor behavior.

The signaling cascade involved in NF-κB activation mediated by HBx was investigated in HepG2 cells in the present study. HBx was found to be a weak inducer for NF-κB activation in vitro. However, NF-κB activation was further enhanced by HBx with cotransfection of IKKβ WT but was inhibited by IKKβ D/N. These data suggest that activation of NF-κB by HBx likely depends on IKKβ activation. Activation of IKKβ requires phosphorylation, and such phosphorylation is achieved by the action of upstream kinases. It has been demonstrated that HBx-

**Fig. 3** NF-κB-luc reporter DNA was cotransfected with IKKβ WT, IKKβ D/N, or HBx expression vector alone or in combination into HepG2. NF-κB luciferase activities were measured 48 h after incubation and obtained by fold induction over mock control transfected with NF-κB-luc reporter alone. Data represented as mean values from three-independent experiments ± SE, and the Ps were calculated by comparison with the mock control. NF-κB, nuclear factor-κB; IKKβ, inhibitor of nuclear factor-κB kinase; WT, wild type; D/N, dominant negative; HBx, hepatitis B viral X protein.

**Fig. 4** HepG2 cells were transfected with expression vector of HBx, IKKβ WT, or IKKβ D/N alone or in combination. HBx, hepatitis B viral X protein; IKKβ, inhibitor of nuclear factor-κB kinase; WT, wild type; D/N, dominant negative; uPA, urokinase plasminogen activator. A, uPA RNA was extracted and detected 48 h after incubation. B, data were obtained by fold induction over mock control and represent mean values from three-independent experiments ± SE. Ps were calculated by comparison with the mock control.
activated NF-κB requires activation of NF-κB inducing kinase and IKKβ through targeting on tumor necrosis factor (TNF) type I receptor, causing degradation of IκBα and eventually leading to NF-κB activation (40). In addition, HBx is capable of inducing cytoplasmic degradation of the two NF-κB inhibitors, IκBα and the precursor/inhibitor p105 through phosphorylation of these proteins (29). HBx has also been demonstrated to associate with newly synthesized IκBα and thus prevents the reassociation with DNA-bound NF-κB (32). Collaboration of HBx with hepatitis B virus X-associated protein is also capable of coactivating NF-κB signaling (33).

In this study, we have demonstrated for the first time that HBx and IKKβ synergistically up-regulate uPA expression in HepG2 cells, through activation of NF-κB signal. These results help to explain the significant correlation we have found among NF-κB activation, HBx expression, and uPA overexpression in human HCCs. It was not surprising that HBx alone failed to induce uPA up-regulation because HBx appeared not to be a strong transactivator. However, uPA expression was enhanced by increased IKKβ level and was synergistically elevated by cotransfection of IKKβ WT and HBx. In fact, such increased level and activation of IKKβ are often found in the liver by up-regulated TNF-α gene expression in inflammatory processes such as acute and chronic HBV infection (41). HBx protein causes liver cell death during viral replication by inflammatory responses such as elaboration of TNF-α (42), and liver regeneration itself also causes an increase of TNF-α (43). Increased level of TNF-α activates IKK complex, which in turn allows NF-κB translocation and activation (44).

To further investigate the association of NF-κB activation and invasive potential of HepG2 cells, cell invasion assay was conducted and showed that HBx and IKKβ WT synergistically
increased the invasiveness of the HepG2 cells, which was abrogated by IKKβ D/N. This suggests that HBx-induced NF-κB activation through IKKβ signaling contributes to the induction of HepG2 cell invasion. Such activation of NF-κB can lead to activation of the target genes governing invasion and metastasis, such as vascular endothelial growth factor, interleukin-8, proteolytic enzymes (such as matrix metalloproteinases, uPA), and cell adhesion molecules (10). In addition, we observed that HepG2 cells, when transfected with HBx alone, also showed increased cell invasiveness, which may be related to elevation of HBx-induced-cyclooxygenase 2 and matrix metalloproteinases expression and activity (34, 35, 45). HBx has also been demonstrated to activate interleukin-8 through NF-κB and CCAAT enhancer-binding protein-like cis-elements, contributing to enhanced metastatic potential (46).

Taken together, our results indicate that NF-κB dysregulation and uPA overexpression may lead to a more aggressive tumor behavior in HCC. In addition, our data suggest that IKKβ plays a critical role in the HBx-activated NF-κB signaling pathway. This study provides new clues for understanding the role of HBx and constitutive activation of IKKβ/NF-κB signaling pathway in HCC. Inhibition of activated NF-κB signaling pathway may provide potential new target treatments against this cancer.

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