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Hepatitis B virus X protein mediates yes-associated protein 1 upregulation in hepatocellular carcinoma

YUZHUO WU[1], JUNHE ZHANG[2], HUAIHONG ZHANG[1] and YUFENG ZHAI[1]

[1]Department of Infectious Diseases, Nanyang City Central Hospital, Nanyang, Henan 473000;
[2]Department of Biochemistry and Molecular Biology, Xinxiang Medical University, Xinxiang, Henan 453003, P.R. China

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Abstract. Hepatitis B virus (HBV) X protein (HBx) is implicated in the development of hepatocellular carcinoma (HCC). Yes-associated protein 1 (YAP) is an important proto-oncogene, which is a downstream effector molecule in the Hippo signaling pathway. The aim of the present study was to investigate the association between HBx expression in HCC samples and YAP expression in the Hippo pathway. A total of 20 pathologically confirmed HCC samples, 20 corresponding adjacent non-tumor liver tissues and 5 normal liver tissue samples were collected. The expression of HBx and YAP in the tissues was analyzed by quantitative reverse transcription-polymerase chain reaction and western blot analysis. The intensity and location of YAP expression were analyzed by immunohistochemistry. YAP mRNA and protein expression levels in HCC samples infected with HBV were significantly higher than those of normal liver tissues. Furthermore, YAP expression was positively correlated with HBx expression in HBV-positive HCC samples. Immunohistochemical staining revealed that YAP was predominantly expressed in the nuclei in HBV-positive HCC tissues. YAP expression was significantly decreased in the normal liver tissue and corresponding adjacent liver tissue when compared with the HCC tissues and by contrast to HCC tissues, YAP was predominantly located in the cytoplasm. In conclusion, these results indicate that the YAP gene is a key driver of HBx-induced liver cancer. Therefore, YAP may present a novel target in the treatment of HBV-associated HCC.

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common type of cancer worldwide, and the incidence of hepatitis B virus (HBV)-related primary HCC is 55 cases/100,000 individuals for each gender (1). HCC can be diagnosed by non-invasive imaging, including computed tomography or magnetic resonance imaging. At present, there are multiple therapeutic methods available for HCC, which depends on the stage of HCC, however, surgical treatment is the most common method. Despite this, the prognosis for HCC is poor (2). HBV is an important pathogenic factor for HCC (3). Hippo is a highly-conserved signaling pathway that has been identified in both drosophila and mammals (4). Yes-associated protein 1 (YAP) is a transcription factor in the Hippo signaling pathway (5), which regulates a number of transcription factors, including erb-b2 receptor tyrosine kinase 4, runt related transcription factor 2, p73, and TEA domain transcription factor 1 (6-9). Furthermore, YAP promotes the proliferation of liver cancer in mice by regulating the transcription of certain target genes, including Ki-67, c-myc, SRY-Box 4 (SOX4), H19 and α-fetoprotein (AFP) (10). In addition, transgenic mice overexpressing YAP exhibited an increased liver size and eventually developed liver cancer (11). It has also been demonstrated that YAP overexpression induces epithelium-mesothelium conversion and anchorage-independent growth in mammary epithelial MCF10A cells (12). A recent study in HCC revealed that YAP overexpression overcame cell contact inhibition and promoted cell growth (13). In addition, the expression and nuclear accumulation of YAP was elevated in prostate, large intestine, breast, esophageal, ovarian and liver cancer (14-16). Clinical studies in HCC have demonstrated that YAP is an independent predictive factor of poor prognosis and overall survival (16). YAP is involved in the development of HCC, indicating that YAP exhibits an extremely important function in HBV-associated HCC (17). However, the mechanism of HBV-induced YAP upregulation remains unclear. In four HBV encoded proteins, HBV X protein (HBx) acts as a multifunctional regulatory protein, which exhibits an important function in HBV-induced HCC (18). Although HBx does not combine directly with DNA, HBx interacts with nuclear transcription factors, including activator protein-1, nuclear factor-κB, specificity protein-1 and cyclic adenosine monophosphate response element-binding protein to regulate transcriptional activity, subsequently affecting the regulation of intracellular signal transduction pathways (19,20). Therefore, it is hypothesized that HBx may be closely associated with YAP upregulation.

Correspondence to: Dr Yufeng Zhai, Department of Infectious Diseases, Nanyang City Central Hospital, 312 Gongnong Road, Nanyang, Henan 473000, P.R. China
E-mail: yufengzhaicn@163.com

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Subjects and methods

Patient samples. A total of 20 HCC tissues and corresponding adjacent non-tumor liver tissues were obtained immediately after surgical resection, performed in Nanyang City Central Hospital (Nanyang, China) between March 2013 and March 2015. Patient's clinical and pathological information was obtained from case records. All patients were known to be HBV-positive. All liver tumor tissue samples were confirmed as primary HCC by at least two pathologists, according to the Barcelona Clinic Liver Cancer staging system (21). No patients had received radiotherapy or chemotherapy prior to surgery. A total of 5 normal liver tissues were obtained from trauma patients during resection. This study was conducted in accordance with the Declaration of Helsinki. This study was conducted with approval from the Ethics Committee of Nanyang City Central Hospital and written informed consent was obtained from all patients.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total cellular RNA was extracted using Trizol reagent (Takara, Dalian, China) according to the manufacturer's instructions. RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, St. Leon-Rot, Germany) was used for cDNA synthesis and PCR amplification. DyNAmo™ Flash SYBR® Green qPCR kit (Finnzymes Oy, Espoo, Finland) was used for qPCR. The primer sequences used for PCR were as follows: Yap forward, 5'-GGGTGTTCATCCATTCTC-3' and reverse, 5'-CCCAGCATCTTGTGTTTC-3'; HBx forward, 5'-GTGGGATGATGACGACG-3' and reverse, 5'-TACGAC CAGAGGCATACAGG-3'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward, 5'-GCGGGAAATCGT GCGTGAC-3' and reverse, CGTCATACTCCTGCTTGC TG-3'. The reaction conditions were pre-degeneration at 94˚C for 30 sec, degeneration at 94˚C for 5 sec, annealing at 94˚C for 30 sec and extension at 72˚C for 40 sec, for 35 cycles, followed by terminal extension for 5 min at 72˚C. PCR was performed according to a previously described protocol (22). The expression levels were calculated and normalized to GAPDH.

Western blot analysis. The liver tissue was homogenized with liquid nitrogen. Cells were lysed using cell lysis solution [0.3% NP40, 1 mM EDTA, 50 mM Tris-Cl (pH 7.4), 2 mM EDTA, 1% Triton X-100, 150 mM NaCl, 25 mM NaF, 1 mM Na_3VO_3, 10 μg PMSF]. The experiment was conducted as described previously (23). The membranes were incubated with monoclonal mouse YAP (1:100 dilution; catalog no. sc-15407; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and rabbit polyclonal HBx (1:200 dilution; catalog no. ab39716; Abcam, Cambridge, UK) primary antibodies, and β-actin mouse monoclonal antibody (1:200 dilution; catalog no., sc-47778; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. The membranes were then washed with phosphate-buffered saline, followed by incubation with goat anti-mouse immunoglobulin G secondary antibody (1:2,000 dilution; catalog no. BA1031; Boster Inc., Wuhan, China) at room temperature for 1 h, and further washing 3 times. Next, the membranes were incubated with enhanced chemiluminescence colored liquid (Boster Inc.). The expression of YAP, HBx and β-actin was quantified using Image J software (version 1.38; National Institutes of Health, Bethesda, MD, USA). The experiment was performed in triplicate.

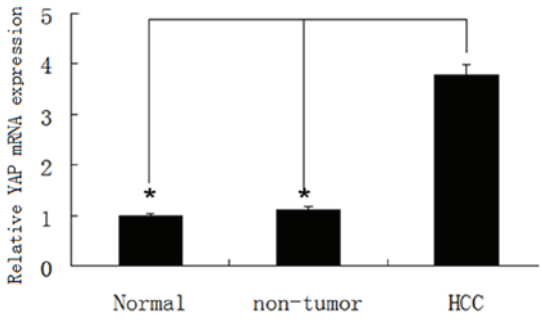

Figure 1. Relative YAP mRNA expression in normal, non-tumor and HCC liver tissues. *P<0.05 vs. HCC. HCC, hepatocellular carcinoma; YAP, yes-associated protein 1.

Immunohistochemical analysis. For routine immunohistochemical analysis, liver tissue was resected, embedded in paraffin and cut into 5-μm thick sections. After the sections were dewaxed and hydrated, the endogenous catalase was removed with 3% hydrogen peroxide. The primary and secondary antibodies used were those applied in the western blotting, with identical incubation times and temperatures. The sections were incubated with DAB, stained with hematoxylin and observed under an optical microscope (Olympus AX80; Olympus Corporation, Tokyo, Japan). YAP immunoreactivity was classified according to the following staining scores: Negative, 0; and positive, 1-3; as described previously (16)

Statistical analysis. All data were analyzed using SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Student's t-test was conducted and the data presented as the mean ± standard deviation. Pearson's correlation coefficient was used to analyze the association between YAP and HBx mRNA expression. The Wilcoxon signed rank test was used to compare YAP expression in tumor tissue and matched adjacent non-tumor tissue. P<0.05 was considered to indicate a statistically significant difference.

Results

YAP and HBx mRNA expression are positively correlated in HBV-positive HCC tumor tissues. YAP mRNA expression was analyzed in 20 paired HCC and adjacent non-tumor liver tissues. The results revealed that YAP mRNA levels in HBV-associated HCC tissues were significantly higher than that of non-tumor liver tissues (P<0.05) (Fig. 1). According to a previous study (24), RT-qPCR identified HBx mRNA in HCC tissue. The present study demonstrated that HBx mRNA expression was identified in all HBV-infected clinical samples by RT-qPCR. Notably, upregulation of YAP was significantly correlated with HBx expression (P<0.05; r=0.719) (Fig. 2). Western blot analysis of YAP expression in HBx-infected liver tissue revealed that HBx expression was positively correlated with YAP upregulation.

HCC tissues exhibit strong nuclear YAP expression. YAP immunoreactivity was classified according to the following staining scores: Negative, 0; and positive, 1-3; as described

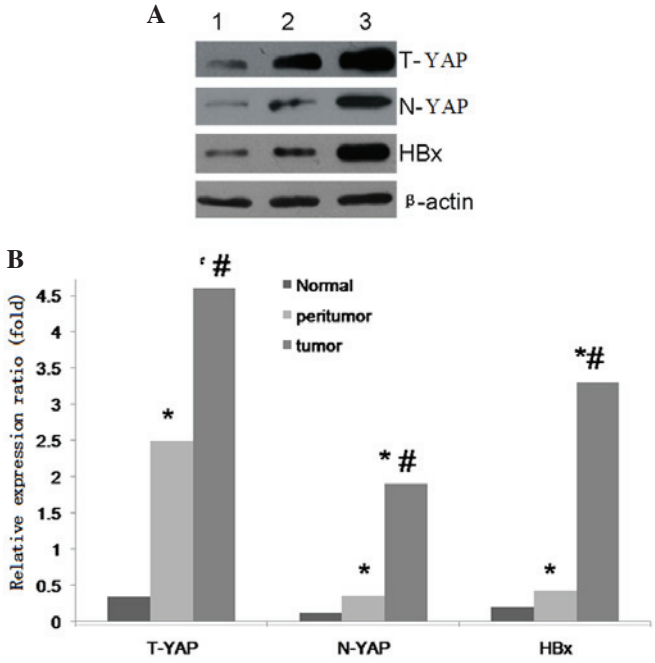

Figure 2. (A) Western blot analysis of T-YAP, N-YAP, HBx and β-actin expression. Lanes 1, 2 and 3 indicate normal, peritumor and HCC tissue, respectively. (B) Relative protein expression of total Yap, nuclear Yap and HBx. *P<0.01 for tumor versus peritumoral or normal tissue; #P<0.01 for tumor versus peritumoral tissue for T-YAP, N-YAP and HBx protein. T-YAP, total yes-associated protein 1; N-YAP, nuclear YAP; HBx, hepatitis B virus x protein.

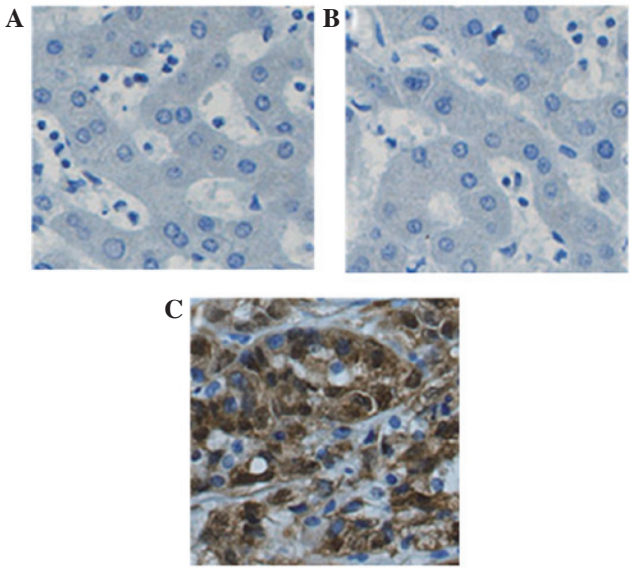

Figure 3. Immunochemical staining for YAP in (A) normal liver, (B) adjacent non-tumor liver tissues and (C) hepatocellular carcinoma tissues (magnification, x200). YAP, yes-associated protein 1.

previously (16). The results confirmed that the expression of YAP was increased in 70% of HCC tissues in comparison to adjacent HCC tissues. A total of 40.0% (8/20), 15% (3/20) and 10% (2/20) of HCC tissues exhibited strong, moderate and low nuclear staining, respectively. The rest of the HCC samples exhibited negative YAP nuclear staining. In HCC tissues, cytoplasmic localization of YAP with strong and moderate immunoreactivity was observed in 35 (7/20) and 25% (5/20) of tissues, respectively. The rest HCC sample showed weak and negative YAP intracellular immunoreactivities. Positive cytoplasmic YAP expression was identified in adjacent non-tumor and normal liver tissues (Fig. 3). Strong YAP staining was observed in HCC cell nuclei, but not in non-tumor cell nuclei.

Discussion

HBx exhibits an important function in HBV-induced hepatocellular carcinoma (18). It has been reported that HBx regulates various genes (25). HBx involves numerous functions, including transcription regulation, signal transduction, cell cycle progression, protein degradation pathways, apoptosis and the interaction between different transcription factors and signal transduction pathway components (19,20,24,26). YAP is an important transcription factor that promotes the proliferation of liver cancer in mice by regulating the transcription of certain target genes, including Ki-67, c-myc, SOX4, H19 and AFP (18). In the present study, YAP expression in 20 primary HCC, 20 adjacent non-tumor liver tissues and 5 normal HBV-negative liver tissues were analyzed by RT-qPCR and western blot analysis. YAP expression was increased in the cell nucleus of primary HCC liver tissue when compared with non-tumor tissues and was positively correlated with HBx expression. Immunohistochemical staining also revealed that YAP was strongly expressed in HBx-induced HCC liver tissue, however, YAP expression was negative or weak in adjacent non-tumor tissue and normal liver tissue, indicating that YAP and HBx expression are positively correlated. We hypothesized that although HBx does not directly combine with DNA, it induces the transcription factor, YAP, in the Hippo pathway to shuttle and translocate to the nucleus. Additionally, YAP forms complexes with other factors to regulate the expression of target genes, such as the AFP protein (18,27). Zhao *et al* (13) reported that 54% of 115 HCC patients exhibited YAP overexpression in the United States and Xu *et al* (16) reported that 62% of 117 HCC patients exhibited YAP overexpression in Hong Kong. Furthermore, YAP was predominantly located in the cell nuclei of liver cancer cells and YAP staining was weak in non-tumor tissues (13,16), which was consistent with the results of the present study. Notably, nuclear YAP expression has also been identified in other tumors, including prostate, colorectal, breast, ovarian and esophageal cancer (13,16). Kaposi's sarcoma-associated herpes virus is an oncogenic virus. The genome-encoded viral G protein-coupled receptor inhibits kinase Last1/2 in the Hippo signaling pathway via Gq/11 and G12/13, which promotes YAP activation leading to cell proliferation and transformation (28). This indicates that certain tumor viruses lead to cell transformation via the Hippo signaling pathway. Therefore, YAP inhibitors may present can potential antiviral or anticancer drug candidates.

In conclusion, YAP is a critical oncogenic protein that is involved in human cancer. The present study revealed that HBx expression upregulates YAP in HCC cells. Therefore, HBx upregulates YAP in liver cancer cells and may contribute to the development of HBV infection-associated HCC, indicating that YAP is a key driver gene of HBx-induced liver cancer. Thus, YAP may present a novel target for the treatment of HBV-associated HCC.

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