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HBV DNA can bind to P53 protein and influence p53 transactivation in hepatoma cells ☆

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ABSTRACT

Hepatitis B virus (HBV) may contribute to hepatocarcinogenesis by blocking *p53* function. A *p53* response element-like binding sequences, TGCCT···TGCCT, was found in HBV genome. To clarify whether HBV DNA can, like some other DNA viruses, bind to P53 protein and form a DNA–protein complex, we used a series of plasmids encoding full-length or mutant HBV or *p53* fragments to determine the binding ability of HBV DNA after cotransfected into cells by electrophoretic mobility shift (and supershift) assay. We found that HBV DNA could bind to P53 protein and form DNA–protein complexes in human hepatoma cell lines. Cotransfection with *p53* and HBV DNA increased the replication of HBV, CAT activity, tumor cell apoptosis, and cytoplasmic P53 accumulation in the hepatoma cells. In conclusions, our observations suggest that the interaction of HBV and p53 at the levels of protein–protein and DNA–protein, which resulted in inactivation of *p53* transactivation.

Introduction

Hepatocellular carcinoma (HCC) is a common malignancy and has become one of the leading causes of death in many countries. Hepatitis B virus (HBV) infection has been proven to be the most prominent factor for the development of hepatocellular carcinoma [1–5]. Tumor suppressor gene *p53* is thought to be closely associated with human hepatocellular carcinoma through involved in cell differentiation, cell cycle regulation and apoptosis [6,7]. Although the molecular pathogenesis of human HCC can involve the somatic mutational inactivation of the *p53* gene [8,9], the absence of *p53* mutation in the majority of HCC cases [10] suggests that the inactivation *p53* can be achieved by other mechanism(s).

Our previous studies showed that P53 protein could bind to hepatitis B virus X protein (HBx) to form a protein–protein complex, resulting in inactivation of *p53* transactivation, and it may contribute to hepatocarcinogenesis by blocking *p53* function [3,11–13]. The same result was also obtained by other laboratories [14]. Wild-type *p53* (wt*p53*) can bind to DNA sequences termed *p53* response elements, TGCCT···TGCCT elements, which abolished the protein is no longer able to direct the activation of specific growth controlling genes both *in vitro* and *in vivo* [15–19]. The *p53*–DNA response element-like binding sequence was found in a number of small DNA tumor viruses [such as the adenovirus type 5 E1B and human papilloma virus (HPV) types 16 E6], which can specifically bind to wild-type *p53* protein, and may result in inactivation of *p53* in virus-transformed cells through complex formation [20–22]. We analyzed the full-length HBV DNA sequence using a computer program, a *p53* response element-like binding sequences was found at the upstream of HBV enhancer I from 1047 to 1059 nucleotides. However, to date the precise role of the binding sequence in human hepatocarcinogenesis remains unclear.

In this study, we further study the relationship between HBV and *p53* and to clarify whether HBV DNA is able to bind P53 protein, forming a DNA–protein complex. We also studied the biological significance of the interaction between HBV DNA with *p53*.

Materials and methods

Cell culture. Human hepatoma cell lines HepG2 (expressing wild-type *p53* with no HBV activity), Hep3B (*p53* disrupted with HBV activity), PLC/PRF/5 (*p53* delated with HBV activity), SKHepG1 (expressing wild-type *p53* with no HBV activity) and Huh7 (*p53* mutated with no HBV activity) were maintained at 37 °C in a

Abbreviations: HBV, hepatitis B virus; HBx, hepatitis B virus X protein; CAT, chloramphenicol acetyl transferase; CMV, cytomegalovirus; HCC, hepatocellular carcinoma.

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humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Synthesizing and labeling of probes of HBV DNA–p53 binding site. Genomic sequence of HBV was analyzed by the Clone software (Geneway Co.). A *p53* response element-like binding site was found in the upstream of HBV DNA enhancer I, spanning nucleotides of 1047–1059. This putative p53 binding sequence was found at the analogous position in all the HBV subtypes examined. The complementary oligonucleotides were synthesized based on the published human HBV sequence [23], namely HBV/p53a (subtype adw), HBV/p53b (subtype ayw), the mutants of the putative HBV/p53 binding sequence form subtype adw (HBV/p53M1) and ayw (HBV/p53M2,). The published wild-type *p53* response element binding sequence was used as control [24]. The sequences of primers are shown in Supplementary material 1.

Plasmids. The HBV, HBx and P53 protein expression vectors pCMV-HBVa, pCMV-HBVb, pCMV-HBx, pCMV-wtp53 and PT7HBc were supplied by the Center for Human Virology of Thomas Jefferson University. pG13-CAT reporter plasmid, its inner control plasmid pSVβ-gal and p21-luc were kindly provided by Vogelstein [23].

Preparation of nuclear extracts. Nuclei protein extraction from hepatoma cells was performed by Dignam's method [25,26].

Electrophoretic mobility shift assay and in situ ultraviolet cross-linking assay. Electrophoretic mobility shift assay (EMSA) and *in situ* ultraviolet cross-linking assay were performed using a DNA–protein binding detection kit (Gibco-BRL) according to the manufacture's protocol, which was shown in Supplementary material 1 [27].

Electrophoretic mobility supershift assay (EMSSA). For monitoring P53 composition in electrophoretic mobility supershift assay (EMSSA), 6 μg nuclei extract of HepG2 cells was incubated with 3 μl anti-P53 monoclonal antibody DO-1 (Oncogene) at 4 °C for 30 min, then a labeled fragment of synthesized complementary oligonucleotides was added, and the mixture was performed without nuclei extract or with anti-Ras antibody in place of anti-P53 antibody. The gel was run as EMSA, and exposed to Kodak X-ray film overnight at −70 °C.

Chloramphenicol acetyltransferase assay (CAT) ELISA and luciferase assays. CAT and luciferase assays were tested according to the manufacturer's instructions as shown in Supplementary material 1 and three independent experiments were done.

Western blot analysis. HepG2 cell pellets were lysed in lysis buffer containing protease inhibitors. Western blot analysis was performed as described previously [28]. The Western blotting detection kit (ECL Plus) was obtained from Santa Cruz Biotechnology (Delaware Avenue Santa Cruz, CA, USA). The antibodies included: anti-p53 (DO-1, 1:1 000 dilution, Oncogene) and anti-p21 (1:200; Dako, Gene Co. Ltd., Shanghai, China). ECL internal control protein GAPDH detection was performed simultaneously.

Immunocytochemical staining of p53 protein. HepG2 cells transfected or cotransfected with pCMV-wtp53 or pCMV-wtp53 + pCMV-HBx were plated on 4-well slides, and allowed to adhere to them overnight. Culture medium was removed and the cells were fixed and reacted with DO-1 (1:50 dilution, Oncogene), then fluorescence-labeled goat anti-mouse IgG (Sigma) was added and the slides were observed under a fluorescence microscope.

HBV DNA and p53 cotransfection and quantitative PCR analysis. HepG2 cells were transfected by the method of calcium phosphate coprecipitation according to the instructions of the manufacture (Promega Inc.). For transfections, pCMV-HBVa, pCMV-HBVb, pCMV-wtp53, pCMV-HBx or pCMV full vector were transfected or cotransfected, respectively. The medium was changed 24 h post transfection. Supernatants and cells were harvested 48 h post transfection. HBV DNA in supernatants and genomic DNA of cells were extracted and purified by standard methods. Real-time PCR was performed to quantify the HBV DNA using a HBV fluorescence detection kit following the manufacturer's protocol (Bioer Technology Co., Ltd.). For measurement of viral DNA, DNA was extracted from the culture supernatant using a QIAamp DNA mini kit. Reactions with no reverse transcriptase enzyme added were performed in parallel.

Cell cycle and apoptosis by flow cytometry. HepG2 cells were harvested at various time points, washed with phosphate-buffered saline, fixed in 70% ethanol, and stained with 0.5 mg/ml of propidium iodide (Sigma) along with 0.1 mg/ml of RNase A (200 KU, Calbiochem, San Diego, CA) for cell cycle and apoptosis analysis by flow cytometry. The annexin V detection kit was used to detect apoptotic cells according to the manufacturer's instructions. Data acquisition and analysis were done in a FACSort Cytometer (FACSCA, USA). For each analysis, 1×10^5 cells were acquired. Each experiment was repeated at least thrice.

Statistical analysis. The results were expressed as mean ± SD. Differences between experimental groups were evaluated by Student's *t*-test. A p value <0.05 was considered statistically significant.

Results

HBV DNA bound to P53 protein formed DNA–protein complex in human hepatoma cell lines in vitro

Firstly, we determined whether p53 and HBV DNA could bind to the nuclear proteins of hepatoma cells. The EMSA results showed that the strongest binding signal was found in HepG2 cells among the cell lines tested, and no signal was detected in Hep3B cells (Fig. 1). Secondly, we investigated whether this binding is specific by using HepG2 cells; stronger binding signals were found with HBV/p53a and HBV/p53b; and this binding signal was associated with nuclear protein in a dose-dependent manner. Because there is a one-base-pair difference between HBV/p53a and HBV/p53b, the signal of HBV/p53b was relatively weaker than that of HBV/p53a. When the NF-κb fragments were used for competition, the binding signals showed no obvious change. But when the unlabeled probes were added for competition, the binding signals were reduced (Fig. 1). *In situ* ultraviolet cross-linking experiments showed that the molecular weight of the protein fractions in the complexes was approximately 53 kDa (Supplementary material 2).

When anti-P53 antibody was allowed to react with nuclei protein, DNA–protein complex bands were supershifted. The density of bands was increased with the increase of anti-P53 antibody dosage. But no supershifting bands were detected when anti-Ras antibody was used in place of anti-P53 antibody or when no nuclei extracts were added (Fig. 2). These results further confirmed that HBV DNA can bind to P53 and form HBV DNA–P53 protein complex *in vitro*.

HBV DNA increased CAT and p21-promoter activity

When transfected with PG13-CAT plasmid alone, HepG2 cells had a weak expression of CAT enzyme (1.402 ± 0.227), which suggests that there is an underlying transcription effect of p53. The CAT activity was increased significantly in HepG2 cells transfected with pCMV-HBVa-PG13-CAT (2.525 ± 0.166), pCMV-p53 (3.122 ± 0.297) or cotransfected with pCMV-p53 and pCMV-HBVa-PG13-CAT (3.419 ± 0.264). However, it was not obvious changed in HepG2 cells transfected with pCMV-HBVb-PG13-CAT, or cotransfected with pCMV-p53 and pCMV-HBVb-PG13-CAT (Supplementary material 3a).

Next, we tested whether HBV DNA or wild-type p53 protein could activate transcription from a p21 promoter. The results showed that the luciferase values was increased significantly in