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Hepatitis B virus X protein inhibits p53-mediated upregulation of mitofusin-2 in hepatocellular carcinoma cells

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ABSTRACT

The hepatitis B virus X (HBx) protein has many significant roles in hepatocellular carcinoma (HCC). Our previous research demonstrated that mitofusin-2 (Mfn2), a potential tumor suppressor gene in HCC, is a novel direct target of p53 that exerts apoptotic effects via the mitochondrial apoptotic pathway. However, the relationship between HBx and Mfn2 expression in the development of HCC is unknown. We found that HBx had little direct effect on the expression of Mfn2 or p53 in HCC cells not treated with doxorubicin. However, HBx inhibited the upregulation of Mfn2 in HBx-transfected HCC cells simultaneously treated with doxorubicin or cotransfected with p53 plasmid, as evidenced by Western Blot and real-time PCR. Through electrophoretic mobility shift analysis, we confirmed that HBx interfered with the binding event of the p53 protein and the p53 binding site-oligo of the Mfn2 promoter. Moreover, luciferase assays revealed that the activity of the Mfn2 promoter did not increase when transfected with HBx plasmid in doxorubicin-treated HepG2 cells. These results indicate that HBx impacts p53-mediated transcription of Mfn2, providing insight into the negative effect of HBx against p53-dependent chemotherapeutic agents, such as doxorubicin, used in the treatment of HCC.

1. Introduction

The mitochondrial GTPase mitofusin-2 (Mfn2) gene, located on chromosome 1p22.3, encodes a mitochondrial membrane protein that participates in mitochondrial fusion and contributes to the maintenance of the mitochondrial network [1,2]. Although it has been reported that Mfn2 might be involved in the pathophysiology of obesity [3] and its mutant could cause Charcot-Marie-Tooth disease type 2A2 [4], the potential apoptotic effect of Mfn2 is gradually being recognized since Chen et al. first described its antiproliferative effects in vascular smooth muscle cells (VSMCs) [5]. Further research indicated that Mfn2 had potential apoptotic effects mediated by the mitochondrial apoptotic pathway [6,7].

Infection with human hepatitis B virus (HBV) is a major risk factor for the development of hepatocellular carcinoma (HCC) [8]. The HBV genome contains four partially overlapping open-reading frames encoding the envelope (pre-S/S), core (pre-C/C), polymerase (P), and X proteins (X). As the smallest of the HBV envelope proteins, the 154 amino acid HBx protein is a multifunctional regulatory protein reported to be associated with hepatocellular carcinogenesis. Several studies have demonstrated that HBx interacts with p53 to inactivate p53-dependent activities via a variety of mechanisms, including transcriptional repression of the p53 gene [9], cytoplasmic retention of the p53 protein [10], and inhibitory effects on p53 sequence-specific DNA binding [11]. Furthermore, HBx downregulates the expression of phosphatase and tensin homolog (PTEN) by interfering with the binding of p53 to the PTEN promoter in liver cells [12]. Interestingly, degradation of HBx can also be promoted by p53 in a proteasome-dependent manner [13]. Therefore, the complicated interactions between HBx and p53 may significantly influence p53-dependent activities such as apoptosis, cellcycle regulation, DNA repair, and tumor suppression.

In our previous research, we demonstrated that Mfn2 is a novel direct target of p53 that could also exert apoptotic effects via Bax-signaling in HCC cells [14,15]. However, it remains unclear whether a relationship exists between Mfn2 and HBx. Thus, this study examines whether HBx affects Mfn2 expression. The results show that whereas HBx had little effect on the expression of Mfn2 or P53, HBx repressed Mfn2 expression mediated by p53 in HCC cell lines, as evidenced by Western Blot and real-time PCR analysis. To further investigate this repression, a luciferase assay was

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employed to evaluate Mfn2 promoter activity after transfection with the HBx plasmid in doxorubicin-treated HepG2 cells. In addition, we used electrophoretic mobility shift analysis (EMSA) to explore the binding of the p53 protein to the Mfn2 promoter in the presence of HBx protein. Doxorubicin treatment in HepG2.2.15, a HCC cell line that was stably transfected with a complete HBV genome, further revealed the interference effect of HBx in Mfn2 expression. These observations purport the view that HBx inhibits p53-mediated upregulation of Mfn2 in HCC cells.

2. Materials and methods

2.1. Cell lines and cell culture

The HCC cell lines HepG2 and Hep3B, which are maintained at our institute, were each cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand land, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma–Aldrich, St. Louis, MO, USA) and 100 U/ml penicillin/streptomycin. HepG2.2.15 cells, also preserved at our institute, were cultured in the presence of 200 mg/l G418 in DMEM containing 10% FBS and 100 U/ml penicillin/streptomycin. All cells were maintained in a humidified atmosphere containing 5% CO2 at 37 °C and were passaged using standard cell culture techniques.

2.2. Transfection and drug treatment

The p53 protein expression plasmid (pcDNA3.1-p53) and the HBx protein (GenBank ID: AAB59970.1) expression plasmid (pcDNA3.1-Myc/HBx) were both stored at our institute. HepG2 and Hep3B cells were plated at a density of 2×10^5 cells per well in six-well plates one day before transfection. Cells were transfected with 4.0 μg pcDNA3.1-p53 or pcDNA3.1-Myc/HBx, or co-transfected with both plasmids, using Lipofectamine 2000 (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Empty pcDNA3.1 vector served as the negative control. Our previous study revealed that the doxorubicin-mediated upregulation of Mfn2 through p53 is specific [14]. For this reason, doxorubicin was used as an endogenous p53 activator. HepG2 and HepG2.2.15 cells were treated with 1.0 μg/ml doxorubicin for 0, 12, 24, and 36 h.

2.3. Western Blot analysis

Using a lysis buffer (Cell Signaling Technologies, Danvers, MA, USA) for 2 h on ice, total protein was isolated from the transfected cells. After centrifugation (12,000×*g*, 4 °C, 10 min), the supernatant was collected, and the protein concentration was measured using the bicinchoninic acid protein assay (Thermo Scientific, Waltham, MA, USA). Equal amounts of protein (50 μg) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking nonspecific binding sites for 2 h with 5% non-fat milk, the membranes were incubated overnight on ice with the primary antibodies against Mfn2, β-actin (Sigma–Aldrich), p53 (Cell Signaling Technologies), Myc-tag, and HBx (Abcam, Cambridge, UK). After washing the membranes several times in Tris-buffered saline and Tween 20 (TBST) while agitating, detection was performed using the appropriate secondary horse radish peroxidase-linked anti-mouse or anti-rabbit antibody. Membrane enhanced chemiluminescence was then conducted using a chemiluminescence detection kit (Biological Industries, Beit Haemek, Israel).

2.4. Real-time polymerase chain reaction (PCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized from total RNA (2 μg) using M-MLV Reverse Transcriptase (Promega, San Luis Obispo, CA, USA). Real-time PCR was performed with an ABIPRISM 7500 Sequence Detection System (Applied Biosystems) using the SYBR Premix DimerEraser kit (Takara Biotechnology, Dalian, Liaoning, China). Amplification reactions included 1 μl cDNA template, 0.3 μl each of the forward and reverse primers (10 μM), 0.2 μl of 50×ROX Reference Dye II (Takara), and 5 μl of 2×SYBR Premix DimerEraserin a total volume of 10 μl. The primers for the two genes were as follows: Mfn2, 5′-AATCTGAGGCGACTGGTGA-3′ (forward) and 5′-AAGACCAGGGACATTGCG-3′ (reverse); and GAPDH, 5′-CTCCTCCTGTTCGACAGTCA-3′ (forward) and 5′-GAGTTAAAAGCAGCCCTGGT-3′ (reverse). Amplification of the transcripts involved an initial denaturation at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 55 °C for 30 s, and 72 °C for 34 s. The comparative threshold cycle (CT) method was used for relative quantification. GAPDH was used as an internal control for normalization. All real-time PCRs were performed in triplicate.

2.5. Plasmids and luciferase assays

Three fragments (P1, nt −2426 to −1826; P2, nt −2252 to −1826; P3, nt −1903 to −1826) of the human Mfn2 promoter were amplified and subcloned into the pGL4.17 firefly luciferase vector (Promega). HepG2 cells were plated onto six-well plates at a density of 6×10^4 cells per well and grown overnight. Using Lipofectamine 2000, cells were cotransfected with or without 1 μg pcDNA3.1 or pcDNA3.1-Myc/HBx plasmid, 250 ng of the indicated Mfn2 promoter-luciferase reporter construct, and 2.5 ng of the control renilla luciferase expression vector, pRL-TK (Promega). After 12 h, the cells were treated with 1.0 μg/ml doxorubicin for an additional 36 h. Luciferase activities of firefly and renilla were assayed using the Dual-Luciferase Reporter Assay System (Promega). Luciferase activity was measured using a luminometer (EG&G Berthold, Bad Wildbad, Germany).

2.6. Electrophoretic mobility shift assay

HepG2 cells were transfected with or without pcDNA3.1 or pcDNA3.1-Myc/HBx, followed by treatment with 1.0 μg/ml doxorubicin for 36 h. At 48 h after transfection, nuclear proteins were extracted using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Consensus binding sequences for p53 [16] and p53 binding oligonucleotides in the Mfn2 promoter (nt −2226 to −2192) were both synthesized by Sangon Biotech (Shanghai, China). The sequences were as follows: p53 oligo (5′-TACA GAACATGTCTAAGCATGCTGGGGACT-3′ and 5′-AGTCCCCAGCATGC TTAGACATGTTCTGTA-3′); wt-Mfn2 oligo (5′-AGGCAAGTGACCAGG ACCTAGACCAATGCTTGCAG-3′ and 5′-CTGCAAGCATTGGTCTAGGT CCTGGTCACTTGCCT-3′) and mut-Mfn2 oligo (5′-AGGTACCTGACCA GGACATACACCAATGATTCCAG-3′ and 5′-CTGGAATCATTGGAGTA TGTCCTGGTCAGGTACCT-3′). The p53 oligo and wt-Mfn2 oligo were 5′-labeled with biotin and used as probes for the electrophoretic mobility shift assay. To obtain double-stranded oligonucleotides as described above, corresponding pairs of oligonucleotides were mixed to a final concentration of 1 pmol/μl with TE buffer (Sigma–Aldrich) and then annealed with a thermocycler at 95 °C for 5 min, followed by −1 °C for 70 min, and then continuously at 4 °C. The EMSA procedure was performed using a LightShift Chemiluminescent EMSA Kit (Thermo Scientific) as described previously [14].

3. Results

3.1. Mfn2 protein express differences in HepG2.2.15 and HepG2 cells treated with doxorubicin

In a previous study, we demonstrated that doxorubicin positively regulated Mfn2 gene expression through the p53 pathway in HepG2 cells, a result that was also confirmed herein (Fig. 1A). To determine whether doxorubicin could upregulate Mfn2 gene expression in other HCC cells, we used HepG2.2.15 cells, a HepG2 cell line stably transfected with a complete HBV genome. Unexpectedly, expression of the Mfn2 protein was not elevated in HepG2.2.15 cells treated with doxorubicin for varying time periods (Fig. 1B). Instead, doxorubicin seemed to downregulate Mfn2 protein expression despite the increase in p53 protein. These results indicated that HBV or its associated proteins, including HBsAg, HBeAg, and HBx [17,18], might inhibit the p53-mediated upregulation of Mfn2 protein in HepG2.2.15 cells.

3.2. HBx inhibits p53-mediated upregulation of Mfn2 gene in HepG2 and Hep3B cells

We examined whether HBx could interfere with Mfn2 expression in HepG2 cells. Western Blot analysis of HepG2 cells transfected with the HBx expression vector (pcDNA3.1-Myc/HBx) revealed that HBx expression had little direct effect on the expression of

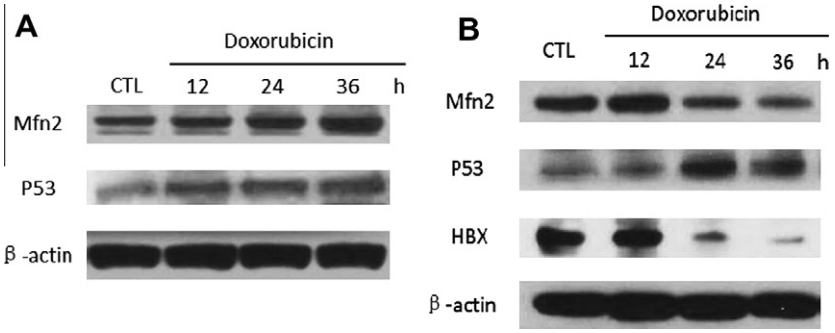


Fig. 1. Expression of Mfn2 treated with doxorubicin in HepG2 and HepG2.2.15. (A) Levels of the Mfn2 and p53 proteins were assessed by Western blot analysis in HepG2 cells treated with or without doxorubicin (1.0 μg/ml) for 12, 24, and 36 h, respectively. β-Actin was used as an internal control. (B) Western Blot analysis of Mfn2 and p53 proteins treated with doxorubicin for the indicated time. Expression of HBx was also assessed using an anti-HBx antibody. β-Actin was used as an internal control.

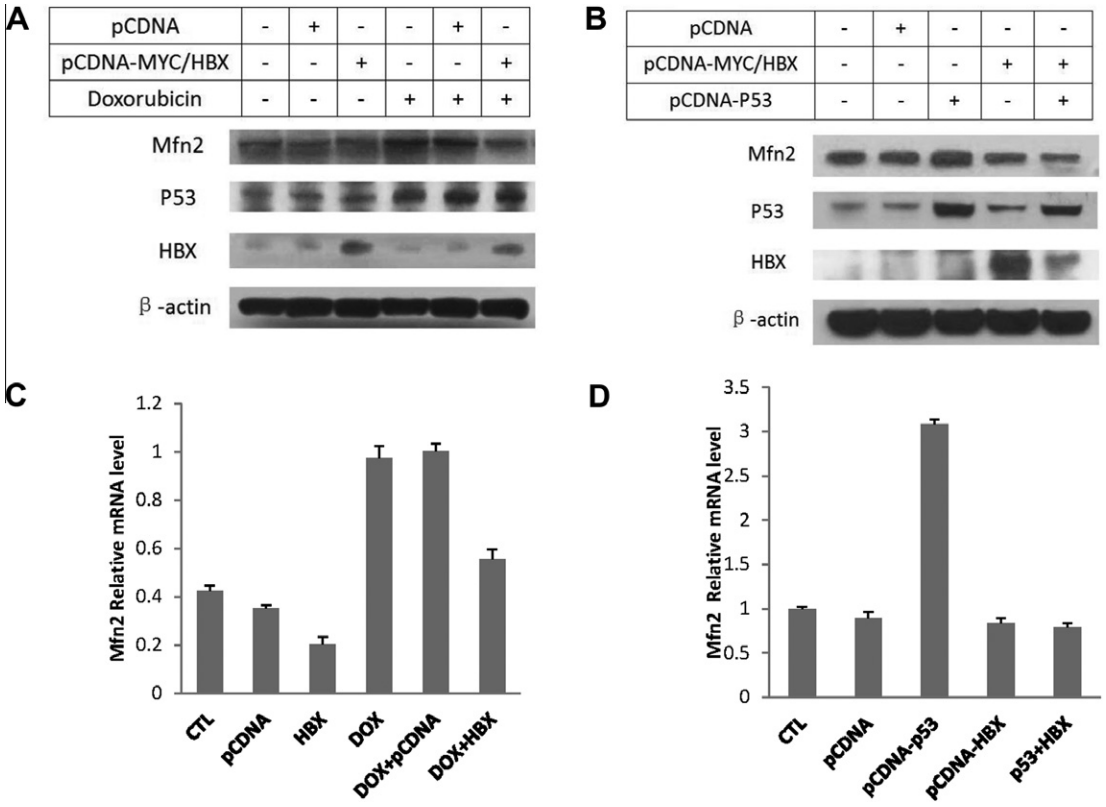


Fig. 2. Expression of Mfn2 protein in HBx transfected cells. (A) Levels of the Mfn2 and p53 proteins were assessed by Western blot analysis in HepG2 transfected with pcDNA3.1 or pcDNA3.1-Myc/HBx. Doxorubicin (1.0 μg/ml) was introduced 12 h after transfection. Lysates of these HepG2 cells were collected 48 h after transfection. Expression of HBx was also detected using an anti-Myc-tag antibody. β-Actin was used as an internal control. (B) Mfn2, p53 and HBx protein levels in lysates were obtained from Hep3B cells transfected with pcDNA3.1, pcDNA3.1-p53 or pcDNA3.1-Myc/HBx, or cotransfected with pcDNA3.1-p53 and pcDNA3.1-Myc/HBx for 48 h. β-Actin was used as an internal control. (C, D) Levels of the Mfn2 mRNA were assessed by real-time PCR under the same conditions described above for Western Blot analysis.

Mfn2 or P53, but it repressed the upregulation of Mfn2 protein under the treatment of doxorubicin, which was expected to be elevated in doxorubicin-treated cells (Fig. 2A). To further assess the interference effect of HBx, Hep3B cells were transfected with the p53 expression vector (pcDNA3.1-p53), pcDNA3.1-Myc/HBx, or cotransfected with both plasmids. Consistent with HepG2 cells, p53 protein was unable to upregulate Mfn2 protein in the presence of HBx protein, although HBx protein had no direct influence on Mfn2 (Fig. 2B). Real-time PCR analysis confirmed that levels of the Mfn2 mRNA paralleled changes in Mfn2 protein expression (Fig. 2C, D). Together, these observations indicated that there was an interference effect of HBx in the course of p53-mediated upregulation of Mfn2.

3.3. Mfn2 promoter activity is counteracted by HBx in doxorubicin-treated HepG2 cells

To investigate whether the transcriptional activity of Mfn2 is regulated by HBx through the p53 binding site, the promoter activity of Mfn2 was examined in doxorubicin-treated HepG2 cells. Three genomic fragments, 612 bp, 438 bp, and 89 bp corresponding to the regions of −2426 to −1826 (p1), −2252 to −1826 (p2), and −1903 to −1826 (p3) of the Mfn2 promoter, respectively, were selected and subcloned into a luciferase reporter plasmid (Fig. 3A). Both p1 and p2 contain the p53 binding site (between −2226 and −2192) in the Mfn2 promoter region, whereas p3 does not. Each plasmid was cotransfected into HepG2 cells with pcDNA3.1 or pcDNA3.1-Myc/HBx under the treatment of doxorubicin. As showed in Fig. 3B, the dual-luciferase reporter assay indicated that doxorubicin-treated cells cotransfected with pcDNA3.1 and either p1 or p2 showed approximately twofold higher luciferase activity than without doxorubicin. However, cells cotransfected with pcDNA3.1-Myc/HBx counteracted the observed luciferase activity increases of p1 and p2 as compared with cells cotransfected with pcDNA3.1. When HepG2 cells were cotransfected with p3 or empty PGL4.17 vector in combination with pcDNA3.1, pcDNA3.1-Myc/HBx, luciferase activity remained essentially unchanged in the presence of doxorubicin. These results suggest that HBx inactivates the promoter activation of Mfn2 in the presence of doxorubicin.

3.4. Physical interactions between p53 and p53 binding site of Mfn2 are affected by HBx

To further confirm that HBx is directly involved in the p53-mediated transcriptional binding event involving the Mfn2 promoter, a 35 bp oligonucleotide sequence (wt-Mfn2 oligo) from the Mfn2 promoter containing the verified p53 response element consensus sequence was used as in EMSA (Fig. 4A). Nuclear lysates were isolated from HepG2, HepG2-pcDNA, and HepG2-pcDNA-x transfected cells, which had been treated with doxorubicin in order to obtain sufficient amounts of p53 protein in the nucleus. The shifted bands were found in the nuclear lysates from HepG2 and HepG2-pcDNA, while there were no obvious bands observed in the nuclear lysates of transfected cells expressing HBx (Fig. 4B). In addition, the formation of an electrophoretically retarded complex was competed only by unlabeled wt-Mfn2 oligo, but not by unlabeled mut-Mfn2 oligo (Fig. 4A). Moreover, the labeled consensus sequence of p53-binding (Biotin-P53 probe) used as a positive control could be observed in parallel positions with the other band

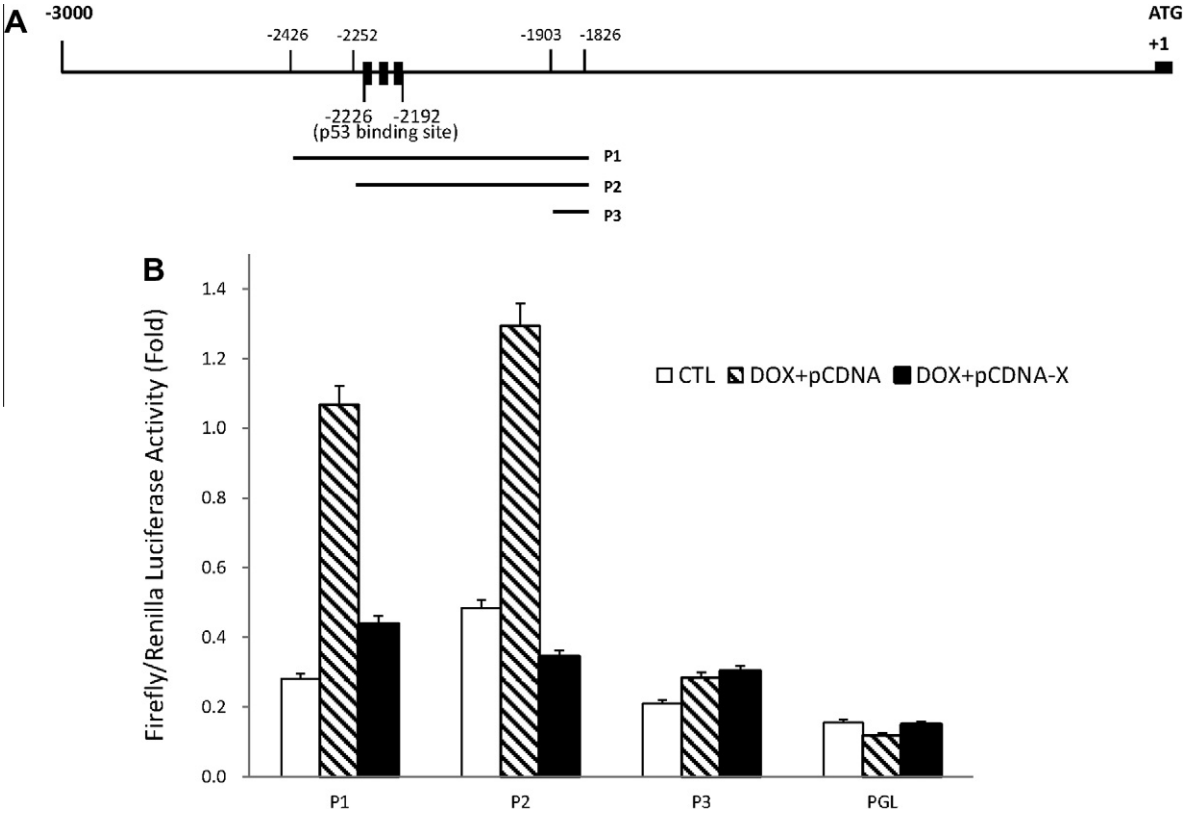
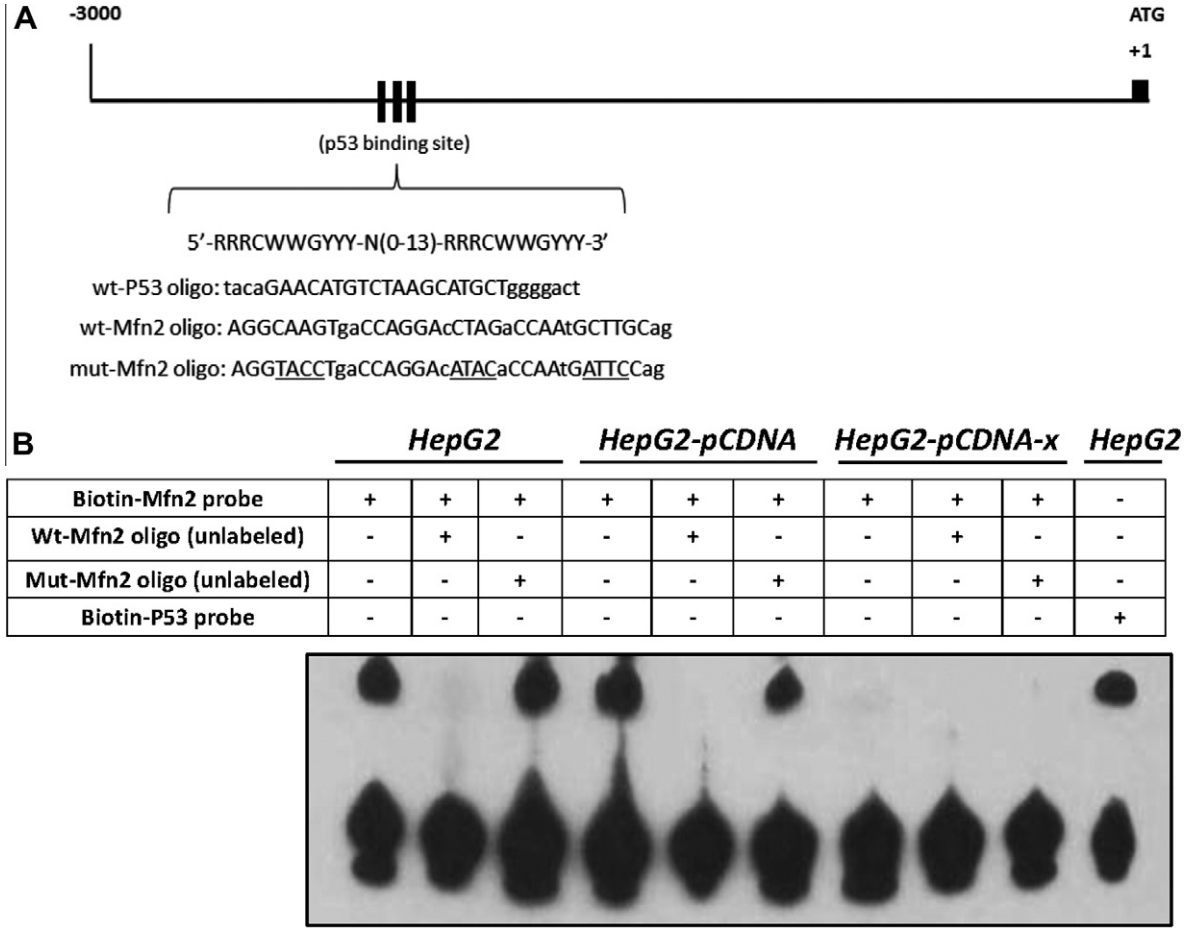


Fig. 3. Regulation of Mfn2 promoter transactivation by HBx. (A) The regions of three fragments, p1, p2 and p3 were illustrated in Mfn2 promoter. They were subcloned into the pGL4.17 firefly luciferase vectors for luciferase assays. (B) HepG2 cells were contransfected with or without 1 μg pcDNA3.1 or pcDNA3.1-Myc/HBx plasmid, 250 ng of the indicated Mfn2 promoter-luciferase reporter construct, and 2.5 ng of the control renilla luciferase expression vector, pRL-TK, using Lipofectamine 2000. After 12 h, the cells were treated with 1.0 μg/ml doxorubicin for additional 36 h. Luciferase activities of firfly and renilla were assayed by using the dual-luciferase reporter assay system. Renilla luciferase activity was used as normalization.



Biotin-Mfn2 probe	+	+	+	+	+	+	+	+	+	-
Wt-Mfn2 oligo (unlabeled)	-	+	-	-	+	-	-	+	-	-
Mut-Mfn2 oligo (unlabeled)	-	-	+	-	-	+	-	-	+	-
Biotin-P53 probe	-	-	-	-	-	-	-	-	-	+

Fig. 4. Physical interactions between p53 and p53 binding site of Mfn2. (A) Oligonucleotides containing a consensus binding sequence for p53, wild-type p53 binding site of Mfn2 promoter and mutant-type oligo. (B) Nuclear extracts were incubated with these oligonucleotides in binding buffer for 20 min at room temperature. Bound DNA complexes were resolved by polyacrylamide gel electrophoresis and transferred to a nylon membrane. The nylon membranes were crosslinked, followed by chemiluminescent.

shifts. These results demonstrate that HBx inhibits the interactions between p53 and the p53 binding site of Mfn2 *in vitro*.

4. Discussion

Mfn2 is a protein located in the outer membrane of the mitochondria. Together with Mfn1, Mfn2 plays an important role in the regulation of mitochondrial morphology and function. Mfn2 also connects the endoplasmic reticulum to the mitochondria, maintaining the stability of the mitochondrial membrane potential [19,20]. Many reports have revealed the relationship between mutations in the Mfn2 gene and Charcot-Marie-Tooth disease type 2A2, a disorder of the peripheral nervous system [3,4]. On the basis of anti-proliferative effects of Mfn2 in vascular smooth muscle cells (VSMCs) [5], we have gradually recognized and demonstrated its apoptotic role in HCC through the mitochondrial pathway [15,21].p53, a well-known tumor suppressor, plays a central role in multiple cellular processes, including the mediation of cell cycle arrest, the suppression of oncogenic transformation, the inhibition of proliferation and promotion of tumor apoptosis [22]. In fact, such functions of p53 are partly obtained by transcriptional activation of some downstream genes, such as WAF1, CIP1, p21, IGF-BP3, mdm2, cyclin G, PCNA, and GADD45 [23]. As a transcription factor, evidence obtained from several experimental systems indicates that the consensus DNA-binding sequence for p53 consists of two copies of the 10 base pair (bp) motif, RRRCWWGYYY (R = A, G; W = A, T; Y = C, T), separated by 0–13 bp [16]. According to this theoretical basis, a 35 bp oligonucleotide sequence in the Mfn2 promoter has been identified in our laboratory (Fig. 4A). Further studies have verified that Mfn2 is one of the downstream proteins regulated by p53, and the effect of doxorubicin in upregulating Mfn2 through p53 is specific [14], thus providing evidence for a direct interaction between p53 and Mfn2 in HCC.

HBx is a multifunctional protein encoded by the HBV genome that increases virus gene expression and replication by transactivating cellular promoters [24]. HBx also causes various morphologic and metabolic alterations in mammalian cells by transactivating several cytoplasmic signaling pathways, such as protein kinase C(PKC), JAK/STAT, PI3K, stress-activated protein kinase (SAPK)/c-Jun NH2-terminal kinase (JNK), ras-raf-MAPK, activator protein-1 (AP-1), AP-2, nuclear factor-κB (NF-κB), Smad, and Wnt [25,26]. Although HBx protein does not bind directly to DNA, it appears to play a critical role by modulating tumor suppressor genes and corresponding signaling pathways in tumor development. Wang et al. [11] reported that there is a direct interaction between p53 and HBx. In HCC cells and transgenic mice expressing HBx, binding of p53 to HBx causes cytoplasmic retention of the p53 tumor suppressor gene product, thus completely blocking the translocation of p53 to the nucleus in HCC development [10,27]. Furthermore, HBx expression modulates the transcriptional activation of PTEN, a gene targeted by p53, in HBx-transfected liver cells [12]. Therefore, p53-mediated upregulation of Mfn2 expression could also be blocked by HBx.

The results show that increased Mfn2 protein expression in the presence of doxorubicin or introduced with pcDNA-p53 can be repressed by HBx in HepG2 and Hep3B cells, respectively, as evidence by Western Blot analysis and real-time PCR (Fig. 2). However, HBx exerts little direct effect on the expression of Mfn2 or p53. In addition, Mfn2 is essentially unchanged without doxorubicin treatment in HBx transfected HepG2 cells, probably because the low levels of p53 and Mfn2 expressed in HepG2 make the indirect influence of HBx to Mfn2 inconspicuous. Treatment with doxorubicin in another HCC cell line, HepG2.2.15, further confirmed the effect of HBx (Fig. 1). In HepG2.2.15 cells, downregulation of HBx expression may due to proteasome-dependent degradation promoted by p53 [13,28]. The reason for decreased Mfn2 expression is unclear, and further studies will be required to determine the specific mechanisms. A luciferase assay proved that transcriptional activity of the Mfn2 promoter is upregulated by p53, but also modulated by HBx (Fig. 3). Moreover, HBx dramatically attenuated the binding of p53 protein to the Mfn2 promoter *in vitro*, as evidence by EMSA (Fig. 4).

In conclusion, this study demonstrates that Mfn2, a novel p53-inducible gene, can be repressed in HBx-expressing HCC cells, thus disabling its pro-apoptotic and anti-proliferative effects. Our findings contribute new evidence to a better understanding of the oncogenic mechanisms of HBx, despite its complicated and controversial role in the anti-apoptotic processes.

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