Original Article

Interferon stimulated exonuclease gene 20 kDa promotes the activity of interferon-alpha on the inhibition of hepatitis B virus replication via its exonuclease activity

Qian Ma1, Bo Qin1, Xi Lu2, Lingna Kong1

1Department of Infectious Diseases, The First Affiliated Hospital of Chongqing Medical University, Chongqing, P. R. China; 2Department of Gastroenterology, The First Affiliated Hospital of Harbin Medical University, Harbin, P. R. China

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Abstract: Aims: The present study is to investigate how interferon stimulated exonuclease gene 20 kDa (ISG20) affects hepatitis B virus (HBV) replication, and its possible role in the anti-HBV process mediated by interferon (IFN)-α. Methods: HepG2.2.15 cells were transfected with human wild-type ISG20 (ISG20-W), D94G mutant ISG20 (ISG20-M), and empty lentivirus vector (NC). Cells that were not transfected were EMPTY group. ISG20 mRNA quantification was conducted by quantitative real-time polymerase chain reaction. Protein expression was measured by Western blotting. HBV DNA was quantified using HBV PCR Fluorescence Quantitative Detection Kit. Enzyme-linked immunosorbent assay was used to measure the concentrations of HBsAg and HBeAg in culture supernatants. ISG20 expression was detected by immunostaining of HepG2.2.15 cells. Results: Expression of ISG20 in HepG2.2.15 cells transfected with ISG20-W or ISG20-M was higher than that in EMPTY and NC HepG2.2.15 cells. ISG20 inhibited the replication of HBV by means of its exonuclease activity. ISG20 exerted dominant positive effect on the antiviral action of IFN-α against intracellular HBV replication. ISG20 protein was located in the nucleus of ISG20-W cells. Conclusions: ISG20, as an IFN-stimulated gene, strongly inhibits the replication of HBV. Differential ISG20 expression is associated with the efficacy of IFN-α treatment on HBV infection. ISG20 may promote the anti-HBV activity of IFN-α via its exonuclease activity.

Keywords: Interferon stimulated exonuclease gene 20 kDa, ISG20, interferon-alpha, IFN-α, hepatitis B virus, HBV, replication

Introduction

Hepatitis B virus (HBV) infection is the leading cause of cirrhosis and hepatocellular carcinoma with up to one million HBV carriers dying of HBV-associated liver diseases annually [1-3]. Interferon alpha (IFN-α) has been widely used for the treatment of chronic hepatitis B (CHB) for decades [1]. Advantages of IFN-α therapy include lack of resistance development, limited treatment course, and even clinical cure with Hepatitis B surface antigen (HBsAg) seroconversion in some patients. However, IFN-α treatment also has some limitations, such as low response rate, expensiveness and inevitable side effects. It is important to identify biomarkers for predicting whether patients with CHB will respond to IFN-α treatment or not [4-6].

In a previous study, microarray gene expression profiling of liver tissues from CHB patients has identified the expression differences of genes between treatment responders and non-responders [7]. Several interferon-stimulated genes (ISGs) and immune-related genes are identified [7]. ISGs are a large family of proteins induced by IFNs that exert antiviral, antitumor, and immunomodulatory effects [8]. One study has demonstrated that ISGs play a key role in response to IFN-α treatment [9]. Further study from the same group shows that differential expression of interferon stimulated exonuclease-
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ase gene 20 kDa (ISG20) in liver cells may have significant effect on the efficacy and prognosis of CHB patients treated with IFN-α [10].

ISG20 is an interferon-inducible 3’-5’ exonuclease that inhibits replication of several human and animal viruses. The substitution of a single conserved aspartic acid by a glycine residue is sufficient to abolish its exonuclease activity [11]. In the present study, we investigate how ISG20 affects HBV replication, and its possible role in the anti-HBV process mediated by IFN-α.

Materials and methods

Cells

HepG2.2.15 cells, obtained from the Second Affiliated Hospital of Chongqing Medical University, were cultured in minimum essential medium supplemented with 10% fetal bovine serum and 380 mg/L antibiotic G-418 sulfate (Promega, Fitchburg, WI, USA), at 37°C in an atmosphere of 5% CO₂. HepG2.2.15 cells were seeded in 24-well plates at 8×10⁴/ml. After 24-48 h, cells were transfected with human wild-type ISG20 (designated as ISG20-W), D94G mutant ISG20 that is deficient in exonuclease activity (designated as ISG20-M), and empty lentivirus vector (designated as NC), following manufacturer’s protocol (Genechem, Shanghai, China). Cells that were not transfected were EMPTY group.

Quantitative real-time polymerase chain reaction (Q-PCR)

Total RNAs of cells were extracted by Trizol reagent according to the manufacturer’s instructions (Thermo Fisher Scientific, Waltham, MA, USA). Total RNA (0.5 μg) was reverse-transcribed to cDNA using PrimeScript RT reagent Kit (Takara, Dalian, China). ISG20 mRNA quantification was conducted by SYBR green-based Q-PCR using primers ISG20-F (5’-CGACGCTTCCACGCAGGCTGTGG-3’), ISG20-R (5’-TCCATCGTTGCCCTCGCATCTTC-3’), β-actin-F (5’-AGCGAGCATCCCCAGAGT-3’), and β-actin-R (5’-GAGCAGGATCCCCAAAGT-3’).

Western blotting

Seventy-two hours after transfection with ISG20-W, ISG20-M and NC, cells were collected and proteins were extracted for assessing ISG20 expression. Total proteins were separated by 10% SDS-PAGE gel and then transferred to polyvinylidene fluoride membrane. The membranes were blocked for 2 hours at room temperature with 3% bovine serum albumin. After washing with Tris-buffered saline with Tween 20 for three times, the membranes were incubated with primary antibody against human ISG20 (Santa Cruz Biotechnology, Dallas, TX, USA) proteins at 4°C overnight. After washing for three times, the secondary antibody was incubated at room temperature for 2 hours. Finally, the membranes were incubated with ECL reagent (Beyotime, Shanghai, China). β-actin (ZSGB-Bio, Beijing, China) was used as control.

HBV PCR fluorescence quantitative detection

Seventy-two hours after transfection, HBV DNA in culture supernatants was collected and then
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Enzyme-linked immunosorbent assay (ELISA)

Seventy-two hours after transfection, the concentrations of HBsAg and HBeAg in culture supernatants were collected and measured by HBsAg and Hepatitis B e antigen (HBeAg) enzyme diagnostic kits according to the manufacturer’s instructions (Autobio, Zhengzhou, China). OD values were recorded at 450 nm using LUMo microplate luminometer (Autobio Diagnostics Co., Ltd., Zhengzhou, China).

Immunostaining

ISG20 expression was immunostained in empty HepG2.2.15 cells (designated as EMPTY), NC and ISG20-W cells. The nucleus was immunostained blue by DAPI, while lentivirus vectors and ISG20 protein were counterstained green and red, respectively.

Statistical analysis

All data were presented as means ± standard deviation. Statistical analysis was carried out using SPSS 13.0 statistical software package (IBM, Armonk, NY, USA), and the significance of each group was verified using one-way analysis of variance (ANOVA) and Student’s t-test. *P value < 0.05 was considered significant.

Results

Expression of ISG20 in HepG2.2.15 cells transfected with ISG20-W or ISG20-M is higher than that in EMPTY and NC HepG2.2.15 cells

To measure the expression of ISG20 in HepG2.2.15 cells, Q-PCR and Western blotting were performed. The ectopically expressed
ISG20-W and ISG20-M were detected in ISG20-W and ISG20-M cells by Q-PCR and Western blotting, but not in HepG2.2.15 cells transfected with empty vector or non-transfected HepG2.2.15 cells (Figure 1). The result suggests that the expression of ISG20 in HepG2.2.15 cells transfected with ISG20-W or ISG20-M is higher than that in EMPTY and NC HepG2.2.15 cells.

**ISG20 inhibits the replication of HBV by means of its exonuclease activity**

To determine how ISG20 affects HBV replication, we compared the ability of ISG20-W, ISG20-M and NC cells in supporting the replication of HBV. Expression of HBV DNA, HBsAg and HBeAg in ISG20-M cells was not significantly different from that in NC cells at 6, 24, 48 and 72 h after transfection (P > 0.05). By contrast, HBV replication was severely impaired in ISG20-W cells. At all time points, the expression levels of HBV DNA, HBsAg and HBeAg in ISG20-W cells were significantly lower than those in ISG20-M or NC cells (P < 0.05) (Figure 2). The result indicates that ISG20 inhibits the replication of HBV by means of its exonuclease activity.

**ISG20 exerts dominant positive effect on the antiviral action of IFN-α against intracellular HBV replication**

To determine whether ISG20 enhances the anti-HBV activity of IFN-α, we pretreated NC and ISG20-W cells with high concentration of human IFN-α (1000 U/ml) to induce cellular antiviral state. Before IFN-α pretreatment, NC and ISG20-W cells showed robust HBV DNA replication, which reached plateau at 48-72 h after transfection (data not shown). IFN-α pretreatment strongly suppressed HBV DNA replication in ISG20-W cells, but was less effective in NC cells. By contrast, HBV replication was severely impaired in ISG20-W cells. At all time points, the expression levels of HBV DNA, HBsAg and HBeAg in ISG20-W cells were significantly lower than that in NC (P < 0.05) (Figure 3). The result suggests that ISG20 exerts dominant positive effect on the antiviral action of IFN-α against intracellular HBV replication.

**ISG20 protein is located in the nucleus of ISG20-W cells**

To observe the localization of ISG20 in cells, immunostaining was performed. The data showed that the nucleus was immunostained blue by DAPI, while lentivirus vectors and ISG20 protein were counterstained green and red, respectively. There was significant overexpression of ISG20 within the nucleus of HepG2.2.15 cells stably expressing wild-type ISG20 (ISG20-W), resembling a nuclear body localization pattern.

**Discussion**

ISG20 is reported to inhibit hepatitis A virus replication [12]. In the liver of chimpanzees
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Acutely infected with HBV, ISG20 production is induced at viral clearance stage [13]. Interestingly, ISG20, when ectopically expressed in HepG2 cells, reduces the secretion of HBs and HBe antigens in transfected HepG2 cells [14]. ISG20 mRNA is highly up-regulated in the liver of chimpanzees infected with hepatitis C virus or following intravenous administration of IFN-α [15]. The broad antiviral activities of ISG20 against multiple, distinct hepatitis viruses (hepatitis A virus, HBV and hepatitis C virus) indicate that ISG20 is an important antiviral effector molecule downstream of IFN-α signaling in innate defenses of the liver. However, how ISG20 acts exactly to inhibit virus infection is not known.

In the present study, we have characterized the antiviral spectrum of ISG20 in vitro by determining the effects of ectopically expressed ISG20 on the replication of HBV. The expression levels of HBV DNA, HBsAg and HBeAg in culture supernatants of ISG20-W cells are decreased significantly compared with NC cells. HBV DNA, HBsAg and HBeAg are markers for HBV replication [16], which indicate that ISG20 strongly inhibits the replication of HBV. Amino acid comparison reveals that endogenous ISG20 protein is present in the nucleus at both nucleolus and Cajal bodies. ISG20 is concentrated in the dense fibrillar component of nucleolus, which is the major site for the early steps of rRNA processing [17]. Cajal bodies are non-membrane-bound nuclear suborganelles implicated in the post-transcriptional maturation of small nuclear and small nucleolar RNAs. Their presence in the cells is the marker of active transcriptional processes [18]. It is well known that many eukaryotic RNAs undergo precise 3′ trimming that accurately processes 3′-extended precursors to mature RNAs [19]. In particular, U3 small nucleolar RNA synthesized in the nucleoplasm is 30-end processed by an unknown exonuclease within the Cajal bodies [20]. Based on that, it is speculated that ISG20 might participate in the maturation of U3 small nucleolar RNA and the final steps of rRNA processing. Exonuclease activity of ISG20 is consistently abrogated by D94G mutation [21]. In the present study, the expression levels of HBV DNA, HBsAg and HBeAg in culture supernatants of ISG20-W cells are decreased significantly compared with ISG20-M cells, indicating that exonuclease activity is required for the antiviral activity of ISG20 against HBV. However, the exact mechanism by which the exonuclease is involved in the cellular defenses against HBV has to be explored in depth in future studies.

Furthermore, we demonstrate that ectopically expressed ISG20 enhances the antiviral effect of IFN-α on the replication of HBV, indicating that ISG20 is a critical component in the antiviral actions of IFN-α against HBV DNA replication. The result is consistent with a previous study [10], which shows that differential expression of ISG20 in liver cells might have significant effect on the efficacy and prognosis of CHB patients treated with IFN-α. It is of note that ISG20 localizes mainly within the nucleus [22, 23]. It is possible that some cellular factors in the nucleus that are essential for HBV replication are targeted by ISG20, indicating that the demonstrated antiviral activities of ISG20 against HBV require its exonuclease activity. In conclusion, ISG20, as an IFN-stimulated gene, could strongly inhibit the replication of HBV. Differential ISG20 expression is associated with the efficacy of IFN-α treatment on HBV infection. ISG20 may promote the anti-HBV activity of IFN-α via its exonuclease activity.

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Disclosure of conflict of interest

None.

Address correspondence to: Bo Qin, Department of Infectious Diseases, The First Affiliated Hospital of Chongqing Medical University, No. 1, Youyi Road, Chongqing, P. R. China. Tel: 86-23-89012887; Fax: 86-23-89012430; E-mail: cqqinbo@126.com

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