S100A9 is Involved in Hepatitis B Virus X Protein-Mediated Proliferation and Migration of HepG2 Cells (Post-Print)

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Abstract

Objective: To investigate the role of S100A9 in hepatitis B virus X (HBx)-mediated proliferation and migration of HepG2 cells. Methods: After infecting HepG2 cells with recombinant adenovirus AdHBx expressing HBx protein, cell proliferation capacity was detected by CCK-8 assay and cell migration capacity was detected by scratch wound healing assay; after transfecting S100A9-siRNA and its control siRNA into HepG2/AdHBx cells, the proliferation and migration capacities of HepG2 cells were detected; in HepG2/AdHBx and control HepG2/AdGFP cells, Real-time PCR and Western Blot were used to detect the expression of S100A9 gene and protein; in HepG2/AdHBx cells, after adding different doses of NF- B inhibitor BAY11-7082, the expression of S100A9 gene and protein in each group was detected. Results: HBx promoted the proliferation and migration of HepG2 cells; after S100A9-siRNA inhibited S100A9 expression, the effect of HBx in promoting HepG2 cell proliferation and migration was reduced, indicating that HBx-mediated proliferation and migration of HepG2 cells were partially dependent on S100A9; S100A9 gene and protein expression were significantly higher in HepG2/AdHBx than in the control group HepG2/AdGFP, indicating that HBx could increase S100A9 expression; after inhibiting NF- B transcriptional activity, S100A9 gene and protein expression in the AdHBx+BAY11-7082 group was significantly lower than in the control group, suggesting that blocking NF- B transcriptional activity could partially inhibit HBx-regulated S100A9 expression. Conclusion: HBx can regulate S100A9 expression and is associated with NF- B activation; S100A9 participates in HBx-mediated proliferation and migration of HepG2 cells.

Full Text

S100A9 is Involved in Hepatitis B Virus X Protein-Mediated Proliferation and Migration of HepG2 Cells

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Abstract

Objective: To investigate the role of S100A9 in hepatitis B virus X protein (HBx)-mediated proliferation and migration of HepG2 cells. **Methods:** HepG2 cells were infected with recombinant adenovirus expressing HBx protein (Ad-HBx), after which cell proliferation and migration were assessed using CCK-8 assay and wound healing assay, respectively. In AdHBx-infected HepG2 cells transfected with S100A9-siRNA or control siRNA, cell proliferation and migration capabilities were evaluated. S100A9 mRNA and protein expression were measured in HepG2/AdHBx and control HepG2/AdGFP cells using real-time PCR and Western blot. Additionally, AdHBx-infected HepG2 cells were treated with varying doses of the NF- B inhibitor BAY11-7082, and S100A9 expression was examined at both gene and protein levels. **Results:** HBx promoted HepG2 cell proliferation and migration. Knockdown of S100A9 via siRNA attenuated HBx-induced proliferation and migration, indicating that HBx-mediated effects were partially dependent on S100A9. Both S100A9 mRNA and protein expression were significantly elevated in HepG2/AdHBx cells compared to HepG2/AdGFP controls, demonstrating that HBx upregulates S100A9 expression. Inhibition of NF- B transcriptional activity markedly reduced S100A9 expression in the AdHBx+BAY11-7082 group compared to controls, suggesting that blocking NF- B activity partially suppresses HBx-regulated S100A9 expression. **Conclusion:** HBx regulates S100A9 expression through NF- B activation, and S100A9 participates in HBx-mediated proliferation and migration of HepG2 cells.

Keywords: S100A9, HepG2 cells, Hepatitis B virus X protein, NF- B

Introduction

Liver cancer is one of the most common malignant tumors worldwide, ranking first in incidence among malignant tumors in Asia, particularly in China [1]. In China, persistent hepatitis B virus (HBV) infection represents a major etiological factor for hepatocellular carcinoma [2]. HBV is a partially double-stranded circular hepatotropic DNA virus containing four partially overlapping open reading frames: pre-S/S, pre-C/C, X, and P regions, which primarily encode viral envelope proteins HBs, core protein HBc, HBx protein, and DNA polymerase,

respectively [3]. Although the HBx protein is only 17 kDa in size, it plays a crucial role in hepatocarcinogenesis. Studies have confirmed that HBx participates in regulating various cellular gene transcriptions, possesses strong transactivation activity, and can activate signaling pathways such as Wnt/ -catenin, NF- B, and NFAT, demonstrating clear oncogenic activity [4,5].

Damage-associated molecular patterns (DAMPs) constitute a large family of cellular structural proteins that are released extracellularly under pathological conditions, initiating and mediating inflammatory responses that transmit danger signals of cellular damage. Examples include HMGB1, HSPs, and S100 proteins [6]. Among these, S100 proteins have been found to be highly expressed in various inflammation-related tumors and promote disease progression by activating TLR4 or RAGE signaling pathways in tumor cells [7]. Current studies have shown that S100A9, a member of the S100 family, is upregulated in liver cancer [8], suggesting that S100A9 may play an important role in the progression from chronic viral hepatitis to hepatocellular carcinoma. Therefore, this study aims to analyze S100A9 expression in HBV-related liver cancer, explore its regulatory relationship with the HBV oncoprotein HBx, and further elucidate its role in HBV-induced hepatocarcinogenesis.

Materials and Methods

1.1 Cell Lines and Plasmids

The HepG2 cell line was purchased from the American Type Culture Collection and maintained in DMEM medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO . HEK293 cells were provided by the Key Laboratory of Clinical Laboratory Diagnostics of the Ministry of Education at Chongqing Medical University. The luciferase reporter plasmid p-Luc-NF- B was a gift from Professor He Tongchuan at the University of Chicago.

1.2 Recombinant Adenovirus and siRNA

The recombinant adenovirus vector expressing HBx (AdHBx) and its control vector, both containing an independent and complete GFP expression system, were gifts from Professor Feng Tao (Center for Molecular Medicine and Cancer Research, Chongqing Medical University). The viruses were amplified in HEK293 cells prior to use. S100A9-siRNA (sense: 5'-GCUUCGAGGAGUUCAUCAUTT-3' and antisense: 3' -CGAAGCUCCUCAAGUAGUATT-5') and control siRNA (sense: 5' -UUCUCCGAACGUGUCACGUTT-3' and antisense: 3' -ACGUGACACGUUCGGAGAATT-5') were synthesized by GenePharma.

1.3 Reagents

Mouse anti-human S100A9 antibody (sc-58706) and mouse anti-human -actin antibody (sc-47778) were purchased from Santa Cruz. Rabbit anti-HBx an-

tibody (ab39716) was from Abcam. DMEM medium and fetal bovine serum were from Hyclone. Trizol reagent and Lipofectamine 2000 were from Invitrogen. ECL detection reagent was from Beijing Zhongshan Biotechnology. Cell Counting Kit-8 was from Dojindo. Real-time PCR reagents were from TaKaRa. Dual-luciferase reporter assay kit was from Promega. NF- B inhibitor BAY 11-7082 was from Beyotime.

2.1 Validation of HBx Recombinant Adenovirus

Recombinant adenoviruses expressing HBx (AdHBx) and control vector AdGFP were amplified and prepared in HEK293 cells. HepG2 cells were infected with these vectors, and HBx protein expression was detected by Western blot 24 hours post-infection.

2.2 CCK-8 Assay for Cell Proliferation

HepG2/AdHBx and HepG2/AdGFP cells were diluted to 0.5 × 10 cells/ml in medium containing 1% FBS and seeded in 96-well plates (100 l per well). After culturing for 24, 48, 72, and 96 hours, 10 l of CCK-8 reagent was added to each well. Absorbance at 450 nm was measured after 4 hours, with experiments repeated three times. Growth curves were plotted with absorbance values on the y-axis and time on the x-axis.

2.3 Wound Healing Assay for Cell Migration

HepG2/AdHBx and HepG2/AdGFP cells were cultured in 6-well plates until confluent. A sterile pipette tip was used to create a scratch in the central region of each well. After washing with serum-free medium to remove detached cells, cultures were maintained in 1% FBS medium for 24 hours. Scratch widths were photographed at 0 and 72 hours under an inverted microscope. The wound healing rate was calculated as: (scratch width at 0 h - scratch width at 72 h) / scratch width at 0 h × 100%.

2.4 Western Blot Analysis of HBx and S100A9 Protein Expression

Logarithmic-phase cells were washed three times with ice-cold PBS, then lysed on ice for 30 minutes in 50 l lysis buffer containing 0.2 l PMSF. After centrifugation at 12,000 rpm for 15 minutes at 4°C, supernatants were collected and total protein concentrations were determined by BCA assay. Equal amounts of protein were separated on 10% SDS-PAGE gels and transferred to PVDF membranes at 4°C. Membranes were blocked with 5% milk for 1 hour at room temperature, then incubated overnight at 4°C with primary antibodies against -actin, HBx, or S100A9 (1:1000 dilution). After three washes with PBST, membranes were incubated with secondary antibody (1:2000) for 2 hours at room temperature, washed again, and visualized using DAB staining. Experiments were repeated three times.

2.5 Real-Time PCR Analysis of S100A9 Gene Expression

HepG2/AdHBx cells and control HepG2/AdGFP cells were cultured and harvested after 24 hours. Total RNA was extracted using Trizol, reverse-transcribed to cDNA, and analyzed by real-time PCR for S100A9 mRNA levels (primers: forward 5' -GGAATTCAAAGAGCTGGTGC-3' , reverse 5' -TCAGCATGATGAACTCCTCG-3'). GAPDH served as an internal control (primers: forward 5' -CAGCGACACCCACTCCTC-3' , reverse 5' -TGAGGTCCACCACCCTGT-3').

2.6 Analysis of Cell Proliferation and Migration After S100A9 Knockdown

HepG2/AdHBx cells were transfected with S100A9-siRNA or control siRNA using Lipofectamine 2000. Cell proliferation was analyzed by CCK-8 assay at 72 and 96 hours post-transfection, and migration was assessed by wound healing assay at 72 hours.

2.7 S100A9 Expression Analysis After NF- B Inhibition

HepG2/AdHBx cells were treated with the NF- B inhibitor BAY11-7082. S100A9 mRNA expression was measured by real-time PCR after 24 hours, and protein expression was detected by Western blot after 48 hours.

2.8 Dual-Luciferase Reporter Assay

HepG2/AdHBx and control HepG2/AdGFP cells were co-transfected with the luciferase reporter plasmid p-Luc-NF- B and pRL-TK. After 48 hours, cells were lysed and luciferase activity was measured in the supernatant. Relative luciferase values were calculated using pRL-TK as an internal control, with GFP set as 1. Each treatment group contained three replicate wells.

2.9 Statistical Analysis

Statistical analysis was performed using SPSS 19.0. Quantitative data are presented as mean ± standard deviation. Comparisons between two groups were analyzed by t-test, while comparisons among three groups were performed using one-way ANOVA with SNK-q test. Statistical significance was defined as $P < 0.05$, **P* < 0.01, and** $P < 0.001$.

Results

1. Establishment of a Stable HBx-Expressing HepG2 Cell Model

HepG2 cells were infected with recombinant adenoviruses AdHBx or control AdGFP. GFP expression was observed by fluorescence microscopy 24 hours post-infection, confirming successful viral infection [Figure 1 Figure 1: see original paper]. Western blot analysis revealed that HBx protein was expressed in

AdHBx-infected cells but not in AdGFP-treated controls [Figure 1(b)]. These results confirm the successful establishment of a stable HBx-expressing HepG2 cell model (HepG2/AdHBx).

Figure 1. Identification of HBx expression in HepG2 cells infected with AdHBx. (a) Fluorescence microscopy observation of HepG2 cells after AdHBx infection (50×). (b) Western blot detection of HBx expression in HepG2 cells infected with AdHBx or AdGFP.

2. HBx Promotes HepG2 Cell Proliferation and Migration

CCK-8 assays demonstrated that HBx significantly enhanced HepG2 cell proliferation. On days 3 and 4, the OD values were 0.68 ± 0.01 and 0.96 ± 0.05 for AdHBx group versus 0.51 ± 0.03 and 0.71 ± 0.04 for AdGFP group, respectively ($P < 0.01$ and $P < 0.001$) [Figure 2 Figure 2: see original paper]. Wound healing assays showed that the migration rate was (79.33 ± 8.03)% in AdHBx group compared to (48.33 ± 5.8)% in AdGFP group ($P < 0.001$) [Figure 2(b and c)]. These findings indicate that HBx promotes both proliferation and migration of HepG2 cells.

Figure 2. Effect of HBx on HepG2 cell proliferation (a) and migration (b, c). P* < 0.01, $P < 0.001$ compared with HepG2 cells infected with AdHBx.

3. Role of S100A9 in HBx-Mediated HepG2 Cell Proliferation and Migration

To determine whether S100A9 mediates the effects of HBx, we knocked down S100A9 expression using siRNA in AdHBx-infected HepG2 cells and assessed proliferation and migration. On days 3 and 4, the OD values were 0.69 ± 0.04 and 0.96 ± 0.06 in AdHBx group versus 0.47 ± 0.03 and 0.64 ± 0.03 in HBx+S100A9-siRNA group ($P < 0.01$ and $P < 0.001$) [Figure 3 Figure 3: see original paper]. Wound healing assays revealed migration rates of (79 ± 6.08)% in AdHBx group versus (66.33 ± 4.67)% in HBx+S100A9-siRNA group ($P < 0.05$) [Figure 3(b)]. These results demonstrate that HBx-mediated proliferation and migration of HepG2 cells are partially dependent on S100A9.

Figure 3. S100A9-siRNA reduces HBx-induced promotion of HepG2 cell proliferation (a) and migration (b, c). $P < 0.05$, **P* < 0.01,** $P < 0.001$ compared with HepG2/AdHBx cells transfected with control siRNA.

4. HBx Regulates S100A9 Expression

Given that HBx-mediated HepG2 cell proliferation and migration were partially dependent on S100A9, we investigated whether HBx regulates S100A9 expression. Real-time PCR and Western blot analysis revealed that both S100A9 mRNA and protein levels were significantly elevated in AdHBx-infected cells

compared to AdGFP controls [Figure 4 Figure 4: see original paper], with statistical significance (P < 0.001). These findings indicate that HBx upregulates S100A9 expression.

Figure 4. S100A9 expression in HepG2/AdHBx and HepG2/AdGFP cells. (a) Real-time PCR analysis. (b) Western blot analysis. $^{**}P < 0.001$ compared with HepG2/AdGFP cells.

5. HBx Regulation of S100A9 Expression Involves NF- B Signaling Activation

NF- B signaling plays a critical role in inflammation-driven tumor progression. We therefore investigated whether HBx regulation of S100A9 expression is associated with enhanced NF- B transcriptional activity. Dual-luciferase reporter assays confirmed that HBx significantly increased NF- B transcriptional activity [Figure 5 Figure 5: see original paper], with statistical significance (P < 0.001). In AdHBx-infected HepG2 cells treated with the NF- B inhibitor BAY11-7082, both S100A9 mRNA and protein levels were significantly reduced compared to untreated AdHBx cells [Figure 5(b and c)], with differences being statistically significant (P < 0.01 or P < 0.001). These results demonstrate that blocking NF- B transcriptional activity partially inhibits HBx-regulated S100A9 expression.

Figure 5. Blocking NF- B transcriptional activity suppresses S100A9 expression. (a) Dual-luciferase reporter assay analysis of HBx effect on NF- B transcriptional activity. (b) Real-time PCR analysis of S100A9 mRNA levels in BAY11-7082-treated HepG2/AdHBx cells. (c) Western blot analysis of S100A9 protein expression in BAY11-7082-treated HepG2/AdHBx cells. **P* < 0.01,** $P < 0.001$ compared with HepG2/AdHBx cells.

Discussion

Hepatocarcinogenesis is a complex, multi-stage process involving multiple factors and genes. Accumulating evidence indicates that chronic HBV infection is a major risk factor for liver cancer development. The HBx protein exhibits diverse biological activities and can regulate numerous cellular processes, including signal transduction, DNA repair, apoptosis, and proliferation, conferring potent malignant transformation capacity that plays a critical role in HBV-mediated hepatocarcinogenesis [9]. Previous studies have reported that HBx promotes liver cancer cell proliferation by upregulating YAP expression and activates the Wnt/ -catenin signaling pathway to enhance tumor invasion and metastasis [10,11]. Our study confirms these findings, demonstrating that HBx promotes HepG2 cell proliferation and migration.

S100A9 is an important member of the calcium-binding S100 protein family that participates in cell growth, differentiation, growth inhibition, apoptosis induction, and inflammatory response mediation. It can bind to Toll-like receptor 4 (TLR-4) to activate JNKs, p38, ERKs, and NF- B signaling pathways,

exerting pro-inflammatory effects [12]. S100A9 expression is elevated in many inflammatory diseases and is associated with rheumatoid arthritis, inflammatory bowel disease, and other human inflammatory conditions [13,14]. Additionally, S100A9 regulates virus-induced inflammatory responses, contributing to pneumonia exacerbation in influenza A virus infection and promoting inflammation in rhinovirus-associated chronic obstructive pulmonary disease [15,16]. Recent studies have identified S100A9 as a danger signaling molecule closely related to tumor diagnosis, prediction, therapy, and prognosis. Notably, S100A9 is upregulated in various infection-related tumors and promotes tumor progression through activation of RAGE and other signaling pathways [17,18]. Previous research has reported S100A9 overexpression in liver cancer, correlating with tumor malignancy [19]. Given that over half of liver cancers are associated with HBV infection, we hypothesized that S100A9 might play a role in the inflammatory response to HBV infection, thereby promoting HBV-related hepatocarcinogenesis.

To elucidate the role of S100A9 in HBV-related liver cancer development, we employed S100A9-siRNA as an intervention tool to investigate its biological effects on HepG2 cells under HBV infection conditions. Our findings reveal that HBx promotes HepG2 proliferation and migration through S100A9, and that interference with S100A9 expression attenuates these HBx-induced effects. These results demonstrate that S100A9 plays an important role in HBx-mediated liver cancer cell proliferation and migration, potentially contributing to tumor invasion and metastasis. This is consistent with studies demonstrating S100A9 involvement in prostate cancer, colorectal cancer, and other malignancies [20,21].

Furthermore, we observed that S100A9 expression was elevated in the presence of HBx but significantly reduced when NF- B was inhibited. These results indicate that HBx upregulates S100A9 expression through NF- B activation. Numerous studies have shown that aberrant NF- B activation during HBV-induced hepatocarcinogenesis regulates multiple transcriptional activities, immune responses, inflammatory reactions, cell growth, differentiation, apoptosis, and gene transcription, serving as a critical bridge between HBV infection and liver cancer. In hepatocellular carcinoma, NF- B binds to the S100A9 promoter to activate transcription, and S100A9 subsequently activates reactive oxygen species-related signaling pathways to protect cancer cells from apoptosis [22]. Our study demonstrates that HBx activates NF- B, which then upregulates S100A9 expression to promote HBV-related hepatocarcinogenesis. We therefore propose that S100A9 may be an NF- B-regulated oncogenic factor and an important molecular link between inflammation and cancer. This suggests that blocking S100A9 expression or its biological activity may provide a novel therapeutic strategy for liver cancer.

In summary, HBx enhances S100A9 expression through NF- B activation, and S100A9 participates in HBx-mediated proliferation and migration of HepG2 cells. S100A9 represents an important oncogenic factor in HBV-related liver cancer with potential applications in tumor diagnosis, therapy, and prognosis.

HBx, NF- B, and S100A9 form a cascade amplification positive feedback loop, wherein HBx activates NF- B, NF- B enhances S100A9 expression, and elevated S100A9 promotes HBx biological functions. This cyclic process drives the development of HBV-related liver cancer. Theoretically, intervention at any point in this loop could inhibit tumor progression, with S100A9, as a secreted factor in the tumor microenvironment, representing the most promising therapeutic target.

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Note: Figure translations are in progress. See original paper for figures.