

HBsAg/ β 2GPI activates the NF- κ B pathway via the TLR4/MyD88/I κ B α axis in hepatocellular carcinoma

XUE JING¹, ZIBIN TIAN¹, PUJUN GAO², HUASHI XIAO^{3,4}, XIAOLONG QI⁵,
YANAN YU¹, XUELI DING¹, LIN YANG¹ and LIANG ZONG^{3,4}

¹Department of Gastroenterology, The Affiliated Hospital of Qingdao University, Qingdao, Shandong 266003;

²Department of Hepatology, The First Hospital of Jilin University, Changchun, Jilin 130021;

³Medical Research Center, Northern Jiangsu People's Hospital; ⁴Department of Surgery, Northern Jiangsu People's Hospital,

Clinical Medical College, Yangzhou University, Yangzhou, Jiangsu 225001; ⁵Department of General Surgery, Nanfang Hospital, Southern Medical University, Guangdong Provincial Engineering Technology Research Center of Minimally Invasive Surgery, Guangzhou, Guangdong 510515, P.R. China

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Abstract. Chronic hepatitis B virus (HBV) infection remains one of the leading causes of hepatocellular carcinoma (HCC) globally. However, the mechanism underlying the mediation by HBV surface proteins (HBsAg) of the early steps in the virus life cycle and following HCC development is unclear. β 2-glycoprotein I (β 2GPI) specifically interacts with HBsAg and demonstrates high expression during the earliest stages of hepatitis B virus infection. In the present study, the assessment of HCC and adjacent tissues revealed that the levels of mRNA and protein of β 2GPI were highly expressed in HBV-related HCC. Previous studies have reported that HBsAg activates the nuclear factor (NF)- κ B pathway via interaction with β 2GPI in HCC. However, the underlying mechanism of how the interaction between HBsAg and β 2GPI confers activation of the NF- κ B pathway is still unclear. The HBsAg is comprised of three carboxyl-co-terminal HB proteins. In the present study, immunofluorescence assay and EMSA consistently revealed that a combination of recombinant small HBV surface antigen (rSHB) and β 2GPI can significantly activate the NF- κ B

signaling pathway. Another study from our team revealed that high expression of β 2GPI enhanced HBsAg binding to cell surfaces and its interaction with Annexin II. However, Annexin II is not a transmembrane protein. Therefore, by a knockdown experiment with TLR2, TLR4 or MyD88 siRNAs using cells with co-incubated HBsAg/ β 2GPI, certain aspects of the mechanism through which the HBsAg/ β 2GPI complex activates the NF- κ B pathway through the Toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88)/I κ B α axis were explained. In the present study, we identified the functional domain of HBsAg co-interaction with β 2GPI for the activation of NF- κ B and revealed the mechanism of the HBsAg/ β 2GPI-activated NF- κ B pathway which could contribute to the treatment of HBV-related HCC. A novel finding of the present study is that HBsAg can bind to β 2GPI. We first identified the functional domain of HBsAg with β 2GPI to activate NF- κ B. Second, by siRNA knockout experiments, we identified the downstream molecules involved in the activation of NF- κ B induced by β 2GPI/HBsAg. In addition, we found that HBsAg/ β 2GPI activated the NF- κ B pathway through the phosphorylation of Ser32/36 by I κ B α .

Correspondence to: Dr Liang Zong, Medical Research Center, Northern Jiangsu People's Hospital, Clinical Medical College, Yangzhou University, Yangzhou, Jiangsu 225001, P.R. China
E-mail: 250537471@qq.com

Abbreviations: HBsAg, hepatitis B surface antigen; β 2GPI, β 2-glycoprotein I; HCC, hepatocellular carcinoma; NF- κ B, nuclear factor κ B; TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; rHBsAg, recombinant hepatitis B surface antigen; rLHBs, recombinant large HBV surface antigen; rSHBs, recombinant small HBV surface antigen; EMSA, electrophoretic mobility shift assay; I κ K, I κ B kinase

Key words: β 2GPI, HBsAg, NF- κ B, hepatocellular carcinoma, Toll-like receptor, myeloid differentiation factor 88

Introduction

HCC is the fifth most common type of cancer and the second leading cause of cancer-related deaths worldwide (1). Notably, 10-20% of HCC cases are caused by HBV infection (2). Although long-term nucleoside analogue therapy produces efficient suppression of HBV viremia, the incidence of HBV caused by HCC remains very high (3). In addition, it has been reported that patients with spontaneous HBV DNA clearance, but residual HBsAg titers >1,000 IU/ml still showed increased risk for HCC (4).

To prevent the development of HCC, it is important to understand the molecular mechanism underlying its carcinogenesis. Accumulation of HBV surface proteins, particularly large HBV surface proteins, has been shown to be involved in HCC carcinogenesis via activation of the NF- κ B pathway (5).

Mehdi *et al* reported that recombinant hepatitis B surface antigen (rHBsAg) binds to β 2-glycoprotein I (β 2GPI) (6). β 2GPI, also known as apolipoprotein H, is a human plasmatic protein primarily synthesized in the liver. They found that the expression of β 2GPI was upregulated in HepG2.2.15 cells, and β 2GPI enhanced the ability of HBsAg to bind to cell surfaces of the liver (7).

In our previous studies, we found that β 2GPI collocated with HBsAg in the cytosol of HepG2.2.15 cells (7). In addition, when we transfected β 2GPI and HBsAg into HCC cells, NF- κ B was activated and transferred from the cytoplasm into the nucleus (8). All these findings indicate that β 2GPI may be involved in the development of HBsAg-caused HCC via the NF- κ B pathway. However, the mechanism through which the interaction between HBsAg and β 2GPI confer activation of the NF- κ B pathway has not yet been elucidated.

In the present study, we first identified the functional domain of HBsAg which co-interacts with β 2GPI for the activation of NF- κ B. Secondly, using a knockdown experiment with siRNA, we identified the downstream molecules that are involved in the β 2GPI/HBsAg-induced NF- κ B activation. Mechanically, we revealed that HBsAg/ β 2GPI activated the NF- κ B pathway through phosphorylation of I κ B α .

Materials and methods

Tissue specimens. HBV-associated HCC tissues (10 patients) and non-cancerous tissues (8 patients) were obtained from HBV-infected patients who underwent partial hepatectomy for HCC between July 2014 and July 2015, at the Department of Pathology, at the Affiliated Hospital of Qingdao University, China. Controls (4 patients) were histologically normal tissues obtained from positive-HBsAg carrier patients who underwent partial hepatectomy for liver hemangiomas or biliary duct diseases. Their ages ranged from 25 to 68 years and there were 14 male and 8 female cases. The diagnosis was established based on commonly accepted clinical and laboratory parameters and typical histologic features. These patients had no other obvious underlying disease, tested positive for HBV markers, and had normal levels of serum transaminase. All tissue samples were collected with the written informed consent of all patients and under the approval of the Ethics Committee of the Affiliated Hospital of Qingdao University.

Cell culture and experimental groups. HCC cells, SMMC-7721 were maintained as a monolayer in Invitrogen™ RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 2 mM L-glutamine, 100 U/ml penicillin and 100 μ g/ml streptomycin at 37°C under 5% CO₂ and were passaged once every 2 days. A stable cell line β 2GPI/SMMC-7721 was generated. pcDNA-3.1(-)-rLHBsAg (recombinant large HBV surface antigen, rLHBs) and pcDNA-3.1(-)-rSHBsAg (recombinant small HBV surface antigen, rSHBs) plasmid were constructed (9).

SMMC-7721 cells were divided into nine groups as follows: i) Group one, where β 2GPI/SMMC-7721 cells were incubated with 5 μ g/ml of serum-derived HBsAg (donated by Professor Gao, The First Hospital of Jilin University) for 48 h; ii) Group two, where SMMC-7721 cells were

incubated by 5 μ g/ml of serum-derived HBsAg for 48 h; iii) Group three, β 2GPI/SMMC-7721 cells; iv) Group four, where SMMC-7721 cells were transiently transfected with pcDNA-3.1(-) vector; v) Group five, SMMC-7721 cells; vi) Group six, β 2GPI/SMMC-7721 cells were transiently transfected with pcDNA-3.1(-)-rLHBs plasmid; vii) Group seven where β 2GPI/SMMC-7721 cells were transiently transfected with pcDNA-3.1(-)-rSHBs plasmid; viii) Group eight, where SMMC-7721 cells were transiently transfected with pcDNA-3.1(-)-rLHBs plasmid; and ix) Group nine, where SMMC-7721 cells were transiently transfected with pcDNA-3.1(-)-rSHBs plasmid.

The transfections were performed using FuGENE® HD transfection reagent according to the manufacturer's instructions (Roche, Basel, Switzerland). Following 48 h of treatment, all cells were washed twice with PBS and incubated for an additional 4–12 h in serum-free culture media to collect secreted β 2GPI, HBsAg and AFP. The procedure was performed as described in a previous study (8).

Hematoxylin and eosin (H&E) staining. The specimens were fixed in 10% formaldehyde, dehydrated, embedded in paraffin and sliced into 4- μ m sections. Following hematoxylin and eosin (H&E) staining, pathological changes in the liver tissues were observed under a light microscope (Olympus BX-53; Olympus Corp., Tokyo, Japan).

Immunofluorescence detection. Detection of the expression of β 2GPI and HBsAg in the tissues and the activated NF- κ B and the phosphorylation site of I κ B α in the cells were performed by immunofluorescence (8). Monoclonal rabbit anti-NF- κ B p65 antibody (1:50 dilution; cat. no. bs-0465R; Beijing Biosynthesis Biotechnology Co., Ltd., Beijing, China), p-I κ B α -Ser32 mouse monoclonal antibody (1:50 dilution; cat. no. SC-7977-R; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), p-I κ B α -Ser32/36 mouse monoclonal antibody (1:50 dilution; cat. no. SC-101713; Santa Cruz Biotechnology, Inc.), FITC-stained β 2GPI protein and RBITC-stained HBsAg protein were used.

The blue (Hoechst-stained DNA), green (FITC-stained β 2GPI protein) and red (RBITC-stained HBsAg protein or p-I κ B α -Ser32/36 or p-I κ B α -Ser32) fluorescence was detected. The activation of NF- κ B was performed by assessments of fluorescence intensity (FI) (8).

Cytokine measurements. The levels of β 2GPI, HBsAg, TNF- α , IL-1 β and AFP in the supernatants of the cells were measured using commercially available ELISA kits (Nanjing KeyGen Biotech. Co., Ltd., Nanjing, China) according to the manufacturer's instructions.

Cell extract preparation and non-radioactive electrophoretic mobility shift assay (EMSA). Nuclear and cytoplasmic extracts from the HCC cell groups were prepared from 1 \times 10⁷ cells, for analysis by EMSA using a non-radioactive EMSA kit (Viagene Biotech Inc., Tampa, FL, USA). The assay was performed according to the manufacturer's instructions (8). The bands were visualized using Viagene Cool Imager (Viagene Biotech Inc.). The band intensities were quantified using Cool Imager.

Table I. Sequences for real-time PCR and si-RNA.

Real-time PCR	Sequences
TLR2	F: 5'-CGTTCTCTCAGGTGACTGCTC-3'
TLR4	R: 5'-CCTTTGGATCCTGCTTGC-3'
	F: 5'-AGCCATGGCCTTCCTCTC-3'
	R: 5'-TTCAGCTCCATGCATTGATAA-3'
MyD88	F: 5'-CTGCTCGAGCTGCTTACCA-3'
	R: 5'-CTTCAAGATATACTTTTGGCAATCC-3'
GAPDH	F: 5'-TGTTGCCATCAATGACCCCTT-3'
	R: 5'-CTCCACGACGTACTCAGCG-3'
si-RNA	
MyD88-homo-316	F: 5'-GCCUGUCUCUGUUCUUGAATT-3'
	R: 5'-UUCAAGAACAGAGACAGGCTT-3'
MyD88-homo-760	F: 5'-GGCAACUGGAACAGACAAATT-3'
	R: 5'-UUUGUCUGUCCAGUUGCCTT-3'
MyD88-homo-987	F: 5'-CCCAUCAGAAGCGACUGAATT-3'
	R: 5'-AUCAGUCGCUUCUGAUGGGTT-3'
TLR2-homo-1375	F: 5'-GCCCUCUCUACAAACUUUATT-3'
	R: 5'-UAAAGUUUGUAGAGAGGGCTT-3'
TLR2-homo-1648	F: 5'-GCAACUCAAAGAACUUUATT-3'
	R: 5'-AUAAAGUUCUUUGAGUUGCTT-3'
TLR2-homo-950	F: 5'-GGUGAAACAAAUUCAUUGATT-3'
	R: 5'-UCAAUGAAUUUGUUUCACCTT-3'
TLR4-homo-1546	F: 5'-GGGCUUAGAACAACUAGAATT-3'
	R: 5'-UUCUAGUUGUUCUAAAGCCCTT-3'
TLR4-homo-1325	F: 5'-CCCACAUUGAAACUCAAUATT-3'
	R: 5'-AUUUGAGUUUCAUGUGGGTT-3'
TLR4-homo-595	F: 5'-CCACCUCUCUACCUUAAUATT-3'
	R: 5'-UAUUAAGGUAGAGAGGUGGTT-3'
Negative control	F: 5'-UUCUUCGAACGUGUCACGUTT-3'
	R: 5'-ACGUGACACGUUCGGAGAATT-3'

F, forward; R, reverse.

Effectiveness of the inhibitors. For the *in vitro* experiments on the inhibition of I κ B α , the cells which showed the highest level of activation of NF- κ B were incubated. I κ B phosphorylation by I κ B kinase (I κ K) prepares I κ B for ubiquitination and subsequent degradation. Pharmacological inhibition of I κ K by Bay 11-7082 or of tyrosine kinase by piceatannol were studied. Bay 11-7082 (CAS 19542-67-7-Calbiochem; Merck Millipore, Darmstadt, Germany) or piceatannol (CAS 10083-24-6-Calbiochem; Merck Millipore) were added and incubated. The nuclear extracts of the cells that were treated by the inhibitors at 15, 30 and 60 min, respectively were isolated. The activation of NF- κ B was analyzed by non-radioactive EMSA.

Co-immunoprecipitation. All cells in the above-mentioned nine groups were prepared and then incubated with a p-Tyr-2C8 mouse monoclonal antibody (1:50 dilution; cat. no. sc-81529; Santa Cruz Biotechnology, Inc.) or p-Ser-4A4 mouse monoclonal antibody (1:25 dilution; cat. no. DAM1460187;

EMD Millipore, Billerica, MA, USA) overnight. The immunocomplex was precipitated with protein A/G-Sepharose beads (Protein G Plus-Agarose Immunoprecipitation Reagent SC-2002; Santa Cruz Biotechnology, Inc.) and then submitted to 7.5% SDS-PAGE. Western blot (WB) analysis was performed using an anti-I κ B α mouse monoclonal antibody (H-4, 1:300 dilution; cat. no. SC-1643; Santa Cruz Biotechnology, Inc.).

RNA interference assay. The cells were transfected with various siRNA directed against TLR4, TLR2 and MyD88 proteins (cat. nos.: si_TLR4-1, TLR4-homo-595; si_TLR4-2, TLR4-homo-1325; si_TLR4-3, TLR4-homo-595; si_TLR2-1, TLR2-homo-950; si_TLR2-2, TLR2-homo-1375; si_TLR2-3, TLR2-homo-1648; si_MyD88-1, MyD88-homo-316; si_MyD88-2, MyD88-homo-760; si_MyD88-3, MyD88-homo-987), and negative siRNA with a random sequence was used as a control (all from Shanghai GenePharma Co., Ltd., Shanghai, China). The siRNA sequences are shown in Table I. The cells were seeded at a density of 5×10^5 cells/well into 6-well dishes

and cultured overnight at 37°C with 5% CO₂ until the cells reached 70% confluency. The cells were co-incubated with the HBsAg protein (5 μ g/ml; Ab73749; Abcam, Cambridge, MA, USA) and β 2GPI protein (10 μ g/ml; 362225; Merck Millipore) for 24 h. Subsequently, the transfections were performed using Invitrogen™ Lipofectamine 2000 reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Gene and protein knockdown efficiencies were examined by qPCR and western blotting.

RNA extraction and quantitative real-time PCR. Total RNAs of tissues and cells in different groups were extracted with TRIzol® reagent (Invitrogen) according to the manufacturer's instructions. RNA integrity was electrophoretically verified by ethidium bromide staining and by OD260/OD280 nm absorption ratio >1.95. Total RNA (2 μ g) was used for reverse transcription to synthesize cDNA (Applied Biosystems High Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific, Inc.). The primers for β 2GPI, TLR4, TLR2 and MyD88 were designed and synthesized by Sangon Biological Engineering Technology and Services Co. Ltd. (Shanghai, China). The PCR primers are listed in Table I. Reaction system (25 μ l) was established and quantitative real-time PCR assays were performed via the Applied Biosystems SYBR®-Green PCR Master Mix (Thermo Fisher Scientific, Inc.) on the Applied Biosystems 7500 Real-Time PCR system (Thermo Fisher Scientific, Inc.). The expression data were normalized to housekeeping gene GAPDH using the $2^{-\Delta CT}/[2^{-(CT \text{ of target genes} - CT \text{ of GAPDH})}]$ method. All PCR experiments were performed in triplicate.

Western blot analysis. The protein concentration of each sample was assayed using the BCA protein assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol as follows: Equal amounts (40 μ l) of protein from each sample were resolved by 10% SDS-PAGE and electrotransferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in TBST containing 5% non-fat dried milk for 1 h at room temperature followed by incubation with primary antibodies at 4°C overnight and incubated with secondary antibodies in TBST at room temperature for 1 h. The primary antibodies used were I κ B α -H-4 mouse monoclonal antibody (1:300 dilution; cat. no. SC-1643; Santa Cruz Biotechnology, Inc.), anti-TLR4 mouse monoclonal antibody (1:200 dilution; cat. no. SC-293072; Santa Cruz Biotechnology, Inc.), anti-TLR2 rabbit polyclonal antibody (1:200 dilution; cat. no. SC-10739; Santa Cruz Biotechnology, Inc.), anti-MyD88 mouse monoclonal antibody (1:200 dilution; cat. no. SC-11356; Santa Cruz Biotechnology, Inc.). The immunoreacted proteins were detected using an enhanced chemiluminescence (ECL) western blotting detection kit (Thermo Fisher Scientific, Inc.). Image Lab v3.0 software (BioRad Laboratories, Inc., Hercules, CA, USA) was used to acquire and analyze imaging signals.

Statistical analysis. Data are presented as the mean \pm standard error of the mean (SEM) or median. Differences between multiple groups were analyzed using one way ANOVA test and the Bonferroni's post hoc test for multiple comparisons. A P-value of <0.05 was considered to indicate a statistically significant difference.

Results

β 2GPI is highly expressed in HBV-related HCC and co-localizes with HBsAg. To determine the expression level of β 2GPI in HBV-related HCC, we estimated the expression level of β 2GPI using HBV-related HCC, adjacent non-cancerous tissues and HBsAg-positive liver tissues. We observed that the protein and mRNA levels of β 2GPI were significantly higher in HBV-related HCC tissues compared with the levels noted in the adjacent non-cancerous and HBsAg-positive liver tissues (Fig. 1A and C). We hypothesized that β 2GPI may co-interact with HBsAg. To test our hypothesis, we conducted an immunohistochemical analysis with double staining to analyze the localization of β 2GPI using HBV-related HCC, adjacent non-cancerous liver and HBV-infected liver tissues. We observed that β 2GPI was localized in the cytoplasm and co-localized with HBsAg (Fig. 1B).

HBsAg interacts with β 2GPI mostly via the S domain to activate the NF- κ B pathway. In our previous study, we found that HBsAg is able to interact with β 2GPI to activate the NF- κ B signaling pathway. To determine which domain of HBsAg co-interacts with β 2GPI, we designed three forms of HBsAg with a different composition: Serum-derived HBsAg, rSHBs and rLHBs. Among them, we observed that a combination of β 2GPI and rSHBs significantly activated the NF- κ B signaling pathway more than the others (Fig. 2A). In addition, by immunofluorescence assay, a combination treatment with β 2GPI and rSHBs induced a transfer of NF- κ B from the cytoplasm to the nucleus (Fig. 2B). To further validate our findings, we analyzed the expression of AFP, known as a downstream target gene of NF- κ B. As displayed in Fig. 2C, the level of AFP was significantly upregulated after a combination treatment with β 2GPI and rSHBs. These data indicate that HBsAg interacts with β 2GPI through the S domain to activate the NF- κ B pathway.

HBsAg/ β 2GPI contributes to the activation of the NF- κ B pathway via the TLR4/MyD88 axis. Generally, NF- κ B activation was reported to be mediated in the MyD88-dependent or -independent pathway. To test whether the activation of NF- κ B by HBsAg/ β 2GPI was mediated by MyD88 dependence or not, we depleted TLR2, TLR4 or MyD88 with three independent siRNAs using the cell line with co-incubated HBsAg/ β 2GPI, and the siRNA with the highest knockdown efficiency was selected (Fig. 3). Notably, we found that both TLR4 and MyD88 depletion mutually downregulated the expression of each other (Fig. 4A-a and b). Both western blotting and real-time PCR revealed that when TLR4 was knocked down, the expression of TLR2 and MyD88 was reduced in addition to TLR4 itself. Based on the results of real-time PCR, the level of TLR4 in the si_TLR4-1 group was significantly different compared with the Negative, Positive and si_TLR2-3 group, $P < 0.05$; the level of TLR2 in the si_TLR4-1 group was significantly different compared with Positive and si_TLR2-3 group, $P < 0.05$; the level of MyD88 in the si_TLR4-1 group was significantly different compared with Positive and si_MyD88-3 group, $P < 0.05$). In addition, when TLR2 was knocked down, the expression of TLR2, TLR4 and MyD88 in the si_TLR2-3 group was significantly decreased compared with the Positive groups ($P < 0.05$). On the contrary, these proteins were not

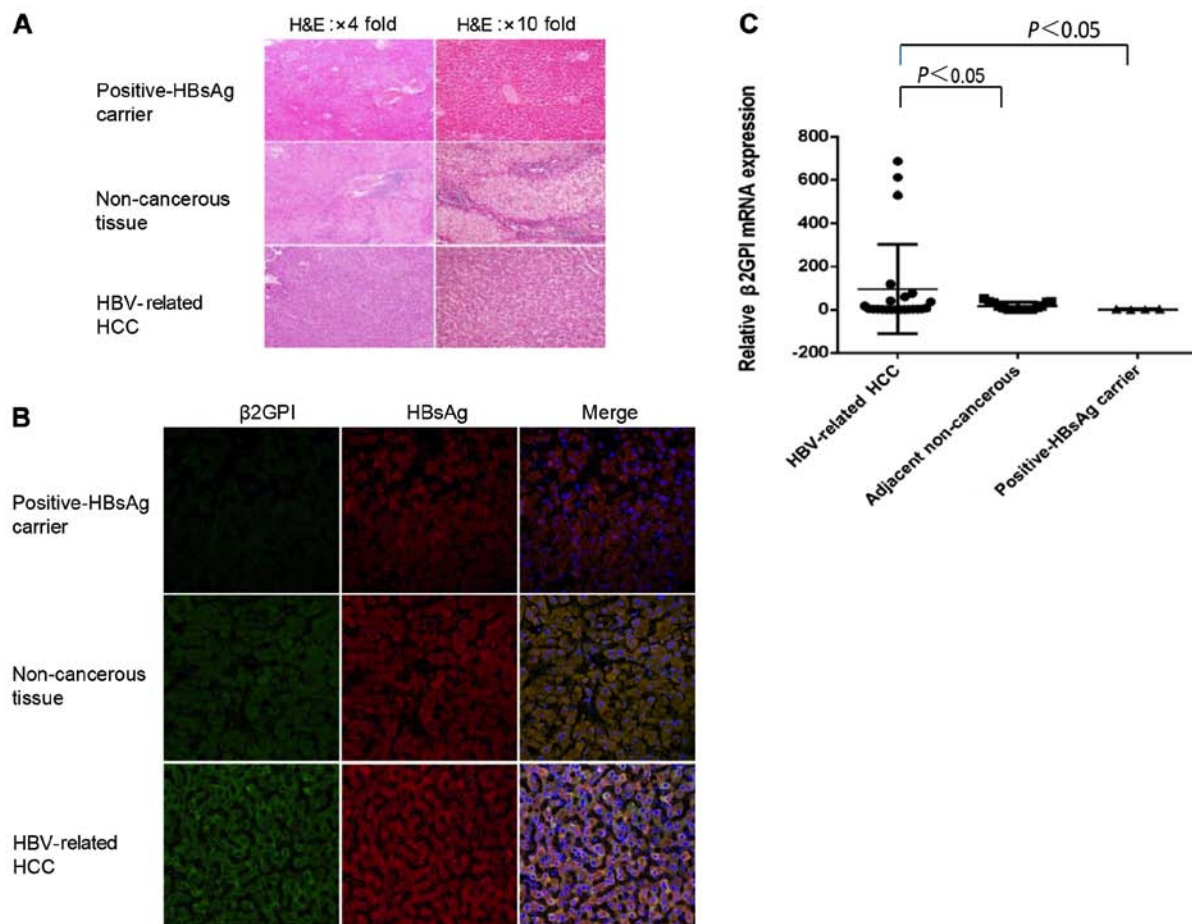


Figure 1. β 2GPI is highly expressed in HBV-related HCC and co-localized with HBsAg. (A) Hematoxylin and eosin (H&E) was used for staining of HBV-related HCC, adjacent non-cancerous and HBsAg-positive liver tissues. Scale bars, 100 μ m. (B) Fluorescent immunohistochemical analysis was performed using β 2GPI pAb (FITG, green color) and HBsAg mAb (RO, red color) for HBV-related HCC, adjacent non-cancerous and HBsAg positive liver tissues. Double-stained tissues are shown (yellow color). Cell nuclei were stained with Hoechst 33258 (blue color). The slides were visualized using an Olympus FluoView FV1000 laser scanning confocal microscope (LSCM). Original magnification: \times 400, and scale bars are 20 μ m. The green fluorescence intensity was strongest in the HBV-related tissue than in adjacent non-cancerous tissues. β 2GPI co-localized with HBsAg in the cytosol of HBV-related tissue and adjacent non-cancerous (It showed white color when double-stained was added to Hoechst stain). (C) Relative analysis of β 2GPI mRNA expression. The mRNA level of β 2GPI of all tissues was quantitated. $P < 0.05$, compared to adjacent non-tumorous liver and positive HBsAg carrier tissues. HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; β 2GPI, β 2-glycoprotein I; HCC, hepatocellular carcinoma.

significantly reduced, when compared with the si_MyD88-3 group. Furthermore, when MyD88 was knocked out, the expression of TLR2, TLR4 and MyD88 in the si_MyD88-3 group was significantly reduced compared with the Positive and si_TLR2-3 groups, but, this was not statistically significant compared with the si_TLR4-1 expected for the expression of MyD88, $P < 0.05$. Furthermore, TLR4 or MyD88 depletion reduced the activation of the NF- κ B pathway (Fig. 4B). In addition, three downstream cytokines, IL-1 β , TNF- α and AFP, were obviously downregulated compared with the Positive and Negative groups when TLR4, TLR2 or MyD88 was depleted. The level of AFP was statistically different among the siRNA groups ($P < 0.05$), which was completely opposite of IL-1 β . The expression of TNF- α among the siRNA groups was statistically different except for the si_TLR4-1 group compared with si_MyD88-3 group ($P < 0.05$) (Fig. 4C). These results indicated that HBsAg/ β 2GPI primarily activated the NF- κ B pathway via the TLR4/MyD88 axis.

HBsAg/ β 2GPI complex activates the NF- κ B pathway through the phosphorylation of I κ B α . It has been reported that NF- κ B

activation is induced by I κ B α degradation. We also hypothesized that the activation of NF- κ B by HBsAg/ β 2GPI is induced by I κ B α degradation. To test this hypothesis and explore the exact mechanism, two pharmacological substance inhibitors (Bay 11-7082 and piceatannol) were used to treat the cells with co-incubated β 2GPI and rSHBs (Group 7). As displayed in Fig. 5A, only Bay 11-7082 clearly abolished the activation of NF- κ B in a time-dependent manner, suggesting that I κ B α degradation plays a key role in the activation of NF- κ B by HBsAg/ β 2GPI. To further determine the mechanism of I κ B α degradation, the phosphorylation of I κ B α in two well-known amino acid positions (p-Ser and p-Tyr) was explored by co-immunoprecipitation using the purified cell extract from the nine groups (Fig. 5B). We observed that the serine phosphorylation of I κ B α protein was markedly increased in group six, seven and nine (β 2GPI+rLHBs, β 2GPI+rSHBs and rSHBs treatment, respectively).

To further explore the site of serine phosphorylation in I κ B α , we analyzed the phosphorylation of I κ B α in two well-known amino acid site (Ser 32/36 and Ser 32) by western blotting using the three groups with higher serine

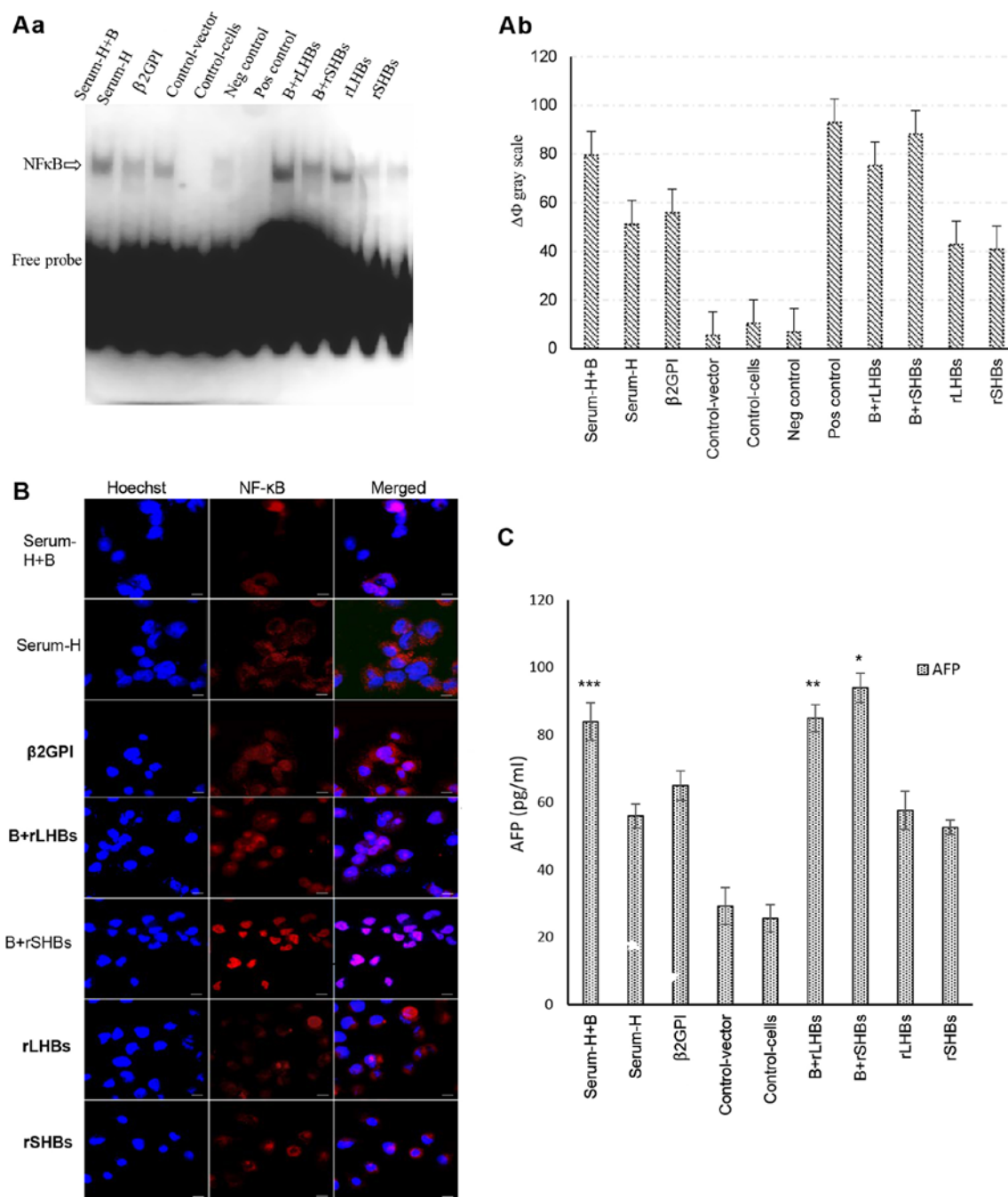


Figure 2. HBsAg interacts with β 2GPI mostly via the S domain to activate the NF- κ B pathway. (A-a) Activation of NF- κ B examined by non-radioactive EMSA. Cell nuclei from each of the nine cell groups were assessed by non-radioactive EMSA. Lane 1: Serum-H+B (group one), β 2GPI/SMMC-7721 cells were incubated with 5 μ g/ml of serum-derived HBsAg; lane 2: Serum-H (group two), SMMC-7721 cells were incubated by 5 μ g/ml of serum-derived HBsAg for 48 h; lane 3: β 2GPI (group 3), β 2GPI/SMMC-7721 cells; lane 4: Control-vector (group 4), SMMC-7721 cells were transiently transfected with pcDNA-3.1(-) vector; lane 5: Control cells (group 5), SMMC-7721 cells; lane 6: Negative control, cold-oligonucleotide; lane 7: Positive control, TNF- α ; lane 8: B+rLHBs (group 6), β 2GPI/SMMC-7721 cells were transiently transfected with pcDNA-3.1(-)-rLHBs plasmid; lane 9: B+rSHBs (group 7), β 2GPI/SMMC-7721 cells were transiently transfected with pcDNA-3.1(-)-rSHBs plasmid; lane 10: rLHBs (group 8), SMMC-7721 cells were transiently transfected with pcDNA-3.1(-)-rLHBs plasmid; lane 11: rSHBs (group 9), SMMC-7721 cells were transiently transfected with pcDNA-3.1(-)-rSHBs plasmid. (b) Gray scale of the nine groups. $\Delta\Phi$ gray scale=total gray scale of each group/blank group gray scale. The combination treatment groups (β 2GPI+serum-derived HBsAg, $\Delta\Phi$ gray scale=79.56, group one; β 2GPI+rLHBs, $\Delta\Phi$ gray scale=75.29, group six) exhibited stronger activation of NF- κ B compared with only β 2GPI or rLHBsAg or rSHBsAg or serum-derived-HBsAg treatment groups. The strongest activation of NF- κ B was observed in the β 2GPI and rSHB treatment group, $\Delta\Phi$ gray scale=88.13. The $\Delta\Phi$ gray scale of positive control was 92.9. (B) Activation of NF- κ B examined by immunofluorescence confocal microscopy. The cells of the nine groups were plated onto glass coverslips in a 24-well plate and were incubated for 36 h, then fixed, permeabilized and stained with the indicated specific antibodies against NF- κ B (red color). Cell nuclei were stained with Hoechst 33258 (blue color). It was suggested that NF- κ B was activated, when it was transferred from the cytoplasm into the nucleus. Positive staining (pink color) in the nucleus was determined in most of the cells, but the most significant positive staining of NF- κ B was shown in the β 2GPI and rSHBs treatment group. The slides were visualized using an Olympus FluoView FV1000 laser scanning confocal microscope (LSCM). Original magnification x600, and scale bars are 20 μ m. (C) The expression of downstream cytokines of AFP was analyzed by ELISA. The expression of AFP was significantly upregulated after a combination treatment with β 2GPI and rSHBsAg. Bars are the mean \pm SEM (n=3). *P<0.05, compared with other groups except Serum-H+B and rLHBs+B; **P<0.05, compared with other groups except Serum-H+B and B+rSHBs; ***P<0.05, compared with other groups except B+rLHBs and B+rSHBs. B+rSHBs: β 2GPI+rSHBs; Serum-H+B: Serum-H+ β 2GPI; rLHBs+B: rLHBs+ β 2GPI. HBsAg, hepatitis B surface antigen; β 2GPI, β 2-glycoprotein I; NF- κ B, nuclear factor κ B.

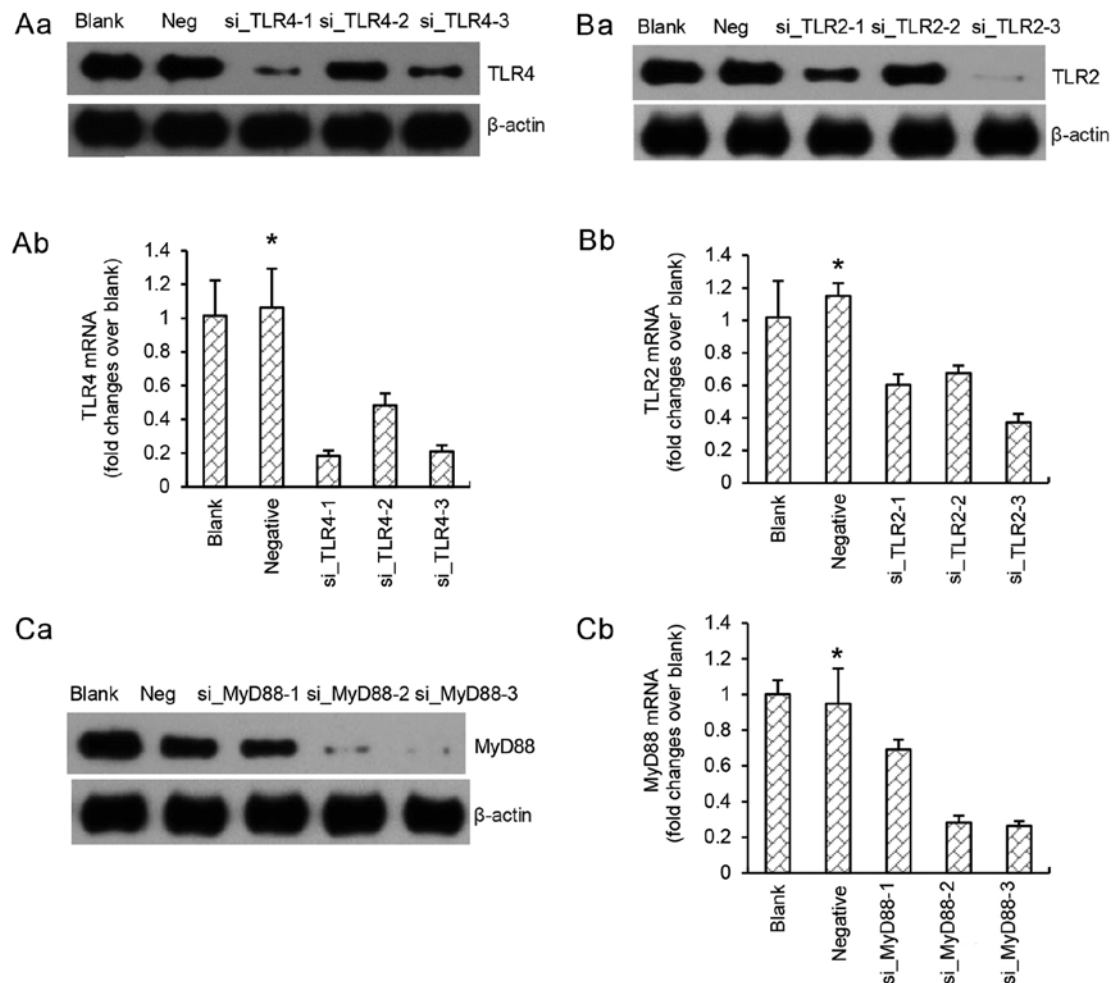


Figure 3. Selected siRNAs with the highest knockdown efficiency. We constructed three independent siRNAs for (A) TLR4, (B) TLR2 and (C) MyD88, named as follows: si_TLR4-1, si_TLR4-2, si_TLR4-3; si_TLR2-1, si_TLR2-2, si_TLR2-3; si_MyD88-1, si_MyD88-2, si_MyD88-3. The siRNA with the highest knockdown efficiency was selected by WB (A-a, B-a and C-a) and real-time PCR expression (A-b, B-b, C-b). si_TLR4-1, si_TLR2-3 and si_MyD88-3 were selected. *P<0.05, negative group compared with siRNA treatment groups. TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; WB, western blotting.

phosphorylation (β 2GPI+rLHBs, group six; β 2GPI+rSHBs, group seven; rSHBs, group nine). We confirmed that the phosphorylation of I κ B α was observed in Ser 32/36 but not Ser 32, and it was more obvious in group seven (Fig. 5C). Subsequently we validated this finding by immunofluorescence using the cells treated with β 2GPI/rSHBs (Fig. 5D). All these data indicated that HBsAg/ β 2GPI activated the NF- κ B pathway through the phosphorylation of I κ B α in Ser 32/36.

Discussion

In the present study, we demonstrated that β 2GPI was highly expressed in HBV-related HCC tissue compared with non-cancerous tissue, and HBsAg-positive carrier normal liver tissues. In our previous studies, we found that HBsAg interacting with β 2GPI conferred activation of NF- κ B, which indicated the probable action of β 2GPI in HBV-induced HCC.

It was reported that HBV envelope proteins consist of three isoforms: Small surface proteins (SHBs), middle surface proteins (MHBs) and large surface proteins (LHBs) (10). SHBs consist of only the S domain, MHBs contain the pre-S2 domain in addition to S, and LHBs contain the pre-S1 domain

in addition to pre-S2 and S (11). However, among these three isoforms, it has been reported that β 2GPI can only bind to the small S proteins of rHBsAg (6). The preS1 can recognize the asialoglycoprotein receptor which is on the surface of human hepatocytes or HCC cells (11), and preS1 not preS2 is essential for virus infection (12). Therefore, in the present study, we designed three different forms of HBsAg: Serum-HBsAg, rSHBs and rLHBs. We found that rSHBs in combination with β 2GPI showed the highest activity for activation of NF- κ B, compared with serum-HBsAg, rLHBs, rSHBs, β 2GPI alone or other combination treatment, indicating that the S domain of HBsAg sufficiently interacts with the β 2GPI-activated NF- κ B pathway. To the best of our knowledge, this is probably due to β 2GPI specially binding to small S protein (6).

Approximately 80% of HCC patients experience chronic liver inflammation, fibrosis or cirrhosis during the development (1). It was reported that liver exposure to various TLR ligands via the portal vein, leads to an uncontrolled activation of innate immunity that may result in inflammatory liver diseases (13). TLRs, a family of transmembrane signaling receptors, contain Toll/IL-1 receptor (TIR) motif to bridge receptors or corresponding intracellular molecules for signal

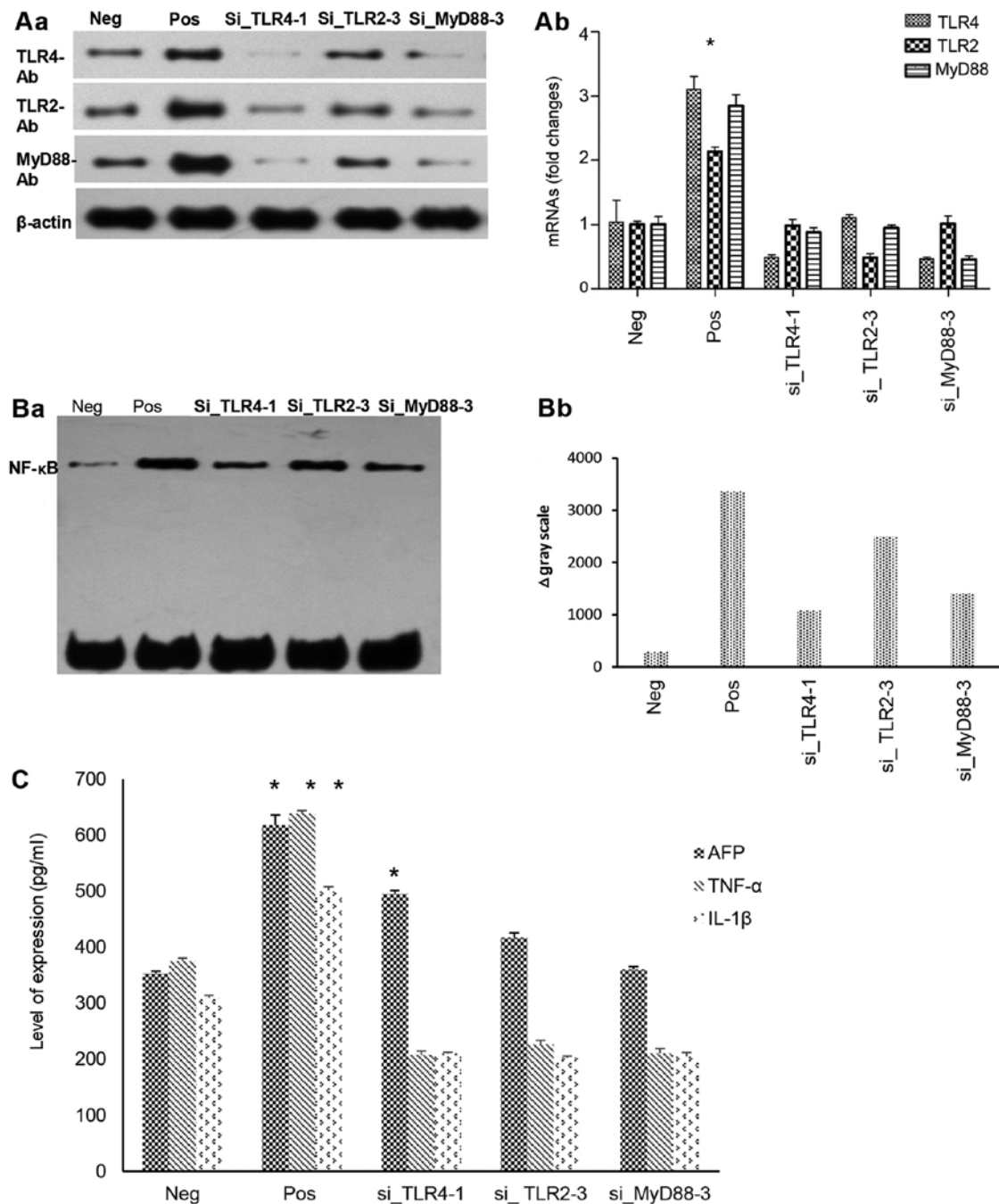


Figure 4. HBsAg/ β 2GPI contributes to the activation of the NF- κ B pathway via the TLR4/MyD88 axis. (A-a) Three independent siRNAs of TLR2, TLR4 or MyD88 using the cell line with co-incubated HBsAg/ β 2GPI. The protein expression of TLR2, TLR4 or MyD88 was analyzed by western blotting with antibodies. (b) The mRNA expression of TLR2, TLR4 or MyD88 in the cell line with co-incubated HBsAg/ β 2GPI was determined by quantitative real-time PCR. Both TLR4 and MyD88 depletion mutually downregulated the expression of each other. * $P < 0.05$, positive group compared with other groups. (B-a) Activation of NF- κ B as examined by non-radioactive EMSA. TLR4 or MyD88 depletion reduced the activation of the NF- κ B pathway. (b) $\Delta\Phi$ gray scale of each treatment group was as follows: The neg control=289.03, the positive control=3,356.6, the siTLR4_1=1,081.8, the siTLR2_3=2,493 and siMyD88_3=1,403.8. (C) The expression of downstream cytokines IL-1 β , TNF- α and AFP was analyzed by ELISA. The result revealed that expression of AFP, TNF- α and IL-1 β in the si_TLR4-1, si_TLR2-3 and si_MyD88-3 groups was distinctly different compared with the Positive and Negative groups ($P < 0.05$). The expression of TNF- α among the si_TLR4-1, si_TLR2-3 and si_MyD88-3 groups was statistically different except the comparison between the si_TLR4-1 and si_MyD88-3 group (* $P < 0.05$). The level of IL-1 β was not statistically different among the si_TLR4-1, si_TLR2-3 and si_MyD88-3 groups. The level of AFP was statistically different among any two groups, * $P < 0.05$, positive group compared with other groups. HBsAg, hepatitis B surface antigen; β 2GPI, β 2-glycoprotein I; TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; NF- κ B, nuclear factor κ B.

transportation (14). It has been reported that TLR2 or TLR4 is upregulated in HCC (15), and knockdown of either of them significantly decreases the proliferation of HCC cells (16,17). MyD88 was found to be an adaptor molecule to TIR, and it can recruit IL-1R-associated kinase (IRAK) and TNF

receptor-associated factor-6 (TRAF6), leading to the activation of NF- κ B (18). In addition, MyD88 was found to be frequently upregulated in HCC, and overexpression of MyD88 was found to promote HCC cell proliferation and invasion *in vitro* (19). Therefore, it appears that TLRs and MyD88

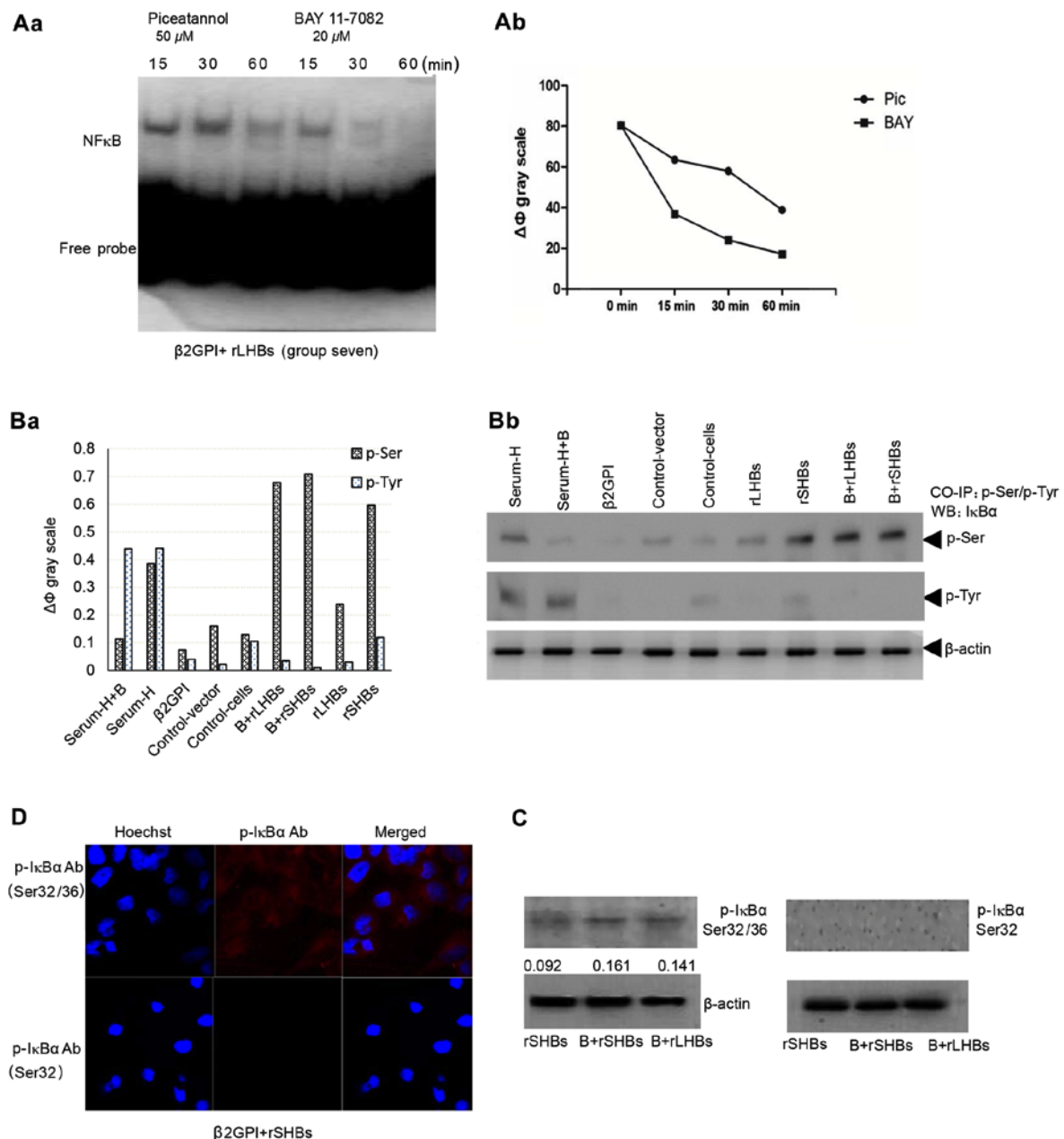


Figure 5. HBsAg/ β 2GPI complex activates the NF- κ B pathway through phosphorylation of I κ B α . (A-a) The pharmacological substance inhibitors (Bay 11-7082 and piceatannol) were used to treat the cells with co-incubated β 2GPI and rSHBs (Group 7). (b) $\Delta\Phi$ Gray scale=total gray scale of each group/back group gray scale. Activation of NF- κ B examined by non-radioactive EMSA. Bay 11-7082 clearly abolished the activation of NF- κ B in a time-dependent manner. (B-b) The p-Ser and p-Tyr were explored by co-immunoprecipitation using the purified cell extract from the nine groups). (a) The $\Delta\Phi$ Gray scale=total gray scale of each group/back group gray scale. The serine phosphorylation of I κ B α protein was markedly increased in group six, seven and nine (β 2GPI+rLHBs, β 2GPI+rSHBs, and rSHBs treatment). (C) The phosphorylation of I κ B α (Ser 32/36 and Ser 32) by western blotting using the three groups with higher serine phosphorylation by WB (β 2GPI+rLHBs, Group six; β 2GPI+rSHBs, Group seven; rSHBs, Group nine). There was no staining in the p-I κ B α -Ser32 group. We confirmed that the phosphorylation of I κ B α was observed in Ser 32/36 but not Ser 32. (D) Then we validated this finding by immunofluorescence using the cells treated with β 2GPI/rSHBs, which were stained with the indicated specific antibodies (p-I κ B α -Ser32 or p-I κ B α -Ser32/36) (red color). Cell nuclei were stained with Hoechst 33258 (blue color). All these data indicated that HBsAg/ β 2GPI activated the NF- κ B pathway through the phosphorylation of I κ B α in Ser 32/36. Original magnification, x600, and scale bars are 20 μ m. B+rSHBs, β 2GPI+rSHBs. HBsAg, hepatitis B surface antigen; β 2GPI, β 2-glycoprotein I; NF- κ B, nuclear factor κ B; TLR, Toll-like receptor; rHBsAg, recombinant hepatitis B surface antigen; rLHBs, recombinant large HBV surface antigen; rSHBs, recombinant small HBV surface antigen; EMSA, electrophoretic mobility shift assay; I κ K, I κ B kinase.

signaling are associated with inflammation-associated liver cancer development (13).

In the present study, we found that knockdown of TLR4 significantly reduced the activation of NF- κ B, but not TLR2. We also provided evidence that activation of NF- κ B was prominently inhibited by the small interfering (si)RNA-directed targeting of MyD88. This finding indicated that the

TLR4-associated MyD88-dependent signaling pathway contributed to the activation of NF- κ B. Notably, we observed that the level of TLR2 protein was decreased by knockdown of TLR4, and the expression of TLR2 and TLR4 proteins were downregulated by si-MyD88. This finding indicated that there was a possible interaction between them. In agreement with our findings, Sweeney *et al* (20) demonstrated that there exists

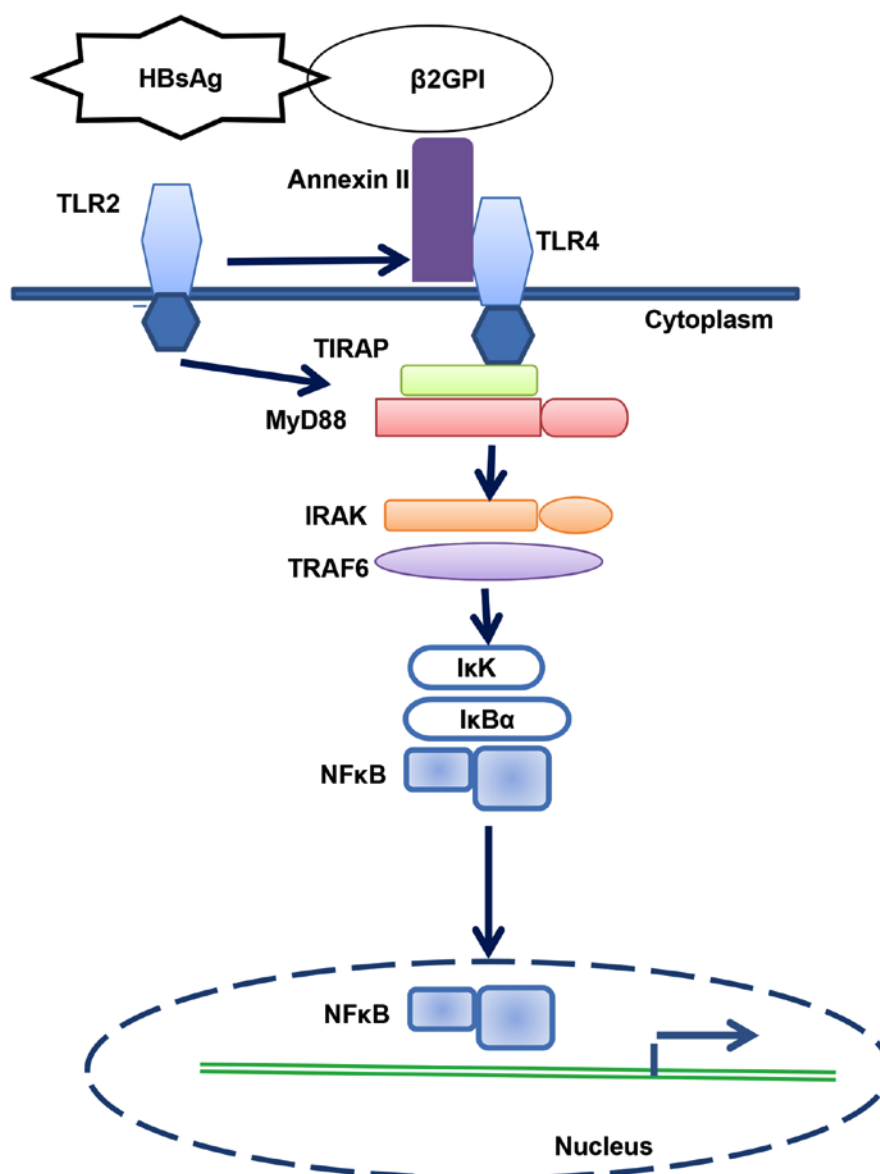


Figure 6. Scheme of the signaling pathway of HBsAg/β2GPI-activated NF-κB through the TLR4/MyD88/IκBα axis. HBsAg, hepatitis B surface antigen; β2GPI, β2-glycoprotein I; NF-κB, nuclear factor κB; TLR, Toll-like receptor; MyD88, myeloid differentiation factor 88; IκK, IκB kinase.

a TLR2/TLR4/TRAM native hepatic protein complex, which may have important implications for the host response to sepsis. Yamamoto *et al* (21) revealed that TLR2/TLR4 shared the MyD88/TIRAP common signaling pathway.

The NF-κB signaling pathway plays a key role in HCC carcinogenesis (22). NF-κB transcription factors consist of five proteins, p65 (Rel A), RelB, c-Rel, p105/p50 (NF-κB1) and p100/52 (NF-κB), which can form a large number of homodimers and heterodimers (23). In unstimulated cells, NF-κB dimers are sequestered in the cytoplasm by inhibitors of IκBs. IκBs are degraded by upstream kinase complex IκB kinase (IKKs) upon activation, thereby allowing nuclear translocation of NF-κB and the subsequent induction of NF-κB response genes and release of NF-κB-dependent interferons and cytokines, including tumor necrosis factors (TNF-α) and IL-1β (5,23,24). In the present study, we observed that the activation of NF-κB by combination treatment of rHBsAg and β2GPI was also evident on the NF-κB canonical pathway,

which depended on the inducible degradation of inhibitor IκB. IκBs are degraded by upstream kinase complex IκB kinases (IKKs), with the site 32/36 of serine of phosphorylation.

In conclusion, we revealed that HBsAg/β2GPI activated the NF-κB pathway through the TLR4/MyD88/IκBα axis (Fig. 6).

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

XJ acquired the data, obtained funding, conceived, designed and drafted the manuscript; ZT and PG studied the supervision; HX, XQ, YY, XD and LY collected the tissue samples and analysed the data and LZ designed the study and drafted the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All tissue samples were collected with the written informed consent of all patients and under the approval of the Ethics Committee of the Affiliated Hospital of Qingdao University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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