Role of STAT1 in the resistance of HBV to IFN-α

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Received February 26, 2020; Accepted February 17, 2021

DOI: 10.3892/etm.2021.9982

Abstract. The objective of the present study was to explore the mechanism of hepatitis B virus (HBV) resistance to interferon (IFN), and the role of signal transducer and activator of transcription 1 (STAT1). HepG2.2.15 cells were stimulated with a long-term (6-24 weeks) low-dose interferon (IFN)α-2b (10-70 IU/ml), so as to construct and screen a HepG2.2.15 cell model resistant to IFNα-2b. The changes of STAT1 and other proteins in the JAK-STAT signaling pathway, before and after drug resistance, were compared. The phosphorylation of STAT1 in HepG2.2.15 cells resistant to IFNα-2b was significantly decreased, and the expression level of 2',5'-oligoadenylate synthetase 1 was downregulated. Decreased phosphorylation of STAT1 in the JAK-STAT signaling pathway a contributor to the development of resistance to IFN-α in HBV.

Introduction

Hepatitis B virus (HBV) is a double-stranded DNA virus that causes acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma, and poses a significant threat to human health (1). It is estimated by the World Health Organization that 257 million people worldwide are chronically infected with HBV, and ~887,000 people die each year from complications associated with HBV/hepatic cell carcinoma (2). Interferon (IFN)-α is a cytokine with immunomodulatory and antiviral effects, which is one of the first choices for clinical treatment of chronic hepatitis B (CHB) (3,4). However, the long-term application of IFN-α is prone to result in drug resistance, which severely limits the effectiveness of IFN drugs (5).

IFN-α exerts anti-HBV effects primarily via the JAK-STAT signaling pathway (6,7). This begins with IFN-α binding the pattern recognition receptor on the cell membrane, which results in heterodimerization of IFNAR1/2, the subunit of the receptor. This subsequently changes the intracellular conformation of the receptor and activates janus kinase (JAK). JAK phosphorylates signal transducer and activator of transcription (STAT) in the cytoplasm, which then forms STAT1/2 heterodimers and is transported to the nucleus to interact with IFN-stimulated response elements (ISRE). This initiates the transcription of IFN-stimulated genes (ISGs), resulting in proteins which exert direct or indirect antiviral effects (8), such as double-stranded RNA-dependent protease (Protein Kinase r; RKR) and 2',5'-oligoadenylate synthetase 1 (OAS1), anti-myxovirus protein (myxovirus resistance protein A; MxA), ISG15. Subsequently, STAT1 can be dephosphorylated by tyrosine phosphatase in the nucleus and translocated to the cytoplasm for reuse.

JAKs are a group of intracellular non-receptor tyrosine kinases, comprising four family members: JAK1, JAK2, JAK3 and TYK2. They mediate the signal transduction of a variety of cytokines and growth factors, participate in immunity and inflammation, and regulate hematopoiesis cell development, differentiation, apoptosis and biological functions (9). In previous years, several JAK kinase inhibitors have been developed, either already on the market or still in clinical trial stages, such as Ruxolitibib an inhibitor of JAK1 and JAK2 (produced by Incyte Corporation) (10) or Tofacitinib, an inhibitor of JAK3 and JAK1 (produced by Pfizer, Inc.) (11). AG-490 is a JAK inhibitor that blocks the phosphorylation of tyrosine or serine at specific sites of JAK, thereby preventing the binding of JAK to STAT and activation of downstream signaling (12). Previous studies of cancer, smooth muscle and intimal proliferative diseases have revealed that AG-490 can inhibit STAT3 activity, thereby reducing the *in vitro* invasiveness of human pancreatic cancer cells (12,13).

STAT1 is a key molecule in the JAK-STAT signaling pathway (14), and the phosphorylation (15-17) and acetylation (18,19) of which have an important impact on the antiviral activity of IFN-α. Concurrently, resistance of the virus to IFN-α is associated with the phosphorylation of STAT1. For example, inhibition of STAT1 phosphorylation can make HCV resistant to IFN-α (15). Also, modification of STAT1 phosphorylation reduces the resistance of HCV to IFN-α (16). Nevertheless, whether the association between STAT1 phosphorylation in HBV is the same as that in HCV is yet to be elucidated. The present study aimed to investigate the resistance of HBV to IFN-α based on a resistant cell model. The HepG2.2.15 cell line is widely used as an *in vitro* cell

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Key words: signal transducer and activator of transcription 1, Hepatitis B virus, interferon α-2b, drug resistance, phosphorylation

model for anti-HBV research (20). Therefore, in the current study, the mechanism of resistance to IFNα-2b and its association with STAT1 was explored using the pre-established IFNα-2b-resistant HepG2.2.15/IFNα-2b cell model (21), so as to provide a basis for finding suitable interventions.

Materials and methods

Induction of drug-resistant cell lines (HepG2.2.15/IFNα-2b). HepG2.2.15 cells (purchased from Shanghai Bodong Biotechnology Co., Ltd.) were routinely cultured in 25-cm^2 culture flasks in Dulbecco's Modified Eagle Medium (DMEM, Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a humidified incubator at 5% CO_2. Cells were divided into 5 groups after counting, and were placed into culture solution, into which IFNα-2b (Anhui Anke Biotechnology Co., Ltd.; at final concentrations of 0, 10, 30, 50 and 70 IU/ml) and G_{418} (Beijing Solarbio Science & Technology Co., Ltd.) (380 mg/l), were added for induction, and cells were cultured at 37˚C for 3-4 days. The culture medium was replaced every 3 days, and the cells were digested and counted every 5-6 days, and were continuously cultured with IFNα-2b for 24 weeks. Then the supernatants and cells were harvested on the 3rd and 6th day, and stored at -80˚C for subsequent experiments. The levels of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) in the supernatant were detected using ELISAs. HBV DNA was tested using PCR-fluorescence probe, the expression of cellular protein was detected via western blotting and the relative expression of mRNA in cells was detected via reverse transcription-quantitative (RT-q)PCR.

Comparison of HepG2.2.15 and HepG2.2.15/IFNα-2b cells. HepG2.2.15 cells and HepG2.2.15/IFNα-2b cells were selected and separately divided into 5 groups, of which group 1 was considered as the control group. The other four groups were treated with the following: i) Group 1, IFNα-2b (1,000 IU/ml); group 2, IFNα-2b (1,000 IU/ml) + AG-490 (1 μM); group 3, IFNα-2b (1,000 IU/ml) + Trichostatin A(TSA) (30 nmol/ml); and iv) group 4, IFNα-2b (1,000 IU/ml) + AG-490 (1 μM) + TSA (30 nmol/ml), respectively, and were cultured at 37˚C for 24 h. Then the supernatant and cells were collected. The levels of HBsAg and HBeAg were detected using enzyme-linked immunosorbent assay (ELISA), and the Quantification Kits for HBsAg (cat. no. S10910113) and HBeAg (cat. no. 20123400740) were provided by Kehua Biotechnology Co., Ltd., China. The HBV DNA was detected by PCR-fluorescence probe, expressions of STAT1, p-STAT1 and OAS1 proteins were detected via western blotting, expression levels of mRNA in β-actin, STAT1, OAS1 and Ubiquitin-Specific Protease 18 (USP18) were detected by quantitative reverse transcription PCR (RT-qPCR).

Western blotting. Cells were lysed in lysis buffer (Beyotime Institute of Biotechnology) for 30 min at 4˚C, and centrifuged at 12,000 r/min for 10 min, then the supernatants (containing soluble proteins) were quantified using the UV-Vis spectrophotometer (SSI). The protein samples (30-50 μg/lane) were separated on 12% SDS polyacrylamide gels at 20 mA. The separated proteins were transferred to PVDF membrane (EMD Millipore; 1.5 h at 160 mA using a Bio-Rad mini-transblotter), and immunoblotted at 4˚C for 12 h with primary antibodies against STAT1 (1:1,000; Cell Signaling Technology, Inc.; cat. no. 9176), p-STAT1 (1:1,000; Cell Signaling Technology, Inc; cat. no. 9167) and OAS1 (1:1,000; Cell Signaling Technology, Inc.; cat. no. 14498), and incubated with horseradish-peroxidase-conjugated secondary antibody (1:10,000; Beijing Zhongshan Jinqiao Biological Technology Co., Ltd.) at room temperature for 1.5 h. Immunoreactive bands were visualized by enhanced chemiluminescence (Beyotime Institute of Biotechnology). The intensities of bands were normalized to β-actin (1:1,000; Beijing Zhongshan Jinqiao Biological Technology. Co., Ltd.) and analyzed using Image J software (Version 1.6.1 for Windows; National Institutes of Health).

Table I. Primers for reverse transcription-quantitative PCR.

Gene	Sequence (5'-3')
β-actin	Forward: GGGAAACTGTGGCGTGAT
	Reverse: AAAGGTGGAGGAGTGGGT
STAT1	Forward: GAACTTACCCAGAATGCC
	Reverse: CTTTCCACCACAAACGAG
OAS1	Forward: AGGTGGTAAAGGGTGGCT
	Reverse: TGCTTGACTAGGCGGATG
USP18	Forward: CAGACCCTGACAATCCACCT
	Reverse: AGCTCATACTGCCCTCCAGA

RT-qPCR. All steps were performed using a sterile technique in designated areas for RNA extraction and RT-qPCR. The specific primer sequences were synthesized by Shanghai Sangon Biotech Co., Ltd., and the sequences are displayed in Table I. Total RNA was extracted according to the instructions of total RNA small quantity kit (Axygen; Corning Inc.). The quantity and purity of RNA were measured by reading the absorbance at 260 and 280 nm with the UV-Vis spectrophotometer (SSI). According to the protocol of the PrimeScript RT Master Mix (Perfect Real Time; Takara Biotechnology Co., Ltd.), the reverse transcription and amplification were performed in a reaction volume of 20 μl, containing 1 μg total RNA. Samples were incubated at 95˚C for 30 sec, and then 40 cycles of amplification were conducted using the following program: 95˚C for 5 sec and 60˚C for 30 sec (22).

qPCR of HBV. HBV DNA was extracted from culture supernatants using a DNA Extraction kit (Da'an gene Co., Ltd, China, https://www.daangene.com), and qPCR was performed in a 96-well Real-Time PCR instrument (Applied Biosystems; Thermo Fisher Scientific, Inc.) using an HBV fluorescent quantitative PCR detection kit (Kehua Biotechnology Co., Ltd.). DNA template (100 ng, 2.0 μl) was added to the amplification tube containing the reaction mixture (PCR reaction solution A, 13.5 μl; PCR reaction solution B, 13.5 μl; PCR reaction solution C, 1.0 μl). After initial denaturation (94˚C for 2 min), and then 40 cycles of denaturation (94˚C for 10 sec) and annealing/extension (60˚C for 30 sec). In this

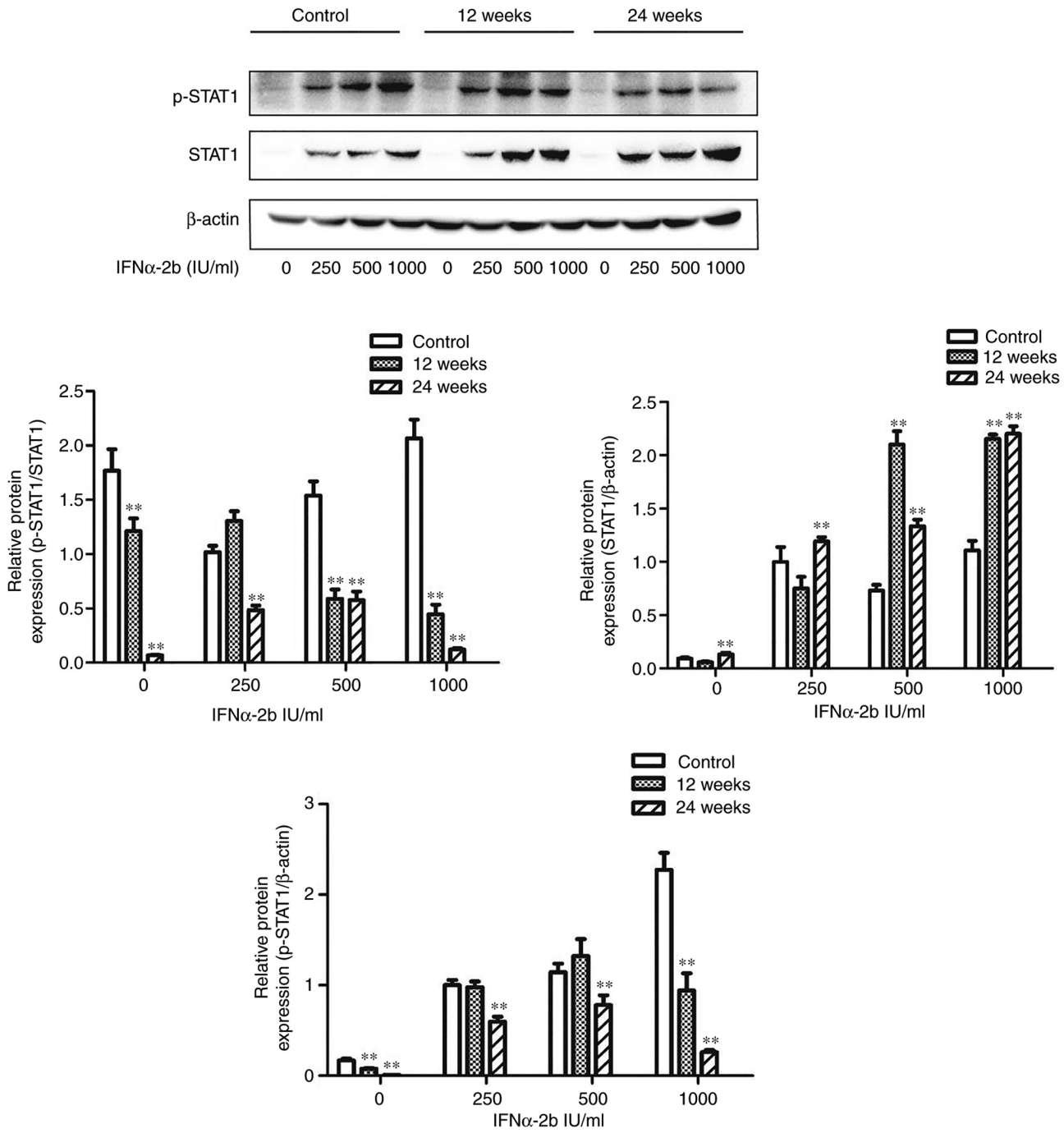

Figure 1. Expression levels of STAT1 and p-STAT1 in HepG2.2.15 cells after long-term stimulation with a low dose of IFNα-2b. The control group, 12-week stimulated group and 24-week stimulated group were treated with (0, 250, 500 and 1,000 IU/ml) IFNα-2b for 72 h. The protein expression levels of STAT1 and p-STAT1 were detected via western blotting. Data were expressed as mean ± SD. Error bars were calculated from three independent experiments. Experiments were performed least in triplicate. The P-values were obtained using the One-way ANOVA followed by Tukey's post hoc test. **P<0.01 vs. control. p-, phosphorylated.

assay, HBV DNA was quantified using a standard curve; the linear range was $1x10^3$-$1x10^8$ copies/ml (23). Each sample was run in duplicate.

Statistical analysis. Statistical analyses were performed using SPSS 17.0 software (SPSS, Inc.). Measurement data were expressed as mean ± SD, and were analyzed using the independent samples t-test. One-way ANOVA followed by Tukey's post hoc test was used for multiple pairwise comparisons, with an inspection level α=0.05. P<0.05 was considered to indicate a statistically significant difference, while P<0.01 was considered to indicate an extremely significant difference.

Results

Expression levels of STAT1 and p-STAT1 proteins in HepG2.2.15 cells after long-term stimulation with a low dose of IFNα-2b. Cells in the control group, 12-week stimulation group and 24-week stimulation group were treated with 0, 250, 500 and 1,000 IU/ml IFNα-2b for 72 h, and the expression

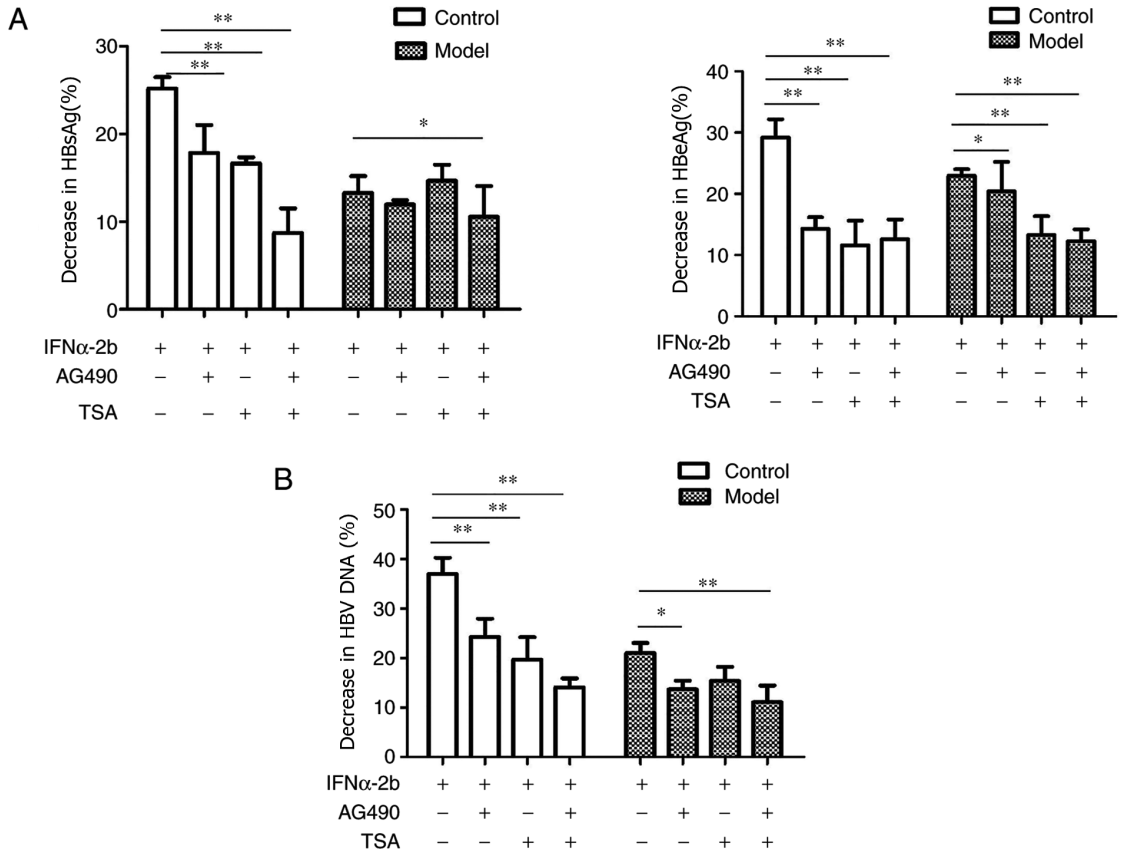

Figure 2. Effects of AG-490 and TSA on HBsAg, HBeAg and HBV DNA of IFNα-2b. The control and model groups were treated with 0, IFNα-2b (1,000 IU/ml), IFNα-2b (1,000 IU/ml) + AG-490 (1 μM), IFNα-2b (1,000 IU/ml) + TSA (30 nmol/ml), IFNα-2b (1,000 IU/ml) + AG-490 (1 μM) + TSA (30 nmol/ml) for 24 h. (A) The levels of HBsAg and HBeAg were detected using ELISA kits. (B) The amount of HBV DNA was detected by PCR-fluorescence probing. Data were expressed as the mean ± SD. Error bars were calculated from three independent experiments. Experiments were performed at least in triplicate. The P-values were obtained using one-way ANOVA followed by Tukey's post hoc test. *P<0.05; **P<0.01. TSA, Trichostatin A; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus.

levels of STAT1 and p-STAT1 proteins were examined using western blotting. The results revealed that compared with the control group, at all concentrations of IFNα-2b, the expression of p-STAT1 protein was downregulated and the expression of STAT1 protein was upregulated, and p-STAT1/STAT1 was significantly decreased in the 24-week stimulation group (P<0.05). In the 12-week stimulation group, the expression of p-STAT1 protein was downregulated when the concentration of IFNα-2b was 1,000 IU/ml, the expression of STAT1 protein was upregulated when the concentration of IFNα-2b was 500 and 1,000 IU/ml, and p-STAT1/STAT1 was significantly decreased (P<0.05) (Fig. 1).

Effects of AG-490 and TSA on IFNα-2b inhibition to HBsAg, HBeAg and HBV DNA. In the control group, the inhibitory effect of IFNα-2b on HBsAg, HBeAg and HBV DNA was significantly decreased after administration of AG-490 (P<0.05). In the model group, after administration of AG-490, the anti-HBsAg effect of IFNα-2b was almost unchanged, while the inhibitory effect on HBeAg and HBV DNA was significant decreased (P<0.05). Notably, the decrease was smaller than that in the control group. Meanwhile, after treatment with TSA, the inhibitory effect of IFNα-2b on HBsAg, HBeAg and HBV DNA was significantly decreased in the control group, after administration of AG-490 (P<0.05). In the model group, after treatment with TSA, the anti-HBsAg and HBV DNA effects of IFNα-2b were almost unchanged, while the inhibitory effect on HBeAg was significantly decreased (P<0.05). Notably, after simultaneous treatment with AG-490 and TSA, the inhibition rates of HBsAg, HBeAg and HBV DNA were significantly decreased in both the control group and model group (P<0.05); however, the decrease was more notable in the control group (Fig. 2).

Effects of AG-490 and TSA on the expression levels of p-STAT1, STAT1 and OAS1 proteins. The expressions of p-STAT1, STAT1 and OAS1 proteins were significantly downregulated in the control group after administration of AG-490 (P<0.05). Similarly, the expressions of p-STAT1, STAT1 and OAS1 proteins were significantly downregulated in the model group after administration of AG-490 (P<0.05), and the decrease of p-STAT1 in the control group was more notable than that in the model group. After treatment with TSA, the expression levels of STAT1 and OAS1 proteins were significantly downregulated in both the control and model groups (P<0.05). After treatment with a combination of AG-490 and TSA, the expressions of p-STAT1, STAT1 and OAS1 were downregulated in the control group, while those of STAT1 and OAS1 were downregulated in the model group (P<0.05) (Fig. 3).

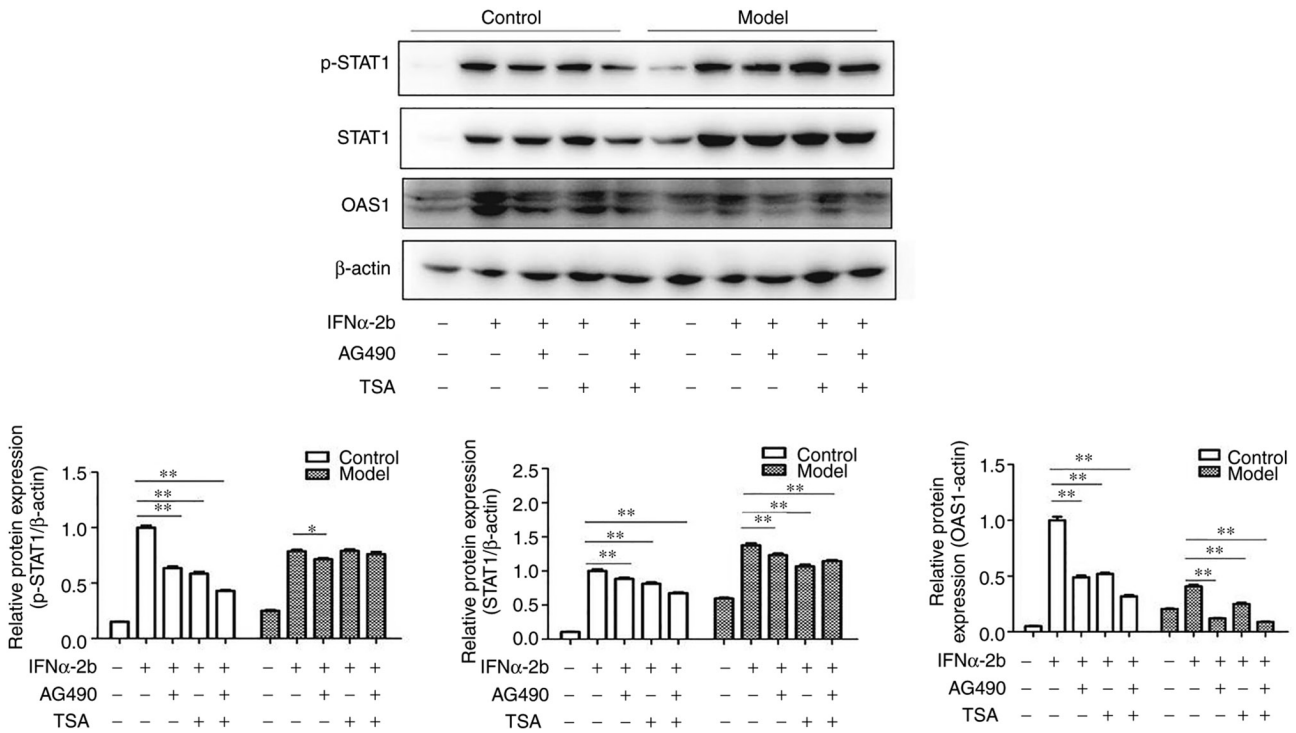

Figure 3. Effects of AG-490 and TSA on the expression levels of STAT1, p-STAT1 and OAS1 with IFNα-2b. The control and model groups were treated with 0, IFNα-2b (1,000 IU/ml), IFNα-2b (1,000 IU/ml) + AG-490 (1 μM), IFNα-2b (1,000 IU/ml) + TSA (30 nmol/ml), IFNα-2b (1,000 IU/ml) + AG-490(1 μM) + TSA (30 nmol/ml) for 24 h. Then, proteins were quantified via western blotting. Data were expressed as the mean ± SD. Error bars were calculated from three independent experiments. Samples were done at least in triplicate. The P-values were obtained using one-way ANOVA followed by Tukey's post hoc test. *P<0.05; **P<0.01. TSA, Trichostatin A; p-, phosphorylated-; OAS1, 2'-5'-oligoadenylate synthetase 1.

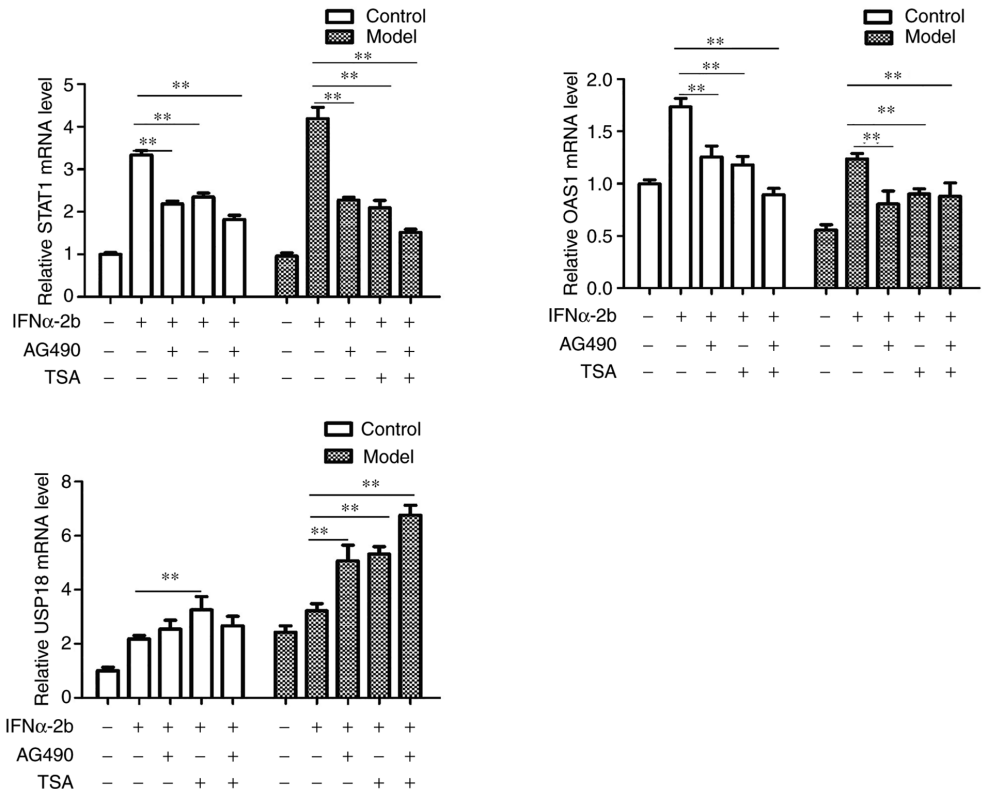

Figure 4. Effects of AG-490 and TSA on the mRNA expression level of STAT1, OAS1 and USP18. The control and model groups were treated with 0, IFNα-2b (1,000 IU/ml), IFNα-2b (1,000 IU/ml) + AG-490 (1 μM), IFNα-2b (1,000 IU/ml) + TSA (30 nmol/ml), IFNα-2b (1,000 IU/ml) + AG-490(1 μM) + TSA (30 nmol/ml) for 24 h. Then, the mRNA expression levels of STAT1, OAS1 and USP18 were quantified via reverse transcription-quantitative PCR. Data were expressed as the mean ± SD. Error bars were calculated from three independent experiments. Experiments were performed at least in triplicate. The P-values were obtained using one-way ANOVA followed by Tukey's post hoc test. **P<0.01. TSA, Trichostatin A; USP18, Ubiquitin-Specific Protease 18; OAS1, 2'-5'-oligoadenylate synthetase 1.

Effects of AG-490 and TSA on the expressions of STAT1, OAS1 and USP18 mRNA. The expression levels of STAT1 and OAS1 mRNA were downregulated in the control group after administration of AG-490 (P<0.05). Meanwhile, the expression levels of STAT1 and OAS1 mRNA were downregulated and the expression of USP18 mRNA was upregulated in the model group after administration of AG-490 (P<0.05). After treatment with TSA, the expression levels of STAT1 and OAS1 mRNA were downregulated, and the expression of USP18 mRNA was upregulated in both the control group and model group, where the difference was statistically significant (P<0.05). After administration of AG-490 and TSA, the expression levels of STAT1 and OAS1 mRNA were downregulated in the control and model groups (P<0.05), while the expression level of USP18 mRNA was significantly upregulated in the model group (P<0.05) (Fig. 4).

Discussion

HBV resistance is the primary factor limiting the clinical application of IFN-α as treatment for patients with HBV (24,25). Establishing an *in vitro* drug resistance cell model is one of the important means to study the drug resistance mechanism. Current methods for establishing drug-resistant cell lines primarily include the *in vitro* drug induction and drug-resistant gene transfection methods. The latter can be achieved by either low-concentration long-term maintenance or intermittent high-dose shock with gradually increased doses (26). The method of low-concentration long-term maintenance is a common method to screen successful and stable drug-resistant strains (27).

In the present study, HepG2.2.15 cell lines were continuously stimulated with low concentrations of IFNα-2b for 24 weeks, and it was revealed that the sensitivity of cells to IFNα-2b was significantly decreased, the inhibition rates of HBsAg, HBeAg and HBV DNA were decreased to varying degrees, and drug resistance was gradually developed. This suggests that IFN-α-resistant HBV cell models can also be constructed using low-dose continuous stimulation.

The method of low-concentration long-term maintenance is a common method to screen successful and stable drug-resistant strains, and was the method used in the present study. IFNα-2b concentrations of 10, 30, 50 and 70 IU/ml were used to treat HepG2.2.15 cells, with the aim of establishing a cell line resistant to IFN-α. We agree that IFNα-2b treatment will only inhibit the replication of the virus in the sensitive cells. But for IFNα-resistant cell model, IFN-α treatment exerts similar effects; however, this effect was significantly reduced compared with sensitive cells. Certainly, for both sensitive and resistant cells, the effect is not killing the cell but merely inhibiting DNA replication.

A number of studies have reported that IFN-α exerts an anti-HBV effect mainly through the JAK-STAT signaling pathway, which induces the expression of antiviral protein OAS1 and produces an antiviral effect (28-31). Phosphorylation of STAT1 serves an important role in this signaling pathway. After stimulation with IFNα-2b, the expression of p-STAT1 protein was decreased in HepG2.2.15/IFNα-2b cells, and the ratio of p-STAT1/total STAT1 was significantly lower than that of the control group, and the expression of antiviral protein OAS1 in the downstream was significantly decreased. Downregulation of STAT1 phosphorylation is part of the mechanism by which HBV is resistant to IFNα-2b. As the phosphorylation level of STAT1 is inhibited, it directly affects the expressions of associated genes and antiviral proteins in the downstream of JAK-STAT signaling pathway, which significantly weakens the antiviral effect of IFNα-2b on HBV. The present study also revealed that expression of USP18 mRNA was significantly increased at the transcriptional level. It has been reported that USP18 negatively regulates the JAK-STAT signaling pathway, which inhibits phosphorylation of STAT1 and prevents nuclear transport of phosphorylated STAT1 (32).

In the present study, HepG2.2.15/IFNα-2b cells and HepG2.2.15 cells were treated with AG-490 and TSA to investigate the changes of their anti-HBV activity after administration of IFNα-2b. It was revealed that the anti-HBV effect of IFNα-2b was weakened to varying degrees after treatment with AG-490 and TSA, which manifested as the decrease of inhibition rates of HBsAg, HBeAg and HBV DNA, downregulation of p-STAT1 and OAS1 protein expressions, as well as corresponding changes in mRNA expressions. Previous studies have reported that phosphorylation-acetylation equilibrium is an important switch regulating STAT1 signaling. Phosphorylation of STAT1 tyrosine and serine is a necessity for STAT1 to serve its role, while acetylation of STAT1 lysine can promote dephosphorylation and inactivation of STAT1 (18,33,34). Whether STAT1 serves its role depends on the balance of intracellular phosphorylation-acetylation. Therefore, the resistance of IFNα-2b to HepG2.2.15/IFNα-2b cells may be associated with the phosphorylation-acetylation balance, which in turn affects the role of STAT1 in the signaling pathway and ultimately changes the antiviral activity.

The present study primarily focused on STAT1 rather than STAT3. While STAT3 regulates growth, proliferation, differentiation and apoptosis in normal cells. Current studies in tumors have reported that inhibiting STAT3 not only directly acts on tumor cells to inhibit tumor growth and metastasis, but also regulates tumor-related immune cells. Investigation using clinical tumor samples confirmed that STAT3 promotes tumor cell proliferation and metastasis and has the function of inhibiting cell apoptosis. *In vivo* and *in vitro* experiments demonstrated that cancer cells highly express STAT3, and this is positively correlated with tumor metastasis (35). Previous studies have also reported that STAT3 inhibits the activity of T lymphocytes in the immune microenvironment and regulates the expression of PD-L1 to promote tumor immune escape (36). STAT3 has become one of the popular targets for anti-tumor and tumor immunotherapy (37). However, whether the expression of STAT3 and its downstream related proteins in the JAK-STAT signaling pathway are associated with the resistance of HBV to IFN-α is yet to be elucidated. Future studies should be designed with related experiments to investigate this issue.

In addition, the present study established an IFN-α-resistant HBV cell model and tested AG-490 and TSA on these resistant cell lines. Nevertheless, whether using a JAK agonist can improve the antiviral effect of IFN-α warrants further investigation, and the result could be very meaningful to clinical

research. Therefore, we also have plan to will also design experiments.

In conclusion, a low level of STAT1 phosphorylation in the JAK-STAT signaling pathway is part of the mechanism underlying HBV resistance to IFN-α, which may be closely associated with high expression of USP18, the balance of STAT1 phosphorylation and acetylation. The specific mechanism underlying HBV resistance to IFN-α remains to be elucidated, for which in-depth research will be continued.

Acknowledgements

The authors would like to thank Professor Lihua Song (Anhui Academic Institute of Biology), Professor Luyan Fan (Department of Pharmacy, The Third Affiliated Hospital of Anhui Medical University) and Mr Kan Qin (Department of Pharmacy, The Third Affiliated Hospital of Anhui Medical University) for their support and leadership.

Funding

The present study was supported by grants from National Natural Science Foundation of China (grant no. 81470165).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BX designed this study. BT and JW performed experiments and statistical analysis. BX and BT wrote the manuscript. BX and BT confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Anhui Medical University (Anhui, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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