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Ubiquitin Specific Protease 18 Inhibits the Anti-Hepatitis B Virus Activity of Interferon-Alpha, but Does Not Inhibit Interferon-Lambda in HepG2.2.15 Cells

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Abstract Interferon alpha (IFN-α) is one of the most widely used anti-hepatitis B virus (HBV) medicine. It has been reported that ubiquitin specific protease 18 (USP18) inhibits the anti-HBV activity of IFN-α, but whether USP18 has an effect on the anti-HBV activity of IFN-λ was unclear. Here we aim to detect the anti-HBV effect of USP18 on IFN-λ in an *in vitro* model. HepG2.2.15 cells were transfected with two different plasmids: the empty vector (pEGFP-N1) and USP18 overexpressed plasmid (pEGFP-USP18), and then were treated with IFN-α and IFN-λ for 24 hours, respectively. Untreated groups served as the negative control. The expression of HBV markers, STAT1/pSTAT1 protein expression and interferon stimulated genes (ISGs) were tested on HepG2.2.15 by Western blotting, quantitative real-time PCR (RT-qPCR) and enzyme-linked immunosorbent assays (ELISA). The results showed that USP18 was successfully overexpressed in the overexpression group compared with both negative control and empty vector control groups ($P < 0.05$), which demonstrated the successful establishment of *vitro* models. In IFN-α treated groups, the expression of hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), hepatitis B core antigen (HBcAg) and HBV-DNA in overexpression groups were significantly higher than empty vectors ($P < 0.05$), while there was little difference in the IFN-λ treatment group. In addition, the expression of ISG15, MxA, IFIT1 and pSTAT1 of the empty vector were obviously higher than those in overexpression groups in the treatment group of IFN-α ($P < 0.05$), but there was also no significant difference in ISGs and pSTAT1 expression in IFN-λ treatment groups. USP18 inhibits the anti-HBV activity of IFN-α by suppressing the activation of the JAK/STAT signaling pathway. In contrast, USP18 has no effect on the anti-HBV activity of IFN-λ via the JAK/STAT signaling pathway.

Key words hepatitis B virus (HBV); ubiquitin specific proteinase 18 (USP18); interferon-alpha; interferon-lambda (IFNλ)

泛素特异性蛋白酶 18 抑制 IFNα 抗 HBV 活性但并不抑制 IFNλ 抗 HBV 的活性

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摘要 干扰素 α(IFN-α)是临床最常用的抗乙型肝炎病毒(HBV)药物之一。泛素特异性蛋白酶 18

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(USP18)被证实是抑制 IFN-α 抗 HBV 活性的因子,但 USP18 是否对干扰素 λ(IFN-λ)抗 HBV 有影响还尚未可知。为了明确 USP18 对 IFNλ 抗 HBV 活性的影响,本研究以 HepG2.2.15 细胞作为乙肝体外模型,采用脂质体转染法分别向细胞转染 pEGFP-USP18、PEGFP-N1 经 48 h,再经 IFN-α 和 IFN-λ 处理 24 h,分为阴性对照组、USP18 过表达 + IFN-α 组、空载组 + IFN-α 组、USP18 过表达 + IFN-λ 组、空载组 + IFN-λ 组。采用 Western 印迹、RT-qPCR 和 ELISA 检测各组的乙肝病毒标志物、STAT1/pSTAT1 和下游的干扰素刺激基因(*ISGs*)的表达。结果显示,与阴性对照组和空载组相比,USP18 蛋白在过表达组明显升高($P<0.05$),过表达细胞模型构建成功;在 IFN-α 处理的两组中,空载组中 HBsAg、HBeAg、HBcAg 及 HBV-DNA 的表达均低于 USP18 过表达组,差异有统计学意义($P<0.05$)。而 IFN-λ 处理组中,乙肝病毒标志物的差异不明显。在 IFN-α 处理组中,空载组的 ISG15、MxA、IFIT1 和 pSTAT1 表达均高于 USP18 过表达组,差异有统计学意义($P<0.05$),而在 IFN-λ 处理组中 ISGs 和 pSTAT1 的表达无明显差异。上述结果证实,USP18 可通过抑制 JAK/STAT 信号通路的激活来减弱 IFN-α 抗 HBV 的活性。研究还证实,IFN-λ 可发挥抗 HBV 的作用,USP18 不通过 JAK/STAT 信号通路抑制其抗 HBV 活性。

关键词　乙型肝炎病毒;泛素特异性蛋白酶 18;干扰素 α;干扰素 λ

中图分类号　R512.6

Chronic hepatitis B (CHB), a worldwide disease, can lead to cirrhosis, liver cancer and other end-stage liver diseases[1,2]. It has been reported that over one million HBV carriers die of HBV-associated liver diseases annually. There are two main antiviral therapies, nucleoside/nucleotide analogues (NAs) and interferon (IFN). NAs have a long course of treatment and it has very high rates of resistance when administered with long-term therapy. IFN has a short course and low rates of resistance. But its major drawback is high frequent side effects and the sustained virological response rate is low[2,3]. For these reasons, it is vital to find a way to improve the response rate of interferon in the treatment of CHB.

In previous work, we found a subset of differentially expressed genes in the liver tissues of treatment responders and non-responders by microarray gene expression profiling. Most of these are *ISGs* and immune-related genes. ISGs were significantly upregulated in the non-responsive group than responsive group[4]. Some studies also indicated that there is differential expression of *ISGs* related to the anti-HBV activity of IFN-α, and regulating these genes can modulate the antiviral effect of IFN-α[5-7].

USP18 is one of these ISGs. High expression of USP18 has been measured in liver, spleen, and thymus and low levels of USP18 expression has been found in bone marrow, adipose and lung tissues[8]. Its gene transcription promoter sequence is located in the interferon sequence response element (ISRE). The body can strongly express USP18 upon IFN induction. USP18 is not only involved in a variety of viral and bacterial infections, it also plays an important role in the development of immune diseases and tumors[9,10]. In addition, USP18 can inhibit the Janus kinase signal transducer and activator of transcription (JAK/STAT), thereby affecting the antiviral effect of interferons[11]. A study found that USP18 knockout rats increased sensitivity to IFN-α and the expression of anti-HBV proteins increased[12]. Our previous work identified that suppression of USP18 significantly increased the antiviral activity of IFN-α[13]. Therefore, USP18 may be an important predictor of IFN-α treatment efficacy.

IFN-λ is a new type of interferon discovered in 2003, including four subtypes of IFN-λ1, IFN-λ2, IFN-λ3 and IFN-λ4. IFN-λ can be incorporated into type III-interferon because its amino acid sequence and specificity recognition receptor are different from other interferons[14-16]. Similar to IFN-α, IFN-λ antiviral effect is also facilitated *via* the JAK-STAT signaling pathway, but its receptors belong to the class II cytokine receptor family (CRF2). It consists of the IFN-lambda receptor 1 (IL-28Ra) and IL-10 receptor beta (IL-10Rβ)[17,18]. A study found that IFN-λ could inhibit the replication of HBV and hepatitis C virus (HCV) in hepatocytes[19]. Although differences exist in the receptors of the two interferons, whether USP18 can affect the antiviral effect of IFN-λ has not been reported.

In this study, we established a USP18 overexpression model in HepG2.2.15 cells to detect the effec of USP18 on anti-HBV activity and the relationship with the JAK/STAT signaling pathway of IFN-α and IFN-λ.

1　Materials and Methods

1.1　Cell culture, construction and transfection of pEGFP-USP18 and pEGFP-N1 plasmid vectors

HepG2.2.15 cell line is derived from the human

hepatoblastoma HepG2 cell line, which was stably transfected with the HBV genome and can secrete HBV-DNA, HBsAg and HBeAg. It has been widely used to explore the virus-host interactions of chronic hepatitis B infection *in vitro*. Our previous study has confirmed that the expression level of USP18 in HepG2.2.15 cells was higher than that of in Huh7, HepG2 and SMMC-7721 cells[13]. Therefore, we selected HepG2.2.15 cells for subsequent experiments. HepG2.2.15 cells (preserved in the Chongqing Key Laboratory of Infectious Diseases and Parasitic Diseases) were cultured in high glucose DMEM (HYCLONE, Utah, USA) with 10% fetal bovine serum (HYCLONE) plus 100 μg/mL penicillin and streptomycin and 380 μg/mL G418 (Sigma-Aldrich, St. Louis, MO, USA) at 37℃ in an atmosphere of 5% CO_2[20]. pEGFP-USP18 plasmid vectors were designed according to the sequences available in GenBank (*NM*_017414.3). Empty plasmid vectors pEGFP-N1 were designed and synthesized as a positive control, and the recombinant vectors were transformed into *Escherichia coli* TOP10 cells and the plasmids were confirmed by DNA sequencing.

HepG2.2.15 cells were seeded into six-well plates at a density 2×10^5 cells/mL 24 hours prior to transduction. An appropriate amount of plasmid DNA (4 μg/well) and DNA transfection reagent (10 μL/well) were diluted into the DMEM (250 μL/well), respectively, and incubated for five minutes at room temperature. The two solutions were mixed thoroughly and stored at room temperature for 20 minutes, then added into HepG2.2.15 cells and incubated at 37°C in 5% CO_2 for 48 hours. HepG2.2.15 cells were harvested 48 hours after transduction for analyzing USP18 expression. After transfection with either pEGFP-USP18 and pEGFP-N1 plasmids for 48 hours, the cells were then treated with IFN-α (100 IU/mL, Roche, Basel, Switzerland) and IFN-λ (50 ng/mL, PEPROTECH, Rocky Hill, USA) for 24 hours, respectively. The cells were then harvested, and the supernatant was centrifuged and filtered and stored at −80℃.

Table 1 Primers for quantitative real-time PCR

Gene		Sequence (5′-3′)
β-Actin	Forward	CGTACAGGTCTTTGCGGATG
	Reverse	CCCTGGAGAAGAGCTACGAG
*USP*18	Forward	CAGACCCTGACAATCCACCT
	Reverse	AGCTCATACTGCCCTCCAGA
*STAT*1	Forward	TGTTTCATTTGCCACCATCCG
	Reverse	ATCCTGAAGATTACGCTTGCT
*ISG*15	Forward	CGCAGATCACCCAGAAGATT
	Reverse	GCCCTTGTTATTCCTCACCA
*IFIT*1	Forward	GCAGCCAAGTTTTACCGAAG
	Reverse	GCCCTATCTGGTGATGCAGT
MxA	Forward	GTTTACCAGACTCCGACACGA
	Reverse	TTCCAGTGCCTTGATTTGCT

1.2 Quantitative real-time PCR analysis

Total RNA was extracted from HepG2.2.15 cells using RNAiso Plus according to the manufacturer's instructions (TaKaRa, Dalian, China). Total RNA was reverse-transcribed to cDNA using all-in-one cDNA Synthesis Super Mix (Bimake, Houston, TX, USA) according to the manufacturer's protocol. Quantitative real-time PCR was performed using a Light Cycler system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using the SYBR Green qPCR Master Mix (Bimake). The forward and reverse primers were shown in Table 1. HBV DNA in the supernatant was extracted by standard methods, using the HBV PCR Fluorescence Quantitative Detection Kit (Da-an, Guangzhou, China) according to the manufacturer's instructions. The average threshold cycle values were used to determine the concentration of HBV-DNA.

1.3 Western blotting

Forty-eight hours after transfection with pEGFP-USP18 and pEGFP-N1, cells were collected and proteins were extracted with RIPA buffers for assessing the expression of USP18. Total proteins were separated with 10% SDS-PAGE and then transfected to polyvinylidene fluoride membranes. The membranes were blocked for 1 hour at room temperature (5% nonfat dry milk, in Tris-buffered saline containing 0.1% Tween-20). After that, the membranes were incubated with rabbit anti-USP18 (CST, Danvers, MA, USA) at 4℃ overnight. After washing with Tris-buffered saline with Tween-20 three times, the secondary antibody was incubated at room temperature

for 1 hour. Finally, the membranes were incubated with an electro chemiluminescent kit (Wanleibio, Shenyang, China).

1.4 Enzyme-linked immunosorbent assay (ELISA)

After treatment with IFN-α and IFN-λ respectively for 24 hours, the concentrations of HBsAg and HBeAg in supernatants were measured with ELISA (Auto bio, Zhengzhou, China) according to the manufacturer's protocols. *A* value was detected using the Lumo microplate luminometer (Auto bio Diagnostics Co., Ltd., Zhengzhou, China).

1.5 Statistical analysis

All data are presented as means ± standard deviation. Statistical analysis was carried out using the SPSS 21.0 statistical software package (IBM, Armonk, NY, USA). The significance of each group was verified using one-way analysis of variance or Student's *t*-test. Statistically significant differences were considered when P value < 0.05.

2 Results

2.1 USP18 was successfully overexpressed in HepG2.2.15 cells

In order to study the biological role of USP18, we constructed an empty vector and a negative control group. HepG2.2.15 cells were transduced with different plasmid vectors, respectively. After incubation for 48 hours, the expression of green fluorescent protein (GFP) was observed under the fluorescence microscopy. The transfection efficiency was >70% (Fig. 1 A). Western blot results indicated that the protein levels of USP18 in the pEGFP-USP18 plasmids and cell lines were significantly increased compared to that of the pEGFP-N1 plasmid and cell lines and negative control group (Fig. 1), indicating that USP18 was successfully overexpressed.

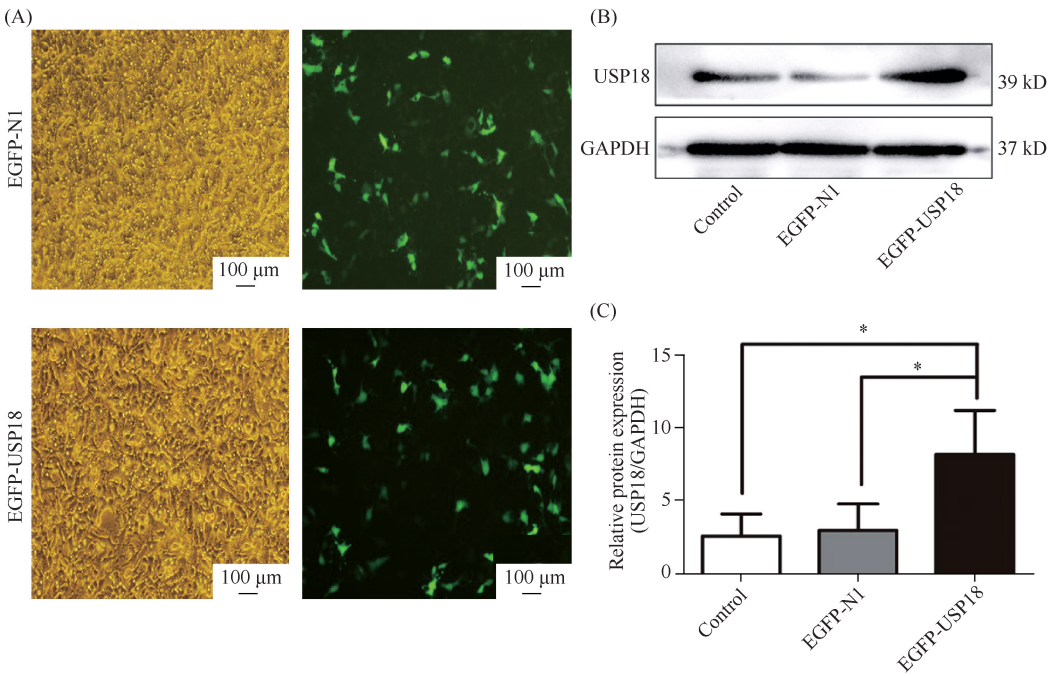

Fig. 1 The protein level of USP18 was significantly increased in pEGFP-USP18 plasmids and cell lines (A) Fluorescence images of HepG2.2.15 cells transfected for 48 hours with pEGFP-N1 and pEGFP-USP18. GFP expression was observed under light and fluorescence microscopy. (B) Cells were transfected with pEGFP-N1 and pEGFP-USP18 for 48 hours, respectively, and then were harvested for Western blotting to detect the protein levels of USP18. GAPDH was used as a loading control. (C) Graphic representation of relative protein expression of USP18 normalized to GAPDH. The results were presented as the means ± *SD* ($n=3$). Error bars indicate *SD*. $^{*}P < 0.05$

2.2 USP18 inhibited the anti-HBV replication ability of IFN-α but not effect IFN-λ

Supernatants were collected and quantified with the HBV PCR detection Kit and ELISA, while intracellular HBcAg was quantified with Western blotting (Fig. 2). In the treatment group of IFN-α, the levels of HBV-DNA, HBsAg and HBeAg, HBcAg in pEGFP-N1 plasmid and cell lines were significantly lower than that in pEGFP-USP18 plasmids and cell lines, indicating that USP18 reduced the antiviral effects of IFN-α against HBV replication. By contrast, in the treatment group of IFN-λ, there was no significant difference in the secretion of HBV markers between pEGFP-N1 plasmids and cell lines and pEGFP-USP18 plasmids and cell lines.

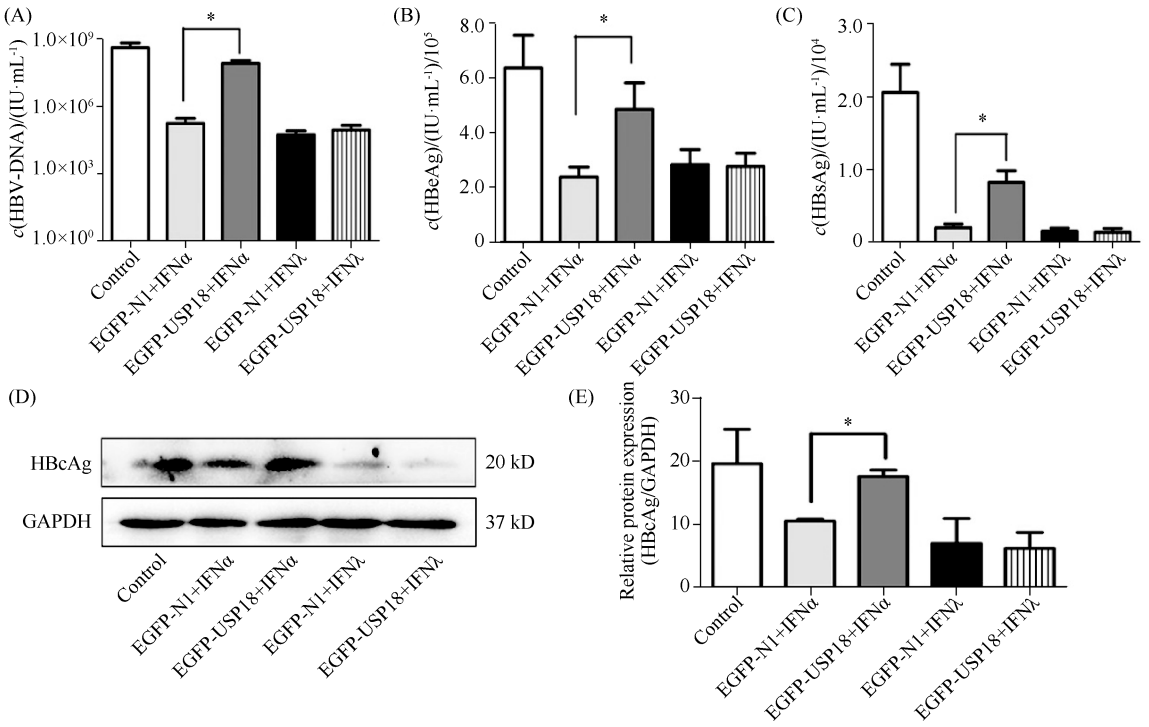

Fig. 2 The expression of HBV markers was significantly increased in pEGFP-USP18 plasmids and cell lines with the IFN-α treatment group but there was no difference on HBV replication in the IFN-λ group Cells and supernatant were collected to detect the expression of HBV markers after IFN-α and IFN-λ treatment in HepG2. 2. 15 cells after either pEGFP-USP18 or pEGFP-N1 transduction. (A) HBV-DNA was detected by the PCR fluorescence quantitative detection Kit. (B and C) HBeAg and HBsAg in the supernatants were detected by ELISA kits. (D) The protein level of intracellular HBcAg was quantified by Western blotting. GAPDH was used as a loading control. (E) Graphic representation of relative protein expression of HBcAg normalized to GAPDH. The results are presented as the means ± *SD* ($n = 3$). Error bars indicate *SD*. $^{*}P < 0.05$

2.3 USP18 attenuated the antiviral activity of IFN-α *via* the JAK/STAT signaling pathway

To clarify the effect of USP18 on the anti-HBV activity of IFN-α and IFN-λ, we examined the effect of USP18 on the classical IFN-induced JAK/STAT signaling pathway. In pEGFP-USP18 plasmid and cell lines, the mRNA and protein levels of ISGs, ISG15, MxA and IFIT1 were decreased compared to pEGFP-N1 plasmid and cell lines after treating with IFN-α, but there was no significant difference in the IFN-λ treatment group (Fig. 3).

In line with this, we evaluated the activation status of JAK/STAT signaling. In pEGFP-USP18 plasmid and cell lines, the protein expression of STAT1 phosphorylation (pSTAT1) was obviously lower than that in pEGFP-N1 plasmids and cell lines after treating with IFN-α (Fig. 4B). In the IFN-λ treatment group, the disparity between two groups was not obvious. No significant difference was observed for STAT1 expression in pEGFP-USP18 plasmids and cell lines and pEGFP-N1 plasmid and cell lines (Fig. 4 A). Taken together, we concluded that USP18 inhibits the anti-HBV activity of IFN-α by targeting the JAK/STAT signaling pathway in HepG2. 2. 15 cells. However, USP18 does not affect the anti-HBV activity of IFN-λ through the JAK-STAT signaling pathway.

3 Discussion

CHB is a serious threat to the health of the people in the world. There are two options for CHB patients, NAs and interferon. IFN-α belongs to type I IFN, it is the most widely used interferon in the clinic. Apart from antiviral effects, IFN-α also enhances cellular immunity. Thus, it plays an important role in the treatment of CHB[21]. In the course of IFN-α treatment, only 30% - 40% of CHB patients can achieve HBeAg seroconversion, which leaves about 60% of patients as non-responders to IFN-α[22-24]. Additional, because of the widely distributed receptors of IFN-α in the body, such as liver cells, bone marrow, hematopoietic cells and fibroblasts, IFN-α has a lot of side effects, including skin rash, muscle soreness and even bone myelosuppression, many patients are unable to bear the side effects and discontinuation of therapy[25]. Therefore, it is necessary to develop effective ways to solve those problems and have a better understanding of the mechanisms of IFN-α resistance.

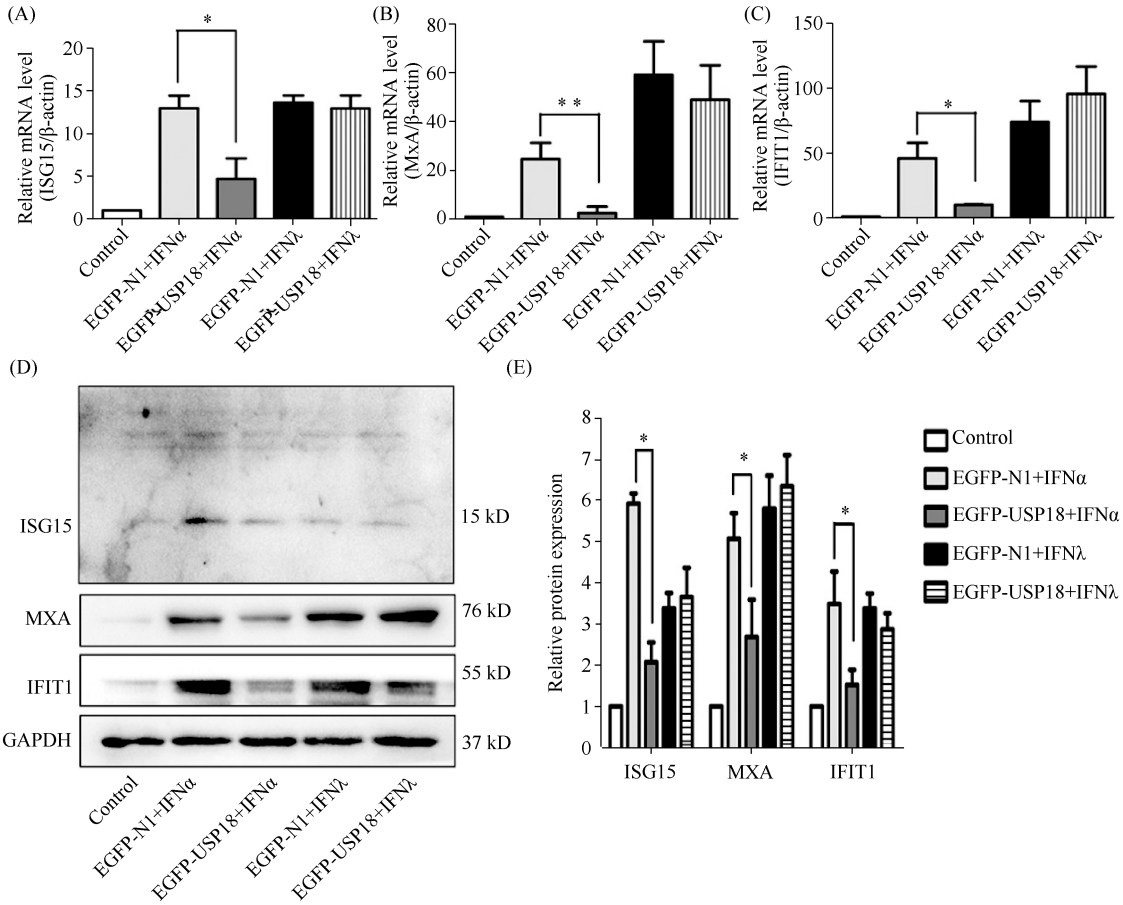

Fig. 3　The levels of ISGs were decreased in pEGFP-USP18 plasmids and cell lines of IFN-α treatment group but USP18 had no impact on ISGs in the IFN-λ treatment group　HepG2. 2. 15 cells were treated with IFN-α and IFN-λ for 24 hours after either pEGFP-USP18 or pEGFP-N1 transduction. (A-C) The mRNA levels of ISGs (ISG15, MxA and IFIT1) were quantified by RT-qPCR. β-actin was used as an internal control. (D) The protein levels of ISGs including ISG15, MxA and IFIT1 were tested by Western blotting. GAPDH was used as loading control. (E) Graphic representation of relative protein expression of ISG15, MxA and IFIT1 normalized to GAPDH. The results are presented as the means ± *SD* ($n = 3$). Error bars indicate *SD*. $^{*}P < 0.05$; $^{**}P < 0.01$

In our previous study, we found that the *USP*18 gene was up-regulated in the non-responders group of IFN-α therapy recipients identified by microarray[4]. It has two major functions. First, USP18 can specifically remove and hydrolyze ISG15 from ISGylation protein conjugates, thus hindering its biological effects[26]. Secondly, USP18 affects the antiviral effect of interferon by competitively binding interferon alpha/beta receptors (IFNAR) with JAK1, thereby blocking the activation of Janus kinase1(JAK1) and downstream signaling pathways[11]. Further studies identified that suppression of USP18 significantly increased the antiviral activity of IFN-α and its influence is associated with the JAK/ STAT signaling pathway[13]. This is similar to the results observed by Chen *et al*[27]. In summary, USP18 expression is an important prognostic marker indicating the success of IFN-α therapy.

IFN-λ, a newly discovered type III-IFN, is generated in various cells *in vivo*, including dendritic and respiratory tract epithelial cells[28, 29]. Its receptor mainly distributed in the epithelial rich tissues of the liver, gastrointestinal tract, while the hematopoietic system and nervous system hardly express it[30]. This restricted expression of the receptors suggests that its side effects may be less than that of IFN-α. Researchers found that IFN-λ has antiviral activity[31]. In addition, it has been proved that IFN-λ can inhibit the activity of human immunodeficiency virus (HIV), rotavirus, respiratory syncytial virus, Sendai virus and influenza virus[32-34]. Further study showed that although IFN-α and IFN-λ have different receptors, IFN-λ also triggers the phosphorylation of STAT1 by activating the JAK/STAT pathway. The combination of the interferon-stimulated gene factor 3 (ISGF3) transcription complex with ISRE promotes ISGs transcription to exert antiviral effects[35]. Studies have found that IFN-λ1 can induce pSTAT1 and inhibit

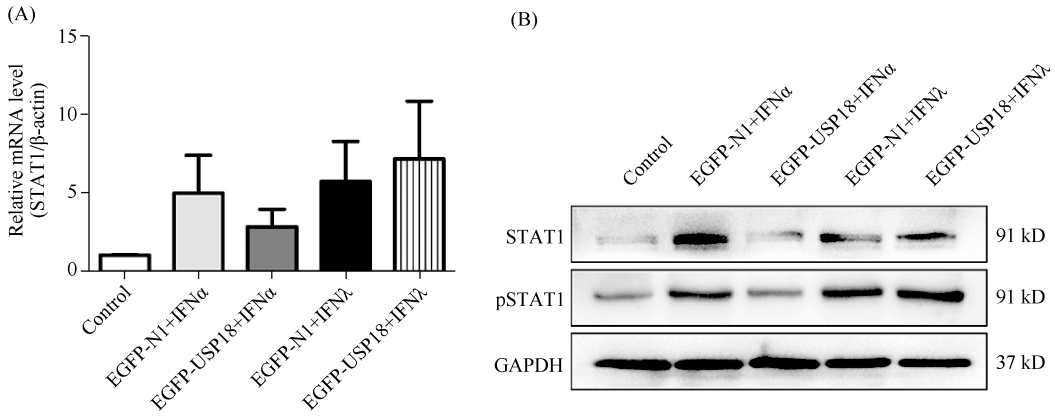

Fig. 4 STAT1 phosphorylation was suppressed by USP18 in the IFN-α treatment group but there was no change of phosphorylation of STAT1 in the IFN-λ treatment group HepG2. 2. 15 cells were transfected with pEGFP-USP18 or pEGFP-N1 for 48 hours and then treated with IFN-α and IFN-λ for another 24 hours, respectively. (A) RT-qPCR analysis was performed to analyze the level of STAT1 mRNA. β-actin was used as an internal control. The results are presented as the means ± *SD* (n = 3). Error bars indicate *SD*. (B) The protein levels of pSTAT1 and STAT1 were quantified by Western blotting. GAPDH was used as loading control

HCV more effectively than IFN-λ2 and IFN-λ3[36]. Previously, IFN-λ has entered a clinical trial for HCV treatment. It has been shown that IFN-λ has mild side effects, and its combination therapy with ribavirin can maintain sustained virological responses for patients with genotype 1 and genotype 4 HCV for 24 – 48 weeks. In conclusion, IFN-λ may play an important role in CHB treatment of in the future, whether USP18 has an effect on the antiviral capacity of IFN-λ is yet to be reported.

On the basis of the successful of cell model construction, to clarify the effect of USP18 on the two kinds of interferons against HBV replication, we detected the HBV markers in the supernatant and cells. The results showed that USP18 significantly inhibited the ability of IFN-α to resist HBV replication, but had no effect on the anti-HBV effect of IFN-λ (Fig. 2).

Interferon activates JAK by binding to its specific receptor, then phosphorylation of STAT, and binding with ISRE to translate into antiviral proteins, including ISG15, MxA and PKR. These antiviral proteins can regulate important proteins in the viral replication cycle, degrade viral RNA, interfere with viral replication, and thus play an important antiviral role. In order to figure out the reasons for the phenomenon shown in Fig. 2, we detected ISGs with Western blotting and RT-qPCR. The results indicated that USP18 significantly inhibited the antiviral activity of IFN-α, but had little effect on the antiviral activity of IFN-λ.

Therefore, we examined the expression of STAT1 and pSTAT1 in cells to clarify the activation status of JAK/STAT signaling pathway. The results showed that the pSTAT1 levels of pEGFP-USP18 plasmids and cell lines were lower than pEGFP-N1 plasmids and cell lines in the IFN-α treatment group, but the protein expression of pSTAT1 in IFN-λ treatment groups was almost no difference (Fig. 4). This suggests that USP18 decreased the phosphorylation of STAT1 induced by IFN-α, thereby affecting downstream ISGs transcription and ultimately reducing the anti-HBV activity of IFN-α. However, IFN-λ could stably induce phosphorylation of STAT1, indicating that USP18 had no effect on the antiviral replication and activity of IFN-λ. We hypothesized that the difference in the receptors between IFN-α and IFN-λ might be responsible for these phenomena. However, further studies are still needed to explore of IFN-λ.

In conclusion, USP18 inhibits the anti-HBV activity of IFN-α by suppressing the JAK/STAT signaling pathway. In contrast, USP18 has no effect on IFN-λ against HBV, indicating that USP18 can be considered as a negative regulator of IFN-α treatment. IFN-λ can be used as a complementary treatment measure to CHB.

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