The response of hepatitis B virus genotype to interferon is associated with a mutation in the interferon-stimulated response element

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Abstract
Genetic variation and genotype of Hepatitis B virus (HBV) are related to the efficiency of interferon alpha (IFN-α)-based antiviral therapy. However, the correlation of variation in interferon-stimulated response element (ISRE) and HBV genotype response to IFN-α therapy remains elusive.

Differences of ISRE between genotype B and C HBV were explored using the HBV sequences retrieved from GenBank, and further investigated by ISRE region cloning and sequencing from 60 clinical samples post-IFN-α therapy. Additionally, ISRE mutants were constructed and their relation to responsiveness of IFN-α was evaluated by real-time PCR and Southern blot analysis.

ISRE pattern between genotype B and C were found based on both clinical sample sequencing and full-length sequence alignment. The primary difference is the fourth base within the ISRE region, with T and C for genotype B and C, respectively. HBV with genotype C-type ISRE had a higher replicative capability as compared to HBV with genotype B-type ISRE after IFN-α treatment in hu7 cells.

Conclusion: Preference of ISRE between genotype B and C HBV are distinct. Single nucleotide difference (C to T) within the HBV ISRE region may link to the efficacy of IFN-α therapy to genotype B and C HBV. Therefore, this study provides a clue for the determination of IFN-α therapy response to HBV treatment.

Abbreviations: (PEG-) IFN-α = peglated-interferon alpha, ALT = alanine aminotransferase, BCP = basal core promoter, DSL = double strand linear HBV DNA, ELISA = enzyme-linked immunosorbent assay, HBeAg = hepatitis B e antigen, HBsAg = hepatitis B surface antigen, HBV = hepatitis B virus (HBV), ISG = IFN-stimulated gene, ISRE = interferon-stimulated response element, ISREM = dysfunctional mutation on ISRE, ISREMcp = extra BCP 1762/1764 double mutation beside ISREMtc, ISREMtc = ISRE mutation to genotype C, IU = international unit, PCR = polymerase chain reaction, qPCR = real-time qualitative PCR, RC = relaxed circular HBV DNA, Rs = response group, S/CO value = signal/cutoff, SS = single strand linear HBV DNA.

Keywords: hepatitis B virus, IFN-α therapy, interferon-stimulated response element, mutation

1. Introduction
Hepatitis B virus (HBV) is the causative agent of hepatitis B. More than 350 million people are chronically infected worldwide.[1,2] The genome of HBV is an enveloped partially double-stranded circular DNA molecule with 3.2 kb in length, encoding 4 overlapping genes (P, C, S, and X). Viral variation is an important characteristic of HBV, due to the lack of proofreading activity of polymerase during replication.[3] Genetic variations of HBV have been declared to be related to the severity of the liver disease, clinical outcomes, and efficacy of antiviral therapy.[1,2]

Interferon alpha (IFN-α) is the first approved and generally used drug for HBV infection, but its efficacy is far less than satisfactory. About one-third of HBeAg-positive CHB patients achieved HBeAg seroconversion after the IFN-α therapy,[4] and less efficacy was observed in HBeAg negative patients.[4] The efficacy was further compromised by accompanying side-effects. Lots of reports have been addressing on efficacy-related factors.[5–9] Among them, the virus itself is a focus. Data showed that genotype A and B are associated with a significantly higher sustained response to IFN-α than genotype C and D, respectively.[5,10–12] In vivo, associations of viral variants with the clinical outcome of therapy of pegylated (PEG) -IFN-α were confirmed.[13–16] And mutations within the HBV precore and/or basal core promoter (PC and/or BCP) region were reported to be related to the efficacy of IFN-α antiviral therapy in HBeAg-positive chronic hepatitis B patients.[6]
An IFN-stimulated response element (ISRE) is involved in the process of IFN-α action by transcriptional enhancement of IFN-stimulated genes (ISGs). ISRE has been identified in the HBV enhancer I/X gene promoter region\(^1\) and the functional role of ISRE was not detected in the initial exploration.\(^2\) Recently, by employing in vitro replication models and in vivo infection system, Belloni et al demonstrated that ISRE mediated IFN-α transcriptional repression of HBV transcription. ISRE mutation (TTTCACTTC to CTTTACCTTC) reduced HBV DNA through reduction of transcription.\(^3\) Recently, Liu reported that ISRE mutation to IFN-α enhanced the reduction of transcription.\(^4\)

A dysfunctional mutation on ISRE, ISREmt, ISRE mutation (ISREmtc), genotype B ISRE mutated to genotype C ISRE; while Pmtbcp primer pair was used to generate genotype C ISRE mutation (ISREmtbcp), extra BCP 1762/1764 double mutation beside ISREmtc.\(^5\) Another forward primer for obtaining ISREmtbcp is Pmtc forward (Fwd).

2.2. Patient grouping and sample collection

The patients were divided into the response group (Rs, \(n=24\)) and the non-response group (NRs, \(n=36\)) according to the follow-up outcomes. HBV DNA and Serum were checked at the initial and at the end of the therapy. In Rs, HBV DNA decreased by less than 1000 copies/ml, the ALT returned to the normal level or HBeAg seroconversion was achieved (loss of HBeAg with or without anti-HBe) at the end of therapy. In NRs, patients did not achieve the criteria mentioned as above.

2.3. DNA extraction and PCR amplification

Virus DNA was extracted from 200 μl of serum using a TIANamp virus DNA kit (Tiangen Biotech Co., Beijing, China) according to the manufacturer’s instructions. HBV ISRE region was amplified using primer pairs (forward 5’-GAGTCCCTTTATGCGGCTGT-3’, reverse 5’-GTAACCAAGGACGTCCCC-GC-3’), which were synthesized by Beijing Genomics Institute (Beijing, China). PCR was performed using Premix PrimeSTAR HS (Takara, Dalian, China). The amplification reactions consisted of an initial denaturation step of 5 min at 98°C, followed by 35 cycles of 10 s at 98°C, 15 s at 60°C, 40 s at 72°C. The reaction was finalized with a 10-minutes extension at 72°C. The PCR products were sequenced by the Beijing Genomics Institute (Beijing, China). The HBV genotype from each sample was characterized using Genbank HBV genotyping tool (http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi).

2.4. Analysis of the pattern of ISRE variation among sequences from GenBank

The complete genome sequences of HBV were downloaded from GenBank using HBV as the search query on May 20, 2018. Only the 3215 bp genotype B and C HBV genome were included in the study. Seven hundred eighty four genotype B and 1287 genotype C HBV were aligned and analyzed with the program Mega Version 6 (Tamura et al. 2013). The HBV ISRE region (nt1089–1098) was extracted and characterized to identify the type of ISRE sequence.

2.5. Plasmid constructs

The original expression plasmid (PCH9–1.1x-HBV-B), containing a CMV-early-promoter-driven HBV genotype B full genome and a 0.1x genome fragment from genotype D, was constructed in our lab by a fragment substitution reaction.\(^6\) To construct different mutant plasmid, site-directed mutagenesis was used. The target mutant region was firstly amplified by mutant primers (Table 2). Pmntc primer pair was used to generate genotype C ISRE mutation (ISREmtc), Pmtbcp primer pair was used to induce an extra BCP 1762/1764 double mutation beside genotype C ISRE mutation (ISREmtbcp), while Pmt primers were used to induce mutation dysfunctional for ISRE involving in

### Table 1

| Feature            | Rs (N=24) | NRs (N=36) | P value |
|--------------------|-----------|------------|---------|
| Age (years)        | 33 (27–51)| 30 (19–49) | .064    |
| Gender (Male/Femal)| 14/10     | 25/11      | .277    |
| HBV DNA (log copies/mL) | 6.48 (5.00–7.70) | 7.068 (5.33–8.31) | .214 |
| ALT (ULN)          | 65.5 (39–113) | 57 (13–233) | .127    |
| AST (ULN)          | 63.5 (26–129) | 40.5 (20–106) | .075    |
| HBeAg (+/-)        | 18/6      | 32/4       | .154    |
| Genotype (B/C)     | 19/5      | 16/20      | .008    |

ALT = Alanine aminotransferase, AST = Aspartate aminotransferase, HBeAg = Hepatitis B virus e antigen, HBV = Hepatitis B virus.

### Table 2

| Primers | Direction (5’→3’) | Mutants |
|---------|------------------|---------|
| Pmntc   | Fwd, GDAAAAACAGGCTTTCATCTTGCTGCC | ISREmtc |
| Pmt    | Rev, GTTGCGGAAATGGAAAGCCCTTGT  | ISREmtc |
| Pmntbcp | Fwd, AGGGTTTTTCACTTGGGCAGACTTA | ISREmtbcp* |
| Pmntc   | Rev, GTCGCGAGAATGGAAAGCCCTTGT  | ISREmtbcp* |

*Another forward primer for obtaining ISREmtbcp is Pmtc forward (Fwd).

ISREmt, a dysfunctional mutation on ISRE. ISREmtc, genotype B ISRE mutated to genotype C ISRE; ISREmtbcp, extra BCP 1762/1764 double mutation beside ISREmtc.
the IFN-α signal cascade (ISREmt). The gel-purified PCR amplicons were used as a metaprimer and the original PCH9–1.1×HBV-B genotype plasmid as a template. The plasmids with specific mutations were constructed by routine molecular cloning. ISRE regions of constructed plasmids (ISREmt, ISREmtbp, ISREmt) were confirmed by sequencing.

2.6. Transfection of Huh7 Cells with HBV expression plasmid

Huh7 hepatoma cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C under 5% CO2 incubator. For transient transfection, Huh7 cells were seeded into 6-well plates 16 to 18 hours before transfection. When the cells were 60% to 70% confluent, the culture medium was replaced with fresh medium and 1.5 μg HBV constructs (PCH9–1.1×HBV-B, ISREmt, ISREmtbp, ISREmt) was transfected into each well using TransIT-LT1 reagent (Mirus Bio, Madison) according to the instructions. After transfection for 24 hours, IFN-α-1b (Sinogen, Shenzhen, China) at a final concentration of 1000 IU/ml was added directly to the culture medium every 24 hours. Four days after transfection, the culture medium was collected and the cells were harvested. All experiments were conducted in triplicate.

2.7. Serological testing of HBsAg and HBeAg by Enzyme-linked immunosorbent assay (ELISA)

The level of HBsAg and HBeAg secreted into the culture supernatants were tested using HBsAg and HBeAg ELISA kits (Ke Hua Co., Ltd., Shanghai) according to the manufacturer’s instructions. The absorbance value at the wavelength of 450nm was read with an Eks800 universal microplate reader (Bio-Tek Instruments, USA). As recommendation, a ratio of S/CO (Signal/ cutoff) value ≥2 was considered a positive response for HBsAg or HBeAg. The relative inhibitory rate was calculated using the following formula: inhibitory rate (%) = (S/COtransfectiongroup-S/COuntreatedgroup)/S/COtransfectiongroup × 100%. The average inhibitory rate was expressed a mean ± SD.

2.8. Quantification of HBV DNA copies by real-time quantitative PCR (qPCR)

HBV intracellular viral core DNA was extracted 4 days after transfection as previously reported.[22] Briefly, cells were rinsed twice with PBS and lysed with 500 μl of lysis buffer (10 mM Tris–HCl [pH 8.0], 1 mM EDTA, 1% NP-40, and 2% sucrose) at 37°C for 10 minutes. After centrifugation at 15,000 g for 4 minutes, the supernatant was transferred to a fresh microcentrifuge tube and 1 M MgCl2 was added to achieve a final Mg2+ concentration of 10 mM. DNase I (Promega, Oakand, USA) was added to digest the contaminating plasmid at a final concentration of 40U/ml. After 3 hours at 37°C, the reaction was terminated with 10 mM EDTA, and then 200 μl of 35% polyethylene glycol 8000 containing 1.5 M NaCl. After incubation for 1 hour on ice the viral nucleocapsids were pelleted by centrifugation at 13,000 g for 10 minutes at 4°C, and then digested overnight in 400 μl of buffer containing 1 mg/ml protease K (Takara, Dalian, China). The digestion mixture was extracted twice with phenol. The DNA was precipitated with ethanol and dissolved in TE buffer (10 mM Tris–HCl [pH 8.0], 1 mM EDTA). The HBV DNA was detected with real-time qPCR with SYBR Green using the PCH9–1.1×HBV-B at different concentrations (5 × 102, 5 × 103, 5 × 104, 5 × 105, 5 × 106 and 5 × 107 copies/μl) as a template to make the standard curve. The HBV-specific primers used for amplification were as follows: 5’- CTTAGTAGTGGTTATGTCAG-3’ (sense) and 5’-TCTATAAGCTGGAGTGAGCGA-3’ (anti-sense). The condition of amplification contained initial denaturation at 95°C for 2 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 50°C for 30 seconds and extension at 72°C for 20 seconds.

2.9. Southern blot analysis

HBV replicative intermediates were extracted from the cells as shown above and then separated on 0.8% agarose gels. The DNA samples were transferred to nylon membranes (Roche Diagnostics, Mannheim, Germany). After ultraviolet cross-linking and prehybridization, the membranes were hybridized with a digoxigenin-labeled full-length HBV DNA probe using the DIG DNA Labeling and Detection Kit (Roche, Mannheim, Germany) according to the manufacturer’s instructions. The signal was detected by exposure on an X-ray film and scanning using the Versa Doc Imaging system (Bio-Rad, Hercules, CA, USA).

The relative inhibitory rate of HBV DNA was calculated using the following formula: inhibitory rate (%)= (S/COtransfectiongroup-S/COuntreatedgroup)/S/COtransfectiongroup × 100%. The average inhibitory rate was expressed as mean±SD. Reduction of HBV DNA levels (Log 10 copies/ml) from baseline in Huh7 cells. Data of groups were compared by one-way ANOVA and further Tukey Least Significant Difference (LSD) to find out which groups differ.

2.10. Statistical analysis

The results of continuous variables were expressed as the median and interquartile ranges. Intergroup differences between the response group and the non-response group were examined by the Chi-Squared test, the Fisher exact test, or Mann–Whitney test where appropriate. HBeAg and HBsAg levels were compared by one-way ANOVA. In all tests, P < .05 was considered statistically significant. All analysis was carried out using GraphPad Prism version 7 for Windows (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. ISRE patterns in HBV sequences from patients’ sera

648-bp region including ISRE was amplified and sequenced for the characterization of ISRE difference among patients. In general, 5 types of ISREs were detected (Table 3). The 2 most common ISRE variants were TTTTACTTTC (37 cases, 61.67%; 16 responders and 21 non-responders) and TTTTACCCT (19 cases, 31.67%; 4 responders and 15 non-responders). The 83% of ISRE variant TTTTACTTTC sequence was from genotype B and all of ISRE variant TTTTACCCT sequence was from genotype C. Thus, we named these 2 ISRE variants as genotype B- and C- ISRE, respectively. Other 3 ISRE variants were TTTTTGTGTTT (1 case, 1.67%; 1 responder), TTTTACCC (2 case, 3.33%; 2 responders), and TTTTACTTTTT (1 case, 1.67%; 1 non-responder).

In total, there were 41 cases with the fourth base T in ISRE (37 cases of genotype B ISRE, 2 cases of TTTTACTTTC, 1 case of TTTTTGTGTTT, and 1 case of TTTTTGTGTTT) and 19 cases with
forth base C (all 19 cases of genotype C ISRE). In the cases with T-type ISRE, 20 cases were in the response group and 21 cases in the non-response group. While in the cases with C-type ISRE, 4 cases were in the response group and 15 cases in the non-response group. Compared to the C-type ISRE patients, the responders within the patients carrying the T-type ISRE showed statistically significant using Chi-Squared test (Chi-Squared value 4.16, \( P = .041 \)), demonstrating that the fourth base T in ISRE region, can predict the response to IFN treatment. In addition, we performed ANOVA to investigate the relationship of HBV genotype and ISRE pattern alone or together to IFN response. These results revealed that the ISRE pattern is significantly related to the response to interferon treatment \( (P = .003) \), but not the genotype \( (P = .424) \). And the ISRE pattern and genotype together significantly relate to the response of interferon treatment \( (P = .003) \).

### 3.3. ISRE pattern of genotype B and C HBV from GenBank database

Most cases with T-type ISRE (85.37%, 35 of 41) were from genotype B HBV, while C-type ISRE (73.68%, 14/19) cases were mainly from genotype C HBV based on the above clinical data. Next, the association of ISRE differences and genotype (B and C) was further investigated by bioinformatics analysis used sequences from GenBank. A set of 2071 Hepatitis B virus (HBV) sequences (784 genotype B and 1287 genotype C) were included in the analysis. There were 15 and 30 ISRE variants for genotype B and C, respectively. The ISRE preference differed significantly between the 2 genotypes \( (P = .000) \). 88.78% of the genotype B ISRE preferred an ISRE sequence of TTTTACTTTC, while 86.71% of the genotype C HBV was preferred the ISRE sequence of TTCACTTTC (Table 4). The ISRE preferences of HBV genotype B and C from published full-length HBV were shown as the same ISRE pattern detected in the study.

### 3.4. Expression and replication of HBV from different ISRE constructs in Huh7 cells

To investigate the relationship of genotype-specific ISRE variants and antiviral response to IFN-\( \alpha \) in vitro, 4 constructs were generated based on HBV-replicating pCH-9/3091 plasmid. Three different mutations within the ISRE region were induced into the plasmid (Fig. 1). ISRE\( \alpha \) represented genotype B-ISRE and wildtype BCP region in original pCH-9/3091 plasmid carrying 1.1 x genotype B HBV. ISRE\( \alpha \) represented the mutation in the ISRE region, which could abolish ISGF3 binding in EMSA assays,[18] but did not alter the HBV polymerase polypeptide sequence. ISRE\( \alpha \) represented the mutation in the ISRE region. ISRE\( \alpha \) had an additional mutation in BCP region (A1762T/G1764A) except ISRE\( \alpha \) as ISRE\( \alpha \) had generated based on HBV-replicating pCH-9/3091 plasmid. Three other mutated constructs (ISRE\( \alpha \), ISRE\( \alpha \), ISRE\( \alpha \)) were 49.67 ± 5.03%, 52.67 ± 7.77%, 51.33 ± 7.57%, respectively. This meant a significant reduction of the inhibitory rate of IFN-\( \alpha \) to HBV constructs carrying original genotype B ISRE (ISRE\( \alpha \)) was 73.6 ± 8.15%, while the inhibitory rate of other 3 HBV constructs (ISRE\( \alpha \), ISRE\( \alpha \), and ISRE\( \alpha \)) were 49.67 ± 5.03%, 52.67 ± 7.77%, 51.33 ± 7.57%, respectively. This meant a significant reduction of the inhibitory rate of IFN-\( \alpha \) to HBV after mutation within ISRE \( (P < .05) \) and the inhibitory rate of BCP mutation beyond ISRE region had no significant difference compared to the other 2 mutations \( (P > .05) \). This result indicated BCP 1762T/G1764A except ISRE\( \alpha \) mutation was detected at a rather low level (data not shown). The inhibitory rate of IFN-\( \alpha \) to HBV constructs carrying original genotype B ISRE (ISRE\( \alpha \)) was 73.6 ± 8.15%, while the inhibitory rate of other 3 HBV constructs (ISRE\( \alpha \), ISRE\( \alpha \), and ISRE\( \alpha \)) were 49.67 ± 5.03%, 52.67 ± 7.77%, 51.33 ± 7.57%, respectively. This meant a significant reduction of the inhibitory rate of IFN-\( \alpha \) to HBV after mutation within ISRE \( (P < .05) \) and the inhibitory rate of BCP mutation beyond ISRE region had no significant difference compared to the other 2 mutations \( (P > .05) \). This result indicated BCP 1762T/G1764A except ISRE\( \alpha \) mutation was detected at a rather low level (data not shown). The inhibitory rate of IFN-\( \alpha \) to HBV constructs carrying original genotype B ISRE (ISRE\( \alpha \)) was 73.6 ± 8.15%, while the inhibitory rate of other 3 HBV constructs (ISRE\( \alpha \), ISRE\( \alpha \), and ISRE\( \alpha \)) were 49.67 ± 5.03%, 52.67 ± 7.77%, 51.33 ± 7.57%, respectively. This meant a significant reduction of the inhibitory rate of IFN-\( \alpha \) to HBV after mutation within ISRE \( (P < .05) \) and the inhibitory rate of BCP mutation beyond ISRE region had no significant difference compared to the other 2 mutations \( (P > .05) \). This result indicated BCP 1762T/G1764A except ISRE\( \alpha \) mutation was detected at a rather low level (data not shown). The inhibitory rate of IFN-\( \alpha \) to HBV constructs carrying original genotype B ISRE (ISRE\( \alpha \)) was 73.6 ± 8.15%, while the inhibitory rate of other 3 HBV constructs (ISRE\( \alpha \), ISRE\( \alpha \), and ISRE\( \alpha \)) were 49.67 ± 5.03%, 52.67 ± 7.77%, 51.33 ± 7.57%, respectively. This meant a significant reduction of the inhibitory rate of IFN-\( \alpha \) to HBV after mutation within ISRE \( (P < .05) \) and the inhibitory rate of BCP mutation beyond ISRE region had no significant difference compared to the other 2 mutations \( (P > .05) \). This result indicated BCP 1762T/G1764A except ISRE\( \alpha \) mutation was detected at a rather low level (data not shown).

### Table 3

**ISRE pattern from sera of patients between response and non-response group**

| HBV ISRE     | No. | Response | Non-response |
|--------------|-----|----------|--------------|
| TTTTACTTTC   | 37  | 15       | 1            |
| TTTCACTTTC   | 19  | 0        | 4            |
| TTTTACGTTT   | 2   | 2        | 0            |
| TTTTACTGT    | 1   | 1        | 0            |
| TTTTACTTGT   | 1   | 1        | 0            |
| Total        | 60  | 19       | 20           |

\* The ISRE pattern is significantly related to the response to interferon treatment \( (P = .003) \), while the genotype is not significant \( (P = .424) \). The ISRE pattern and genotype together significantly relate to the response of interferon treatment \( (P = .003) \).

\( g_{TB} = \) genotype B, \( g_{TC} = \) genotype C, HBV = Hepatitis B virus, ISRE = interferon stimulated response element.

### Table 4

**ISRE preference of genotype B and C**

| ISRE type   | HBV-B | HBV-C |
|-------------|-------|-------|
| TTTTACTTTC  | 88.78%| 4.97% |
| TTTCACTTTC  | 4.72% | 86.71%|
| Other       | 6.50% | 8.31% |

\* The set of 2071 Hepatitis B virus (HBV) sequence (784 genotype B and 1287 genotype C) HBV are included in the analysis. There are 15 and 30 ISRE variants for genotype B and C, respectively. Above are shown the 2 most common ISREs. HBV-B, Genotype B hepatitis B virus; HBV-C, Genotype C hepatitis B virus.

\* B-type ISRE and C-type ISRE differs significantly between the 2 genotypes \( (P = .000) \).
enhancement of HBV replication could antagonize the inhibitory effect of IFN-α. The inhibition of HBV replication by IFN-α may happen at the transcriptional level, as reported previously.\(^{23,24}\)

The enhancement of HBV replication by ISRE mutation needs further investigation. All the results demonstrate that mutation of ISRE from genotype B-type to genotype C-type results in reduced inhibition of HBV replication by IFN-α and may relate to, at least partially, the response of genotype B and C to IFN-α in the clinical sample.

4. Discussion

In the study, ISRE variation among HBV genotype B and C was explored, and the association of ISRE variation with antiviral response of IFN-α was further evaluated. Results showed that a single nucleotide variation at the fourth base of ISRE for genotype B and C might explain, at least partially, the different response of IFN-α therapy among patients infected with HBV genotype B and C.

Investigation of the correlation of genetic variation to IFN-α antiviral efficacy is beneficial for us to predict the clinical outcome of antiviral drugs. IFN antiviral response is partially genotype-dependent. The PEG-IFN-α response rate of genotype B is significantly higher than that of genotype C in HBeAg-positive patients in the Asian population, within which genotype B and C HBV are dominating.\(^{1,25}\) Other variations such as mutations in PreC/BCP region and ISREs are also involved in the difference of the antiviral response. Interestingly, a single mutation within ISRE is reported to relate to the outcome of IFN-α therapy.\(^{26}\)

However, no HBV genotyping information within the patients was offered. ISRE was reported to be associated with IFN-α-mediated suppression of viral gene expression of HBV,\(^{17,19,20,26}\) probably through affecting its binding stability to ISGF3 complexes, as well as IRF3.\(^{27}\)

To address the effect of ISRE mutation on IFN-α, we mutated the genotype B ISRE and found the first and fourth base mutation of genotype B ISRE (ISREmt) reduced the inhibitory effect of IFN-α on HBV DNA replication in huh7 cells. The result is consistent with previous reports.\(^{26}\) Further, we changed genotype B ISRE to genotype C ISRE, and found this change reduced the viral replication under the treatment of IFN-α. This pattern coincides with the clinical findings that genotype B HBV has a relatively higher response of IFN-α therapy.\(^{1}\) Thus, the proportion of genotype B and C in the clinical sample may explain the difference of antiviral response of IFN-α therapy in our sample, as well as the previous report.\(^{20}\)

Dynamic changes of HBV sequences could affect the outcome of therapy during the IFN-α therapy.\(^{11,13}\) An earlier study concluded that the IFN-α resistance to HBV was related to genotype C with a higher frequency of BCP A1762T/G1764A mutation than genotype B.\(^{28}\) In vitro, PC and BCP mutations resulted in IFN-α resistance.\(^{29,30}\) However, clinical data reveal the inconsistency of PC and/or BCP mutations with IFN-α response.\(^{11,14,15}\) In this study, HBV with additional BCP A1762T/G1764A mutation showed no distinct difference in levels of antigen expression and HBV replicative intermediate in vitro. In the study, 1.1× HBV full-length expressing plasmid was employed, while 3.2kb linear HBV monomers were employed in the previous work.\(^{28}\) This difference may be an explanatory factor for the inconsistency.

Extensive genetic heterogeneity exists within individual HBV patient. One limitation of our study is that the plasmid represents only a single variation. An unbiased method for overall phenotype achievement might be transfected with all kinds of variants. It is theoretically feasible but unrealistic. Recently developed colony methods might provide an alternative way for HBV population phenotype analysis.\(^{31}\) Further study on genetic correlates of IFN-α resistance may consider genetic heterogeneity.

![Figure 1](image1.png)

**Figure 1.** Sequence around ISRE and BCP region of HBV 4 constructs used in the study. ISREwtb, wildtype genotype B ISRE; ISREmt, a dysfunctional mutation on ISRE; ISREmtc, genotype B ISRE mutated to genotype C ISRE; ISREmtbcp, extra BCP 1762/1764 double mutation beside ISREmtc.

![Figure 2](image2.png)

**Figure 2.** Inhibition on the expression of extracellular HBsAg and HBeAg of different constructs by IFN-α in Huh7 cells after transfection for 72h. The relative inhibitory rate was calculated as described in methods. The average inhibitory rate was expressed as mean±SD. The inhibitory rate of IFN-α to different constructs was around 50% and 25% for HBsAg (A) and HBeAg (B), respectively. No statistical significance in response to IFN-α treatment was shown.
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Figure 3. Expression of intracellular HBV replicative intermediates of different plasmid constructs with or without IFN-α treatment (1000 U/ml) in HuH7 cells. Reduction of HBV DNA level (Log10copies/ml) from baseline in HuH7 cell measured by qPCR (A), and southern blot (B). Results were shown as mean values from 3 independent experiments. * indicates a significant difference, P < .05. RC, relaxed circular HBV DNA; DSL, double strand linear HBV DNA; SS, single strand linear HBV DNA.
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