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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 huh7 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 = pegylated-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 virus surface antigen, HBV = hepatitis B virus (HBV), ISGs = IFN-stimulated gene, ISRE = interferon-stimulated response element, ISREmt = dysfunctional mutation on ISRE, ISREmtbcp = extra BCP 1762/1764 double mutation beside ISREmtc, ISREmtc = ISRE mutation to genotype C, IU = international unit, NRs = No response group, PC = precore promoter, 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]

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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[17] and the functional role of ISRE was not detected in the initial exploration.[18] 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 the reduction of transcription.[19] Recently, Liu reported that ISRE mutations may reflect the response of interferon treatment for hepatitis B patients.[20] However, the association of ISRE mutation to IFN-α efficacy remains elusive and deserves further investigation. In the study, the pattern of ISRE mutation and its association to efficacy were investigated.

2. Materials and methods

2.1. Patients

Sixty patients with chronic HBV infection admitted in the First and Second Affiliated Hospital of Chongqing Medical University between September 2009 and February 2014 were enrolled in this study. The inclusion criteria were as follow:

1. All the patients were adult;
2. Patients were all positive for HBsAg for more than 6 months;
3. Patients were HBsAb negative;
4. Patients had elevated serum median alanine aminotransferase (ALT) of $>2 \times$ ULN (Upper limit of normal) but $<10 \times$ ULN and detectable HBV DNA of $>100\,000$ copies/ml.

The exclusion criteria were that patients were positive for HAV, HCV, HDV or HIV and exhibited evidence of the preexistence of liver disease. The clinical data of included patients were shown in Table 1. For treatment, all the patients received subcutaneous injection of 5 MU recombinant IFN-α (IFN-α-1b, Sinogen, Kexing Biotech Co. Ltd. Shenzhen, China) 3 times per week for 24 weeks. All patients gave written informed consent and the study was approved by the ethics committee of the university.

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'-GAGTCCCTT-TATGCCGCTGT-3'; reverse 5'-GTAAACAAAGGACGTCCC-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 0.1× genome fragment from genotype D, was constructed in our lab by a fragment substitution reaction.[21,22] To construct different mutant plasmid, site-directed mutagenesis was used. The target mutant region was firstly amplified by mutant primers (Table 2). Pmtc 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

Clinical features of hepatitis B patients at baseline.

	The response group (N = 24)	The Non-response group (N = 36)	*P* value
Age (years)	33 (27–51)	30 (19–49)	.064
Gender (Male/Femal)	14/10	25/11	.377
HBV DNA (log copies/mL)	6.48 (5.00–7.70)	7.068 (5.33–8.31)	.214
ALT (IU/L)	65.5 (39–113)	57 (13–233)	.127
AST (IU/L)	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 for construction of different HBV constructs using PCH9–1.1×-B PCH9 plasmids.

Primers	Direction (5'-3')	Mutants
Pmtc	Fwd, GCAA*AACAGGCTTTCACTTTCTCGC*	ISREmtc
	Rev, GTTG*GCGAGAAAGTGAAAGCCTGTT*	
Pmt	Fwd, AGGCCTTTACCTTCTCGCCAACTTA	ISREmt
	Rev,GTTGGCGAGAAGGTAAAGGCCTGTT	
Pmtbcp	Rev,CTCCTAATACAAAGATCATTAACCTAATC	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 (ISREmtc, ISREmtbcp, 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% CO_2 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, ISREmtc, ISREmtbcp, 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 450 nm was read with an Elx800 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 (%) = $(S/CO_{transfectiongroup}\text{-}S/COIFN\text{-}_{treatedgroup})/S/CO_{transfectiongroup} \times 100\%$. 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 minuts, the supernatant was transferred to a fresh microcentrifuge tube and 1 M $MgCl_2$ was added to achieve a final Mg^{2+} concentration of 10 mM. DNase I (Promega, Oakland, 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 proteinase 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 (5×10^2, 5×10^3, 5×10^4, 5×10^5, 5×10^6 and 5×10^7 copies/μl) as a template to make the standard curve. The HBV-specific primers used for amplification were as follows: 5'- CCTAGTAGTCAGTTATGTCAAC-3' (sense) and 5'-TCTATAAGCTGGAGGAGTGCGA-3' (antisense). 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 (%) = $(S/CO_{transfectiogroup}\text{-}S/CO_{IFN\text{-}treatedgroup})/S/CO_{transfectiongroup} \times 100\%$. 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 TTTCACTTTC (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 TTTCACTTTC sequence was from genotype C. Thus, we named these 2 ISRE variants as genotype B- and C- ISRE, respectively. Other 3 ISRE variants were TTTTTCGTTT (1 case, 1.67%; 1 responder), TTTTACGTTC (2 case, 3.33%; 2 responders), and TTTTACTTTT (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 TTTTACGTTC, 1 case of TTTTACTTTT, and 1 case of TTTTTCGTTT) and 19 cases with

Table 3

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

HBV ISRE	No.	Response		Non-response	
		gtB	gtC	gtB	gtC
TTT***T***ACTTTC	37	**15**	**1**	**16**	**5**
TTT***C***ACTTTC	19	0	4	0	15
TTT***T***ACGTTC	2	2	0	0	0
TTT***T***TCGTTT	1	1	0	0	0
TTT***T***ACTTTT	1	1	0	0	0
Total	60	19	5	16	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 ($P=.003$).

gtB = genotype B, gtC = genotype C, HBV = Hepatitis B virus, ISRE = interferon stimulated response element.

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 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 analysis 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 ($P=.003$).

3.2. 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 GeneBank. A set of 2071 HBV full-length sequences consist of 784 genotype B and 1287 genotype C were included in the analysis. There were 15 and 30 ISRE variants for genotype B and genotype C, respectively. The ISRE preference differed significantly between the 2 genotypes ($P=.000$). 88.78% of the genotype B HBV preferred an ISRE sequence of TTTTACTTTC, while 86.71% of the genotype C HBV was with an ISRE sequence of TTTCACTTTC (Table 4). The ISRE preferences of HBV genotype B and C from published full-length HBV showed the same as the ISRE pattern detected in the study.

Table 4

ISRE preference of genotype B and C*.

ISRE type	HBV-B	HBV-C
T<u>TTT</u>ACTTTC†	88.78%	4.97%
T<u>TTC</u>ACTTTC†	4.72%	86.71%
Other	6.50%	8.31%

* A 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 differed significantly between the 2 genotypes ($P=.000$).

3.3. 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-α 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). ISREwtb represented genotype B-ISRE and wildtype BCP region in original pCH-9/3091 plasmid carrying 1.1× genotype B HBV. ISREmt 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. ISREmtc converted genotype B ISRE to genotype C ISRE with point mutation in ISRE region. ISREmtbcp had an additional mutation in BCP region (A1762T/G1764A) except ISRE mutation as ISREmtc. Huh7 cells were transiently transfected with the genotype B pCH-9/3091 construct (ISREwt) and 3 other mutated constructs (ISREmtc, ISREmt, ISREmtbcp).

HBsAg and HBeAg in supernatants were measured 4 days after transfection by ELISA. HBsAg was detected in all the samples transfected with different HBV ISRE constructs, while HBeAg was detected at a rather low level (data not shown). The inhibitory rate of IFN-α to different HBV ISRE constructs was around 50% and 25% for HBsAg and HBeAg, respectively (Fig. 2A and 2B). However, no statistical significance in response to IFN-α treatment between the 4 HBV constructs was shown.

Intracellular HBV replicative intermediates were measured by qPCR and southern blotting, and the results showed reduced HBV DNA levels in all constructs after IFN-α treatment was given. The inhibitory rate of IFN-α to HBV constructs carrying original genotype B ISRE (ISREwtb) was $73.6\pm8.15\%$, while the inhibitory rate of other 3 HBV constructs (ISREmt, ISREmtC, and ISREmtbcp) were $49.67\pm5.03\%$, $52.67\pm7.77\%$, $51.33\pm7.57\%$, respectively. This meant a significant reduction of the inhibitory rate of IFN-α 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 1762/1764 double mutations in the study showed no impact on the HBV response to IFN-α in vitro (Fig. 3A). HBV replication was further quantified by southern blotting analysis (Fig. 3B). Distinct reductions in HBV replicative intermediates were observed in transfected Huh7 cells treated by IFN-α with different HBV plasmids as compared to the IFN-α-treatment naïve group. In contrary with the IFN-α-treatment naïve group, the mutant HBV constructs showed a higher level of replication, indicating a single ISRE mutation at the forth base (from T to C) enhanced the HBV replication. The

```
            <------------------------------------------------------------->
            1090        1100            1100     1760      1770
ISREwtb     CAGGCTTTTACTTTCTCGCCA........TTAAAGGTCTTT
ISREmt      CAGGCCTTTACCTTCTCGCCA.......TTAAAGGTCTTT
ISREmtc     CAGGCTTTCACTTTCTCGCCA.......TTAAAGGTCTTT
ISREmtbcp   CAGGCTTTCACTTTCTCGCCA.......TTAATGATCTTT
                 [-----ISRE------]             [BCP]
```

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.

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.[1,4,13] 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.[6,11,14,15] 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 3.2 kb linear HBV monomers were employed in the previous work.[29] 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.

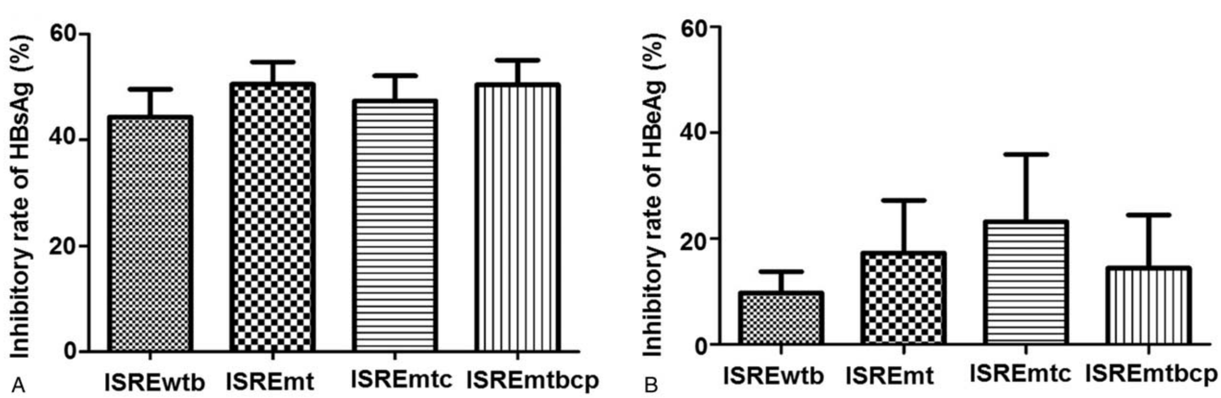

Figure 2. Inhibition on the expression of extracellular HBsAg and HBeAg of different constructs by IFN-α in Huh7 cells after transfection for 72 h. The relative inhibitory rate was calculated as described in methods. The average inhibitory rate was expressed a mean±SD. The inhibitory rate of IFN-α to 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.

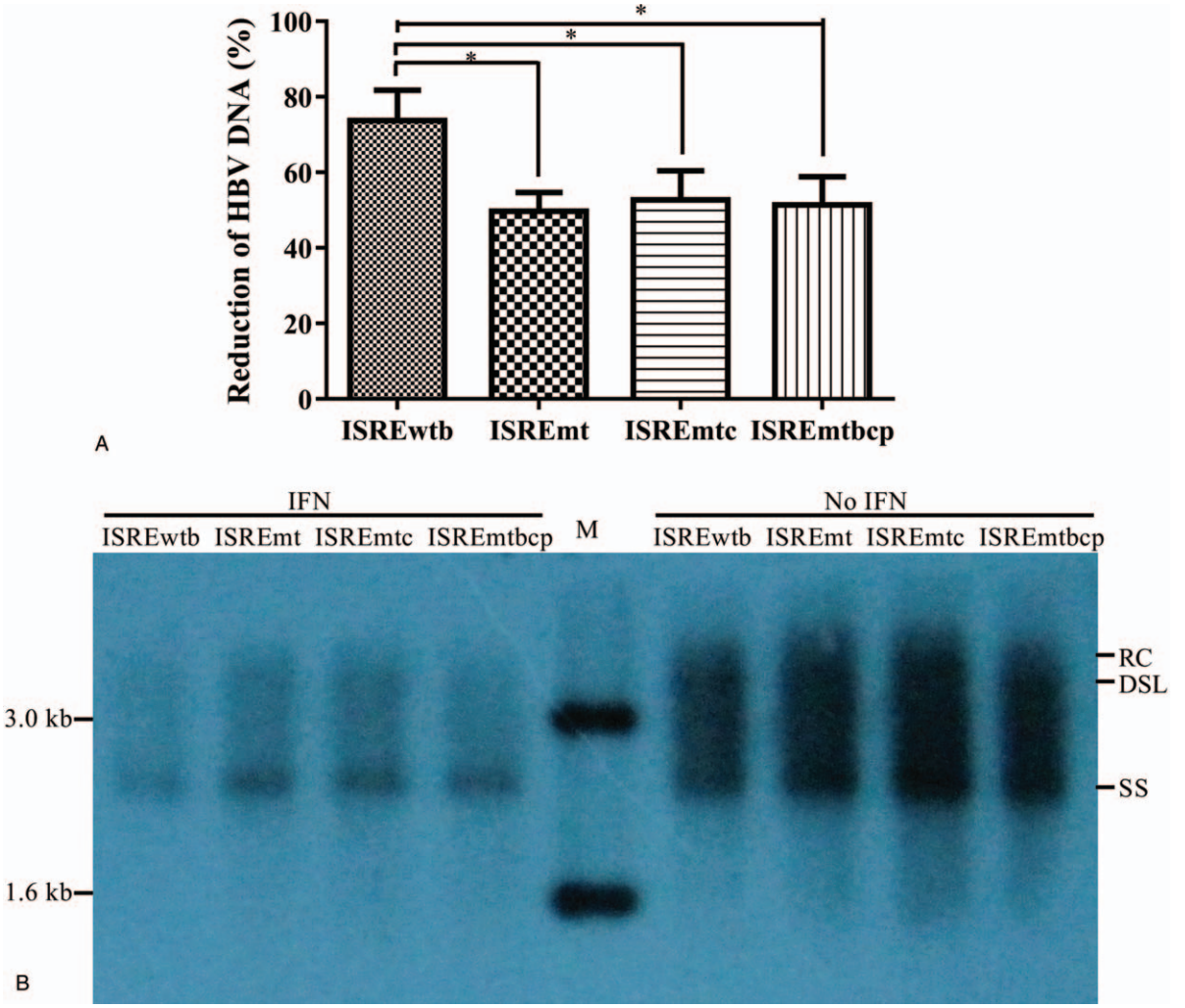

Figure 3. Expression of intracellular HBV replicative intermediates of different plasmid constructs with or without IFN-α treatment (1000 U/ml) in Huh7 cells. Reduction of HBV DNA level (Log10 copies/ml) from baseline in Huh7 cell measured by qPCR (A), and southern blot (B). Results were shown as mean 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.

Author contributions

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