Erratum to ‘Novel role of MHC class II transactivator in hepatitis B virus replication and viral counteraction’ [Clin Mol Hepatol 2024;30:539-560]

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In the original publication of the article, the Figures 1 to 3 were published in wrong order. The Figs. 1, 2, 3 should appear as in this correction.

The original article has been corrected as of 16th July 2024.

RESULTS

CIITA suppresses HBV replication in HCC cell lines and mediates the anti-HBV activity of IFN-γ

Initially, through new analysis of previously obtained gene expression profiles, we identified a correlation between HBV infection and CIITA gene expression. The specimens used for this analysis were obtained from HBV-negative normal liver donors (or patients who underwent liver resection for hemangioma) and donors with HBV-associated acute liver failure.26 While CIITA levels remained unchanged in the normal liver or non-HBV-related hemangioma patients, we observed a significant increase (between 5- to 15-fold increase) in the gene expression of CIITA in eight patients with acute liver failure caused by HBV (Fig. 1A). According to previous reports, CIITA expression is induced by IFN-γ, and IFN-γ, along with TNF-α, is associated with HBV viral clearance.27 Therefore, to determine whether CIITA expression is actually induced by IFN-γ in hepatocytes, HepG2, Huh7, and PHH cells were treated with different cytokines; CIITA induction levels were measured. Semi-quantitative RT-PCR results showed that CIITA expression was induced by IFN-γ in the two HCC cell lines as well as PHH (Fig. 1B-right). Interleukin-32γ (IL32) gene expression levels were measured as the positive control.28 In the tissues isolated from a PHH donor and Huh7 cells, CIITA mRNA levels were substantially increased, as demonstrated by real-time PCR (Fig. 1B-left). To explore the anti-HBV activity of CIITA, Huh7 cells were co-transfected with the HBV 1.2 (+) replicon and the myc-CIITA plasmid. The
Figure 1. CIITA inhibits HBV replication in hepatoma cells and mediates the anti-HBV activity of IFN-γ. (A) Comparison of the gene expression of CIITA in liver biopsies between normal and patients with chronic hepatitis B (CHB) (accession no. GSE14668); data were obtained from a previously published gene expression profiling analysis. (B) Primary human hepatocytes (PHH) were isolated from a 64-year-old female patient donor. PHH, HepG2, and Huh7 cells were seeded in 6-well plates for 24 h, then type I IFNs (IFN-α, IFN-β, and IFN-γ) and TNF-α were added at 1,000 units/mL and 50 ng/mL, respectively. After 48 h, cells were harvested and analyzed using semi-quantitative RT-PCR. IL32 gene expression levels were measured as the control. (C) Huh7 cells were transfected with HBV 1.2-mer (HBV 1.2 (+)), myc-CIITA and empty vector plasmids (pcDNA3.1) using lipofectamine 2000. After 72 h, cells and supernatants were harvested. (D) HBV replication was determined using Southern blot analysis (left side) and the expression levels of CIITA were determined using western blot analysis of the same samples. β-actin was used as the loading control. DNA was quantified, and the data was plotted (right side). (E) Relative HBeAg and HBsAg were measured using ELISA of the cell supernatant. (F) Huh7 cells were transfected with siCIITA using lipofectamine RNA iMAX. IFN-γ was added at 1,000 U/mL twice and total RNA was extracted with TRIzol. Cell lysates were subjected to semi-quantitative RT-PCR. GAPDH was used as the control for each lane. (G) Huh7 cells were transfected with HBV 1.2 (+) for 6 h, and then siRNAs were transfected using lipofectamine RNA iMAX. After 12 h, IFN-γ was added twice at 24-h intervals. After 60 h, cells and supernatants were harvested. (H) Southern blot analysis was conducted to confirm the presence of capsid-associated HBV DNA and the signals were quantified. (I) Huh7 supernatants were collected, and relative HBeAg and HBsAg levels were measured using ELISA. Data were obtained from three independent experiments (mean±SD). The statistical significance was assessed using the Student’s t-test: *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001. CIITA, class II transactivator; TNF-α, tumor necrosis factor-alpha; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; ELISA, enzyme-linked immunosorbent assay.

The experimental scheme is shown in Figure 1C. As shown in Figure 1D, CIITA reduced HBV replication in a dose-dependent manner as determined by Southern blotting. Similarly, the ELISA results showed that the levels of HBeAg and HBsAg secreted in the cell supernatant significantly decreased (Fig. 1E).

After evaluating the function of the siRNA by measuring CIITA RNA levels (Fig. 1F), we silenced the expression of CIITA using 20 nM of siCIITA (Fig. 1F) and treated the Huh7 cells with IFN-γ, as shown in the scheme in (Fig. 1G).
The co-transfection efficiency was examined by confocal microscopy (Supplementary Fig. 1). FN-γ-mediated reduction of HBV replication was reversed by CIITA depletion (Fig. 1H). Moreover, HBeAg and HBsAg levels in the cell supernatant were similarly restored (Fig. 1I). Collectively, these findings suggest that CIITA inhibits HBV replication and partially mediates IFN-γ-induced anti-HBV activity in HCC cells.

**CIITA exhibits antiviral activity in the HBV infection system**

Next, we validated the effects of CIITA in a biologically relevant HBV infection system by isolating PHHs from two liver tissue donors (Fig. 2A). In both donor samples, CIITA diminished HBV DNA replication levels in a dose-dependent manner, consistent with the decline in HBsAg and HBeAg levels (Fig. 2B–E). A similar experiment was conducted using HepG2-NTCP cells, which are a well-established infection model, to explore the anti-HBV effects of CIITA. In line with the results in PHHs, the intracellular cap-sid-associated HBV DNA and secreted antigen levels were reduced in a concentration-dependent manner (Fig. 2F–I). Collectively, these findings indicate that CIITA displays antiviral properties against HBV in an actual infection system.

**CIITA impairs HBV at the transcriptional level but does not exert regulatory control over cccDNA levels**

Considering that the conventional function of CIITA is as a major regulator of MHC class II transcription, we wondered whether CIITA inhibited the transcriptional activity of HBV. The experimental flowchart of the mechanistic study is summarized in Supplementary Figure 2. Following the ectopic expression or knockdown of CIITA, HBV transcripts were examined using Northern blotting. CIITA overexpression decreased pgRNA, preS1/S mRNA, and X mRNA levels by more than 50% (Fig. 3A, B). In addition, after silencing CIITA, IFN-γ failed to efficiently reduce HBV RNA levels (Fig. 3C, D), suggesting that CIITA partially mediates the anti-HBV activity of IFN-γ. These results imply that CIITA
Figure 2. HBV replication is restricted by CIITA in an HBV infection system. (A) PHH cells were isolated from two patient donors and seeded in 6-well plates. HBV infection was induced with 4% PEG and 2% DMSO. The plasmid expressing Myc-CIITA and the mock vector plasmid were transfected at 2 dpi and 5 dpi, respectively. Cells and the supernatant were harvested at 7 dpi. (B, D) HBV replication was determined using Southern blot analysis. CIITA protein levels were measured using Western blot analysis of the same sample. (C, E) HBeAg and HBsAg levels in the supernatant were measured using ELISA. (F) HepG2-NTCP cells were seeded in 6-well plates and were infected using the same protocol. Myc-CIITA was transfected into the cells at 4 dpi and cells were harvested three days later. (G) HBV intracellular-capsid-associated levels were determined using Southern blotting and (H) real-time PCR. (I) HBV-secreted antigen levels in the cell supernatants were quantified. dpi: days post-infection. HBV, hepatitis B virus; CIITA, class II transactivator; PHH, primary human hepatocytes; ELISA, enzyme-linked immunosorbent assay; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen. *P<0.05, **P<0.01.
Figure 3. CIITA suppresses HBV transcription but does not regulate HBV cccDNA levels. (A and C) Huh7 cells were co-transfected with HBV 1.2(+) myc-CIITA, and the mock vector control (pcDNA3.1) plasmid using lipofectamine RNAiMAX reagent. (C) Cells were treated with IFN-γ for 8 h before harvesting. HBV RNA was extracted and analyzed using northern blotting. The signals were normalized to the quantity of 18S and 28S ribosomal RNA. (B and D) Relative HBV RNA levels were quantified and plotted in a graph. Data were obtained from three independent experiments (mean±SD). The statistical significance of the differences was assessed using the Student’s t-test: *p<0.05; **p<0.01; ***p<0.001. (E) HepAD38 cells were seeded into a 6-well plate. Tetracycline (Tet) was removed from the media at the indicated time points. (F) HBV DNA was obtained using hirt DNA extraction. The samples shown in the left panel were electrophoresed immediately. (G) Samples were boiled at 88°C for 5 min and were incubated with EcoRI restriction enzymes at 37°C for 30 min prior to electrophoresis. (H and I) The upper panel presents the experimental scheme. (Bottom panel) HepAD38 cells were treated with 2 mM PFA between day 2 to day 6 following Tet removal. Cells were transfected with CIITA plasmid on the indicated days. Lysates from HepAD38 cells were harvested for hirt DNA extraction and Southern blotting. (J) One day after seeding, HepG2-NTCP cells were infected with HBV inoculum and were washed the next day. Plasmid transfection was performed 72 h before harvesting. (K) Samples were prepared for Southern blotting analysis of cccDNA levels. mtDNA served as the loading control. mtDNA: mitochondrial DNA. HBV, hepatitis B virus; CIITA, class II transactivator; PFA, phosphonoformic acid; cccDNA, covalently closed circular DNA.
induces HBV resistance by affecting viral gene expression and is involved in IFN-γ-mediated anti-HBV activity.

Since HBV RNA levels were significantly decreased in cells transfected with CIITA, we sought to determine whether the impact of CIITA extended to cccDNA alterations. After investigating the kinetics of cccDNA in HepAD38 cells (Fig. 3E–G), a suitable strategy for the transfection of the CIITA plasmid was employed (Fig. 3H and I, upper panel). The robust formation of cccDNA was detected at 7 d and reached a plateau at 9 d after Tet-off (Fig. 3F, G). Therefore, we harvested the cells 7 and 10 days after Tet-off to determine the impact of CIITA on cccDNA formation and destabilization. Furthermore, PFA was used to arrest HBV DNA synthesis, which allows for the time-dependent formation of cccDNA. Intriguingly, CIITA had no noticeable effect on cccDNA formation or destabilization (Fig. 3H and I bottom). We confirmed these results in an HBV infection model using HepG2-NTCP cells (Fig. 3J, K). Nonetheless, the levels of HBV cccDNA remained constant despite transient transfection with increasing doses of CIITA plasmid. These observations imply that CIITA may regulate HBV transcription rather than directly affect cccDNA abundance.