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HBV-Induced Increased N6 Methyladenosine Modification of PTEN RNA Affects Innate Immunity and Contributes to HCC

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BACKGROUND AND AIMS: Epitranscriptomic modification of RNA has emerged as the most prevalent form of regulation of gene expression that affects development, differentiation, metabolism, viral infections, and most notably cancer. We have previously shown that hepatitis B virus (HBV) transcripts are modified by N6 methyladenosine (m^6A) addition. HBV also affects m^6A modification of several host RNAs, including phosphatase and tensin homolog (PTEN), a well-known tumor suppressor. PTEN plays a critical role in antiviral innate immunity and the development of hepatocellular carcinoma (HCC). Reports have shown that PTEN controlled interferon regulatory factor 3 (IRF-3) nuclear localization by negative phosphorylation of IRF-3 at Ser97, and PTEN reduced carcinogenesis by inhibiting the phosphatidylinositol-3-kinase (PI3K)/AKT pathway.

APPROACH AND RESULTS: Here, we show that HBV significantly increases the m^6A modification of PTEN RNA, which contributes to its instability with a corresponding decrease in PTEN protein levels. This is reversed in cells in which the expression of m^6A methyltransferases is silenced. PTEN expression directly increases activated IRF-3 nuclear import and subsequent interferon synthesis. In the absence of PTEN, IRF-3 dephosphorylation at the Ser97 site is decreased and interferon synthesis is crippled. In chronic HBV patient biopsy samples, m^6A-modified PTEN mRNA levels were uniformly up-regulated with a concomitant decrease of PTEN mRNA levels. HBV gene expression also activated the PI3K/AKT pathway by regulating PTEN mRNA stability in HCC cell lines.

CONCLUSIONS: The m^6A epitranscriptomic regulation of PTEN by HBV affects innate immunity by inhibiting IRF-3 nuclear import and the development of HCC by activating the PI3K/AKT pathway. Our studies collectively provide new insights into the mechanisms of HBV-directed immune evasion and HBV-associated hepatocarcinogenesis through m^6A modification of the host PTEN mRNAs. (Hepatology 2020;0:1-15).

HBV infection leads to chronic hepatitis and carries the risk for the development of hepatocellular carcinoma (HCC).[1] HCC is the fourth leading cause of cancer-related deaths worldwide. Hepatitis B virus (HBV) is a member of *Hepadnaviridae* family. HBV genome codes for the

Abbreviations: bp, base pair; FTO, fat mass and obesity–associated protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HBx, hepatitis B x protein; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IFN, interferon; IP, immunoprecipitation; IRF-3, interferon regulatory factor 3; m^6A, N6 methyladenosine; MeRIP, methylated RNA immunoprecipitation; miRNA, microRNA; n.s, not significant by unpaired Student t test; pgRNA, pregenome RNA; PHH, primary human hepatocyte; PI3K, phosphatidylinositol-3-kinase; PIP_3, phosphatidylinositol-3, 4, 5-triphosphate; PIP2, phosphatidylinositol-2, 4, 5,-triphosphate; PTEN, phosphatase and tensin homolog; qRT-PCR, quantitative real-time PCR; siRNA, small interfering RNA; UTR, untranslated region; YTHDF, YTH-domain family protein.

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following proteins: hepatitis B surface antigen (HBsAg), hepatitis B e antigen, and hepatitis B core antigen (HBcAg) proteins, and polymerase (pol, a reverse transcriptase) and hepatitis B x protein (HBx). Although HBV is a DNA-containing virus, it replicates through an RNA intermediate termed pregenome RNA (pgRNA) to produce a relaxed circular DNA that ultimately transforms into a covalently closed (cccDNA) in the nucleus.[2] Although there is an effective vaccine available for HBV, there are about 350 million people infected with HBV worldwide with 600,000 deaths reported annually.[2] The antiviral treatment of HBV has limited efficacy. Once the drug is withdrawn, HBV infection can resume as cccDNA, which is unaffected by antivirals, maintains its presence, can resume the infectious process.[3] The inability to totally eliminate HBV infection poses a great challenge today. HBV can trigger changes in host gene-expression profile to optimize conditions for viral replication and increase long-term survival of the virus.[3] Chronic hepatitis B evolves into liver cirrhosis and HCC through the regulation of changes in the host gene expression. In 8%-20% of patients with chronic hepatitis B, the infection progresses to liver cirrhosis within 5 years, and 2%-8% of those with cirrhosis advance to HCC.[4] The exact mechanism(s) or trigger that leads to HBV-related HCC remains to be defined.

Phosphatase and tensin homolog (PTEN) is widely known as a tumor suppressor with reduced expressions in several tumors.[5] PTEN is a metabolic regulator as well as a negative regulator of cell growth signaling pathways. It is a dual phosphatase capable of both protein and lipid phosphatase activities.[5] PTEN regulates PI3K/AKT signaling pathways by dephosphorylating phosphatidylinositol-3, 4, 5-triphosphate (PIP_3). PTEN knockout in the transgenic mice model developed HCC.[6] In addition to its role as a tumor suppressor, PTEN regulates innate immune response activated by viral infections. PTEN induces interferon regulatory factor 3 (IRF-3) dephosphorylation at the Ser97 site, promoting IRF-3 nuclear translocation. The silencing of PTEN activity using small interfering RNAs (siRNAs) decreased p-IRF-3 nuclear import, which leads to reduce interferon (IFN) signaling pathway.[7]

Functional relationships between host gene expression and viral infections have important roles in modulating the viral life cycle. Viral infections induce alterations of cellular regulatory networks of host proteins. Altered host gene expression induced by viruses paves the way for successful establishment of the infectious process and subsequent disease pathogenesis.[8,9] Most notably, viruses affect cellular transcription, translation, and host gene expression. Several chemical modifications on cellular RNA are known to regulate RNA stability and turnover. Among the RNA chemical modifications, the N6-methyladenosine modification (m^6A) is the most prevalent internal mRNA modification of eukaryotic cells.[10] The recent development of detection methods with high sensitivity combined with high-throughput sequencing revealed informative profiles of m^6A RNA modifications in cells. Over 7,000 transcripts contain m^6A modifications that are typically enriched in the 3′-untranslated region (UTR) and near the stop codons of cellular mRNA. These modifications have been linked to various biological processes, including sex determination, stem cell differentiation, circadian clock, meiosis, stress response, and cancer.[10] The m^6A modification is a dynamic co-transcriptional process that is reversibly catalyzed

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by m^6A "writers," such as METTL3, METTL14 and WTAP, and "erasers," such as fat mass and obesity–associated protein (FTO) and ALKBH5.[10] The m^6A-modified mRNA directly interacts with the YTH-domain family proteins (YTHDF1, YTHDF2, and YTHDF3). Functionally, YTHDF1 enhances m^6A-modified mRNA translation, while YTHDF2 promotes target mRNA degradation. The YTHDF3 regulates degradation and translation of m^6A-modified mRNA through cooperation with YTHDF1 and 2.[11] RNA genomes of several RNA viruses, as well as the RNA transcripts of DNA viruses, have been reported to be m^6A modified.[12-17] m^6A modification of viral RNAs has been shown to affect various aspects of the viral life cycle and associated pathogenesis. m^6A forming enzymes (methyltransferases), METTL3/14, and reader proteins (YTHDFs) play important roles in regulating the life cycle of both DNA and RNA viruses.[12-17] While m^6A modification of viral transcripts has been identified, their effects on viral replication and translation are being characterized.

We previously reported that HBV transcripts are modified by m^6A and this modification played a dual role in regulation of the viral life cycle.[14,18] In this study, we demonstrate that HBV alters host gene expression by regulating m^6A modification of cognate RNAs. We found that m^6A modification of host RNA is either increased or decreased by HBV gene expression. Among those altered genes, we focused on PTEN mRNA, because PTEN has a critical role in innate immune response as well as liver carcinogenesis. We further demonstrate that HBV increases m^6A modification of PTEN mRNA, which leads to the destabilization of PTEN mRNA, and blocks the IFN signaling pathway. Reduction in PTEN expression may contribute to the development of HCC. Altogether, our results highlight a mechanism of HBV immune evasion and possible role in HBV-associated HCC through m^6A modification of the host PTEN mRNAs.

Experimental Procedures

PLASMIDS, ANTIBODIES, AND REAGENTS

HBV 1.3-mer was a kind gift from Dr. Wang-Shick Ryu (Yonsei University, Seoul, South Korea) and obtained from the Addgene (Cambridge, MA). FLAG-YTHDF1, 2, and 3 plasmids were a kind gift from Dr. Stacy M. Horner (Duke University Medical Center, Durham, NC).[15] Antibodies were obtained as follows: anti-HBcAg antibodies from Dr. Haitao Guo (University of Pittsburgh, Pittsburgh, PA),[19] anti preS2, anti-YTHDF3; anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibodies from Santa Cruz Biotechnology (Santa Cruz, CA); anti-METTL3 antibody from Proteintech Group (Rosemont, IL); anti-METTL14 antibody from Sigma-Aldrich (San Jose, CA); anti-FTO and anti-YTHDF2 antibodies from Abcam (Cambridge, MA); and anti-FLAG, anti-PTEN, anti-IRF-3, and anti-phospho-IRF-3 antibodies from Cell Signaling Technology (Danvers, MA).

CELL CULTURE AND TRANSFECTION

The Huh7 and HepG2 cells were obtained from ATCC (Manassas, VA). The HepAD38 cells were provided by Dr. C. Seeger (Philadelphia, PA).[20] The cells were maintained as described previously.[21] Primary human hepatocytes (PHHs) were obtained from Gibco (Waltham, MA) and cultured according to the manufacturer's protocol. HepG2 cells were transfected with plasmids and poly (I:C) using Mirus TransIT-LT1 and the Mirus TransIT-mRNA kit (Madison, WI) according to the manufacturer's protocol, respectively. siRNAs were transfected using Lipofectamine RNAiMAX reagent (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol.

VIRUS PRODUCTION AND CELL INFECTION

HBV particles were harvested from the culture medium of HepAD38 cells. The supernatants were incubated with 5% polyethylene glycol (PEG) 8000 overnight at 4°C, and then centrifuged at 4,000 rpm for 30 minutes at 4°C. Pellet was redissolved in a serum-free culture medium. PHHs were incubated for 24 hours with HBV particles, which were diluted in culture medium with 4% PEG 8000. After incubated with HBV particles, the cells were washed with culture medium. *In vitro*–transcribed full-length hepatitis C virus (HCV) RNA was electroporated into Huh7 cells. At 72 hours following electroporation,

the culture medium was passed through a 0.45-μm filter. For infection, Huh7 cells were incubated with filtered culture medium for 4 hours. Human immunodeficiency virus (HIV) particles were collected from pNL4.3 DNA–transfected HEK293T cells. Infectious HIV-1 particles were used for infection in Jurkat cells.

QUANTITATIVE REAL-TIME PCR

Total RNA was isolated using Trizol reagent (Invitrogen, Waltham, MA). The quantitative PCR was assessed with SsoAdvanced Universal SYBR Green supermix (Bio-Rad, Hercules, CA). Each mRNA expression level, normalized to GAPDH, was analyzed using the ΔΔCt method. The primers for quantitative real-time PCR (qRT-PCR) are provided in Supporting Table S1.

METHYLATED RNA IMMUNOPRECIPITATION SEQUENCING

Methylated RNA immunoprecipitation (MeRIP) sequencing was performed as previously described.[14] Libraries were sequenced to 1 × 50 base pair (bp) reads on the Illumina HiSeq2500 at the Weill Cornell Medicine Epigenomics Core Facility. Reads were aligned to a combined human genome reference sequence (hg38) using the Spliced Transcripts Alignment to a Reference method. Mean coverage was plotted for all three replicates using CovFuzze (https://github.com/al-mcintyre/covfuzze). The raw data discussed in this publication have been deposited in the National Center for Biological Information's Gene Expression Omnibus and are accessible through Gene Expression Omnibus Series accession number GSE114486. The MeRIP qRT-PCR followed the same protocol, except that total RNA was not fragmented. Eluted RNA was reverse-transcribed into complementary DNA and subjected to qRT-PCR.

WESTERN BLOTTING AND IMMUNOPRECIPITATION

Cell pellets were lysed in NP-40 lysis buffer (1% NP-40, 50 mM Tris-HCl, pH 8.0, 150 mM NaCl) supplemented with a protease inhibitor and a phosphatase inhibitor (Thermo Fisher Scientific). Purified cell lysates were incubated with anti-FLAG M2 Magnetic Beads (Sigma-Aldrich) for 2 hours on a rotator at 4°C. Immunoprecipitates or lysates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad). Membranes were incubated by using various primary antibodies.

SUBCELLULAR FRACTIONATION

Cytoplasmic and nuclear protein fractions were prepared with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) according to the manufacturer's protocol.

ETHICS STATEMENT

Human liver biopsy specimens were collected from St. Mary's Hospital, Catholic University, Seoul, South Korea, from 2 healthy volunteers, 3 anti-HBV-negative patients with HCC, and 2 anti-HBV-positive patients with HCC. Liver biopsy samples were collected according to the institutional review board (IRB) protocols. The IRB has approved this study as a whole. The IRB number of the human biopsie used in this study is KC12SIS0207.

STATISTICAL ANALYSIS

All results are representative of three independent experiments. For each result, error bars represent the ±SD from at least three independent experiments. The *P* value was calculated using a one-tailed unpaired Student *t* test.

Results

HBV INDUCES m^6A MODIFICATION OF PTEN 3′-UTR

To determine whether HBV regulates m^6A modification of host cellular RNA, we performed MeRIP assay with m^6A-specific antibody in HepG2 cells expressing HBV 1.3-mer plasmid. Immunoprecipitated RNAs were analyzed by high-throughput sequencing. We found that HBV transfection either increased or decreased m^6A modification of host cellular RNAs (Supporting Tables S2 and S3). The list represents a profile of 70 host cellular RNAs with either the higher or lower levels of m^6A modification induced by HBV.

Among the various host RNAs with m^6A methylation levels altered by HBV, we focused on PTEN mRNA, which displayed a 2-fold increase in m^6A modification.

Most m^6A modification is found within a consensus motif DRACH (where D = A, G, or U; R = G or A [A = denotes methylated adenosine]; C = C; and H = A, C, or U) and enriched in 3′-UTR of cellular mRNAs.[22] Because all DRACH motifs are not m^6A-methylated, we aligned m^6A reads to the PTEN 3′-UTR sequence to identify which DRACH motifs of the PTEN mRNA were m^6A-modified. We identified that PTEN 3′-UTR has eight different DRACH motifs, and m^6A peaks are enriched in 41-239 bp of 3′-UTR (Fig. 1A). Interestingly, HBV increased m^6A peaks in 300-330 bp and 521-550 bp of PTEN 3′-UTR (Fig. 1B) and as shown in Fig. 1C, m^6A modification of PTEN mRNA was significantly increased in HBV-expressing cells. We also checked the methylated PTEN mRNA levels in immunoprecipitated RNA by m^6A-specific antibody using the qRT-PCR method. In the MeRIP-qRT-PCR assay (Fig. 1D), m^6A-modified PTEN mRNA levels were increased by HBV. *CREBBP* (a cellular RNA known to contain m^6A) and *HPRT1* (a cellular RNA that does not contain m^6A) were used as positive and negative controls, respectively.[23]

To determine whether m^6A methylation of PTEN mRNA affects its RNA and protein levels, we depleted both METTL3 and METTL14 or FTO in HepG2 cells using siRNAs. The silencing of METTL3/14 elicited a 60% ± 10.90% increase in PTEN mRNA levels and a 41% ± 4.22% increase in PTEN protein levels. In contrast, the PTEN mRNA and protein levels were decreased by FTO siRNA (45% ± 9.57% mRNA decrease; 53% ± 8.34% protein decreases; Fig. 1E). These results suggest that m^6A modification of PTEN mRNA negatively regulates its RNA and protein expression levels.

The stability and translation of m^6A-modified cellular RNAs are regulated by the "reader proteins" (YTHDFs).[10] Therefore, we next investigated whether the m^6A-modified PTEN mRNA is recognized by YTHDFs. We performed immunoprecipitation (IP) experiments using cell lysates from the HepG2 cells in which FLAG-YTHDF1, FLAG-YTHDF2, or FLAG-YTHDF3 was ectopically expressed. Figure 1F shows that PTEN mRNAs were enriched in FLAG-YTHDF immunoprecipitated samples compared with control. *CREBBP* (positive) and *HPRT1* (negative) RNAs were analyzed as control (Fig. 1G). The m^6A-modified PTEN mRNA level recognized by YTHDF3 was a greater than 4-fold increase compared with YTHDF1 and YTHDF2. This result is supported by the finding that before YTHDF1 and YTHDF2 bind to m^6A-modified mRNAs, the target mRNAs are first recognized by YTHDF3.[11]

After determining that the m^6A machinery regulates levels of PTEN mRNA and protein, we next analyzed whether the PTEN RNA and protein levels could be similarly affected by the m^6A reader proteins. In HepG2 cells transiently expressing the YTHDF2 or YTHDF3, decreased PTEN mRNA (48% ± 9.26% and 55% ± 2.84% of control, respectively) and protein levels (46% ± 6.55% and 44% ± 14.64% of control, respectively) were observed (Fig. 1H), whereas knockdown of either YTHDF2 or YTHDF3 dramatically increased levels of PTEN mRNA (>3-fold and >2-fold increases compared with control, respectively) and protein (162% ± 12.55% and 154% ± 14.64% compared with control, respectively; Fig. 1I). Taken together, these results reveal that PTEN mRNA is m^6A-modified, and this m^6A modification is induced by HBV. In particular, m^6A modification of PTEN mRNA reduces its RNA and protein levels.

HBV DOWN-REGULATES PTEN mRNA STABILITY

Having found that HBV regulates m^6A modification of PTEN mRNA, we analyzed whether HBV affects PTEN mRNA stability. The m^6A-modified PTEN mRNA levels were dramatically induced in chronic HBV patient samples (339% ± 50.53%, 468% ± 60.33%, and 1,066% ± 164.25% increases in liver biopsies HBV-1, HBV-2, and HBV-3, respectively, from patients infected with HBV) and PHHs infected with HBV (195% ± 12.43%; Fig. 2A,C), while the total PTEN mRNA expression levels were reduced (47% ± 2.91%, 46% ± 9.59%, and 86% ± 9.59% decreases [Fig. 2B] and 48% ± 3.89% [Fig. 2D]). We confirmed these results using hepatoma cell lines transiently expressing HBV 1.3-mer, and HepAD38 cells stably expressing HBV. Decreased PTEN mRNA and protein levels were observed in HBV-expressing cells (Fig. 2E,F and Supporting Fig. S1). HBV-induced HepAD38 cells elicited a decrease of 55 ± 3.2 PTEN mRNA level

and a 57% ± 6.0% decrease in PTEN protein level. HBV 1.3-mer transfection decreased 54% ± 7.2% of PTEN mRNA and 52 ± 5.2 PTEN protein levels in HepG2 cells, and 58 ± 5.5% of PTEN mRNA and 57 ± 7.8 PTEN protein levels in Huh7 cells. We then determined the half-life of PTEN mRNA in HepG2 cells transfected with the HBV 1.3-mer following actinomycin D treatment. Fig. 1G shows

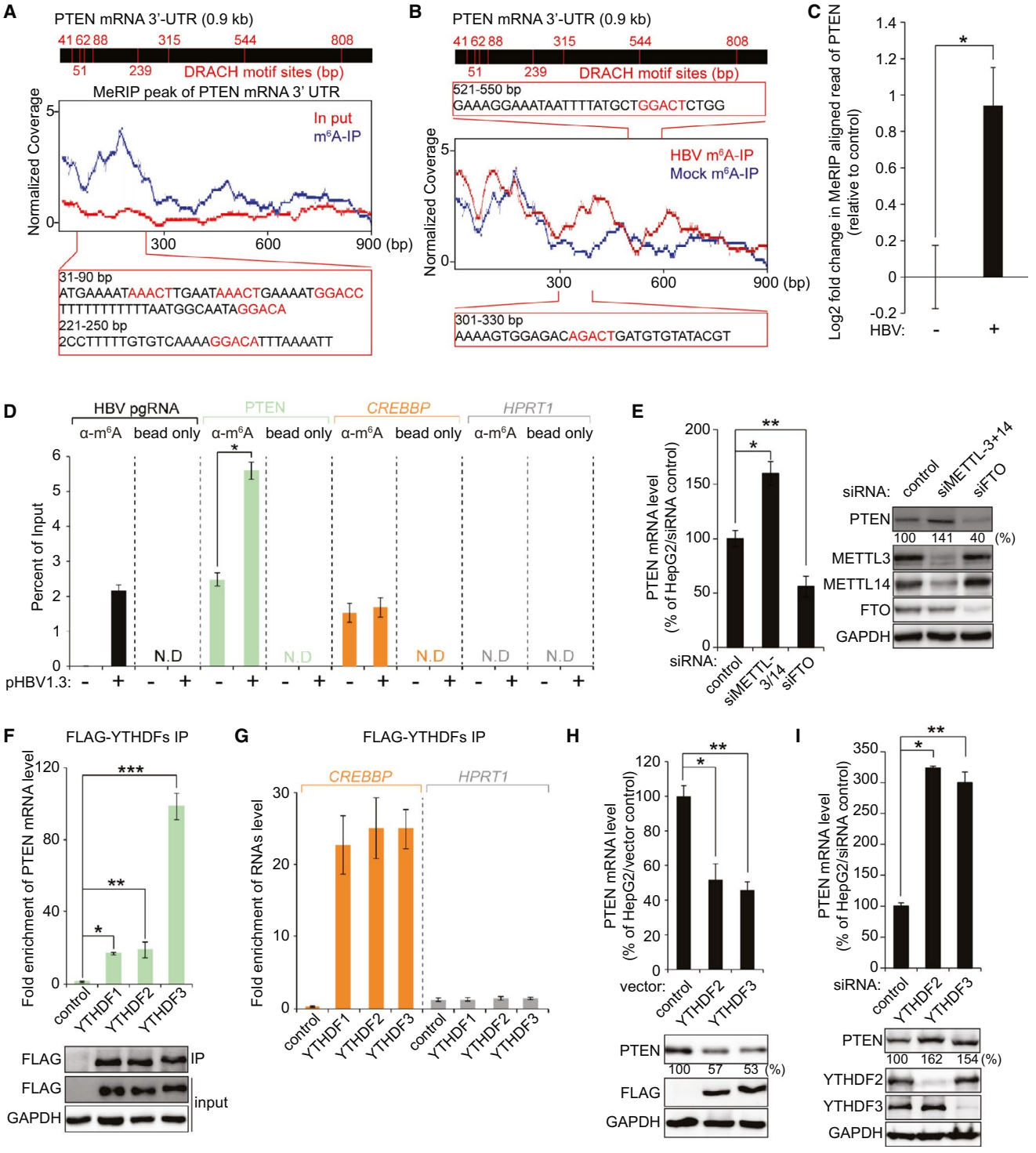

FIG. 1. PTEN 3′-UTR contains the m^{6}A modification, and its modification is regulated by HBV transfection. (A,B) Map of m^{6}A-sites in the PTEN 3′-UTR region by MeRIP sequencing (representative of three independent samples) from HBV-expressing HepG2 cells. Blue coverage, normalized to the total number of reads mapping to the PTEN mRNA for each experiment, is in blue for MeRIP sequencing and red for input MeRIP sequencing. Eight DRACH motif sites were identified in PTEN 3′-UTR. Several m^{6}A peaks were analyzed after normalizing for coverage. The *Inset* presents 31-90 bp and 221-250 bp of the PTEN 3′-UTR region, with the m^{6}A sites highlighted by red text (A). Read coverage, normalized to the total number of reads mapping to the PTEN mRNA for each experiment, is in red for HBV-expressing cells RNA sequencing and in blue for control cells RNA sequencing. The *Inset* presents 301-330 bp and 521-550 bp of the PTEN 3′-UTR region, with the m^{6}A sites in HBV-expressing cells highlighted by red text (B). (C) Fold change (log2) of the MeRIP reads of PTEN mRNA in the HBV-expressing cells compared with the control HepG2 cells. Plotted in (C) are the relative reads number of PTEN (mean ± SD estimated from three independent samples). $^*P = 0.0058$. (D) MeRIP-qRT-PCR of m^{6}A-modified HBV transcripts and PTEN mRNA from the total RNA extracted from HepG2 cells transfected with HBV 1.3-mer plasmid. *CREBBP* and *HPRT1* serve as positive and negative controls, respectively. $^*P = 0.00029$. (E) Relative PTEN mRNA level in HepG2 cells transfected with siMETTL3 + siMETTL14 or siFTO at 2 days following transfection. PTEN protein expression was assessed by immunoblotting. $^*P = 0.0015$; $^{**}P = 0.0035$. (F) Enrichment of PTEN mRNA following IP of FLAG-tagged YTHDFs from extracts of HepG2 cells after 48 hours of transfection. Enriched PTEN mRNA was quantified by qRT-PCR as the percentage of input and graphed as fold enrichment relative to control. Immunoblot analysis of FLAG-YTHDFs in the input and IP is shown in the bottom panels. $^*P = 0.00004$; $^{**}P = 0.0021$; $^{***}P = 0.00022$. (G) RNA-IP from FLAG-YTHDFs-transfected HepG2 cells using anti-FLAG antibody, with qRT-PCR analysis of *CREBBP* and *HPRT1* quantified as relative enrichment RNA level. (H,I) HepG2 cells were transfected with FLAG-YTHDF2/3 or siRNAs of YTHDF2/3. After 48 hours, cells were harvested to assess expression levels of PTEN mRNA and proteins levels. $^*P = 0.0015$; $^{**}P = 0.00013$; E, $^*P = 0.00024$; $^{**}P = 0.0065$. In (C)-(I), the error bars indicate the SDs of three independent experiments, each involving triplicate assays. Statistical significance of the difference between groups was determined using an unpaired Student *t* test.

that transient HBV expression reduced the half-life of PTEN mRNA from about 9.2 ± 0.98 hours to 7.2 ± 0.82 hours, and PTEN protein expression levels also decreased in HBV expressing cells at each time point, compared with control cells. These results suggest that HBV negatively regulates PTEN mRNA stability in both chronic HBV patients and cultured HBV-expressing cells.

To verify whether HBV affects PTEN mRNA stability by increasing m^{6}A modification of PTEN mRNA, we performed co-transfection with siMETTL3/14 and HBV 1.3-mer. Fig. 2H demonstrates that HBV transfection destabilized PTEN mRNA, resulted in decreased PTEN protein level; however, in the absence of METTL3/14, HBV did not affect PTEN mRNA and protein levels. Together, these results reveal that PTEN mRNA stability regulation is mediated by up-regulating the m^{6}A modification of PTEN mRNA by HBV.

HBV EXPRESSION INHIBITS IRF-3 NUCLEAR IMPORT, WHICH SPECIFICALLY IS REGULATED BY PTEN PROTEIN EXPRESSION

Recent studies have shown that IRF-3 has two phosphorylation sites. The S396 residue of IRF-3 is a positive phosphorylation site for IRF-3 activation, and the newly identified negative phosphorylation site S97 dictates IRF-3 nuclear import. PTEN can dephosphorylate IRF-3 S97 residue and facilitate its nuclear import for the IFN signaling pathway.[7] Here, we sought to test the possibility that HBV affects PTEN protein expression to disrupt the IFN signaling pathway. First, we analyzed IFN-β mRNA levels in PTEN-depleted cells. As expected, in the absence of PTEN, we observed that the IFN-β mRNA level induced by poly (I:C) treatment was significantly reduced (< 4-fold decrease), and IRF-3 nuclear import was substantially inhibited (Fig. 3A,B). Next, we tested whether PTEN protein directly interacts with activated IRF-3. We performed co-IP experiments using cell lysates from the HepG2 cells in which the FLAG-tagged PTEN protein was ectopically expressed. Fig. 3C illustrates the ability of the PTEN protein to interact with activated IRF-3. Poly (I:C) treatment strongly induced the interaction between PTEN and phosphorylated IRF-3.

After determining that the PTEN protein regulates nuclear import of IRF-3, we next investigated whether HBV expression affects the IFN signaling pathway by inhibiting IRF-3 nuclear import by the down-regulating PTEN mRNA stability. The IFN-β mRNA level induced by poly (I:C) was decreased in HBV-expressing cells (<2-fold decrease; Fig. 3D), but HBV failed to reduce IFN-β synthesis in PTEN-depleted cells. The results presented in Fig. 3D indicate that IRF-3 nuclear import was inhibited by HBV expression, whereas HBV

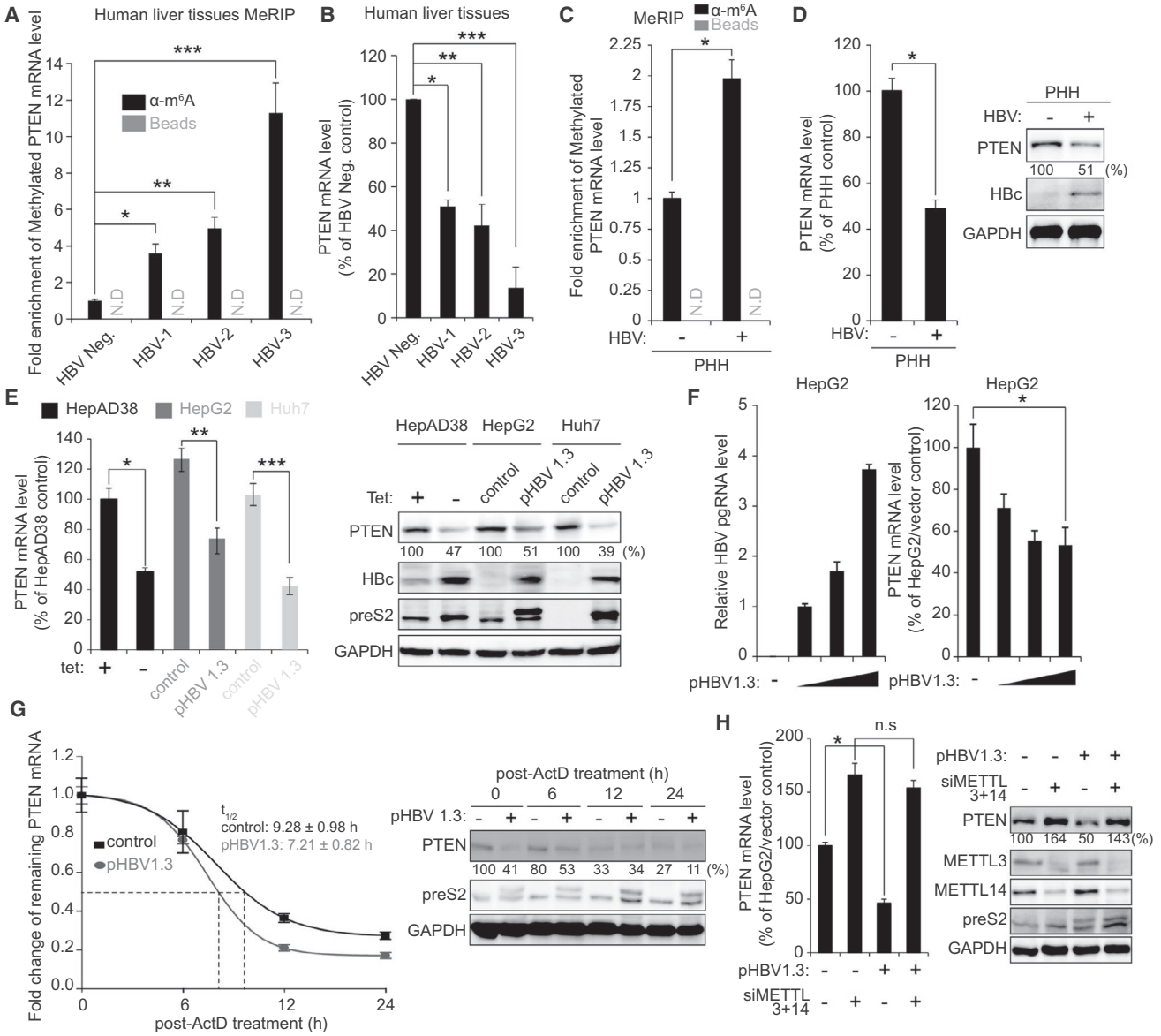

FIG. 2. HBV reduces PTEN mRNA stability by regulating m^{6}A modification. (A) MeRIP-qRT-PCR analysis of total RNA from patients with chronic HBV (HBV-1 to HBV-3) and healthy control (HBV-negative) using primers specific to PTEN mRNA. RNA was immunoprecipitated with anti-m^{6}A antibody and eluted RNA was quantified by qRT-PCR. **P* = 0.00051; ***P* = 0.00043; ****P* = 0.0011. (B) qRT-PCR quantification of PTEN mRNA levels normalized to GAPDH RNA in liver biopsies in (A). **P* = 0.0036; ***P* = 0.0061; ****P* = 0.00027. (C,D) PHHs were infected with HBV. After 72 hours, PHHs were harvested to assess the expression levels of m^{6}A modified PTEN mRNA, PTEN mRNA, and protein. **P* = 0.0047 (C); **P* = 0.0025 (D). (E) Relative PTEN mRNA levels in HBV-induced HepAD38 and hepatoma cell lines (HepG2 and Huh7) transfected with HBV 1.3-mer plasmid at 2 days following transfection. Shown in the right panels are the results of immunoblotting analysis for the indicated proteins in cell lysates. **P* = 0.0044; ***P* = 0.0054; ****P* = 0.0059. (F) HepG2 cells were transfected with increased amounts of HBV 1.3-mer (1, 5, and 10 μg). After 48 hours, cells were harvested to assess the expression levels of PTEN mRNA. **P* = 0.00387. (G) qRT-PCR analysis of PTEN mRNA relative to GAPDH in the HBV 1.3-mer expressing HepG2 cells. The HBV 1.3-mer transfected HepG2 cells were treated with actinomycin D at 24 hours following transfection. Cells were harvested at 0, 6, 12, and 24 hours following actinomycin D treatment, and relative levels of remaining PTEN mRNA and proteins were analyzed. (H) The HBV 1.3-mer transfected HepG2 cells were depleted for METTL3 and METTL14 by siRNAs. After 48 hours, total RNA and lysates were extracted to assess the expression levels of PTEN mRNA and protein. **P* = 0.0039. In (C)-(H), the error bars indicate the SDs of three independent experiments, each involving triplicate assays. *P* values were calculated using an unpaired *t* test. Abbreviations: ActD, actinomycin D; n.s, not significant by unpaired Student *t*-test.

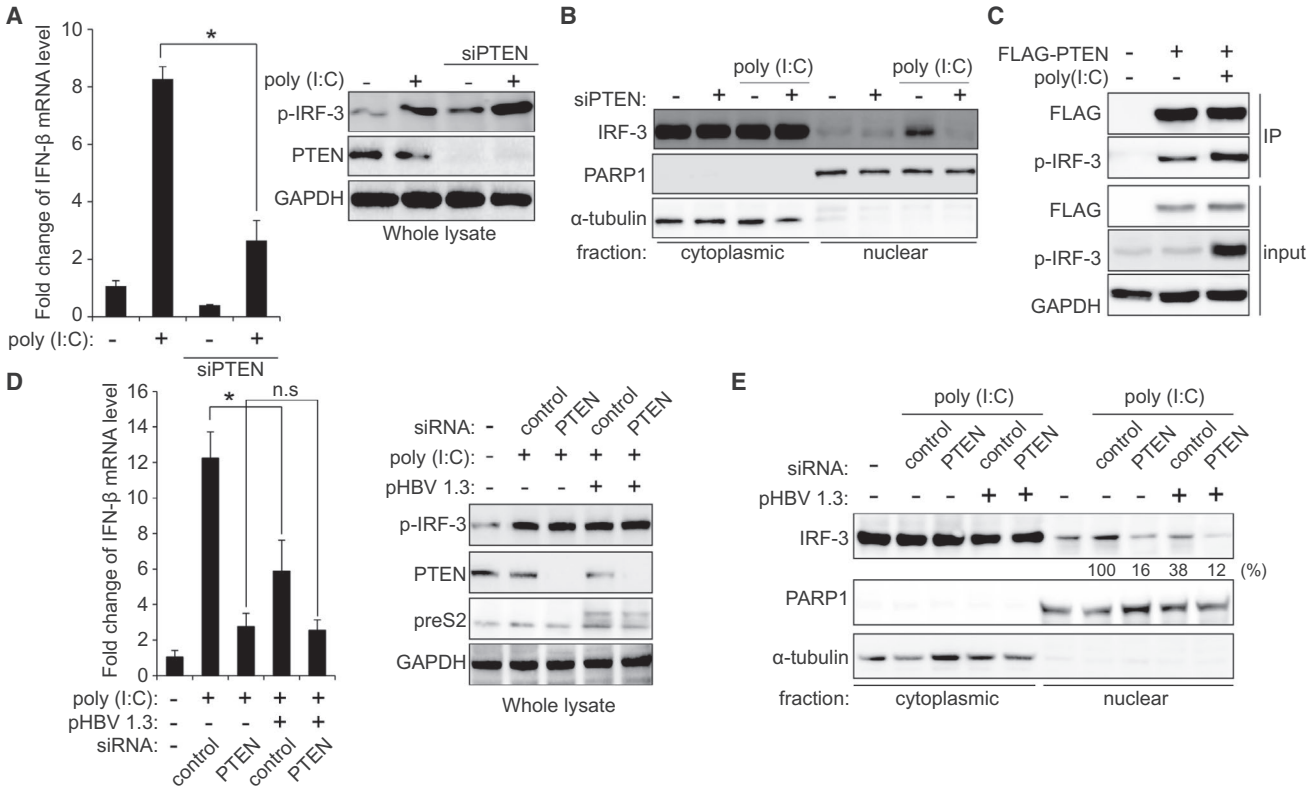

FIG. 3. HBV inhibits IFN-β synthesis by regulating IRF-3 nuclear import. (A,B) HepG2 cells transfected with siPTEN or control siRNA were treated with poly (I:C). The IFN-β mRNA levels were determined by qRT-PCR at 12 hours following poly (I:C) treatment, normalized with GAPDH. The p-IRF-3 protein levels were analyzed by immunoblotting in whole-cell lysates. **P* = 0.00023. (B) Immunoblot analysis of isolated nuclear and cytoplasmic biochemical fractions from extracts of HepG2 cells co-transfected with siPTEN and poly (I:C). (C) HepG2 cells were transfected with FLAG-PTEN plasmid. After 36 hours, cells were treated with poly (I:C) for 12 hours. PTEN protein was immunoprecipitated using M2 FLAG antibody followed by immunoblotting with anti-p-IRF-3 and FLAG antibody. (D,E) HBV 1.3-mer expressing HepG2 cells were transfected with the control or PTEN-specific siRNAs. After 36 hours, the cells were stimulated with poly (I:C) for 12 hours, and cells were harvested to assess the expression levels of IFN-β mRNA by qRT-PCR. Levels of p-IRF-3 and PTEN proteins were analyzed by immunoblotting. GAPDH was used as an internal control for loading. **P* = 0.0088. (E) The IRF-3 protein levels were determined by immunoblotting from isolated nuclear and cytoplasmic biochemical fractions (PARP1, nuclear marker; α-tubulin, cytoplasmic marker). All experiments were performed in triplicate. Immunoblots shown are representative of three independent experiments. *P* values were calculated using an unpaired *t* test. Abbreviation: PARP1, poly(adenosine diphosphate ribose) polymerase 1.

did not affect IRF-3 nuclear translocation in PTEN-depleted cells. The results demonstrate that HBV inhibits IRF-3 localization to the nucleus through destabilization of PTEN mRNA as a strategy of immune evasion.

HBV ACTIVATES THE PI3K/AKT PATHWAY THROUGH THE UP-REGULATION OF m^{6}A MODIFICATION OF PTEN mRNA

It has been reported that PTEN inhibits cell survival and apoptosis signaling by blocking the PI3K pathway, leading to its inactivation-enabling carcinogenesis.[5] We sought to test the possibility that decreased PTEN protein expression by HBV affects liver tumorigenesis. Therefore, we analyzed the PTEN mRNA and m^{6}A-modified PTEN mRNA levels in human liver biopsy specimens from a healthy individual (N-1), HBV-negative patients with HCC (HCC-1 to HCC-3), and HBV-positive patients with HCC (HCC-4 and HCC-5). PTEN mRNA expression levels were down-regulated in both HBV-positive and HBV-negative patients with HCC (34% ± 4.45%, 46% ± 2.55%, and 40% ± 4.92% decreases in liver biopsies HCC-1, HCC-2, and HCC-3, respectively, from patients with HCC; and 64% ± 2.30% and

58% ± 7.82% decreases in liver biopsies HCC-4 and HCC-5, respectively, from HBV-positive patients with HCC; Fig. 4A). In contrast, m^6A-methylated PTEN mRNA levels were significantly increased in all samples of patients with HCC (291% ± 4.51%, 376% ± 55.08%, and 289% ± 8.4% increases in liver biopsies HCC-1, HCC-2, and HCC-3; and 441% ± 20.52% and 403% ± 26.48% increases in liver biopsies HCC-4 and HCC-5; Fig. 4B). In particular, in HBV-positive patients with HCC, the m^6A modification of PTEN mRNA was on average about 28% higher than HBV-negative patients with HCC. These results collectively indicate that decreased PTEN mRNA stability by m^6A methylation is a critical factor in the development of HCC.

PI3K phosphorylates phosphatidylinositol-2, 4, 5,-triphosphate (PIP_2), forming lipid-signaling second messenger PIP_3, which in turn phosphorylates and activates AKT. PTEN disrupts the activity of PI3K by dephosphorylating PIP_3 to generate PIP_2.[6] To verify whether HBV affects PI3K activity by regulating PTEN expression, we checked phosphorylated AKT levels from HBV-expressing cells (Fig. 4C). Because p-AKT expression levels were low in HepAD38 cell lines and HepG2 cells, we treated HBV-expressing cells with lipopolysaccharide, a PI3K activator. Interestingly, the PI3K/AKT pathway was activated by HBV expression, suggesting that HBV activates the PI3K pathway by decreasing PTEN expression levels, which acts as a negative regulator of the PI3K pathway. These results imply that HBV infection can contribute to the development of HCC through the down-regulation of PTEN mRNA stability.

CHRONIC HBV REGULATES m^6A METHYLATION OF PTEN mRNA BY INDUCING METTL3 EXPRESSION

We investigated whether chronic HBV infection has any effect on host m^6A machinery. We observed that METTL3 mRNA levels were increased in chronic HBV patient samples and PHHs infected with HBV, whereas FTO mRNA levels were not changed (Fig. 5A,B). The METTL14 mRNA level was dramatically increased in the HBV-3 sample, whereas increased METTL14 mRNA levels were not observed in samples from other patients with HBV (HBV-1 and HBV-2) and HBV-infected PHHs. These results correlate with Fig. 2A,B, which shows that m^6A-methylated PTEN mRNA was the highest in the HBV-3 patient. Because the METTL14 mRNA expression level was regulated in only HBV-3 biopsy and this phenomenon was not

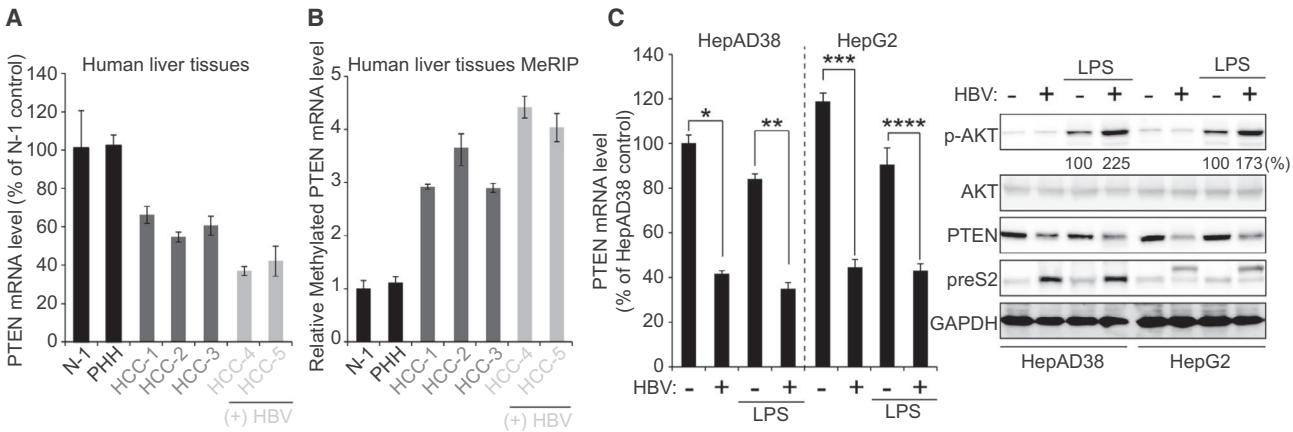

FIG. 4. HBV activates the PI3K/AKT pathway by regulating m^6A modification on PTEN mRNA. (A) qRT-PCR quantification of PTEN mRNA levels normalized to GAPDH in liver biopsies from HBV-negative patients (HCC-1 to HCC-3) or HBV-positive (HCC-4 and HCC) patients with HCC and healthy controls (N-1 and PHH). (B) MeRIP-qRT-PCR analysis of total RNA from liver biopsy samples in (A) using primers specific to PTEN mRNA. (C) HBV-expressing HepG2 cells (HepAD38, HBV off and on, and transfected with HBV 1.3-mer) were treated with lipopolysaccharide. Sixteeen hours following treatment, the cells were harvested to assess the expression levels of PTEN mRNA and indicated proteins by qRT-PCR and immunoblotting. **P* = 0.00065; ***P* = 0.00010; ****P* = 0.0017; *****P* = 0.00078. Abbreviation: LPS, lipopolysaccharide.

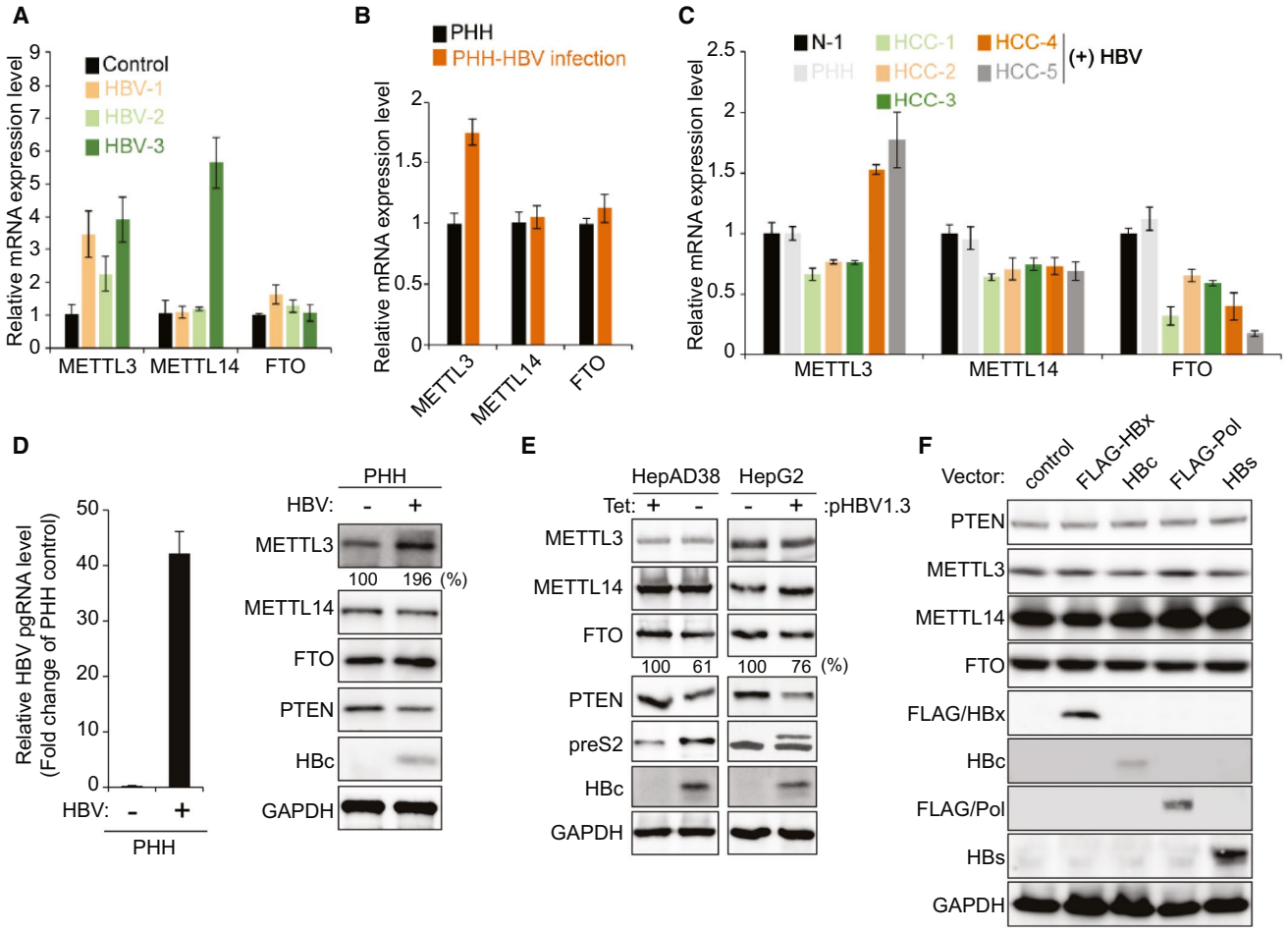

FIG. 5. HBV infection induces METTL3 expression levels. (A-C) METTL3/14 or FTO mRNA levels in liver biopsies from patients as described in Figs. 2A and 4A, and PHHs infected with HBV. The METTL3, METTL14, and FTO mRNA levels were determined by qRT-PCR. (D) PHHs were infected with HBV. After 72 hours, PHHs were harvested to assess the expression levels of indicated proteins. (E) The indicated proteins were analyzed in lysates from HBV-expressing HepG2 cells (HepAD38 and HBV 1.3-mer transfected cells). (F) The indicated proteins were analyzed in lysates from HepG2 cells that individually transfected with FLAG-HBx, HBcAg, FLAG-Pol, and HBsAg.

observed in the HBV-1 and -2 samples, suggesting that HBV infection regulates METTL3 expression, but METTL14 expression may be regulated by other factors.

In HBV-positive HCC samples, increased METTL3 mRNA expression levels were also observed, whereas in HBV-negative patients with HCC, METTL3 was not induced (Fig. 5C). The FTO expression levels were significantly reduced in both HBV-negative and HBV-positive HCC samples. Next, we confirmed m^6A methyltransferases and demethylases protein expression levels in PHHs infected with HBV and HBV expressing cell lines. In particular, METTL3 protein expression levels were induced only in HBV-infected PHHs and not increased in HBV-expressing hepatoma cell lines (Fig. 5D,E). Decreased FTO protein expression levels were observed in HBV-expressing cell lines, but not HBV-infected PHHs. In HBV-infected PHHs and chronic HBV patients (non-HCC), METTL3 expression was increased, while in liver cancer cell lines and patients with HCC, FTO expression was decreased by HBV. These results suggest that HBV regulates host m^6A machinery in different ways in HCC tissues/cell lines and non-HCC tissues/PHH, respectively.

To determine whether HBV proteins affect host m^{6}A machinery, we analyzed the PTEN protein expression levels in cells expressing individual HBV genes (HBx, HBsAg, HBcAg, and HBV pol; Fig. 5F). PTEN protein expression levels were not altered in any of these HBV gene-expressing cells. We also observed that the FTO expression level was not affected by each HBV protein expression. These results suggest that m^{6}A modification of PTEN might be regulated during HBV infection in a life cycle–dependent manner but not by any specific HBV gene product.

PTEN NEGATIVELY REGULATES HBV REPLICATION

We investigated whether PTEN has any effect on HBV replication. HBV transcripts and protein levels were reduced in FLAG-PTEN plasmid-transfected cells (Fig. 6A). Next, we assessed whether the silencing of PTEN can up-regulate HBV replication, but we did not observe any changes in HBV pgRNA and protein levels in PTEN-depleted cells (Fig. 6B). In fact, HepG2 cell lines were established from hepatoblastoma cells. Thus, the PTEN expression levels in HepG2 cell lines are expected to be lower compared with primary hepatocytes. As expected, we found that PTEN expression levels were dramatically decreased in HepG2 cells compared with PHHs (Supporting Fig. S2). Therefore, we checked whether knockdown of PTEN in PHHs affects HBV replication and found that HBV pgRNA and viral protein expression levels were significantly increased in PTEN-depleted PHHs (Fig. 6C).

Next, we investigated whether the inhibitors of PTEN phosphatase activity affect HBV pgRNA and protein levels. We found that the treatment with PTEN inhibitors (VO-OHpic and SF1670) could rescue the inhibitory effects of ectopically PTEN protein expression on HBV replication (Fig. 6D), whereas PTEN inhibitors did not affect the regulation of HBV replication and translation in untransfected

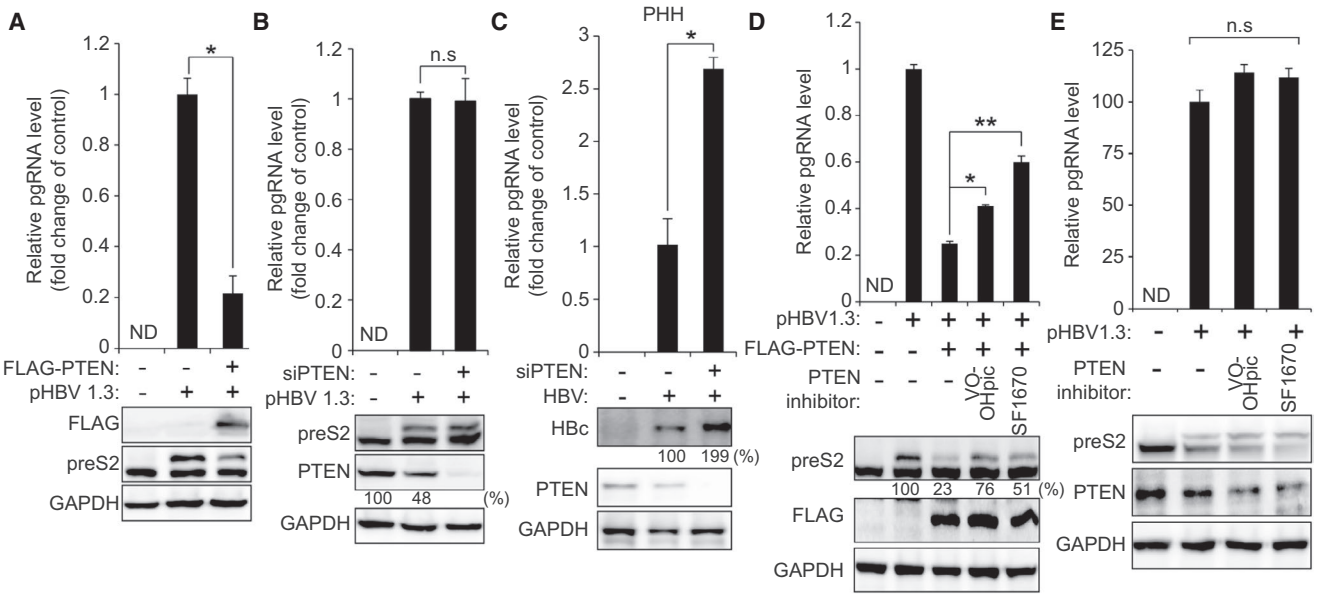

FIG. 6. PTEN negatively regulates HBV replication and translation. (A) Relative HBV pgRNA level in HepG2 cells co-transfected with HBV 1.3-mer and FLAG-PTEN plasmid was determined 2 days after transfection. The HBV preS2 protein level was analyzed by immunoblotting. **P* = 0.00010. (B) Relative HBV pgRNA level in PTEN-silenced cells was analyzed by qRT-PCR at 48 hours following siRNA transfection. Levels of PTEN and HBV preS2 proteins were assessed by immunoblotting. (C) PTEN-silenced PHH were infected with HBV. After 72 hours, the total RNA and proteins were extracted to assess the expression levels of HBV pgRNA and protein. **P* = 0.0067. (D) HepG2 cells were co-transfected with the HBV 1.3-mer and FLAG-PTEN plasmid. (E) HepG2 cells were transfected with HBV 1.3-mer. Twelve hours after transfection, cells were treated with PTEN inhibitors (46 nM VO-OHpic or 2 μM SF1670), and 48 hours later the cells were harvested. The HBV pgRNA level was analyzed by qRT-PCR, and the HBV preS2 protein level was analyzed by immunoblotting. **P* = 0.00015; ***P* = 0.00026.

cells (Fig. 6E). These results demonstrate that PTEN negatively regulates HBV replication, implying that HBV reduces PTEN expression to up-regulate pro-viral activity.

Discussion

In this study, we examined the mechanism by which the cellular abundance of PTEN mRNA is down-regulated by HBV infection. Diverse regulatory mechanisms, which include the transcriptional regulation and posttranscriptional turnover control of cellular mRNAs, might explain the differential expression of host mRNAs in HBV-infected cells. HBV affects m^6A modification of multiple cellular mRNAs in addition to PTEN mRNA by increasing or decreasing the m^6A modification (Supporting Tables S2 and S3). These cellular mRNAs did not have any conserved sequences, suggesting that the selection of the altered m^6A modification of cellular mRNA by HBV lacks any specificity. In addition to the role of viral infection on changing host mRNA expression, the HBV is also known to be involved in the altering of microRNA (miRNA) expression.[24] Because miR-21 is known to be induced by HBV[25] and the miR-21 sequence has the DRACH motif, we analyzed whether HBV affects miR-21 expression through the regulation of m^6A modification (Supporting Fig. S3). We found that miR-21 expression was increased in HBV-transfected cells by reducing m^6A modification. Thus, HBV-mediated change in miRNAs expression might be determined by m^6A cellular machinery regulated by HBV. Interestingly, PTEN is the known target of miR-21, and it has been shown that miR-21 suppresses PTEN protein expression in several cancer cell lines.[26] These results further suggest that HBV may regulate PTEN expression by altering the m^6A modification of miR-21.

Because PTEN protein has a critical role in liver cancer by functioning as a tumor suppressor,[5] we focused on PTEN among the m^6A-modified host mRNAs affected by HBV. We found that HBV reduced PTEN protein expression by increasing m^6A methylation of PTEN mRNA (Figs. 2 and 4). The tumor-suppressor role of PTEN has been well-characterized in numerous cancers.[6] Thus, our results suggest that HBV may contribute to the development of HCC by activating the PI3K/AKT pathway, by decreasing PTEN mRNA stability. Decreased PTEN expression during the chronic HBV infection may in part explain the oncogenic phenotype previously observed in cell culture and human liver chimeric mice expressing HBV genes.[27] Furthermore, the transgenic mice carrying the HBV gene and PTEN knockout mice are characterized by liver inflammation and fibrosis, followed by the development of HCC with age,[5,27] further supporting the role of the HBV infection in liver disease pathogenesis through its ability to cause PTEN mRNA destabilization by inducing increased m^6A modification. In addition to liver cancer, PTEN expression levels are also reduced in various cancer tissues such as brain, breast, lung and prostate cancer, but it is not clear whether PTEN expression-level reductions are mediated by m^6A machinery in those cancer tissues.[28] Thus, our results also imply that decreased demethylases (FTO) expression in various cancers may cause tumorigenesis by reducing PTEN expression (Fig. 5C). It remains to be investigated whether the m^6A modification of PTEN is regulated in several cancer tissues by decreasing FTO expression. If true, then induced m^6A modification of PTEN by decreasing FTO expression can be a possible mechanism of reducing PTEN expression in cancer tissues. This hypothesis is supported by the finding that in several human cancer tissues, expression levels of m^6A methyltransferases and demethylases are altered to regulate tumor cell proliferation, differentiation block, tumorigenesis, and metastasis.[29]

HCV infection is also associated with the risk of the development of HCC.[30] Decreased PTEN expression was observed in HCV-infected cells through the up-regulation of m^6A modification, but HIV, a non-oncogenic virus, did not affect m^6A modification of PTEN (Supporting Fig. S4), suggesting that both hepatitis viruses (HBV and HCV) induce HCC by a shared mechanism(s) of destabilizing PTEN mRNA through altering the m^6A modification. PTEN inhibition can be one of the mechanisms accelerating tumor development by chronic virus infection.

Given that HBV transfection altered cellular m^6A profiling, the important question remains as to how HBV alters m^6A modification of cellular RNAs. Interestingly, we found that HBV infection up-regulates METTL3 expression level in patients with chronic HBV, HBV-positive patients with HCC, and PHHs infected with HBV (Fig. 5), raising the

possibility that HBV-mediated liver tumors might be caused by the up-regulation of METTL3 expression. Supporting this hypothesis, we analyzed METTL3 expression levels in HBV-negative HCC patient samples and HepG2 transfected with HBV. METTL3 expression levels were not changed in these samples (Fig. 5). Notably, decreased FTO expression was observed in both HBV-positive and HBV-negative patients with HCC, but not in patients with chronic HBV (non-HCC) and PHHs infected with HBV. These results clearly suggest that HBV induces METTL3 expression in normal hepatocytes and promotes m^6A modification of PTEN mRNA to reduce its RNA stability and affect protein levels, which could aid in the development of liver neoplasia.

Despite the activation of RIG-I signaling by pgRNA, little is known about the molecular mechanism explaining how HBV infection does not induce IFN-α/β. Our laboratory has previously shown that mitochondrial dynamics is altered by HBV, in which Parkin translocation to the mitochondria triggers massive ubiquitination of mitochondrial antiviral signaling protein, thus crippling downstream IFN signaling.[31,32] In addition to its role as a tumor suppressor, the PTEN protein is also known to be involved in innate immune response activated by viral infections.[7] The function of PTEN in innate immunity is well characterized in virus-infected cells. PTEN causes p-IRF-3 nuclear translocation by dephosphorylation at the IRF-3 Ser97 residue, to activate the IFN signaling pathway. Thus, our observations explain that the reduced PTEN protein expression during HBV infection causes inhibition of p-IRF-3 nuclear import, which leads to the disruption of the IFN signaling pathway (Fig. 3). HBV-induced elimination of PTEN can be one of the mechanisms of HBV immune evasion. Furthermore, overexpression of PTEN substantially decreased HBV replication, and the negative effect of PTEN on HBV replication was rescued by inhibitors of PTEN phosphatase activity (Fig. 6). These results together suggest that HBV reduces PTEN expression to evade innate immunity and positively regulates the viral life cycle.

In summary, our results unravel mechanisms by which HBV controls host gene expression to maintain persistent infection by regulating the host m^6A modification machinery. HBV infection suppresses innate immunity by the effects of PTEN in the IFN signaling pathway. Finally, the HBV-induced m^6A modification of PTEN, leading to its reduced levels of expression, may contribute to the virus-associated hepatocarcinogenesis. Furthermore, this work expands the role of m^6A modification in HCC.

Author Contributions: G.W.K.: Conceptualization; methodology; validation; formal analysis; investigation; Writing - Original Draft; Writing - Review & Editing. H.I.: Validation; investigation. M.K.: Validation; investigation. S.A.M.: Validation; investigation. S.J.K.: Resources. S.K.Y.: Resources. W.H.: Resources. A.S.: Conceptualization; methodology; Writing - Original Draft; Writing - Review & Editing; Supervision; project administration; funding acquisition.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.31313/suppinfo.