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HBV/Pregenomic RNA Increases the Stemness and Promotes the Development of HBV-Related HCC Through Reciprocal Regulation With Insulin-Like Growth Factor 2 mRNA-Binding Protein 3

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BACKGROUND AND AIMS: HBV-pgRNA (pregenomic RNA) has been proposed for predicting the response of nucleos(t)ide analogue (NA) treatment, guiding discontinuation of NA therapy and monitoring the emergence of viral mutations. However, the contributions of HBV-pgRNA to HCC remain open for study.

APPROACH AND RESULTS: Double-center cohorts of serum samples with undetectable serum HBV-DNA (below the lower limit of detection) were obtained from long-term NA-treated (≥48 weeks) HBV-related HCC patients. The correlation between serum pgRNA concentration and the prognosis of HCC were analyzed. The role pgRNA played in HCC development was assessed both *in vitro* and *in vivo*. Our findings revealed that for patients who underwent long-term NA therapy with undetectable serum HBV-DNA, patients with high serum pgRNA expression had a poorer overall survival rate and higher cumulative recurrence rate after hepatectomy. Experiments demonstrated that pgRNA promotes proliferation, stemness, and tumorigenicity of HCC cells. Mechanistically, we found that pgRNA could up-regulate the expression of insulin-like growth factor 2 mRNA-binding protein 3 (IGF2BP3), a well-proven oncoprotein, at the posttranscriptional level. Furthermore, interferon (IFN)-α-2a could degrade the stability of pgRNA through increasing its N6-methyladenosine (m6A) RNA modification. Collectively, our findings uncover that serum pgRNA could serve as a potential biomarker for predicting the prognosis and recurrence of HCC in patients who received long-term NA therapy with undetectable serum HBV-DNA; and the pgRNA-IGF2BP3 axis plays an important role in the development of HBV-related HCC. Moreover, IFN-α-2a could reduce the stability of pgRNA by increasing its m6A RNA modification level, thereby suppressing the development of HBV-related HCC.

CONCLUSIONS: In conclusion, our studies reveal a significance and mechanism of HBV-pgRNA in increasing stemness features and offer a potential prognostic marker and a therapeutic target for HBV-related HCC. (Hepatology 2021;74:1480-1495).

HCC is one of the most prevalent and lethal malignancies globally,[1] especially in Asia where chronic hepatitis B (CHB) infection is the most predominant etiology.[2] Since the extensive

Abbreviations: cccDNA, covalently closed circular DNA; CCK-8, Cell Counting Kit-8; CHB, chronic hepatitis B; CRR, cumulative recurrence rate; EdU, 5-ethynyl-2′-deoxyuridine; FISH, fluorescence in situ hybridization; FTO, fat mass and obesity-associated protein; IFN-α-2a, interferon alpha 2a; IGF2BP3, insulin-like growth factor 2 mRNA-binding protein 3; m6A, N6-methyladenosine; METTL3, methyltransferase-like protein 3; METTL14, methyltransferase-like protein 14; miRNA, microRNA; NAs, nucleos(t)ide analogues; OS, overall survival; pgRNA, pregenomic RNA; RIP, RNA immunoprecipitation.

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application of HBV vaccine, the incidence of HBV new infections has been significantly decreased, and the availability of potent antiviral therapy also leads to the inhibition of HBV replication and reduction of the incidence of serious complications effectively. Still, 240 million people are chronically infected with HBV and remain with a high risk of developing liver cirrhosis and HCC.[3]

To date, radical hepatectomy remains the most effective treatment with curative potential. However, merely one fifth of HCC patients are suitable for surgical treatment.[4] In addition, patients always suffer from a high rate of HCC relapse after hepatectomy. Previous studies have illustrated that the high viral load of HBV-DNA is a vital risk factor for the recurrence of HBV-related HCC, and nucleos(t)ide analogue (NA) therapy reduces HCC recurrence and improves overall survival after radical hepatectomy.[5] However, because NA exert their antiviral function through inhibiting viral reverse transcription, HBV cannot be completely eliminated as a result of the persistence of covalently closed circular DNA (cccDNA) in the liver reservoir. So, the processes of viral transcription and translation remain intact.[6] Because of the low level of intrahepatic viral products and residual ongoing viral replication, patients with NA therapy still have a higher risk of HCC and end-stage liver disease compared to the normal population.[7]

HBV is a member of the hepadnaviruses family. The genome of HBV is a circular, partially double-stranded DNA genome that replicates through reverse transcription from pregenomic RNA (pgRNA). HBV-pgRNA plays a dual role during HBV infection, given that it serves not only as the mRNA for the translation of the HBcAg and the viral polymerase, but also as the template for reverse transcription.[8] Given that quantifying cccDNA requires liver biopsy, which was an invasive procedure, and pgRNA, as a direct transcription product of cccDNA, can reflect its activity to a certain extent, more and more researches have focused on pgRNA in recent years. Lately, studies have shown that HBV-RNA can also be detected in the serum of CHB patients, and serum HBV-RNA is in the form of pgRNA.[9] Additionally, serum pgRNA

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level reflects intrahepatic viral transcriptional activity and is associated with liver disease progression during NA therapy, even in patients for whom the majority of viral replication is suppressed.[10] Furthermore, serum pgRNA has also been reported as a potential biomarker for monitoring NA-resistant mutants,[11] predicting the efficacy of NA treatment,[12, 13] and guiding the safe discontinuation of NA therapy.[9] However, in patients who underwent long-term NA therapy with undetectable serum HBV-DNA, whether the level of serum pgRNA correlates with the prognosis of HCC remains open for study. Moreover, given that the persistence of pgRNA and low levels of viral replication may lead to liver disease progression in patients who have undergone long-term NA therapy, it is worthy of further exploration to illustrate the role of pgRNA in the progression of liver disease.

Interferon-alpha (IFN-α), with immunomodulatory, antiproliferative, and viral-inhibitory properties, has the advantages of finite treatment duration, absence of viral resistance, and potential of eliminating HBsAg compared to NA.[14] IFN-α has been reported to inhibit HBV replication by blocking of RNA-containing core particle formation, accelerating decay of replication-competent core particles, and inhibiting the transcription of HBV-pgRNA.[15,16] Previous studies have suggested that adjuvant IFN therapy after hepatectomy prevents the recurrence of HBV-related HCC patients and improves their overall survival.[17,18] Whether IFN-α prevents HCC development and recurrence through inhibiting pgRNA remains open for study.

In the present study, we aimed to determine the value of serum pgRNA in evaluating the prognosis of HCC in patients who have undergone long-term NA therapy with undetectable serum HBV-DNA. Then, we further explored the role of pgRNA in the development of HCC. Finally, we investigated the underlying mechanism of IFN-α-2a in prognosis improvement of HBV-related HCC.

Materials and Methods

PATIENTS AND SAMPLES

All samples were collected with the informed consent of the patients, and the study protocol was approved by the ethics committee of Eastern Hepatobiliary Surgery Hospital (Shanghai, China) and Mengchao Hepatobiliary Surgery Hospital (Fuzhou, China). Patients and samples are detailed in the Supporting Information.

QUANTIFICATION OF HBV-pgRNA IN SERUM AND LIVER TISSUES

All serum and tissue samples were stored at −80°C until use. Serum HBV-pgRNA was detected by the RNA simultaneous amplification testing method (HBV-SAT) based on real-time fluorescence detection of RNA transcription-mediated nucleic acid amplification using the HBV-SAT kit (Rendu Biotechnology, Shanghai, China), according to the manufacturer's recommendations, as described.[19] The lower limit of detection of serum pgRNA is 50 copies/mL. Quantification of tissue HBV-pgRNA is described in the Supporting Information.[20]

CELL CULTURE AND TRANSFECTION

The human liver cancer cell line, Huh7, the stable HBV-expressing human liver cancer cell line, HepG2.2.15, and human embryo kidney cell line HEK293T were all cultured at 37°C in an atmosphere containing 5% CO_2 in DMEM supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA), 2 mM of L-glutamine, and 1% penicillin-streptomycin. All cell lines were identified by short tandem repeat typing to exclude HeLa cell-line contamination. Lentiviruses were transfected into cells, followed by puromycin selection, according to the manufacturer's instructions. Plasmids and the mimics and inhibitor of microRNA (miRNA) were transfected into cells by Lipofectamine 3000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

RNA PULL-DOWN ASSAY

RNA pull-down assay was performed as described.[21] Briefly, pgRNA and its antisense RNA were *in vitro* transcribed from vector pSPT19-pgRNA, biotin labeled with the Biotin RNA Labeling Mix (Roche Diagnostics, Indianapolis, IN) and T7/SP6 RNA polymerase (Roche), and purified with an RNeasy Mini Kit (Qiagen, Valencia, CA). One milligram of protein from HepG2.2.15

cells was then mixed with 50 pmol of biotinylated RNA, incubated with streptavidin agarose beads (Invitrogen), and washed. Retrieved proteins were resolved by SDS-PAGE, then silver stained, and the specific bands were excised and analyzed by mass spectrometry.

RNA IMMUNOPRECIPITATION

RNA immunoprecipitation (RIP) was performed as described.[22] Briefly, a Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Merck, Darmstadt, Germany) was used according to the manufacturer's instructions. Anti-IGF2BP3 antibody (5 μg; Abcam) or a corresponding control anti-IgG (rabbit IgG) was conjugated to protein A/G magnetic beads (Thermo Fisher Scientific, Waltham, MA) by incubation for 4 hours at 4°C, followed by washing three times and incubation with precleared protein extraction in RIP lysis buffer. Input and coimmunoprecipitated RNAs were detected by RT-qPCR. Gene-specific primers used for detecting pgRNA are presented in Supporting Table S2.

GENE-SPECIFIC N6-METHYLADENOSINE qPCR

N6-methyladenosine (m6A) RNA modifications on pgRNA was determined using Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore), following the manufacturer's instructions as described before.[22] Anti-m6A antibody-conjugated (Synaptic Systems) or control IgG-conjugated (mouse IgG) beads were incubated with precleared extraction of HepG2.2.15 cells treated with or without IFN-α-2a in RIP lysis buffer. The related enrichment of m6A RNA modification in each sample was analyzed by qPCR using a pgRNA-specific primer (Supporting Table S2).

ANIMAL STUDY

Animal experiments in this study were approved by the Institutional Animal Care and Use Committee of Second Military Medical University (Shanghai, China). Additional details are described in the Supporting Information. The limiting dilution subcutaneous tumor growth assays were implemented as described.[23]

STATISTICAL ANALYSIS

All statistical analyses were performed using GraphPad Prism (version 5; GraphPad Software Inc., La Jolla, CA) and SPSS software (version 21.0; SPSS, Inc., Chicago, IL). The Student *t* test, chi-squared test, Mann-Whitney U test, Wilcoxon signed-rank test, and Pearson's correlation test were used for statistical analysis as appropriate. Survival curves were calculated by the Kaplan-Meier method. The Cox proportional hazards model was used to detect the independent factors, which were based on the variables selected by a univariate analysis. Statistical significance was indicated by P values <0.05 ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

The remaining methods are described in the Supplementary Data in the Supporting Information.

Results

SERUM HBV-pgRNA SERVES AS A PROGNOSTIC FACTOR FOR HCC PATIENTS WITH UNDETECTABLE SERUM HBV-DNA

First, we detected the level of HBV-pgRNA in serum and corresponding T and NT tissues of patients in cohort A. HBV-pgRNA was both detectable in T and NT tissues in 107 of 136 patients. Consistent with the previous study,[20] we found that pgRNA level was slightly higher in NT than in T samples (Fig. S1A; n = 107; $P = 0.003$), and the relevance of pgRNA level between serum and corresponding NT or T tissues in patients showed that serum HBV-pgRNA level was strongly correlated with intrahepatic pgRNA level in both NT (Supporting Fig. S1B; n = 107; $r = 0.6345$; $P < 0.001$) or T (Supporting Fig. S1C; n = 107; $r = 0.4753$; $P < 0.001$) tissues.[10] Next, we investigated the association between serum pgRNA and the clinicopathological characteristics of patients both in cohort A and cohort B. According to the median of serum pgRNA level (2.025 $\log_{10}$ copies/mL), patients were divided into the pgRNA high-expression group and low-expression group. High serum pgRNA expression was significantly associated with lower degree of tumor differentiation, positive HBeAg, more-severe liver necroinflammation and fibrosis degree, and cirrhosis (Table 1). Moreover, high serum

TABLE 1. Clinical Characteristics of Patients in Cohort A and Cohort B

	Cohort A			Cohort B		
	pgRNA > 2.025 log_{10}copies/mL	pgRNA < 2.025 log_{10}copies/mL		pgRNA > 2.025 log_{10}copies/mL	pgRNA < 2.025 log_{10}copies/mL	
Variable	n = 68	n = 68	*P* Value	n = 69	n = 70	*P* Value
Age, years	51(45-58)	54.5 (47-60)	0.102	58 (49.0-63.5)	56 (49-63)	0.566
Sex (male/female)	62 (91.2%)/6 (8.8%)	54 (79.4%)/14 (20.6%)	0.053	58 (84.1%)/11 (15.9%)	57 (81.4%)/13 (18.6%)	0.682
Edmondson's grade (3, 4/1, 2)	55 (80.9%)/13 (19.1%)	42 (61.8%)/26 (38.2%)	0.014*	42 (60.9%)/27 (39.1%)	29 (41.4%)/41 (58.6%)	0.022*
Inflammation degree (G ≥ 2/G < 2)	53 (77.9%)/15 (22.1%)	38 (55.9%)/30 (44.1%)	0.006*	31 (44.9%)/38 (55.1%)	15 (21.4%)/55 (78.6%)	0.003*
Fibrosis stage (S ≥ 2/S < 2)	49 (72.1%)/19 (27.9%)	35 (51.5%)/33 (48.5%)	0.013*	59 (85.5%)/10 (15.5%)	48 (68.6%)/22 (31.4%)	0.018*
Cirrhosis (with/without)	39 (57.4%)/29 (42.6%)	27 (39.7%)/41 (60.3%)	0.04*	46 (67.6%)/22 (32.4%)	33 (47.1%)/37 (52.9%)	0.015*
TNM stage (II + III/I)	30 (44.1%)/38 (55.9%)	27 (39.7%)/41 (60.3%)	0.602	47 (68.1%)/22 (31.9%)	40 (57.1%)/30 (42.9%)	0.181
Tumor diameter (≥5cm)	26 (38.2%)/42 (61.8%)	28 (41.2%)/40 (58.8%)	0.726	26 (37.7%)/43 (62.3%)	19 (27.1%)/51 (72.9%)	0.184
Tumor no. (multiple/single)	14 (20.6%)/54 (79.4%)	10 (14.7%)/58 (85.3%)	0.368	18 (26.1%)/51 (73.9%)	12 (17.1%)/58 (82.9%)	0.200
Tumor encapsulation (incomplete/complete)	59 (86.8%)/9 (13.2%)	56 (82.4%)/12 (17.6%)	0.477	49 (71.0%)/20 (29.0%)	49 (70.0%)/21 (30.0%)	0.896
Microvascular invasion (with/without)	20 (29.4%)/48 (70.6%)	21 (30.9%)/47 (69.1%)	0.852	40 (58.0%)/29 (42.0%)	36 (51.4%)/34 (48.6%)	0.439
AFP, μg/L (>400/≤400)	14 (20.6%)/54 (79.4%)	16 (23.5%)/52 (76.5%)	0.679	23 (33.3%)/46 (66.7%)	14 (20.0%)/56 (80.0%)	0.075
HBeAg (positive/negative)	26 (38.2%)/42 (61.8%)	11 (16.2%)/57 (83.8%)	0.004*	34 (49.3%)/35 (50.7%)	11 (15.7%)/59 (84.3%)	<0.001*
TBIL, μmol/L (>17/≤17)	43 (63.2%)/25 (36.8%)	32 (47.1%)/36 (52.9%)	0.058	25 (36.2%)/44 (63.8%)	29 (41.4%)/42 (58.6%)	0.530
ALB, g/L (<35/≥35)	23 (33.8%)/45 (66.2%)	21 (30.9%)/47 (69.1%)	0.714	9 (13.0%)/60 (87.0%)	8 (11.4%)/62 (88.6%)	0.771
ALT, U/L (>44)	31 (45.6%)/37 (54.4%)	25 (36.8%)/43 (63.2%)	0.296	18 (26.1%)/51 (73.9%)	11 (15.7%)/59 (84.3%)	0.132
Late recurrence (≥2 years)	12 (27.3%)/32 (72.7%)	2 (7.10%)/26 (92.9%)	0.035*	11 (20.4%)/43 (79.6%)	0 (0%)/32 (100%)	0.006*

According to the median of the patient's serum HBV expression level.
*$P < 0.05$ by χ^2 test.
Abbreviations: AFP, alpha-fetoprotein; ALB, albumin; ALT, alanine aminotransferase; TBIL, total bilirubin; TNM, tumor node metastasis.

pgRNA expression is also strongly correlated with late tumor recurrence. Next, a Kaplan-Meier analysis was used to evaluate the prognostic value of serum pgRNA expression. Remarkably, patients with high serum pgRNA expression had poorer overall survival (OS; cohort A, $P = 0.0121$; cohort B, $P = 0.0065$; Fig. 1A,C) and higher cumulative recurrence rate (CRR; cohort A, $P = 0.0243$; cohort B, $P < 0.001$) after hepatectomy (Fig. 1B,D). Furthermore, Cox multivariate proportional hazards analysis indicated that a high level of serum pgRNA was an independent predictor for HCC recurrence (CRR: cohort A, HR = 2.114; 95% CI = 1.296-3.450; $P = 0.003$; cohort B, HR = 1.646; 95% CI = 1.042-2.599; $P = 0.033$; Supporting Tables S6 and S8. Additionally, patients of the two cohorts were divided into the serum pgRNA-positive group and serum pgRNA-negative group (below the lower limit of detection), and the prognosis of them was further analyzed. The result showed that patients with negative serum pgRNA had better OS ($P < 0.001$) and CRR ($P < 0.001$; Fig. 1E,F).

In conclusion, higher or positive expression of serum pgRNA predicted the clinical progression of the disease, including recurrence and survival, in patients with HBV-related HCC in two independent cohorts from different centers.

HBV-pgRNA UP-REGULATES PROLIFERATION, STEMNESS, AND TUMORIGENICITY OF HCC CELLS *IN VITRO* AND *IN VIVO*

To assess the biological function of HBV-pgRNA, we knocked down its expression in HepG2.2.15 cells (Supporting Fig. S1D). Proliferation and clone formation abilities of HCC cells were inhibited when silencing pgRNA expression (Fig. 2A-C). The flow cytometry analysis showed a reduction in

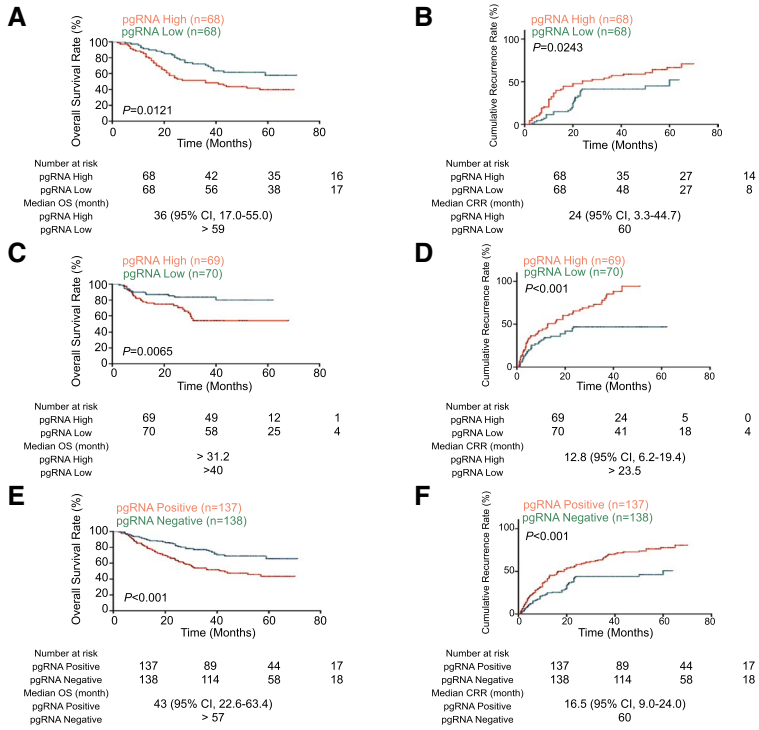


FIG. 1. Serum HBV-pgRNA serves as a prognostic factor for HCC patients with undetectable serum HBV-DNA. High serum pgRNA expression group had a worse OS (A) and CRR (B) than the low serum pgRNA expression group from patients in cohort A (n = 136). OS (C) and CRR (D) curves from cohort B (n = 139). OS (E) and CRR (F) curves based on positive serum pgRNA of the patients from two cohorts. The Kaplan-Meier method and log-rank test were used.

the percentage of cells in the S phase (Supporting Fig. S2A-C) and an increasing percentage of apoptotic cells after pgRNA knockdown (Supporting Fig. S2D-F). Sphere formation ability and expression of well-proven liver stemness biomarkers (CD44, CD90, and CD133) were also decreased in pgRNA knockdown cells (Fig. 2D,E). Moreover, the limiting dilution assays *in vitro* and *in vivo* showed that the stemness and tumorigenicity of pgRNA knockdown cells were significantly down-regulated (Supporting Fig. S2G and Fig. 2F).[23] Furthermore, when representative tumors that had developed after inoculation of 1×10^6 cells were dissected and measured at termination, the volume and growth of tumor were found to be significantly inhibited by pgRNA knockdown (Fig. 2F).

Next, we succeeded in overexpressing pgRNA in HepG2.2.15 and Huh7 cell lines and further obtained stable pgRNA overexpression of HepG2.2.15 and Huh7 clones (Supporting Fig. S1E, F). Consistently, overexpression of pgRNA significantly promoted the proliferation and clone formation of HCC cells (Fig. 3A-C and Supporting Fig. S4A-E). The flow cytometry analysis revealed the inhibition of apoptosis (Supporting Figs. S3A-C and S5A-C) and the increased proportions of cells in the S phase (Supporting Figs. S3D-F and S5D-F) after overexpressing pgRNA. Similarly, sphere formation ability and expression of stemness biomarkers were also up-regulated when overexpressing pgRNA (Fig. 3D,E and Supporting Fig. S4F-H). Stemness, tumorigenicity, and growth of representative tumors were also significantly up-regulated after pgRNA overexpression (Fig. 3F and Supporting Figs. S3G, S5G, and S4I-K).

To further confirm that proliferation, stemness, and tumorigenicity of HCC cells are regulated by pgRNA, rather than HBV other proteins, we determined the expression of HBV X protein, HBsAg, and HBeAg in pgRNA knockdown or overexpression cells. The result showed that the expression of these proteins was not significantly affected (Supporting Fig. S5H-L).

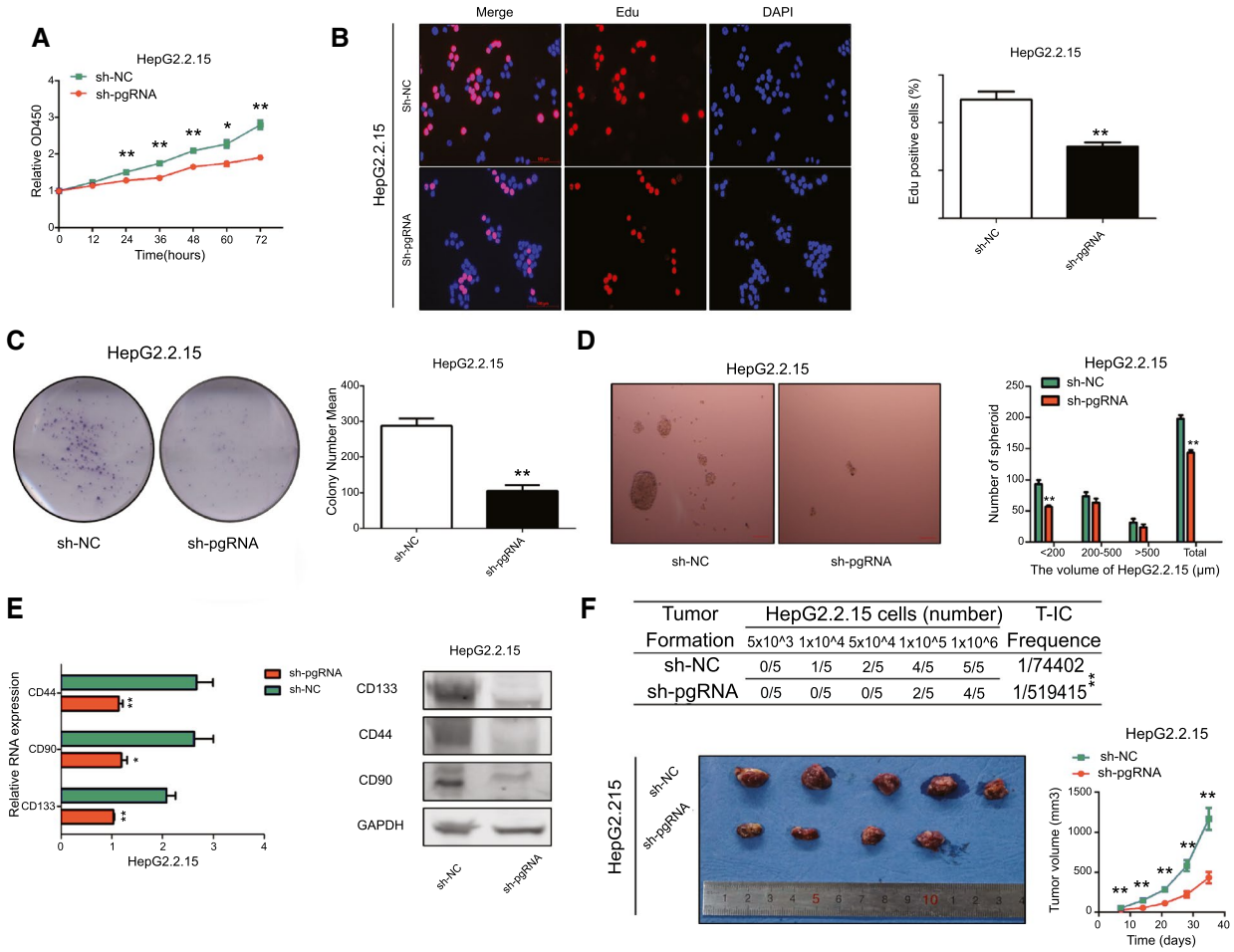


FIG. 2. Knockdown of pgRNA inhibits the proliferation, stemness, and tumorigenicity of HCC cells *in vitro* and *in vivo*. CCK-8 assays (A), EdU staining (B), and the clone formation experiment (C) were performed to measure cell viability. Blue fluorescence represents DAPI staining, and red represents EdU staining. Scale bar, 100 μm. (D) Stemness of HCC cells was measured by spheroid formation assays. Scale bar, 100 μm. (E) Expression of CD44, CD90, and CD133 was examined by RT-qPCR and western blot. (F) Stemness, tumorigenicity, and growth of pgRNA knockdown cells were performed by limiting dilution assays *in vivo*. Tumor volume was calculated as $1/2 \times A \times B^2$ (A, longest diameter; B, shortest diameter). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; OD450, optical density at 450 nm.

IGF2BP3 SPECIFICALLY BINDS TO pgRNA AND ENHANCES ITS STABILITY IN A m6A-INDEPENDENT MANNER

As reported before, various kinds of proteins, such as transcription factors, splicing factors, and RNA binding proteins, could bind to the mRNAs of HBV, regulating viral replication.[21,24,25] Subsequently, proteins interacting with HBV-pgRNA were detected by RNA pull-down assays, followed by silver staining and mass spectrometry analysis. The result from mass spectrometry analysis showed a series of proteins (Supporting Table S9), and among the top of the list we found IGF2BP3, which was closely related to HCC stemness (Fig. 4A). Furthermore, using western blotting, specific interaction of IGF2BP3 was confirmed in the pull-down extract of pgRNA (Fig. 4B). Additionally, the result of the RIP assay showed a significantly higher enrichment of pgRNA with the IGF2BP3 antibody (Fig. 4C). Evidence of intracellular colocalization was also obtained through immunofluorescence staining of IGF2BP3 and fluorescence *in situ* hybridization (FISH) against pgRNA by confocal microscopy in HepG2.2.15 cells (Fig. 4D). These results suggested that HBV-pgRNA is indeed specifically associated with IGF2BP3.

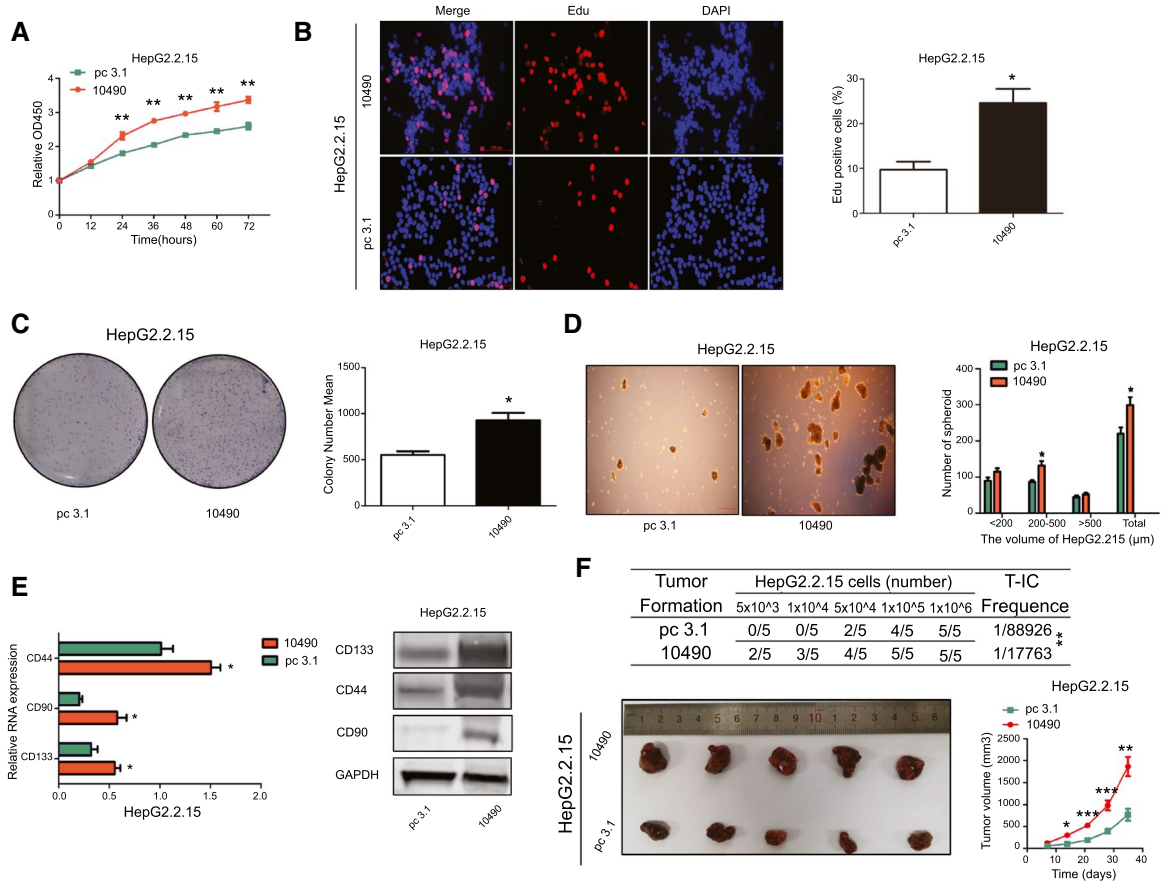


FIG. 3. Overexpressing pgRNA increases the proliferation, stemness, and tumorigenicity of HCC cells *in vitro* and *in vivo*. CCK-8 assays (A), EdU staining (B), and the clone formation experiment (C) were performed to measure cell viability. Scale bar, 100 μm. (D) Stemness of HCC cells was measured by spheroid formation assays. Scale bar, 100 μm. (E) Expression of CD44, CD90, and CD133 is detected by RT-qPCR and western blotting. (F) Stemness, tumorigenicity, and growth of pgRNA-overexpressing cells were performed by limiting dilution assays *in vivo*. Tumor volume was calculated as $1/2 \times A \times B^2$ (A, longest diameter; B, shortest diameter). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; OD450, optical density at 450 nm.

m6A RNA modification plays a vital role in regulating the life cycle of HBV.[26] Given that IGF2BP3 was reported to recognize m6A-modified mRNAs and promote the stability of potential mRNAs in an m6A-dependent manner,[22] we speculated on whether IGF2BP3 would bind to pgRNA by recognizing its m6A consensus motif. To further confirm this hypothesis, we made certain mutations of the m6A consensus motifs in pgRNA and repeated RNA pull-down and western blotting assays. Surprisingly, the specific interaction of IGF2BP3 in each of these constructs had no difference, compared to the wild-type plasmid (Fig. 4E). Given that IGF2BP3 was reported to enhance the stabilization of targeting encoding mRNAs,[27] we subsequently tested the effect of IGF2BP3 on the stability of pgRNA. Interestingly, we observed a corresponding higher expression of pgRNA in the IGF2BP3 overexpression cells (Supporting Fig. S7A). The result of actinomycin D treatment also showed that IGF2BP3 overexpression increased the half-life of pgRNA from 6.013 to 15.01 hours (Fig. 4F). We next explored whether the stability of IGF2BP3 would be influenced after it bound to pgRNA, followed by cycloheximide treatment, and no remarkable changes of IGF2BP3 stability were observed after pgRNA overexpression (Supporting Fig. S8D).

To further verify the specific binding site of pgRNA and IGF2BP3, we predicted pgRNA structure using the

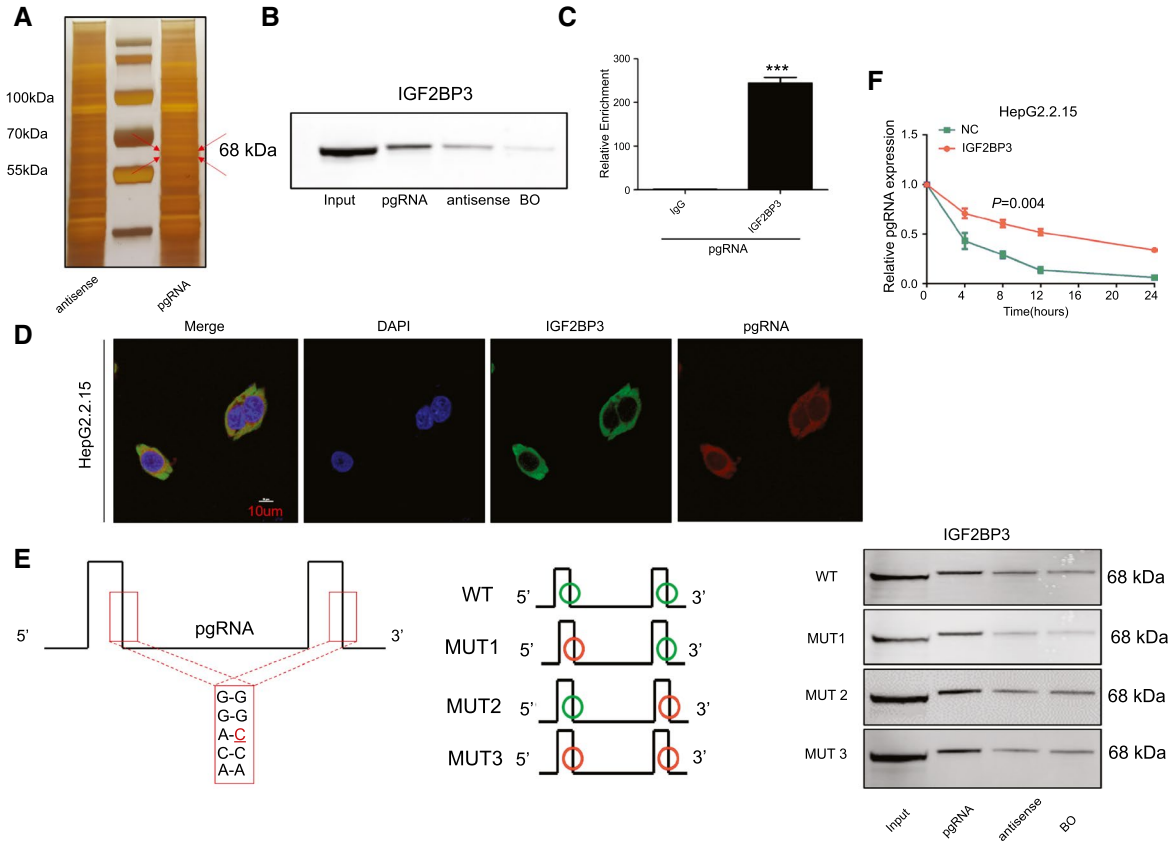


FIG. 4. IGF2BP3 specifically binds to HBV-pgRNA and regulates pgRNA expression through enhancing its stability in an m6A-independent manner. Silver staining (A) and western blotting (B) showed selective pulldown of IGF2BP3 protein (68 kDa) in the extract of pgRNA, but not in controls (using antisense of HBV-pgRNA and empty beads). (C) RIP assays were performed using an antibody against IGF2BP3 on extracts from HepG2.2.15 cells with IgG as a negative control. Enrichment of the pgRNA was normalized to the input. (D) Colocalization of IGF2BP3 (green) and pgRNA (red) was observed through immunofluorescence staining and FISH. Scale bar, 10 μm. (E) Schematics indicate the location of the mutations in pgRNA. Green circles indicate m6A RNA modification whereas red circles represent absence of m6A RNA modification (attributable to mutation) in pgRNA. Mutations of both 5′ and 3′ ends (MUT3), only 3′ ends (MUT2), or only 5′ (MUT1) ends of the m6A RNA modification motifs on pgRNA and their effect on the specific combination with IGF2BP3. (F) RT-qPCR analysis of the relative level of remaining pgRNA at indicated times following by 24 hours of actinomycin D treatment in IGF2BP3-overexpressed HepG2.2.15 cells (15.01 ± 1.183 hours, IGF2BP3; 6.013 ± 1.009 hours, NC). Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; WT, wild type.

RNA Structure (version 5.3). According to the predicted pgRNA structure, we found that the secondary structure of pgRNA mainly existed in the 3′ terminal. So, we truncated pgRNA (S1; 0.0-3.5 kb) into fragments S2 (0.0-3.0 kb), S3 (0.0-2.5 kb), and S4 (0.0-2.0 kb) and repeated RIP assay. Our results showed a significantly higher enrichment of S1 fragment with the IGF2BP3 antibody. However, when we truncated S1 fragment into shorter S2, S3, or S4 fragments, the binding ability of these fragments to IGF2BP3 was significantly decreased, which revealed that the 3,000- to 3,326-nucleotide (nt) fragment at the 3′ end of pgRNA might mediate binding to IGF2BP3 (Supporting Fig. S8A-C).

To sum up, our data revealed that IGF2BP3 specifically binds to pgRNA and enhances its stability in an m6A-independent manner.

HBV-pgRNA FUNCTIONS AS A SPONGE FOR miR-let-7e-5p, UP-REGULATING IGF2BP3 EXPRESSION AT THE POSTTRANSCRIPTIONAL LEVEL

Next, we sought to determine the functional association between pgRNA and IGF2BP3 and analyzed the correlation between their expression. The

result illustrated the positive correlation between pgRNA and IGF2BP3 in pgRNA knockdown cells (Supporting Fig. S7B), pgRNA overexpressing clones (Supporting Fig. S7C,D), and in pgRNA-xenograft tumor (Supporting Fig. S7E,F; $r = 0.757$; $P < 0.001$), as well as in patients' NT tissues of cohort A (Supporting Fig. S7G; n = 107; $r = 0.3106$; $P = 0.0011$). These data together indicated that pgRNA might contribute to IGF2BP3 up-regulation at the transcriptional or posttranscriptional level.

We next investigated whether pgRNA up-regulates the expression of IGF2BP3 at the transcriptional level. The result of dual-luciferase reporter assays showed no significant change of luciferase activity between pgRNA overexpression and control group (Fig. 5A). Given that HBV-pgRNA has been reported to

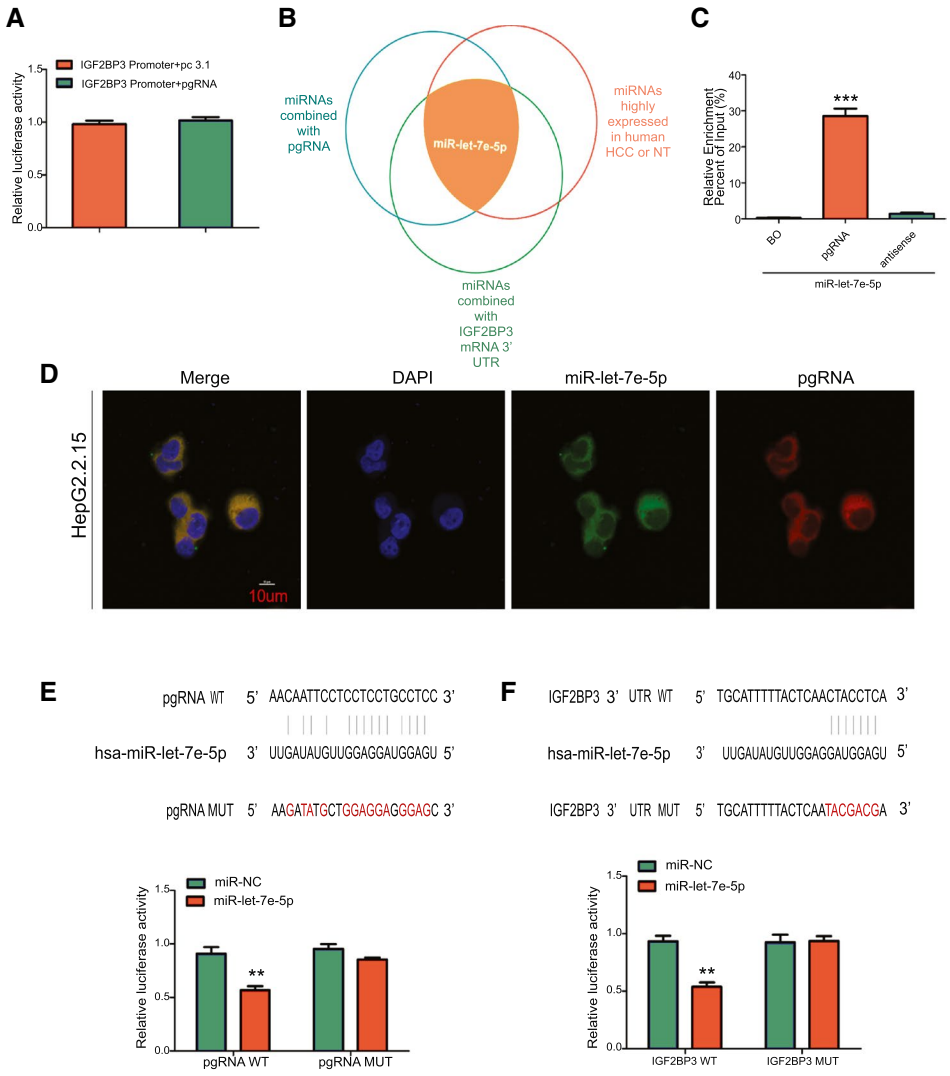


FIG. 5. HBV-pgRNA functions as a sponge for miR-let-7e-5p and up-regulates IGF2BP3 expression at the posttranscriptional level. (A) The luciferase activity of LUC-*IGF2BP3* promoter in HEK293T cells after cotransfection with pgRNA full-length or pcDNA3.1 vector. (B) Identification of potential miRNA by miRNA target prediction tools (miRanda, PITA, RNAhybrid, and TargetScan) and microarray analysis (expression of miRNAs between paired T and NT tissues). (C) Confirmation of pgRNA-specific binding miR-let-7e-5p by RNA pull-down assays. (D) Colocalization of miR-let-7e-5p (green) and pgRNA (red) was observed through double FISH assays in HepG2.2.15 cells. Scale bar, 10 μm. (E) Relative luciferase activity of LUC-pgRNA-WT or LUC-pgRNA-MUT in HEK293T cells after cotransfection with miR-NC or miR-let-7e-5p. (F) Relative luciferase activity of LUC-*IGF2BP3*-5'-UTR-MUT-WT or LUC-*IGF2BP3*-5'-UTR-MUT in HEK293T cells after cotransfection with miR-NC or miR-let-7e-5p. Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; NT, nontumor; T, tumor; WT, wild type.

promote the carcinogenesis of HCC by the sequestration of host miRNAs as an miRNA sponge,[8] we next explored whether pgRNA could combine with certain miRNAs, regulating IGF2BP3 expression at the post-transcriptional level. Using miRNA target prediction tools miRanda, PITA, and RNAhybrid, 292 repeated candidates that could bind to pgRNA were found, and 74 of these miRNAs are highly expressed in human HCC and NT tissues according to a recent genome-wide study (GSE76903). We subsequently used Targetscan (http://www.targetscan.org) to predict highly conserved miRNAs targeting IGF2BP3/3'-untranslated region (3'-UTR), and screened out 12 miRNAs that met the condition of context ++ score percentile >90 and Pct >0.9; then, these 12 miRNAs were further overlapped with the 74 miRNAs selected above. Finally, we identified hsa-miR-let-7e-5p, because it could not only be sponged by pgRNA, but also combined to *IGF2BP3* 3'-UTR with high stringency (Fig. 5B). By RNA pull-down assays, double FISH assays, and dual-luciferase reporter assays, miR-let-7e-5p was found to selectively bind to pgRNA (Fig. 5C-E). Moreover, dual-luciferase reporter assays (Fig. 5F) and the transfection of miR-let-7e-5p mimics or inhibitor (Supporting Fig. S9A-F) further confirmed the specific combination of miR-let-7e-5p and *IGF2BP3* 3'-UTR. However, the expression of miR-let-7e-5p showed no significant changes when silencing or overexpressing pgRNA (Supporting Fig. S9G-I).

In summary, we found that HBV-pgRNA may function as a sponge for miR-let-7e-5p, up-regulating IGF2BP3 expression at the post-transcriptional level.

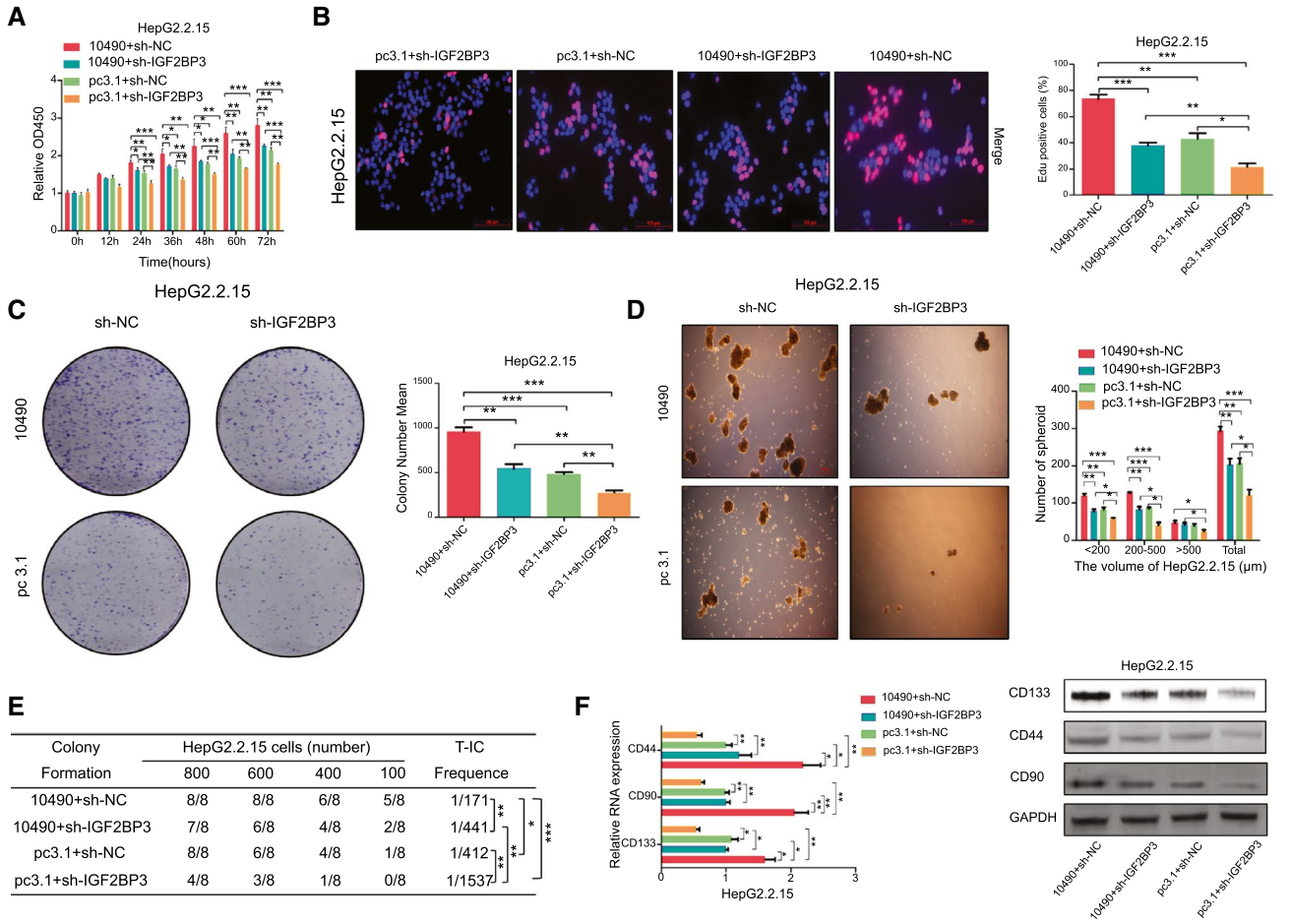
HBV-pgRNA PROMOTES PROLIFERATION, STEMNESS, AND TUMORIGENICITY OF HCC CELLS THROUGH UP-REGULATING IGF2BP3

IGF2BP3 is the main form of IGF2BPs family highly expressed in human cancer. Recent studies have provided functional evidence for the carcinogenic role of IGF2BP3 in HCC.[22,28] Given that the promotion of proliferation, stemness, and tumorigenicity of HCC cells caused by pgRNA might be dependent on up-regulating of IGF2BP3, we subsequently knocked down the expression of IGF2BP3 in pgRNA overexpression clones to perform a rescue experiment. The results showed that silencing IGF2BP3 could significantly suppress the expression of IGF2BP3, whereas the suppression was retarded after overexpression of pgRNA (Supporting Fig. S10). The Cell Counting Kit-8 (CCK-8) assays, 5-ethynyl-2′-deoxyuridine (EdU) incorporation assays, and clone formation assays showed that the increased proliferation and clone formation ability of pgRNA overexpression clones was reversed by IGF2BP3 knockdown (Fig. 6A-C and Supporting Fig. S11A-E). Effects of pgRNA overexpression on apoptosis and cell cycle were also reversed when silencing IGF2BP3 (Supporting Figs. S12 and S13). Furthermore, the enhanced sphere formation ability and augmented expression of stemness markers observed in pgRNA overexpression clones were also abrogated by knockdown of IGF2BP3 (Fig. 6D-F and Supporting Fig. S11F-I). To further prove that pgRNA, rather than other HBV mRNAs, is involved in the regulation of IGF2BP3, we transfected other lengths of HBV transcripts (2.4, 2.1, and 0.7 kb) into HepG2.2.15 and Huh7 cells and detected their expression of IGF2BP3. The result illustrated that no significant changes were detected (Supporting Fig. S6).

Collectively, our results showed that pgRNA promoted proliferation, stemness, and tumorigenicity of HCC through up-regulating IGF2BP3. The HBV-pgRNA-IGF2BP3 axis plays an essential role in the development of HBV-related HCC.

IFN-α-2a DECREASES THE STABILITY OF pgRNA BY INCREASING ITS m6A RNA MODIFICATION AND FURTHER DOWN-REGULATES IGF2BP3 EXPRESSION, THUS INHIBITING THE DEVELOPMENT OF HCC

Currently, IFN treatment has been recognized as a vital antiviral strategy for CHB, with the function of enhancing immune clearing of HBV and inhibiting HBV directly. CHB patients under IFN-α-2a treatment had a better rate of viral inhibition, higher rates of HBeAg disappearance and serological conversion, and a lower HBV recurrence rate after drug discontinuation.[29] It has also been reported that postoperative



Colony Formation	HepG2.2.15 cells (number)				T-IC Frequence
	800	600	400	100	
10490+sh-NC	8/8	8/8	6/8	5/8	1/171
10490+sh-IGF2BP3	7/8	6/8	4/8	2/8	1/441
pc3.1+sh-NC	8/8	6/8	4/8	1/8	1/412
pc3.1+sh-IGF2BP3	4/8	3/8	1/8	0/8	1/1537

FIG. 6. Knockdown of IGF2BP3 counteracts the promotion effects of pgRNA overexpression on HCC cells. Cell viability was measured using CCK-8 assays (A), EdU staining (B), and the clone formation experiment (C). Scale bar, 100 μm. Stemness of HCC cells was measured by spheroid formation assays (D) and limiting dilution assays (E). Scale bar, 100 μm. (F) Expression of liver stemness biomarkers (CD44, CD90, and CD133) is examined by RT-qPCR and western blotting. Abbreviations: GAPDH, glyceraldehyde 3-phosphate dehydrogenase; OD450, optical density at 450 nm.

adjuvant IFN therapy postponed the recurrence of HBV-related HCC.[4] However, the mechanism of how IFN-α-2a prevents recurrence of HCC remains open for study. Therefore, we next investigated whether IFN-α-2a prevents the recurrence of HCC by inhibiting the pgRNA-IGF2BP3 axis.

We first examined the impact of IFN-α-2a treatment on HCC cells. HepG2.2.15 cells were treated with the half-inhibitory concentration of IFN-α-2a against it (2,000 IU/mL; Supporting Fig. S14A), and the expression of pgRNA and IGF2BP3 was qualified. The results suggested that IFN-α-2a significantly down-regulated the expression of pgRNA and IGF2BP3 after IFN-α-2a treatment (Fig. 7A). However, the expression of IGF2BP3 in HepG2 cells (cells with the same genetic background as HepG2.2.15 cells, but not expressing HBV) was not affected after IFN-α-2a treatment (Fig. 7B), which reflected that the down-regulation of IGF2BP3 was attributable to the decreased expression of pgRNA caused by IFN-α-2a treatment. Additionally, when treating pgRNA overexpression clones with IFN-α-2a, we found that the inhibitory effect of IFN-α-2a on the proliferation, stemness, apoptosis, cycle, and expression of IGF2BP3 of HepG2.2.15 cells can be partially restored by overexpressing pgRNA (Supporting Figs. S14 and S15). In summary, our data illustrated that IFN-α-2a decreases the expression of the pgRNA-IGF2BP3 axis, thus inhibiting the development of HCC.

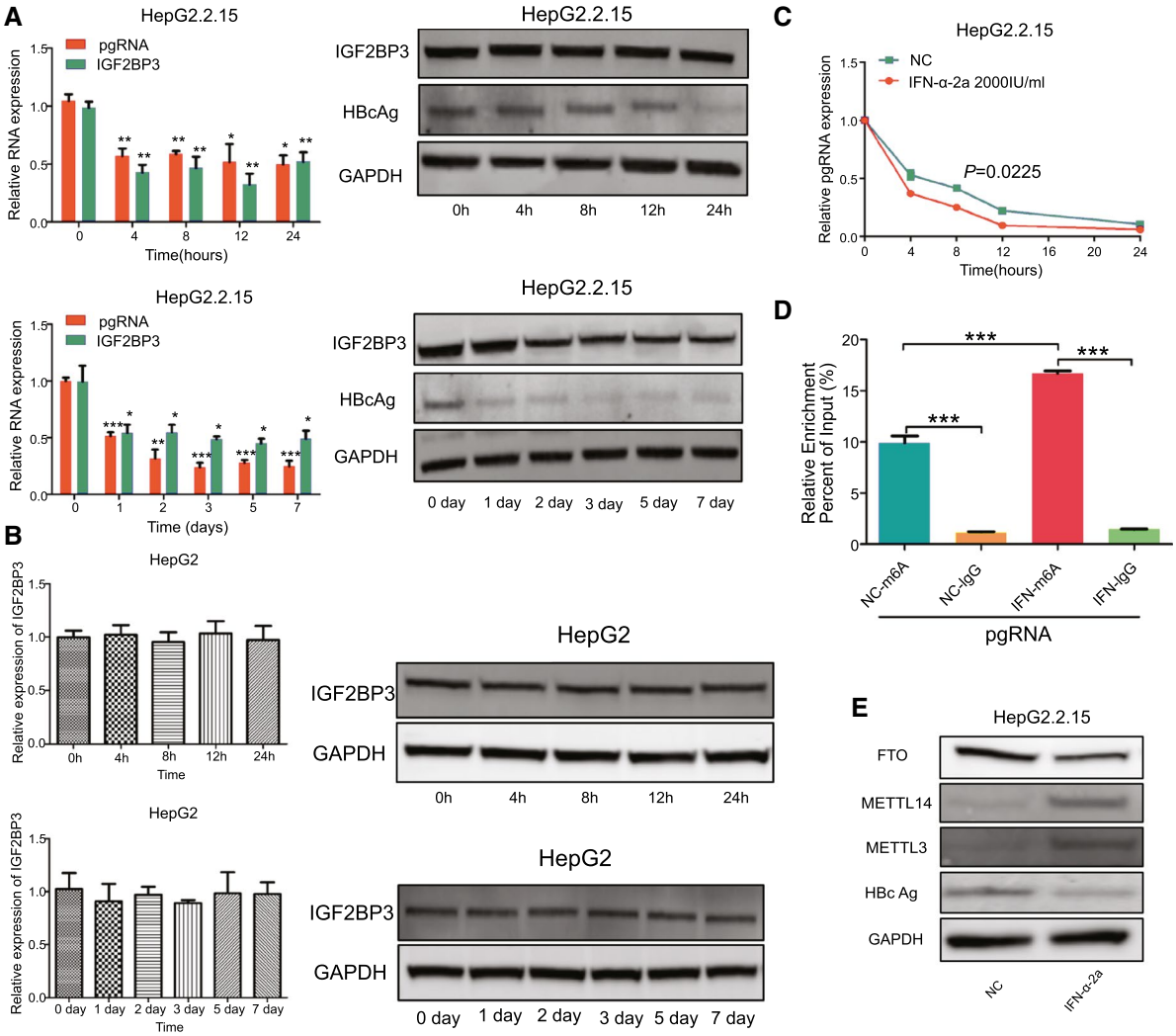


FIG. 7. IFN-α-2a decreases the stability of pgRNA by increasing its m6A RNA modification and further down-regulates the expression of pgRNA-IGF2BP3 axis, thus inhibiting the development of HCC. (A) Expression of pgRNA and IGF2BP3 in HepG2.2.15 cells was analyzed by RT-qPCR and western blotting after IFN-α-2a treatment for the indicated time. (B) Expression of IGF2BP3 in HepG2 cells was analyzed by RT-qPCR and western blotting after IFN-α-2a treatment for the indicated time. (C) RT-qPCR analysis of the relative level of remaining pgRNA at indicated times following 24 hours of actinomycin D and IFN-α-2a treatment on HepG2.2.15 cells. (D) Enrichment of m6A RNA modification in pgRNA after 48 hours of IFN-α-2a treatment was detected by a gene-specific m6A qPCR assay. (E) Expression of HBcAg, METTL3, METTL14, and FTO was analyzed by western blotting after IFN-α-2a treatment for 48 hours. Abbreviation: GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Next, the potential mechanism of IFN-α-2a to inhibit pgRNA was further explored. Actinomycin D transcriptional inhibition assays were performed, and the declining stability of pgRNA was observed after IFN-α-2a treatment (Fig. 7C). Because it has been reported recently that m6A RNA modification can lower the stability of HBV-RNA transcripts, we subsequently investigated the effect of IFN-α-2a treatment on m6A RNA modification of pgRNA through a methylated RNA immunoprecipitation assay, followed by an RT-qPCR test using primers that recognize a shared sequence presented in pgRNA.[26] Furthermore, increase of m6A RNA modification of pgRNA after IFN-α-2a treatment was observed (Fig. 7D). Next, the expression of methyltransferase-like protein 3 (METTL3), methyltransferase-like protein 14 (METTL14), and fat mass and obesity-associated protein (FTO) in HepG2.2.15 cells after IFN-α-2a treatment was further examined. The results showed a significant

up-regulation of METTL3 and METTL14, but an obvious decreasing of FTO, in IFN-α-2a treated HepG2.2.15 cells (Fig. 7E), which suggests that the increase of m6A RNA modification after IFN-α-2a treatment might be regulated by METTL3, METTL14, and FTO.

In conclusion, our result indicated that IFN-α-2a could degrade the stability of pgRNA through up-regulating its m6A RNA modification level, thus reversing its promotion effect of HCC cells by inhibiting the pgRNA-IGF2BP3 axis.

Discussion

HBV-pgRNA plays an important role in the life cycle of HBV. Given that HBV-pgRNA carries genetic information and the quantity of serum pgRNA is available, more and more studies in recent years have focused on pgRNA. Serum pgRNA has been considered as a biomarker for cccDNA, and it was also reported that the level of serum pgRNA could not only reflect the intrahepatic viral transcriptional activity, but also be associated with the inflammation and fibrosis degree of liver, even in patients under long-term NA therapy whose viral replication is suppressed.[9,10] Furthermore, serum pgRNA has also been reported as a potential biomarker for predicting NA antiviral treatment response, guiding the discontinuation of NA therapy,[8] and monitoring viral mutations.[11]

In this study, we aimed to investigate the clinical significance of serum pgRNA and further explored the effect of pgRNA in the development of HCC in patients who had received long-term NA therapy before surgery with undetectable serum HBV-DNA. The data showed that for most of the long-term NA-treated patients with their viral replication effectively suppressed, serum pgRNA remained detectable and was correlated with intrahepatic pgRNA level. Furthermore, high or positive serum pgRNA expression was significantly associated with poorer prognosis and was an independent predictor for the recurrence of HBV-related HCC patients from two independent cohorts.

Our data illustrated that the promoting effect of pgRNA in the development of HBV-related HCC and the mechanism of pgRNA to the promotion of HCC might be dependent on IGF2BP3. We uncovered the reciprocally regulatory relationship between pgRNA and IGF2BP3: On one hand, IGF2BP3 could specifically combine with pgRNA and increase its stability; on the other hand, pgRNA could work as a sponge absorbing miR-let-7e-5p, up-regulating IGF2BP3 expression at the posttranscriptional level. Human IGF2BP3 has been reported to enhance the development of HCC with the ability of recognizing m6A-modified mRNAs and stabilizing the potential mRNAs of indicated target genes in an m6A-dependent manner.[22] Interestingly, our data showed that the stabilizing function of pgRNA by IGF2BP3 was not in an m6A-dependent manner. Furthermore, the result of the RIP assay revealed that the 3,000- to 3,326-nt fragment at the 3' end of pgRNA might mediate binding to IGF2BP3. So, we illustrated a mechanism for the combination of IGF2BP3 with certain mRNA in addition to its recognition of m6A RNA modification.

Recently, suggestions have proposed that for CHB patients who received long-term NA therapy with undetectable serum HBV-DNA while positive serum pgRNA, switching from NAs to IFN-α or combination of NAs and IFN-α might reduce the risk of HBV viral rebound after withdrawal of NA treatment, given that IFN-α has been identified to decrease the transcription of HBV-pgRNA.[9,29] Furthermore, many randomized control trials have demonstrated that adjuvant IFN-α treatment for HBV-related HCC patients following curative resection reduces the rate of recurrence and improves their OS.[17] However, the mechanism is still unclear. In our study, we found that IFN-α-2a could increase the m6A RNA modification of pgRNA, thereby promoting its degradation, and the prevention of HCC recurrence by IFN-α is owing to, at least partly, its inhibition function of the pgRNA-IGF2BP3 axis.

In conclusion, our study revealed that serum pgRNA could serve as a noninvasive biomarker for prognosis judgement and recurrence prediction of HCC in patients who received long-term NA therapy with undetectable serum HBV-DNA. Furthermore, we initially identified that the pgRNA-IGF2BP3 axis plays an important role in the development of HBV-related HCC, which may shed light on the molecular mechanisms of HBV contributing to HCC development. We also illustrated that IFN-α-2a could down-regulate the expression of pgRNA by reducing its stability through increasing its m6A RNA modification

and preventing the development of HCC by inhibiting the pgRNA-IGF2BP3 axis. Therefore, for those patients who have undergone long-term NAs therapy with undetectable serum HBV-DNA but positive serum pgRNA, switching to or combining with IFN-α-2a may prevent the recurrence, and improve the long-term survival of, these HCC patients. However, problems are still in evidence. First, our study focuses on the stemness of HCC cells through increased IGF2BP3 expression regulated by pgRNA, thus promoting the development of HCC. The role of other HBV mRNAs and proteins in promoting the progression of HCC was not excluded. Other HBV mRNAs and proteins, especially HBcAg, may also promote the development of HCC, which needs further research to prove. Second, multicentered, large-scale, cohort, randomized controlled trials are needed to further testify on the feasibility of pgRNA as a biomarker for prognosis judgement and the validity of IFN-α-2a treatment of HBV-related HCC.

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Supporting Information

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