Original Article

Hepatitis B Virus Surface Antigen Promotes Stemness of Hepatocellular Carcinoma through Regulating MicroRNA-203a

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Abstract

Background and Aims: Patients with persistent positive hepatitis B surface antigen (HBsAg), even with a low HBV-DNA load, have a higher risk of hepatocellular carcinoma (HCC) than those without HBV infection. Given that tumor stemness has a critical role in the occurrence and maintenance of neoplasms, this study aimed to explore whether HBsAg affects biological function and stemness of HCC by regulating microRNA, and to explore underlying mechanisms. ***Methods:*** We screened out miR-203a, the most significant down-regulated microRNA in the microarray analysis of HBsAg-positive samples and focused on that miRNA in the ensuing study. *In vitro* and *in vivo* functional experiments were performed to assess its regulatory function. The effect of miR-203a on stemness and the possible correlation with BMI1 were analyzed in this study. ***Results:*** MiR-203a was significantly down-regulated in HBsAg-positive HCC with the sharpest decrease shown in microarray analysis. The negative correlation between miR-203a and HBsAg expression was confirmed by quantitative real-time PCR after stimulation or overexpression/knockdown of HBsAg in cells. We demonstrated the function of miR-203a in inhibiting HCC cell proliferation, migration, clonogenic capacity, and tumor development *in vivo*. Furthermore, the overexpression of miR-203a remarkably increases the sensitivity of tumor cells to 5-FU treatment and decreases the proportion of HCC cells with stem markers. In concordance with our study, the survival analysis of both The Cancer Genome Atlas database and samples in our center indicated a worse prognosis in patients with low level of miR-203a. We also found that *BMI1*, a gene maintains the self-renewal capacity of stem cells, showed a significant negative correlation with miR-203a in HCC specimen ($p<0.001$). Similarly, opposite *BMI1* changes after overexpression/knockdown of miR-203a were also confirmed *in vitro*. Dual luciferase reporting assay suggested that miR-203a may regulate *BMI1* expression by direct binding. ***Conclusions:*** HBsAg may promote the development of HCC and tumor stemness by inhibiting miR-203a, resulting in poor prognosis. miR-203a may serve as a crucial treatment target in HBsAg-positive HCC. More explicit mechanistic studies and animal experiments need to be conducted as a next step.

Introduction

Hepatocellular carcinoma (HCC) is the most common fatal primary liver cancer.[1] As in many other solid tumors, cancer stem cells (CSCs) have an essential role in the progression of HCC.[2,3] According to CSC hypothesis, a minority cell population in cancer with the property of extensive self-renewal contributes to tumor growth and heterogeneity. With regard to HCC, CSCs are identified by canonical cell surface markers including CD133, CD90, CD44, *BMI1*, EpCAM and oval cell marker OV6, etc.[4–6] HBV infection is a critical risk factor for liver cirrhosis and HCC,[7] as it accounts for about 80% of all HCC cases worldwide and increases the risk of HCC approximately by 20 times.[8,9] Although HBV infections are effectively controlled by long-term nucleoside analog therapy, they are a public health problem in HCC carcinogenesis, with risk paralleled by the HBV virus burden.[10] It has been reported that patients with residual hepatitis B virus surface antigen (HBsAg) titers higher than 1,000 IU/mL are much more likely to suffer from HCC.[11] However, the exact influence of HBsAg on HCC is not well documented.

Chisari *et al*.[12] reported a greater probability of HCC in HBsAg transgenic mice. Recently, we found that the stem cell marker, oncogene B cell-specific Moloney murine leukemia

virus integration site 1 (*BMI1*) was overexpressed in HBsAg transgenic mouse liver and HBsAg-positive human HCC tissue.[13] We suspected that *BMI1* be an underlying mechanism in HBsAg-induced HCC, as *BMI1* is an important cofactor of polycomb repressive complex 1 associated with cell cycle regulation, cell apoptosis, and maintenance of stem cell self-renewal.[14] Increased expression of *BMI1* was observed to maintain the tumor-initiating ability of human HCC,[15–18] consistent with our findings in HCC with bile duct tumor thrombi.[19] We also demonstrated that *BMI1* knockdown decreased proliferation, colony formation, and invasiveness of human HCC cells *in vitro* and significantly increased its chemosensitivity.[20,21] Most importantly, we showed that forced expression of *BMI1* promoted the malignant transformation of rat liver progenitor cells into liver CSCs.[22,23] The findings indicated the importance of *BMI1* in the maintenance of stemness and the potential role in initiating cancer.

Micro RNAs (miRNAs) are short noncoding RNAs with 18–22 nucleotides that regulate gene expression by interfering with endogenous RNA machinery.[24] Cumulative evidence has demonstrated that miRNAs can function as tumor promoters or suppressors, and regulate biological processes including apoptosis, invasion, proliferation, and stemness.[25–27] This study investigated the biological function of miRNAs in HCC with high HBsAg titers.

Methods

Patients and tissue specimens

A cohort of 55 paired frozen liver tumor and normal adjacent tissues four fresh tumor tissues were obtained from HCC patients undergoing surgical resection at Sun Yat-sen Memorial Hospital. Patients who had received radiation therapy or chemotherapy prior to radical tumor resection were excluded. The difference and significance of HBsAg, miR-203a, and BMI1 expression in these patients were investigated. Routine histological examination of hematoxylin and eosin stained tissue confirm the HCC diagnosis.

Microarrays

Four fresh HCC tissues with or without HBV were obtained from HCC patients undergoing surgical resection at Sun Yat-sen Memorial Hospital. Total RNA was extracted with TRIzol reagent (TaKaRa, Shiga, Japan). RNA was purified with mir-Vana miRNA isolation kits (Ambion, Austin, TX, USA), tailed with polyadenosine-acidified polymerase, combined with biotinized DNA dendritic polymers, and then hybridized to Affymetrix GeneChip miRNA arrays using FlashTag Biotin RNA labeling kits (Genisphere, Hatfield, PA, USA). We used an Affymetrix GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA, USA) to scan slides and the miRNA QC Tool to analyze miRNA data.

Cell lines and cell culture

Huh-7, HepG2, HepG2.2.15, HepG2.117 HCC cell lines stably transfected with HBV genome[13,28] and LO2 human liver cells were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's Modified Eagle Medium (Gibco, Shanghai, China) supplemented with 10% fetal bovine serum (Biological Industries, Beit HaEmek, Isreal) and 1% penicillin-streptomycin (New Cell & Molecular Biotech, Suzhou, China) at 37°C in a humidified incubator with 5%

CO_2. G418 was needed for stably screening of HepG2.2.15 and hygromycin was used for HepG2.117 culture.

HBV preparation and stimulation

We collected HBV-containing supernatants with 6% poly-ethylene glycol 8000 (Sigma, Darmstadt, Germany) precipitated at 4°C overnight, and concentrated by centrifugation at 12,000 *g* for 60 m at 4°C. The supernatants of HepG2 cells collected by the same procedure served as the non-particle control. HCC or LO2 cells were seeded in 24-well plates and incubated with HBV particles (MOI>1,000) for 24, 48, 72 h, and 4 days. At the end of the incubation, cells were further cultured in normal maintenance medium.

Exogenous HBsAg protein stimulation

We stimulated Huh7 and HepG2 cells 500 ng/mL and LO2 cells with 250 ng/mL recombinant HBsAg adr (HBS-875, PROSPEC, Israel) for 5 days, with daily change of the medium and addition of HBsAg adr to maintain the concentration. The procedures followed the supplier's instructions, using isovolumetric phosphate buffered saline (PBS) as the control. RNA was collected 5 days after stimulation for further use.

Transfection

HBsAg-encoded plasmid pCDNA-HBsAg and pCDNA3.1 vector, which were provided by Professor Mengji Lu, were transiently transfected into three cell lines (HepG2, Huh-7, and LO2).[29] SiRNA-HBsAg was obtained from GenePharma (Shanghai, China) and transiently transfected into HepG2.2.15 HCC cells transfected with the total HBV genome. Hsa-miR-203a-mimics (5'-GUGAAAUGUUUAGGACCACUAG-3'); hsa-miR-203a-inhibitors (5'-CUAGUGGUCCUAAACAUUUCAC-3'), were transiently transfected into two HCC cell lines (HepG2 and Huh-7) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) following the manufacturers' recommendations.[22] The cells were harvested 48 h after transfection for subsequent procedures.

Quantitative RT-PCR (qPCR)

Total RNA was extracted with RNAiso Plus reagent (TaKaRa). The primers are shown in Supplementary Table 1. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the housekeeping gene and relative expression was calculated with the $2^{-\Delta\Delta}$ cycle threshold methods. qPCR was performed following the manufacturer's recommendations as previously described.[23]

Western blotting

Cell pellets were lysed with RIPA buffer (Beyotime, Beijing, China). Protein concentration was determined using a bicinchoninic assay kit (Beyotime). Samples were denatured in 5× loading buffer at 95°C for 10 m. Proteins were separated by 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% dried skimmed milk in tris-buffered saline with Tween (TBST) at room temperature for 1 h and incubated with primary antibodies recognizing BMI1 (1:300) and GAPDH (1:1,000) overnight at 4°C. The membranes were incu-

bated with the appropriate HRP-conjugated secondary antibodies (1:5,000) next day after washed by TBST three times. The proteins were visualized with by enhanced chemiluminescence plus reagents (Beyotime). The primary and secondary antibodies are listed in Supplementary Table 2.

Cell proliferation assay

HCC cells were seeded into 96-well plates at the density of 2×10^3/well and four repetitive wells for each group. After 6–8 h the cells were treated with 10% cell counting kit-8 (CCK-8; Dojindo Molecular Technologies) in an incubator for 1 h. The absorbance at 450 nm was measured with an ELISA plate reader and cell growth (%) was calculated.

Colony formation assay

Suspensions of 1,000 HCC cells were seeded into six-well plate and incubated in complete medium, which was changed every 3 days. After 14 days, the cells were fixed with 10% formaldehyde and stained with 0.1% crystal violet for colony counting.

Cell invasion assay

An aliquot of 1×10^5 cells in 0.1 mL serum-free medium was placed in the upper chamber of 6.5 mm, 8 μm pore size polycarbonate membrane that was precoated with extracellular matrix gel (Corning, NY, USA). The lower chamber was loaded with 0.5 mL of medium containing 20% fetal bovine serum. The cells were fixed with 10% paraformaldehyde after 48 h incubation and then counterstained with 0.1% crystal violet. Cells migrating to the lower chamber were observed by light microscopy, and the number of migrating cells was calculated.

Flow cytometry

HCC cells were resuspended in PBS at a density of 1×10^7 cells/100 μL and stained with annexin V-FITC, propidium iodide-phycoerythrin (Abcam, Cambridge, UK), anti-human CD133-PE antibody and CD90-PE antibody (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), followed by incubation for 20 m on ice. The respective isotype controls were set up at the same concentrations. The cells were analyzed on by flow cytometry (FACSVerse; BD Biosciences, Franklin Lakes, NJ, USA).

Aldehyde dehydrogenase (ALDH) assay

ALDEFLUOR kits (Stem Cell Technologies, Vancouver, Canada) were used following the manufacturer's instructions. HCC cells were suspended in Aldefluor assay buffer containing ALDH substrate (BAAA, BODIPY amino acetaldehyde, 1 mmol/L) at 1×10^6 cells/mL for 30 m, with or without the specific ALDH inhibitor diethylamino benzaldehyde (1 mmol/L). DEAB was used as an internal negative control for each individual experiment to distinguish between high ALDH activity (ALDH positive) cells and cells with low ALDH activity (ALDH negative). Analysis and sorting were conducted fluorescence-activated cell sorting. Aldefluor was excited at 488 nm and fluorescence emission was detected at 530/30. The data were analyzed by Cell Quest Pro and FlowJo (Ashland, KY, USA).

Generation of stably transfected HCC cell lines for constitutive miR-203a expression

The HCC cell lines HepG2 and Huh7 cells were infected with lentiviruses and negative control purchased from GenePharma. Virus-containing medium was replaced with fresh culture medium after 12–24 h of infection. Virus-containing medium was replaced with fresh culture medium after 12–24 h of infection and then replaced again with fresh media containing puromycin after 48–96 h of infection to select for stably transfected cells. *BMI1* mRNA and protein expression were verified by qPCR and western blotting.

In vivo subcutaneous xenografts

All animal procedures were performed following protocols approved by the Institutional Animal Care and Use Committee at Sun Yat-sen University and were performed at Sun Yat-sen University (Guangzhou, China). Briefly, 5×10^6 HCC cells overexpressing miR-203a and control cells were suspended in 100 μL PBS-Matrigel (1:1) and injected subcutaneously into 3-4-week-old mice (Balb/c nu/nu), beginning 7 days after injection, tumor volumes were calculated every second day after measuring the length and width with calipers. Mice were sacrificed 5 weeks after injection, and the tumors were removed and measured. Volume in cm^3 was calculated as $(width^2)\times length/2$.

Luciferase reporter assay

The region of the human BMI13'-untranslated region (UTR; bases 8,334–10,276) contained three putative miR-203a-binding sites that were predicted by miRDB, miRmap, and TargetScan. A wildtype (WT) and four mutant (mut) 3'-UTR fragments of human BMI1 mRNA were amplified and subcloned to *Xho*I and *Not*I restrictive sites in the psiCHECK-2 vector (Applied Biosystems, Foster City, CA, USA), downstream of the fluorescent enzyme reporter gene. The primer sequences of BMI1 3'-UTR amplification are listed in Supplementary Table 3 (BMI1-3'-UTR-mut4, all three putative miR-203a-binding sites were mutated by double mutation). All clones were sequenced to verify the correctness of the nucleotide sequences. Luciferase activity was assayed with a dual luciferase reporter assay system (Promega, Madison, WI, USA).

Statistics

The statistical analysis was performed with SPSS 17.0 (SPSS, Chicago, IL, USA). *In vitro* data were analyzed with Student's *t*-tests; Mann-Whitney U-tests and log-rank tests were used to analyze *in vivo* data and clinical parameters. *P*-values <0.05 were considered statistically significant. All cell culture experiments were repeated at least three times independently, with three multiple wells at a time. The results were reported as means±SD.

Results

MiR-203a is down-regulated in HBsAg-positive HCC tissues

Genome-wide miRNA expression profiles of HBV-positive and HBV-negative HCC tissues were utilized to evaluate HBV-related miRNAs. Among the expression patterns, we
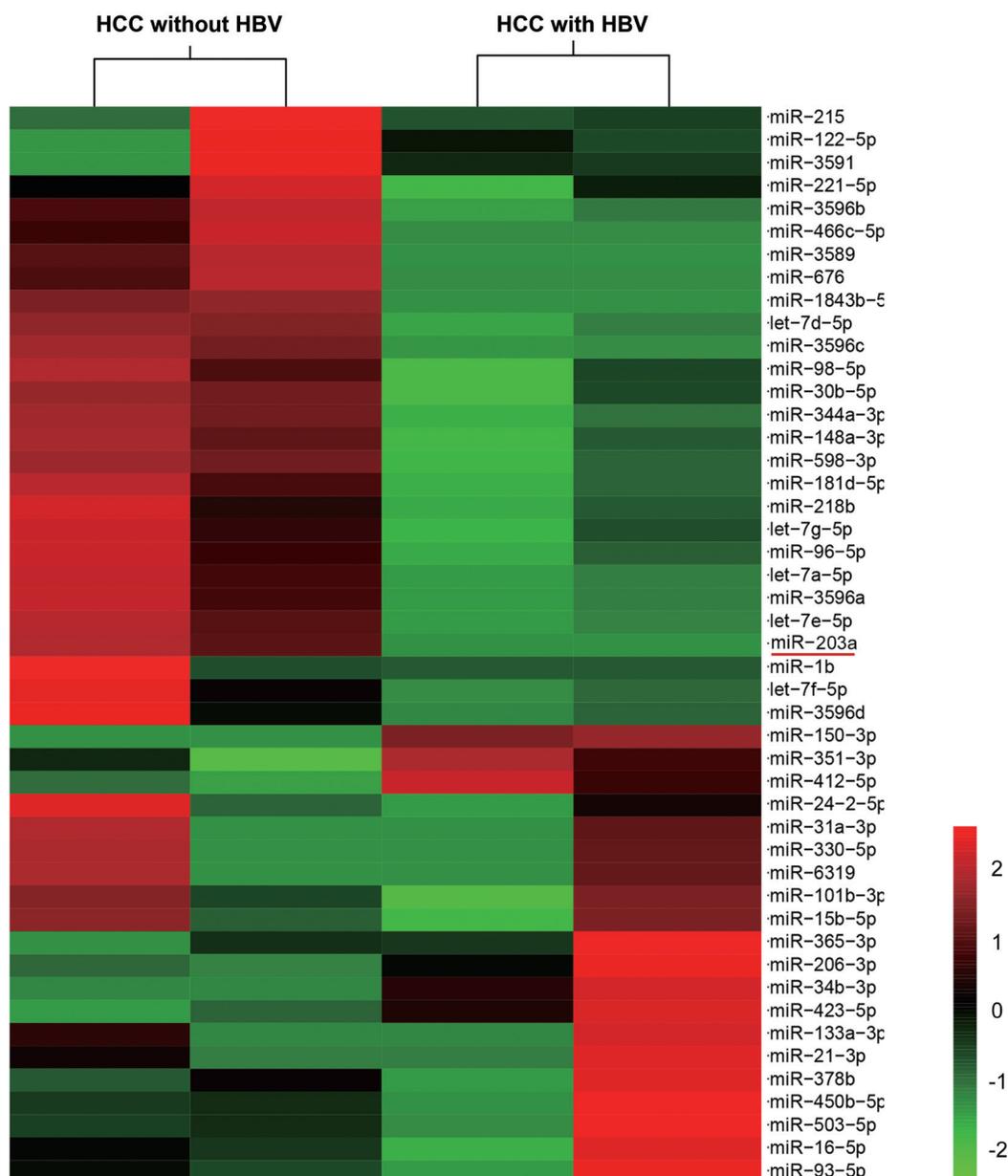
Fig. 1. The most upregulated/down-regulated microRNAs in HBV-positive and HBV-negative liver cancer tissues identified by microRNA microarray.

found miR-203a was significantly decreased in HBV-positive HCC tissues (Fig. 1). Further validation using qPCR confirmed the alteration of miR-203a in tissue specimens from 55 HCC patients. As shown in Figure 2A, miR-203a expression was significantly lower in HBV-positive than in HBV-negative tissues ($p<0.01$). We also found a negative correlation between HBsAg level and miR-203a expression in HBV-infected HCC tissues, which prompted exploration the underlying regulatory mechanism and the potential clinical transformation (Fig. 2B, $p=0.029$).

HBsAg decreases the expression of miR-203a in liver cells and HCC cells

qPCR confirmed that HBV inhibited the of miR-203a expres-

sion in LO2 and Huh7 and HepG2 HCC cells (Fig. 2C–E). Stimulation by exogenous HBsAg protein, and overexpression, and knockdown experiments showed that HBV down-regulated miR-203a through HBsAg in HCC. Five days after stimulation with recombinant HBsAg, qPCR revealed decreased miR-203a expression in LO2 ($p=0.0025$), Huh7 ($p=0.0043$), and HepG2 cells ($p<0.0001$, Fig. 2F–H). HBsAg-coding plasmid was transfected into LO2 liver cells and HepG2 and Huh-7 HCC cells. siRNA-HBsAg was transfected into HepG2.2.15 HBV-related HCC cells. qPCR results showed that miR-203a was down-regulated after transfection with HBsAg plasmid forced overexpression in both HCC cell lines and in liver cells (Fig. 2F). miR-203a was upregulated in HepG2.2.15 cells by transcription with HBsAg-siRNA, compared with the control (Fig. 2G). The findings indicate that miR-203a expression in HCC cells was decreased by HBsAg in vitro ($p<0.01$).
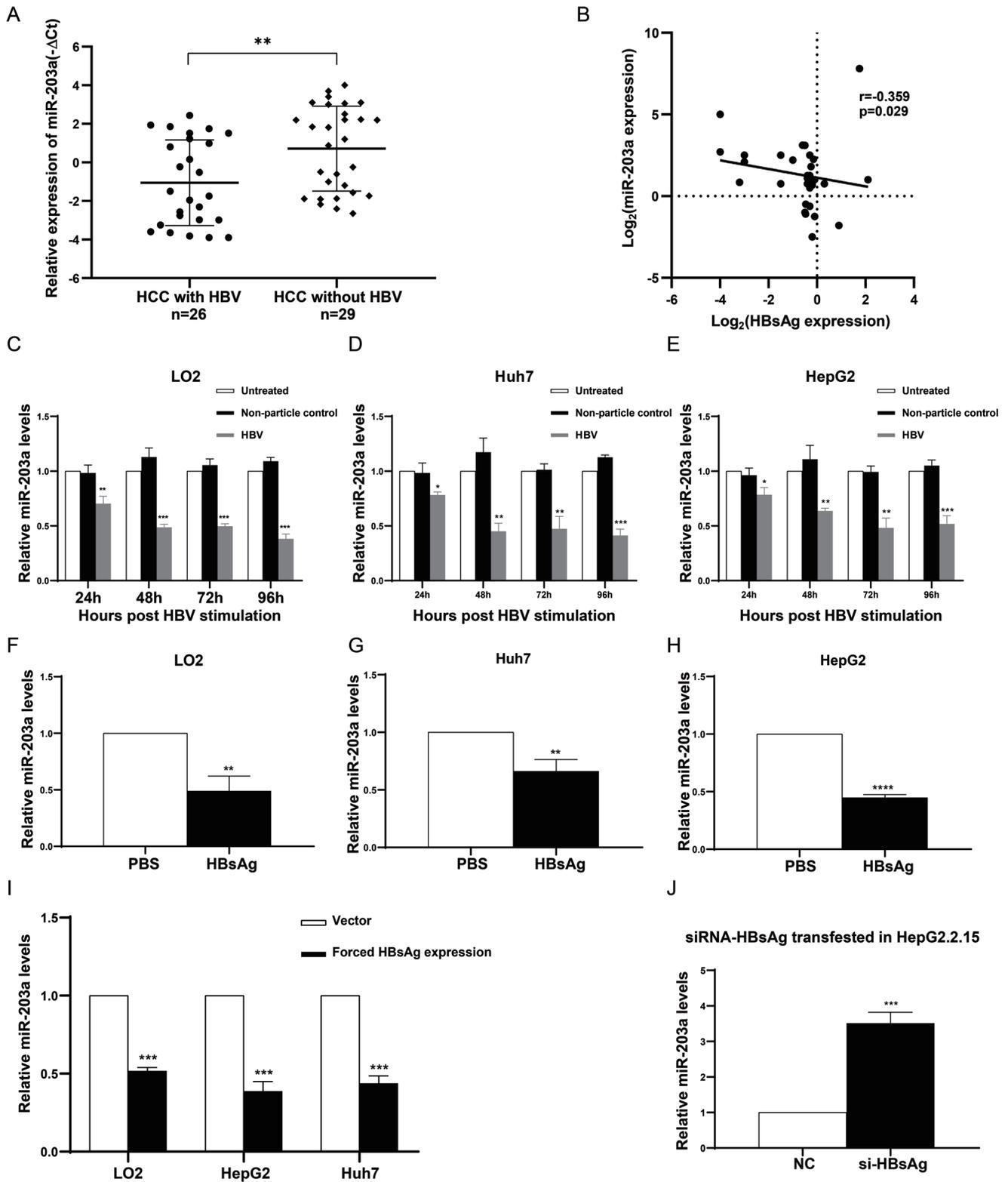
Fig. 2. HBsAg regulates the expression of miR-203a in liver cells and HCC cells. qPCR results of miR-203a in HCC tissues with or without HBV infection (A). HBV-HCC, *n*=26; HCC without HBV, *n*=29). Spearman correlation between expression of miR-203a and HBsAg (B). qPCR results of miR-203a in LO2 liver cells (C) and HepG2 and Huh-7 HCC cells (D, E) stimulated with HBV particles or a non-particle control. qPCR results of miR-203a in LO2 liver cells (F) and HepG2 and Huh-7 HCC cells (G, H) stimulated with recombinant HBsAg adr. qPCR results of miR-203a in HCC cells with HBsAg overexpression (F) and inhibition (G). *$p<0.05$, **$p<0.01$. HBsAg, hepatitis B surface antigen; HCC, hepatocellular carcinoma.
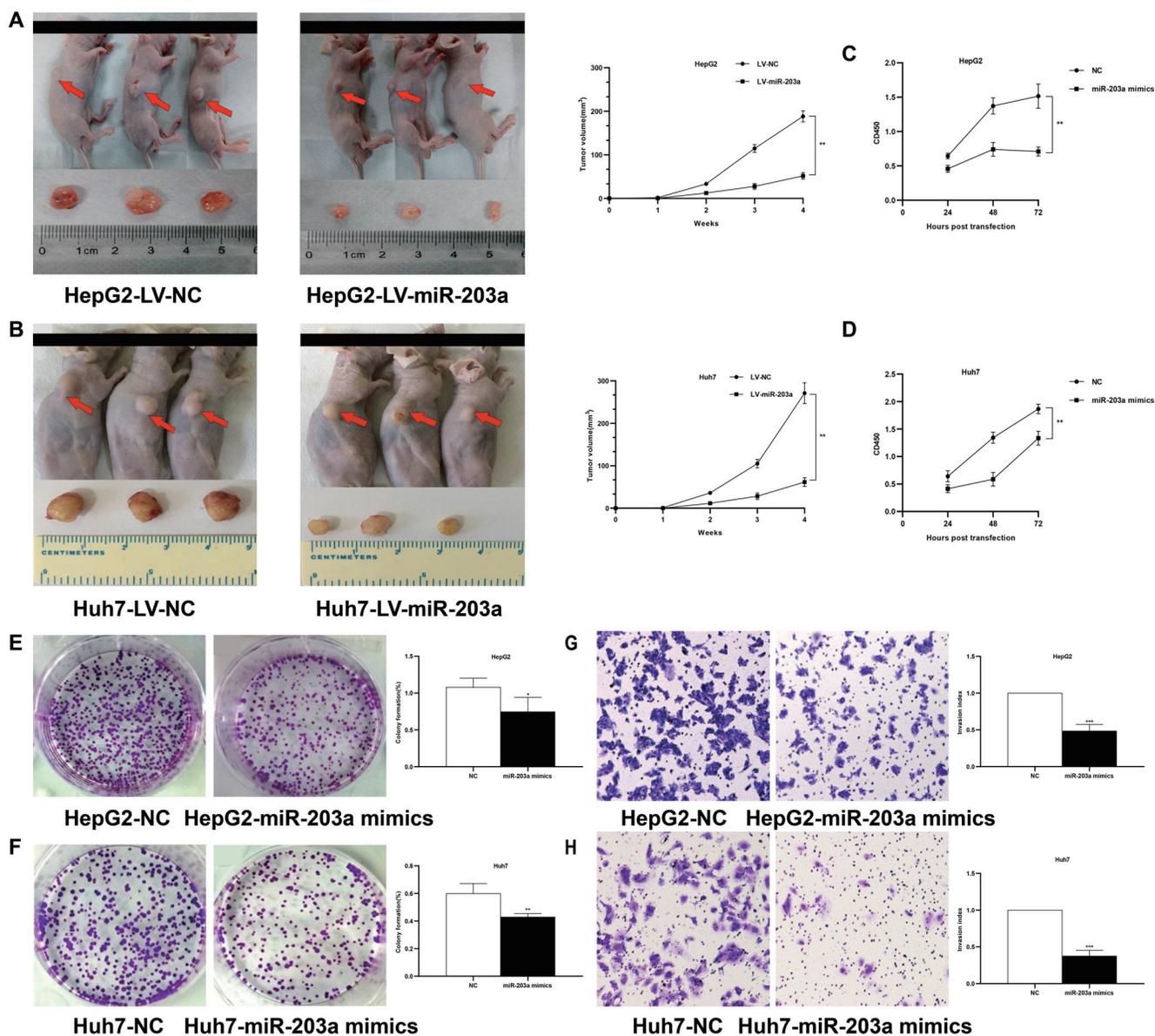
Fig. 3. MiR-203a affects tumor formation *in vivo* and tumor progression *in vitro*. Tumor formation in a nude mouse xenograft model with HepG2 and Huh7 HCC cells in which miR203a were overexpressed and control (A, B). Cell growth ability was determined by CCK-8 assay (C, D). Colony formation of HepG2 and Huh7 HCC cells overexpressing miR203a and in control cells (E, F). Invasion of HepG2 and Huh7 HCC cells overexpressing and in control cells (G, H). *$p<0.05$. HCC, hepatocellular carcinoma; CCK-8, cell count kit-8.

MiR-203a inhibits the function of HCC

Inspired by the significant down-regulation of miR-203a expression in HCC tissues and HCC cells and the clinical correlation, we investigated the biological significance of miR-203a in hepatocarcinogenesis. Control lentiviral vector-infected cells and miR-203a mimic vector-infected cells were injected subcutaneously into nude mice in opposite flanks. The tumor volumes were significantly smaller in in nude mice injected with cells overexpressing miR-203a (Fig. 3A, B, $p<0.01$). CCK-8 cell proliferation assays revealed that miR-203a mimics significantly decreased the proliferation of HCC cells (Fig. 3C, D). As shown in Figure 3E, F, miR-203a mimic-transfected HCC cells formed fewer and smaller colonies compared with the control group. Similarly, the invasiveness of

miR-203a overexpressing cells was significantly attenuated (Fig. 3G, H). Overall, the results showed that endogenous overexpression of miR-203a inhibited the tumorigenicity of HCC cells both *in vitro* and *in vivo*, implying that miR-203a suppressed the self-renewal and invasiveness of HCC cells.

MiR-203a impairs the stem cell properties of HCC

To assess the stem cell properties in HCC, classical markers CD133, and CD90 were evaluated by flow cytometry. The results revealed that forced expression of miR-203a decreased the percentage of CD133-positive Huh7 cells (28.34%±1.82% vs. 91.17%±3.83%, $p<0.01$). Representative images are shown in Figure 4A. However, there was
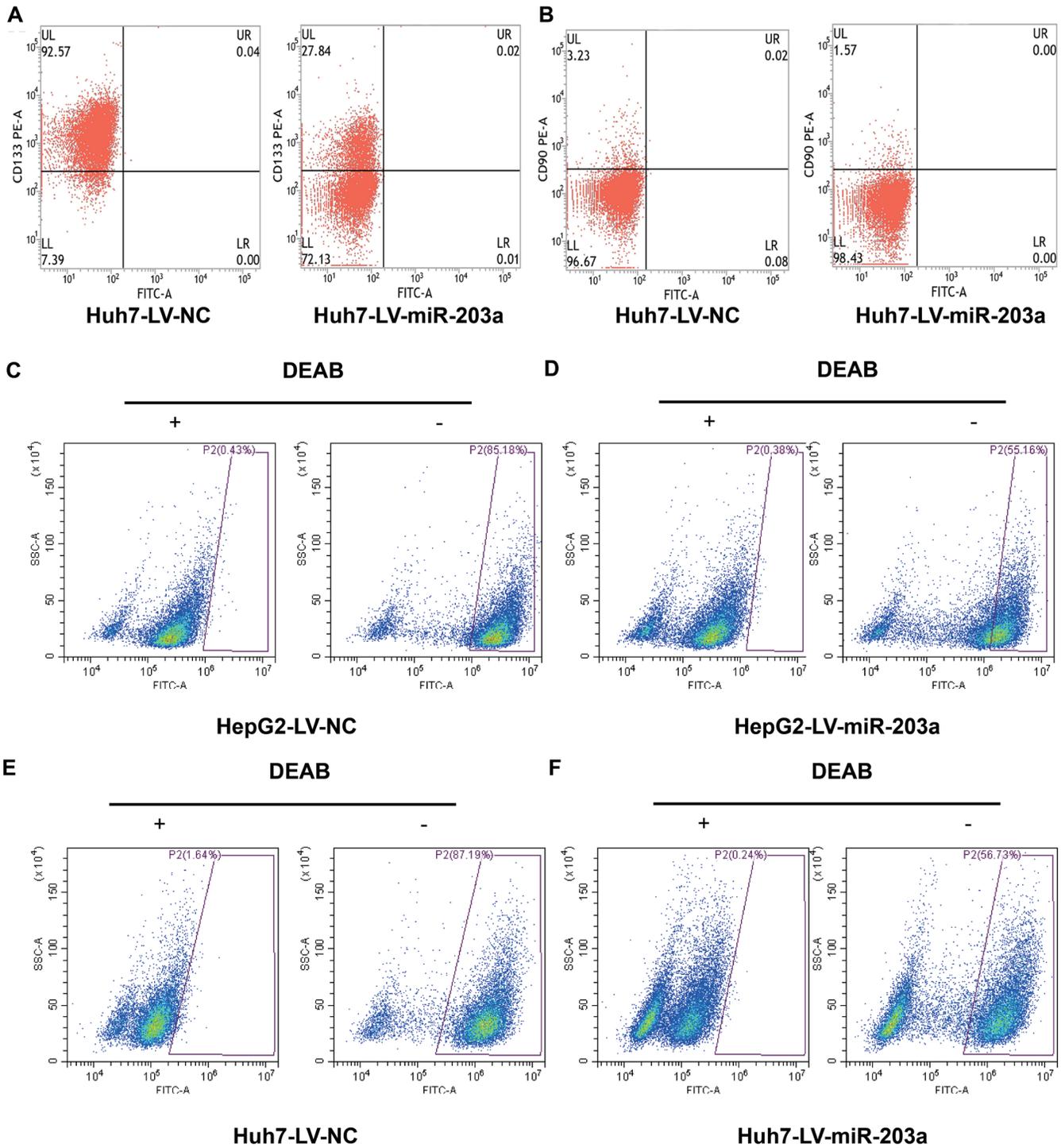
Fig. 4. MiR-203a affects the expression levels of stem cell markers of HCC cells. Flow cytometry of CD133-positive ratio in Huh7 cell with and without miR-203a overexpression (A). Flow cytometry of CD90-positive ratio in Huh7 cell with and without miR-203a overexpression (B). ALDH activity in HCC cells by Aldefluor assay, with and without DEAB (C–F). *$p < 0.05$. ALDH, aldehyde dehydrogenase; DEAB, diethylamino benzaldehyde.

no significant change in CD90 positivity in Huh7 cells that overexpressed miR-203a (3.53%±0.32 vs. 2.07%±0.33%, Fig. 4B). The ALDH assay results found that the average percentage of ALDH positive malignant stem cells was 63.02±6.29% in Huh7 cells overexpressing miR-203a and 85.74±1.46% in control cells ($p < 0.01$). In HepG2 cells the 64.44±9.28% of the miR-203a upregulated cells and 83.49±1.69% of the control cells were ALDH positive ($P = 0.0249$). Representative fluorescence-activated cell sorting images are shown in Figure 4C–F. Overall, the findings provide evidence of the regulation of hepatic CSC-like phenotypes of HCC cells by miR-203a.
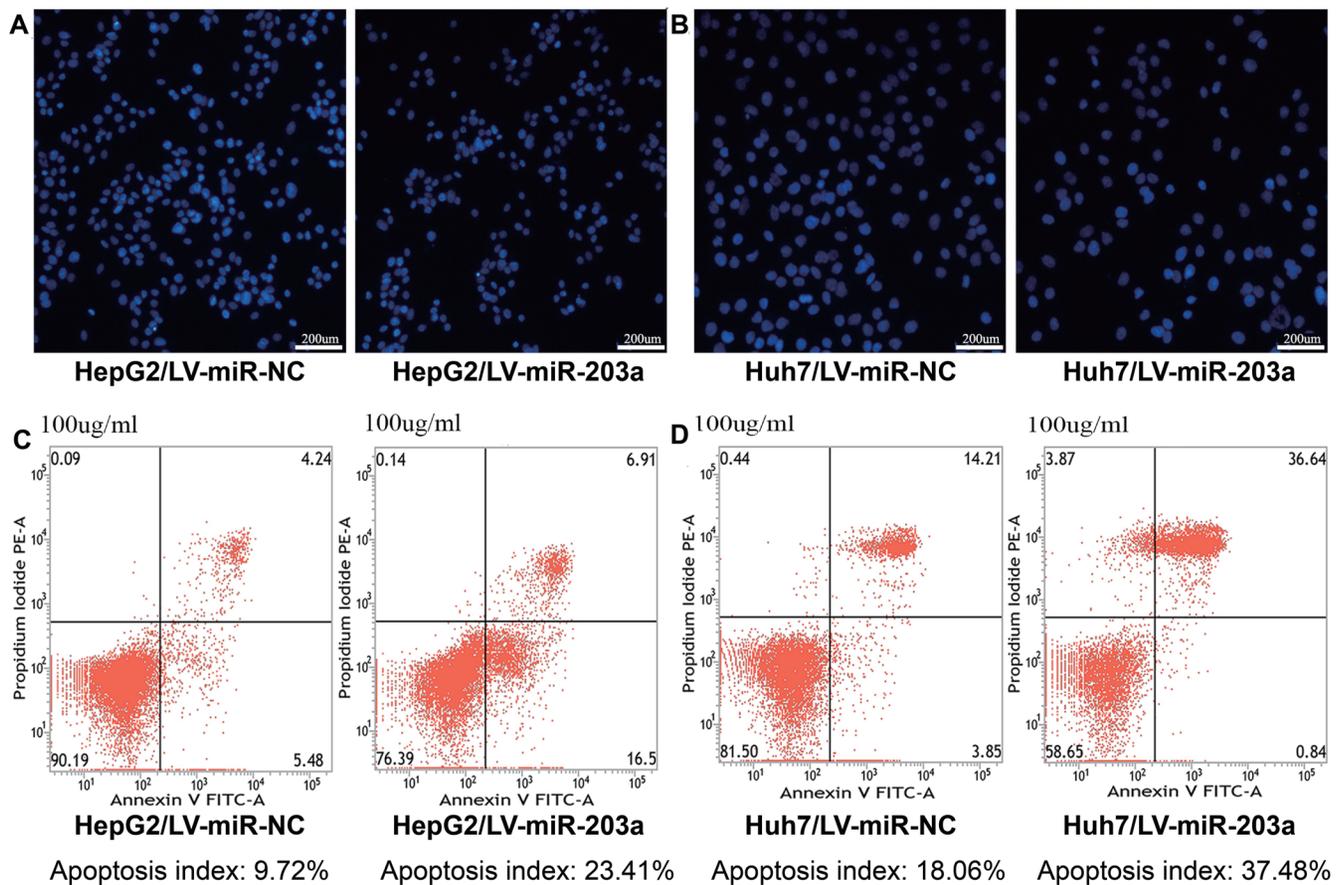
Fig. 5. 5-FU-induced apoptosis in HCC cells is influenced by miR-203a. Viability of HCC cells with and without miR-203a overexpression stained with DAPI (A, B). Flow cytometry assay of apoptosis index of HCC cells with and without miR-203a overexpression (C, D). *$p<0.05$. 5-FU, fluorouracil; DAPI, diamidino-phenylindole.

MiR-203a sensitizes HCC to 5-fluorouracil (5-FU)-induced apoptosis

To determine whether miR-203a up-regulation increased the sensitivity of HCC cells to chemotherapy, we studied the effect of miR-203a on cell apoptosis after 5-FU administration. Compared with the controls, miR-203a overexpression reduced HCC cell viability after 5-FU treatment (Fig. 5A, B). There was a great increase in the apoptosis rate of HCC cells transfected with miR-203a mimics in response to 5-FU, from 23.41±2.03% and 37.48±2.97%. The corresponding rates in NC-transfected HCC control cells were 9.72±1.2% and 18.06±2.07%. Representative images are shown in Figure 5C, D. The findings indicate that miR-203a overexpression sensitized HCC cells to chemotherapy drug-induced apoptosis ($p<0.01$).

BMI1 expression is negatively correlated with miR-203a in human HCC tissues

We previously reported that the expression of BMI1 was significantly upregulated in human HCC tissues and in four human HCC cell lines.[13,22] In consideration of its role in stemness maintenance and the biological effect consistent with miR-203a, we asked whether *BMI1* was involved in miR-203a regulation. qPCR results indicated a negative correlation between expression of *BMI1* mRNA and miR-203a in human HCC tissues (Fig. 6A, $r=-0.469$, $p=0.04$). Analysis of Starbase data[30]

also found a significant negative correlation of *BMI1* expression and miR-203a (Fig. 6A, $r=-0.242$, $p<0.001$).

BMI1 may be a direct target gene of miR-203a and is negatively regulated by miR-203a in human HCC cell lines

To find the underlying effect of miR-203a on *BMI1*, we examined the change of BMI1 in cells with altered miR-203 expression. The results of western blot analysis suggested that miR-203a overexpression significantly decreased the level of BMI1 in HCC cells, and miR-203a knockdown increased BMI1 expression (Fig. 6B). The luciferase assay was used to determine whether BMI1 was a target gene of miR-203a. Cotransfection of miR-203a mimics decreased luciferase expression of the BMI1-3'-UTR-WT reporter but had no effect on luciferase activity in the four BMI1-3'-UTR mutant reporters and the psiCHECK-2 control reporter. Similarly, only the luciferase expression of the BMI1-3'-UTR-WT reporter was elevated after cotransfection of miR-203 inhibitor (Fig. 6C). The results indicate that miR-203a served as a direct negative regulator of *BMI1* expression in HCC cells.

MiR-203a is a prognostic factor in HCC patients and is related to clinical characteristics
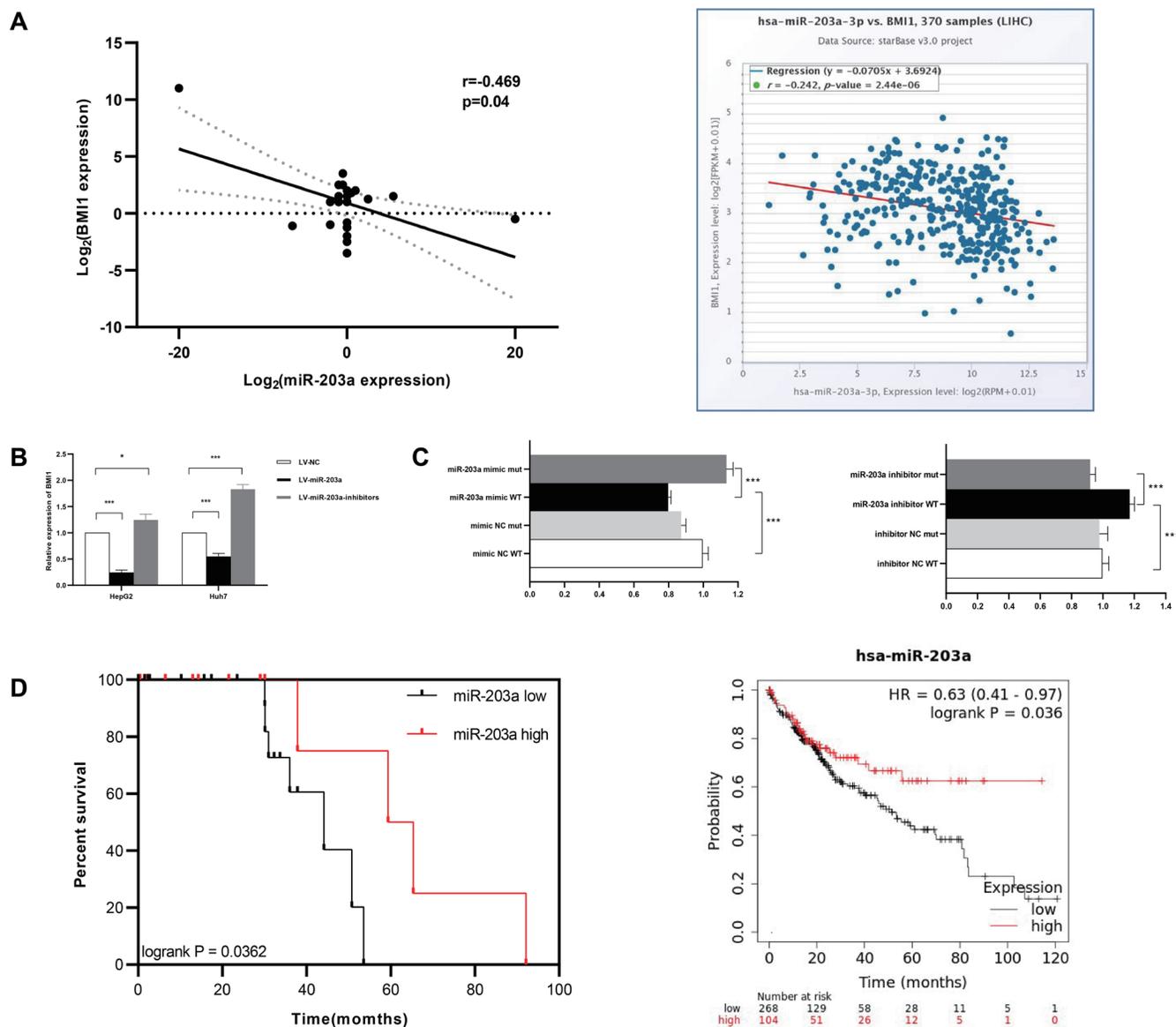
To explore the role of miR-203a in the development of hu-

Fig. 6. Relationship of MiR-203a and *BMI1* and the role of miR-203a in prognosis. Dot plots of *BMI1* and miR-203a in tissue from a single patient at our center and from TCGA database (A). Western blot results of *BMI1* in HCC cells with miR-203a overexpression and inhibition (B). Luciferase assay of HCC cells with miR-203a WT and mutant overexpression and inhibition (C). Kaplan-Meier analysis of overall survival in HCC patients with high and low miR-203a levels including TCGA database and patients at our center (D). *p<0.05. TCGA, The Cancer Genome Atlas; HCC, hepatocellular carcinoma.

man liver cancer, we assayed miR-203a levels in HCC tissues. miR-203a expression was significantly lower in human HCC tissues than in adjacent normal tissues (Supplementary Fig. 1E, n=29, p<0.01). Analysis of the correlation of miR-203a expression and clinical parameters in clinical cases found that loss of miR-203a expression was correlated with larger tumor size (Table 1, p=0.03). Kaplan-Meier analysis (Fig. 6D) found that miR-203a was an independent prognostic factor of the overall survival of HCC patients (p=0.0362), consistent with the results in the database (Fig. 6D, p=0.036).[31]

Discussion

Despite advanced medical interventions, HBV remains a

major threat to public health as it is difficult to completely eliminate, persists in the liver, and plays multiple roles in different stages of hepatitis, liver cirrhosis, and liver cancer. Recently, evidence have accumulated that microRNAs regulate tumor-associated genes and are an integral part in some pathways, implying critical involvement in the progression of cancer.[32] To distinguish microRNAs essential for the development of HBV-HCC, we designed a sequencing protocol to compare the microRNAs differentially expressed in HBV-positive and HBV-negative HCC tumor tissues, excluding paratumoral tissues. Most candidate microRNAs selected from the microarray were down-regulated in HBV-positive HCC tissues, including miR-30b-5p, miR-98-5p, miR-148a-3p, and miR-221-5p, which have been shown to regulate the development of HCC.[33–37] miR-203a, with the most significant difference, has been reported to in-

Table 1. Correlation between miR-203a expression HCC in 35 patients

Characteristic	N	Group 203a+	Group 203a−	*p*-value
Sex				0.635
Male	33	7	26	
Female	2	0	2	
Age (years)				0.280
>50	19	5	14	
≤50	16	2	14	
Tumor size				0.03
>5 cm	23	2	21	
≤5 cm	12	5	7	
Vascular invasion				0.453
Yes		4	19	
No		3	9	
Histological grade				1
G1+G2	14	3	11	
G3+G4	21	4	17	
Tumor number				0.335
Single	25	6	19	
Multiple	10	1	9	
Tumor recurrence				0.21
Yes	11	5	6	
No	24	2	22	
Serum AFP				0.02
25 ng/mL	16	7	9	
≤25 ng/mL	19	0	19	
CA19-9				0.340
>35 μ/mL	9	3	6	
≤35 μ/mL	26	4	22	

HCC, hepatocellular carcinoma; AFP, alpha fetoprotein; CA 19-9, carbohydrate antigen 19-9. Chi-square test (Fisher's exact test).

hibit the growth and metastasis of HCC by regulating molecules such as interleukin-24 and matrix metalloprotein-2, and also negatively regulates the epithelial-mesenchymal transition process caused by HCV core protein.[38–41] However, the relationship between HBV and miR-203a has not yet been described, and most studies have focused on cell proliferation and cell cycle changes rather than on tumor stemness.

Among the well-established proteins of HBV, HBsAg is often underestimated and is considered to possess only weak cancer promoting activity, but increasing evidence shows that HBsAg is an independent risk factor for HCC. The aim of this study was to explore whether miR-203a was regulated by HBsAg and affect the tumor stemness of HCC. First, we used clinical specimens to demonstrate that miR-203a was associated not only with HBV infection but was also negatively correlated with HBsAg titers. Furthermore, miR-203a expression was negatively regulated by HBsAg, as shown by the results of HBV particle stimulation, exogenous HBsAg protein stimulation, and HBsAg overexpression in cell lines

without HBV infection and in HBsAg knockdown of cell lines with intact HBV genomes.

In the next step, we validated the tumor suppressor effect of miR-203a by functional experiments *in vitro* and *in vivo* and explored its influence on HCC stemness. In the evaluation of tumor stemness, we preferred the traditional markers CD133 and CD90. CD133 is one of the earliest markers of human hepatic CSCs in HCC. Not only does it increase the incidence of HCC, but drives it to a tumor with poor differentiation and promotes cell proliferation by regulating cell cycle-related pathways. It has been found that tissues with viral hepatitis shared the same expression level of CD133 as HBV-HCC, which leaves us a clue that HBV infection might enhance the stemness of HCC through CD133. Furthermore, CD133 is proven to act widely on cancer stemness-related genes, including *NANOG*, the *SOX* family, and *BMI1*, which provides a basis for our research on the role of BMI in HBV-HCC.[42] On the other hand, normal hepatic progenitor cells express CD90, and its abnormal elevation affects the interaction and adhesion of cells, further accelerates the development of HCC, and increases its tolerance to chemotherapy.[43] It has also been reported that the PreS1 of HBV genome increases the CD133+/CD90+ ratio in HCC.[44] These two markers are thus used to assess HCC stemness. As shown in Supplementary Figure 2, the RNA levels of stemness-related genes in LO2 were increased after recombinant HBsAg adr stimulation, and were elevated in Huh7 and HepG2, but the differences were not significant. The reason may be that 5-day stimulation was too short for the alteration of cancer stemness. Given that tumor stemness only existed in a small fraction of cells, which was possibly masked by PCR or western blotting. Thus, we chose stably transfected cell lines to evaluate the effect of miR-203a on cancer stemness and utilized a more accurate method, flow cytometry, to detect changes in the proportion of stem marker-positive cells. The stemness of tumors can affect multiple aspects of biological function, and the one most closely related to clinical treatment is chemosensitivity.[45–47] The effect of miR-203a on chemosensitivity of HCC was preliminarily verified with a traditional chemotherapeutic agent 5-FU, which inspired us to further investigate the underlying mechanism.

The human *BMI1* gene is a core component of polycomb inhibition complex 1 (PRC1) and mediates gene silencing by monoubiquitination of histone H2A. It has been reported to promote tumorigenesis by regulating the cell cycle inhibitory genes, p16 and p19 and is regarded as a marker of tumor stemness.[48–50] We previously showed that BMI1 expression was upregulated in HBsAg-positive HCC.[13,22] In this subsequent study, we showed that miR-203a inhibited BMI1 expression and the direct binding sites of miR-203a and BMI1 by dual luciferase reporter assay. The data supported the hypothesis that miR-203a affected HCC stemness through BMI1, but further studies are required to draw definite conclusions.

Finally, we emphasize the translational value of our research, as miR-203a might be a prognostic marker of HBs-HCC. Clinical specimens from our center and patients in The Cancer Genome Atlas (TCGA) database, both indicated shorter overall survival of patients with low miR-203a expression. Our analysis of clinical features also found larger tumor diameters in those with low level miR-203a expression. However, patients with high miR-203a expression presented higher alpha fetoprotein (AFP) levels, which implied that miR-203a may affect tumor differentiation and warrants further investigation. In conclusion, our findings indicate that HBsAg promotes stemness and chemoresistance in HCC by regulating the microRNA-203a/BMI1 axis. Our data provide a hint of the mechanism of HBsAg in promoting HCC, but more evidence is required for confirmation.

Funding

Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Designed the study (RZ, HML, CL), Conducted the experiments and analyzed the data (HWF, WRW), Interpreted the results (LBX, XLX, YFQ, ZYZ), Drafted the manuscript with contributions from all authors (RZ, CL), Revised the paper (RZ, HWF). All authors have read and agreed to the published version of the manuscript.

Ethical statement

The protocol was approved by the ethics committee of Sun Yat-sen Memorial Hospital and written informed consent was obtained from either the patients or their guardians. All animal procedures were performed following protocols approved by the Institutional Animal Care and Use Committee at Sun Yat-sen University and were performed at Sun Yat-sen University (Guangzhou, China).

Data sharing statement

The database used in support of the findings of this study have been deposited in the TCGA repository. The additional data used in support of the findings of this study are included within the supplementary information file(s) accompanying this publication in the Journal of Clinical and Translational Hepatology. The experiments used in support of the findings of this study are available from the corresponding author at zhangr95@mail.sysu.edu.cn upon request.

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