miRNA-548p suppresses hepatitis B virus X protein associated hepatocellular carcinoma by downregulating oncoprotein hepatitis B x-interacting protein

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Aim: miR-548p is a recently identified and poorly characterized miRNA. However, its role of miR-548p in tumorigenesis and progression remains poorly understood. Here, we aimed to investigate the biofunction of miR-548p in hepatocellular carcinogenesis.

Methods: The expression levels of miR-548p were detected by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The role of miR-548p in hepatocellular carcinoma (HCC) was determined by colony formation, flow cytometry assay and nude mice xenograft experiments. miR-548p target genes were analyzed by miRNA target predication programs and verified by qRT–PCR, western blotting assay and dual-luciferase reporter assay.

Results: miR-548p is repressed by hepatitis B virus X protein (HBx) in HCC tumor tissues and hepatoma cells, and inhibited cell growth by inhibiting cell proliferation and promoting cell apoptosis. miR-548p directly downregulated the expression of hepatitis B x-interacting protein (HBXIP) by binding to the 3′-untranslated region of HBXIP mRNA. Further study showed that hepatocyte nuclear factor-4a (HNF4A) promoted the expression of miR-548p and inhibited the transcription of HBXIP. HNF4A is a dominant transcriptional regulator of hepatocyte differentiation and hepatocellular carcinogenesis, and is shown to be repressed by HBx.

Conclusion: We proposed the model for HBx/HNF4A/miR-548p/HBXIP pathway that controls hepatoma cell growth and tumorigenesis of HCC. miR-548p was identified as a tumor-suppressor in HBx-associated hepatocellular carcinogenesis.

Key words: hepatitis B virus X protein, hepatitis B x-interacting protein, hepatocellular carcinoma, hepatocyte nuclear factor-4a, miR-548p

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is the sixth most common cancer and the third most common cause of cancer-related death worldwide.1,2 The major risk factor for HCC is chronic viral hepatitis B or C, which accounts for 80–90% of all cases of HCC worldwide.3 The hepatitis B virus X (HBx) protein as the main pathopoiesis factor of hepatitis B virus (HBV) is involved in the tumorigenesis and progression of HCC.4–6 miRNA are endogenous, small non-coding RNA consisting of 20–25 nucleotides. They are responsible for modulating gene expression by promoting mRNA degradation or repressing protein translation through sequence-specific interaction with the 3′-untranslated region (UTR) of targeted mRNA.7 miRNA play important roles in physiological processes, such as cell apoptosis, proliferation and differentiation.8 Accumulating evidence indicates that dysregulated miRNA expression is a common feature of human tumors.9 Recent studies showed that more and more miRNA participated in the development or progression of HBx-related HCC. Depending on the target gene, miRNA can function as tumor suppressor genes or oncogenes.10,11 miR-548p is a recently identified and poorly characterized miRNA (chr5, 100 816 565–100 816 482 [—]; miRBase accession number, MI0006420).12 According to the network analysis, Li et al. observed that miR-548p not only conferred significant explanatory power to mRNA expression changes in acute myeloid leukemia but also
targeted a large number of leukemia-related genes. However, the role of miR-548p in tumorigenesis and progression remains ambiguous.

In this study, we investigated the expression and biofunction of miR-548p, and found that miR-548p was downregulated in HBV positive HCC tumor tissues compared with HBV negative HCC tumor tissues and the adjacent non-tumor tissues. Then, we proposed a model of HBx/hepatocyte nuclear factor-4a (HNF4A)/miR-548p/hepatitis B x-interacting protein (HBXIP) illustrating a possible molecular mechanism and functional basis for HBx-associated hepatocellular carcinogenesis. In this axis, miR-548p was proved to be a tumor-suppressor in HBx-associated HCC.

METHODS

Tissue specimens

A total of 33 pairs of (including 12 pairs of HBV negative and 21 pairs of HBV positive patients) HCC tumor tissues and the matched adjacent non-tumor tissues were collected during the curative resection at Liver Cancer Department, Nanfang Hospital, Southern Medical University (Guangzhou, China) between January 2012 and August 2014. All of the tissue specimens were stored at −80°C for RNA or protein isolation. The concentrations of serum HBV DNA and hepatitis B surface antigen of all patients were detected before curative resection surgery (Table S1). All the clinical samples were collected from patients after obtaining informed consent in accordance with a protocol approved by the Ethics Committee of Nanfang Hospital, Southern Medical University (Guangzhou, China).

Cell lines and cell culture

In this study, a non-transformed hepatic cell line, LO2, and four hepatoma cell lines (HepG2, SMMC-7721, HepG2.2.15 and Hep3B) were used. HepG2 and Hep3B cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). LO2, SMMC-7721 and HepG2.2.15 cells were purchased from the Cell Bank of Typical Culture Preservation Committee of Chinese Academy of Science, Shanghai, China. Stable HBx overexpressing LO2, HepG2 and SMMC-7721 cells were obtained by infection with pLVX-mCMV-ZsGreen-puro-HBx, followed by selection with 5 μg/mL puromycin for 2 weeks (Figure S1).

Transient transfection

miR-548p mimics, inhibitors and miRNA negative control (miR-NC) (RIBOBIO, Guangzhou, China) were transfected into hepatic and hepatoma cell lines by Lipofectamine 2000 (Invitrogen, San Diego, CA, USA). The duplexes of siRNA targeting HNF4A mRNA (HNF4A siRNA) and the negative control (non-silencing siRNA; QIAGEN, Valencia, CA, USA) were transfected into hepatic and hepatoma cell lines by RNAiMAX (Invitrogen). The sequences are listed in Table S2.

Quantitative reverse transcription polymerase chain reaction (qRT–PCR)

The total RNA containing miRNA were extracted from the HCC tissues and cell lines with cold TRIzol (Invitrogen) as follows: (i) the frozen tissues were ground in liquid nitrogen; (ii) the cold TRIzol was added into the fragment tissues for lysing; (iii) chloroform and the cold isopropanol were used to isolate the total RNA; and (iv) following the washing step by cold ethanol, the purified RNA were resolved in the DNase/RNase-free water and detected immediately or stored at −80°C. The expression levels of miRNA were measured by miRNA qRT–PCR using an All-in-One miRNA Q-PCR detection kit (GeneCopoeia, Rockville, MD, USA) on a LightCycler 480 system (Roche, Basel, Switzerland). snRNA U6 was used as the endogenous control. The primers of mirRNA and snRNA U6 used for miRNA quantitative PCR were purchased from GeneCopoeia. The mRNA expression levels were measured with LC480 SYBR Green Master kit (Roche, Switzerland) by qRT–PCR. β-Actin was used as the endogenous control. The primers were synthesized by Invitrogen (sequences are shown in Table S2). The relative quantification value of the target, normalized to a control, was calculated by the comparative Ct methods. The products of qRT–PCR were verified by sequencing.

Western blotting analysis

Total protein from tissues and cell lines were extracted using RIPA lysis buffer with proteinase inhibitor. The protein concentration in the lysates was measured with the Protein BCA Assay Kit (Bio-Rad, Hercules, CA, USA), and 50 μg of the total protein mixed with 1 × sodium dodecyl sulfate (SDS) loading buffer was loaded per lane. The proteins in the lysates were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). To block non-specific binding, the membranes were incubated at room temperature for 1 h with 5% skim milk powder. The PVDF membranes were then incubated for 12 h at 4°C with anti-serum containing antibodies against HBXIP (LS-C111379, 1:1000 dilutions; LifeSpan BioSciences, Seattle, WA, USA), HNF4A (SC-6556, 1:1000 dilutions; Santa Cruz Biotechnology, CA, USA), HBXIP (LS-C111379, 1:1000 dilutions; LifeSpan BioSciences, Seattle, WA, USA), HNF4A (SC-6556, 1:1000 dilutions; Santa Cruz Biotechnology, CA, USA), HNF4A (SC-6556, 1:1000 dilutions; Santa Cruz Biotechnology, CA, USA), and HBXIP (LS-C111379, 1:1000 dilutions; LifeSpan BioSciences, Seattle, WA, USA). The membranes were then incubated with anti-mouse IgG (ZSGB-BIO, Beijing, China) and anti-rabbit IgG (ZSGB-BIO, Beijing, China) secondary antibodies for 1 h each at room temperature. The PVDF membranes were washed with TBST for 10 min and then developed using the ECL Plus system (GE Healthcare, Chicago, IL, USA) and exposed to X-ray film (Kodak, Rochester, NY, USA).
and photographed. Numbers of colonies with more than 50 cells were counted and stained with 0.1% crystal violet for 30 min. The 96-well plate and cultured to 50% 60% confluence. Cells were seeded in six-well plates at 200 cells/well. Two weeks later, colonies were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 30 min. The numbers of colonies with more than 50 cells were counted and photographed.

Assessment of cell proliferation by colony formation assay
Cells were seeded in six-well plates at 200 cells/well. Two weeks later, colonies were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet for 30 min. The numbers of colonies with more than 50 cells were counted and photographed.

Cell cycle and apoptosis analyzing by flow cytometry
Cell cycle analysis was conducted by flow cytometry using a propidium iodide (PI) cell cycle detection kit (Beyotime Institute of Biotechnology, Shanghai, China). Cell apoptosis analysis was performed with the FITC Annexin V Apoptosis Detection Kit 1 (BD Biosciences, San Jose, CA, USA). The HBXIP expression was detected by immunohistochemistry with HBXIP antibody (LS-C111379; LifeSpan Biosciences, San Antonio, TX, USA). The differences between groups were analyzed using ANOVA for comparing more than two groups or one-way ANOVA for comparing more than two groups. The correlation between HBXIP and miRNA were analyzed by Spearman’s rank correlation coefficient. Statistical significance was set at *P < 0.05 and **P < 0.01. P < 0.05 was considered statistically significant.

RESULTS

miR-548p is significantly downregulated in HBV-related HCC tumor tissues and hepatoma cell

According to the miRNA qRT–PCR analysis, the expression level of miR-548p was significantly downregulated in HCC tissues compared with adjacent non-tumor tissues (t = 3.001, P < 0.01, Fig. 1a). Further analysis showed the miR-548p levels of both tumor tissues and the adjacent non-tumor tissues in HBV-associated HCC patients were obviously lower than those of HBV negative patients (P < 0.01, Fig. 1b), which suggested that the expression of miR-548p was inhibited by HBV. Collectively, these data suggest that miR-548p is frequently downregulated in HBV-associated HCC tumor tissues.
Then, we detected the expression of miR-548p in five liver cell lines including a non-transformed hepatic cell line LO2 and four established HCC cell lines (HepG2, SMMC-7721, HepG2.2.15 and Hep3B). The result showed that the expression of miR-548p in HepG2.2.15 and Hep3B cells (with endogenously driven HBV replication) were lower than HepG2, SMMC-7721 and LO2 cells ($P < 0.05$, Fig. 1c). Further studies revealed that the expression level of miR-548p in HBx overexpression cells including LO2-HBx, HepG2-HBx and SMMC-7721-HBx cells were significantly lower the negative control cells ($P < 0.01$, Fig. 1d). These data suggest that HBV infection inhibits miR-548p expression may through the HBx expression.

miR-548p inhibits cell proliferation and enhances cell apoptosis

Hepatitis B virus X protein is known to be is a multifunctional regulatory protein and involved in tumorigenesis; here, we wonder whether miR-548p is playing a negative role in HCC tumorigenesis or not. The effect of miR-548p on cell proliferation was detected by colony formation assay. As shown in Figure 2(a,b), the number of colonies was considerably lower for cells transfected with miR-548p mimic than the respective miR-NC groups ($P < 0.05$). On the contrary, miR-548p inhibitor significantly increased cell colony number compared with miR-NC groups ($P < 0.05$). To assess whether this effect was mediated through perturbation of the cell cycle, cell phase distribution analysis was performed by flow cytometry. Compared with the miR-NC groups, cells treated with miR-548p mimic increased the percentage of cells staying at G1 phase ($P < 0.05$) and decreased the percentage of cells entering into the S phase ($P < 0.05$). Oppositely, the percentage of cells re-entering into the S phase were increased when treated with miR-548p inhibitor ($P < 0.05$, Fig 2c,e). These results revealed that miR-548p may inhibit hepatoma cell proliferation by
miR-548p inhibited cell proliferation and enhanced cell apoptosis. The miR-548p mimic, inhibitor and miRNA negative control (miR-NC) were introduced to HepG2 and HepG2.2.15 cell lines to analyze the cell apoptosis and proliferation capacities in vitro. (a) Cell colony formation assay showed the different proliferation capability of HepG2 and HepG2.2.15 cells transfected with miR-548p mimic, inhibitor and miR-NC. (b) The cell colony numbers among three different groups were analyzed by one-way ANOVA. (c) Cell cycle was analyzed by flow cytometry. (d,e) The percentage of HepG2 and HepG2.2.15 cells in G1 phase, G2 phase and S phase were compared among miR-548p mimic, inhibitor and miR-NC groups by one-way ANOVA. (f) The cell apoptosis levels were detected and analyzed by flow cytometry. (g) The cell apoptosis index among three groups of HepG2 and HepG2.2.15 cells were analyzed by one-way ANOVA. All values shown are mean ± standard deviation of triplicate measurements and have been repeated at least three times with similar results (*P < 0.05).
arresting the G1 to S phase transition in the cell cycle. Then, the apoptotic index was calculated using flow cytometry. The results showed that the apoptotic index was significantly increased in cells transfected with miR-548p mimic compared with the miR-NC and miR-548p inhibitor groups, suggesting that miR-548p enhanced cell apoptosis ($P < 0.05$, Fig. 2f,g). Taken together, these data suggest that miR-548p suppressed hepatoma cell growth by inhibiting cell proliferation and promoting cell apoptosis procedures.

miR-548p downregulates HBXIP by directly targeting 3′-UTR of HBXIP mRNA

In order to explore the possible mechanism of miR-548p suppressing HCC tumorigenesis, we predicted the putative target genes of miR-548p using publicly available databases, including TargetScan, PicTar, Miranda and RNAhybrid. Finally, HBXIP was selected as the candidate target gene because of the conserved site nt 236–242 in 3′-UTR of HBXIP which matched with miR-548p (Fig. 3a). Luciferase activity in HepG2 cell co-transfected with the miR-548p mimic and the pEZX-MT05-HBXIP-WT vector was significantly decreased compared with the pEZX-MT05-HBXIP-Mut group and miR-NC group ($P < 0.05$, Fig. 3b), which proved that the 3′-UTR of the HBXIP transcript is a direct target of miR-548p. Then, the mRNA of HBXIP in 33 pairs of HCC tumor tissues and the matched adjacent non-tumor tissues were detected by qRT-PCR (Table S1). The expression level of HBXIP mRNA was significantly increased in HCC tissues versus...
adjacent non-tumor tissues ($t = -4.335$, $P < 0.01$, Fig. 3c). Also, the HBXIP mRNA levels both of tumor tissues and the adjacent non-tumor tissues in HBV positive HCC tissues were much higher than the relevant tissues in the HBV negative patients ($P < 0.05$, Fig. 3d). The further analysis showed that the expression of miR-548p was negatively correlated with the levels of HBXIP mRNA in HCC tissues ($r = -0.466$, $P < 0.01$, Fig. 3e).

In order to verify the regulation function of miR-548p on the expression of HBXIP, the miR-548p mimic and miR-NC were transfected into LO2, HepG2, Hep3B and SMMC-7721 cells, respectively. Both the mRNA and protein expression levels of HBXIP were obviously decreased when miR-548p mimic was added into cells for 48 h compared with miR-NC (Fig. 3f).

In the following study, the miR-548p mimic and miR-NC were transfected into the HBx overexpressed cell lines to observe the co-function of HBx and miR-548p on the expression of HBXIP. Compared with the negative control cell lines, the expression levels of HBXIP were increased in HBx overexpressed cell lines and decreased in miR-548p mimic transfected cell lines. Interestingly, when miR-548p mimic was transfected into the HBx overexpressed cell lines, the upregulation of HBXIP induced by HBx was reversed by miR-548p mimic (Fig. 3g).

miR-548p downregulated the expression of HBXIP and suppressed tumor growth in vivo

To further examine the effect of HBx and miR-548p on the tumor growth in vivo, HepG2-HBx and HepG2-NC cell lines were respectively co-transfected with pEZX-MT04-pre-miR-548p (miR-548p overexpression, miR-548p OE) and pEZX-MT04-miR-NC and divided into four groups, as follows: group 1, HepG2-HBx-miR-548p OE; group 2, HepG2-HBx-miR-NC; group 3, HepG2-NC-miR-548p OE; and group 4, HepG2-NC-miR-NC. There were seven mice for each group. As expected, the average volume of tumors generated by the HepG2-HBx-miR-NC cells (group 2) was significantly larger than the HepG2-NC-miR-NC group (group 4). The average tumor volume of the HepG2-NC-miR-548p OE group (group 3) were noticeably smaller than the HepG2-NC-miR-NC group (group 4). Comparing group 1 and group 2, we observed that miR-548p overexpression inhibited the tumors growth which were induced by HBx (Fig. 4a,b). Then, the expression of miR-548p and HBXIP of subcutaneous tumors were detected by qRT–PCR and immunohistochemistry analysis. Consistent with the in vitro results in HCC cell lines, the tumors in mice inoculated with HBx overexpressed HepG2 cell lines (group 2) had reduced levels of miR-548p (Fig. 4c) and enhanced the expression of HBXIP (Fig. 4d,e). The miR-548p overexpression decreased the expression of HBXIP which was induced by HBx (group 1). These data suggest that HBx enhances liver cell growth may be through inhibiting miR-548p and promoting the expression of HBXIP.

HNF4A participates in HBV-induced miR-548p downregulation and HBXIP upregulation

Hepatitis B virus X protein was reported to regulate gene expression through its interaction with host transcriptional factors. To further investigate the mechanism of HBV-mediated expression of miR-548p and HBXIP, we analyzed the sequence of the promoter regions of miR-548p and HBXIP via ChIP Base and UCSC Genome Browser on Human. Consequently, there exists three predicted HNF4A-binding sites in the upstream region of the miR-548p promoter (Fig. 5a), and two predicted HNF4A-binding sites located in 5′-UTR and the first exon of HBXIP, respectively (Fig. 5b). To investigate the mechanism by which HNF4A regulates miR-548p and HBXIP, we examined the level of miR-548p and HBXIP mRNA in HepG2 cells treated with HNF4A siRNA and the siRNA negative control sequence. As shown in Figure 5(c), the transcripts of miR-548p were decreased by HNF4A knockdown, suggesting that HNF4A positively modulates the transcription of miR-548p. Conversely, HBXIP mRNA levels were increased in HNF4A siRNA transfected cells compared with the negative control group, which indicted that HNF4A negatively regulates the transcription of HBXIP (Fig. 5d). In the further study, miR-548p inhibitor and HNF4A siRNA were co-transfected into HepG2 cells to observe the regulation of HNF4A on the expression of miR-548p and HBXIP excluding the post-transcriptional regulation by miR-548p (Fig. 5e,f). The results showed that the mRNA level of HBXIP in HepG2 cell co-transfected with miR-548p inhibitor plus HNF4A siRNA was significantly higher than that of cells treated with miR-548p inhibitor. Moreover, the luciferase assays show that knockdown of HNF4A robustly stimulated the activity of the luciferase reporter containing the putative HNF4A-binding site in the first exon, but not the reporter with the mutated binding site or with other regions (Fig. 5g). These results suggest that HNF4A directly downregulates the transcription of HBXIP by binding to the first exon, which is not dependent on the post-transcriptional regulation pathway by miR-548p.

DISCUSSION

miR-548p is a recently identified and poorly characterized miRNA. miR-548p belongs to the miR-548 family, which is primate/human-specific and derived...
miR-548p downregulated the expression of hepatitis B x-interacting protein (HBXIP) and suppressed tumor growth in vivo. (a) Xenografts were established by s.c. injecting HepG2 cells stably expressing HBx, miR-548p, HBx plus miR-548p, or the corresponding negative control cells into nude mice for 4 weeks. When inoculated for 20 days, the subcutaneous tumors were removed and total RNA of subcutaneous tumors were purified for detecting the expression levels of HBXIP and miR-548p by reverse transcription polymerase chain reaction (RT-PCR). (b) The average tumor volumes of each group. (c) The relative expression levels miR-548p in the subcutaneous tumors. (d) The average mRNA expression of HBXIP of subcutaneous tumors. (e) Immunohistochemistry analysis showed the expression of HBXIP in subcutaneous tumors. The boxed areas in the upper images are magnified in the lower images. Scale bars, 500 μm (upper), 50 μm (lower). All values shown are mean ± standard deviation of triplicate measurements and have been repeated three times with similar results (*P < 0.05, **P < 0.01).
from repetitive elements in the genome.\textsuperscript{18} It was reported by Hu et al. that a sum of 19 members of the miR-548 family (representing 29 mature miR-548 members) in 30 pairs of human lung cancer tissues were expressed lower than in their adjacent non-tumor tissues. The relative levels of the sum of the miR-548 family members in 19 tumor cell lines were significantly lower than that in five normal cell lines.\textsuperscript{19} Similarly, we found that the expression levels of miR-548p in HCC tumor tissues were significantly lower than their matched adjacent non-tumor tissues, which indicated that the decreased miR-548p is related to the malignant pathology in liver tissues. Moreover, miR-548p expression levels were decreased in HBV positive HCC tumor tissues and adjacent non-tumor tissues compared with the HBV negative samples, which suggested that the expression of miR-548p was inhibited by HBV infection.

Then, we observed that miR-548p inhibited tumorigenesis and growth both in vitro and in vivo. To identify the underlying molecular mechanisms of miR-548p inhibiting tumorigenesis, we predicted the putative target genes of miR-548p using publicly available databases and focused on HBXIP, because of the conserved site nt 236–242 in the 5′-untranslated region (UTR) of HBXIP.
3′-UTR of HBXIP mRNA which bound with miR-548p. HBXIP is a new oncoprotein which was originally identified by its interaction with the C-terminus HBx. The function of HBXIP protein is to negatively regulate HBx activity and thus to alter the replication life cycle of the virus. However, recent studies proved HBXIP to be an oncoprotein and promote tumorigenesis and progression. HBXIP may form a complex with survivin, an anti-apoptotic protein, resulting in the suppression of cell apoptosis through the mitochondrial/cytochrome pathway. In addition, as a regulator of centrosome dynamics and cytokinesis, HBXIP is necessary for bipolar spindle formation in human HeLa carcinoma cells. HBXIP could promote cell proliferation via stimulating the transcription and expression of hTERT and increasing the activity of telomerase. Besides, it was reported that HBXIP upregulated Lin28B via activating TFIID to promote proliferation of breast cancer cells. Moreover, HBXIP promoted the proliferation of cancer cells by increasing the expression of c-Myc and inhibiting p27. In this study, we discovered that miR-548p directly downregulated the expression of HBXIP. Thus, we presume that miR-548p inhibits tumor growth through downregulating HBXIP expression. However, whether miR-548p suppresses tumorigenesis through another pathway besides inhibiting HBXIP cannot be excluded. In further study, we will complete the underlying mechanism of miR-548p effecting on tumorigenesis regulation by the knockdown of HBXIP.

Hepatitis B virus X protein has been shown to interact with some transcription factors or repress the transcriptional activity of the transcription factors, which regulate miRNA transcription. For example, HBx inhibited miR-148a transcription by repressing the transcriptional activity of p53 which induced miR-148a expression by binding to the miR-148a promoter. Besides, another known mechanism of miRNA downregulation is aberrant hypermethylation of the miRNA promoter. In our study, we found that the expression levels of miR-548p were downregulated in hepatoma cells with endogenously driven HBV replication (HepG2.2.15 and Hep3B cells) and HBx overexpressed cells, which suggested that HBV inhibited the expression of miR-548p mediated by HBx. To further investigate the mechanism of HBx-mediated miR-548p downregulation and HBXIP upregulation, we analyzed the sequence of the promoter regions of miR-548p and HBXIP via ChiP Base and UCSC Genome Browser on Human. Consequently, we found that HNF4A upregulated expression of miR-548p and inhibited the transcription of HBXIP. As miR-548p directly downregulated the expression of HBXIP by degrading the mRNA, in order to identify whether HNF4A directly regulated the transcription of HBXIP, miR-548p inhibitor and HNF4A siRNA co-transfecting assay as well as luciferase assay were performed in HepG2 cells, respectively. Both results suggested that HNF4A directly represses the transcription of HBXIP by binding to the putative binding site in the first exon but not in the 5′-UTR, which is not dependent on the post-transcriptional regulation pathway by miR-548p.

Hepatocyte nuclear factor-4a is a dominant transcriptional regulator of hepatocyte differentiation and hepatocellular carcinogenesis. HNF4A is a key regulator of hepatocyte differentiation and maintenance of the adult hepatocyte-differentiated phenotype, whose loss is determinant for HCC onset and progression. Xie and colleagues demonstrated that forced expression of HNF4A inhibited the proliferation of HCC in vitro and reduced the tumor formation in mice by inducing differentiation and that HNF4A overexpression in fibrotic livers blocked the activation of myofibroblasts, thus preventing HCC occurrence. HNF4A has been found to actively repress the EMT program through a direct transcriptional repression of the EMT master genes Snail and Slug. Recent studies reported that HBx inhibits the expression and transcription activity of HNF4A by activating AKT in primary hepatocytes. On the basis of the above results, we propose a model of HBx/HNF4A/miR-548p/HBXIP illustrating a possible molecular mechanism and functional basis for HBx-associated hepatocellular carcinogenesis (Fig. 5h).

In conclusion, our results reveal a novel association between HBV infection and the miR-548p/HBXIP pathway in the tumorigenesis of HBV-associated HCC. In this way, miR-548p serves as a suppressor in HBx-associated cell growth and hepatocarcinogenesis. Our study proved that miR-548p is a new potential therapeutic target for HBV-associated HCC.

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Figure S1  HBx overexpressed cell lines. (a) LO2, HepG2 and SMMC-7721 cell lines were selected to infect with lentivirus vectors pLVX-mCMV-ZsGreen-puro-HBx and pLVX-mCMV-ZsGreen-puro-NC, respectively. (100×) (b) The relative expression of HBX mRNA in HBx over-expressed cells and the negative control cells.

Figure S2  The expression of miR-548p and HNF4A in transient transfected cells. (a) Expression levels of miR-548p in cells treated with miR-548p mimic, inhibitor and miR-NC. MiR-548p mimic, inhibitor and miR-NC were transfected into cells by lipotac2000. (b) Expression levels of HNF4A mRNA in HepG2 cells transfected with HNF4A siRNA and nonsilencing siRNA (NC). (c) Expression levels of HNF4A protein in HepG2 cells transfected with HNF4A siRNA and nonsilencing siRNA (NC).

Table S1  The hepatitis B virus (HBV) status and relative expression levels of hepatitis B x-interacting protein (HBXIP) and miR-548p in 33 hepatocellular carcinoma (HCC) patients.

Table S2  miR-548p mimic and inhibitor, primers for quantitative reverse transcription polymerase chain reaction (qRT-PCR) and siRNA sequences used in this study.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web site.


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