



miR-135a inhibits the proliferation of HBV-infected hepatocellular carcinoma cells by targeting *HOXA10*

Jianjun Lin¹, Xiang Lian², Shihang Xue³, Lian Ouyang⁴, Lihui Zhou⁴, Yuyang Lu⁵, Longteng Xie²

¹Clinical Laboratory Department, Xiangshan First People's Hospital, Ningbo Fourth Hospital, Ningbo, China; ²Hepatology Department, Xiangshan First People's Hospital, Ningbo Fourth Hospital, Ningbo, China; ³Department of General Surgery, Xiangshan First People's Hospital, Ningbo Fourth Hospital, Ningbo, China; ⁴Department of Orthopaedic Surgery, Xiangshan First People's Hospital, Ningbo Fourth Hospital, Ningbo, China; ⁵Xiangshan County Center for Disease Control and Prevention, Ningbo, China

Contributions: (I) Conception and design: L Xie, Y Lu; (II) Administrative support: L Xie, Y Lu; (III) Provision of study materials: J Lin, X Lian, S Xue; (IV) Collection and assembly of data: L Ouyang, L Zhou; (V) Data analysis and interpretation: L Ouyang; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Yuyang Lu. No. 162, Danfeng East Road, Dandong Street, Xiangshan County, Ningbo 315700, China. Email: yuyang000666@126.com; Longteng Xie. 291 Donggu Road, Dandong Street, Xiangshan County, Ningbo 315700, China. Email: xfxlt@126.com.

Background: The incidence of hepatocellular carcinoma (HCC) in patients with hepatitis B virus (HBV) is extremely high. MicroRNAs (miRNAs) are a type of endogenous non-coding small RNA with novel molecular therapeutic mechanisms that plays an important role in the occurrence and development of cancers. This study aimed to explore the regulation mechanism of *miR-135a* and *HOXA10* in the proliferation, invasion, and apoptosis of HCC cells.

Methods: Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis was used to detect the expression level of *miR-135a*. Overexpression of *miR-135a* was used to measure the roles of *miR-135a* in the proliferation, invasion, and apoptosis of HCC cells. A dual luciferase experiment was performed to assess the relationship between *HOXA10* and *miR-135a*. Western blot was applied to observe the protein levels of p-p38, p-ERK, and p-JNK.

Results: The expression levels of *miR-135a* were significantly decreased in HCC tissues and cells. Overexpression of *miR-135a* inhibited the proliferation and invasion but promoted the apoptosis of HCC cells. Importantly, our results confirmed that *HOXA10* was a direct target of *miR-135a*. Under HBV infection, silencing of *HOXA10* significantly blocked the proliferation and invasion and promoted the apoptosis of HCC cells. In addition, *miR-135a/HOXA10* regulated the expressions of p-p38, p-ERK, and p-JNK through the *miR-135a/HOXA10* axis, thereby inhibiting the activation of the MAPK pathway.

Conclusions: HBV promoted the proliferation and invasion, and inhibited the apoptosis of HCC cells by regulating the *miR-135a/HOXA10* pathway. These findings provide a theoretical basis for improving the treatment of HBV-infected HCC patients.

Keywords: Hepatitis B virus (HBV); hepatocellular carcinoma (HCC); *miR-135a*; *HOXA10*

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Introduction

At present, the incidence of hepatocellular carcinoma (HCC) is very high (1,2). Hepatitis B virus (HBV) infection is the main cause of type B HCC onset. Globally, 55% of

HCC patients are infected with HBV, while in Asia and Africa, this proportion is as high as 80% (3). Adults with chronic hepatitis B will eventually develop cirrhosis and/or HCC. Cirrhosis and HCC were responsible for 887,000

HBV deaths in 2015 (4). HBV is an easily mutated virus with genetic diversity. There are more than 9 genotypes of HBV. Different genotypes of HBV have different prevalence, geographic distribution, natural history, disease progression, and treatment outcomes (5). Although the specific mechanism through which the HBV virus induces hepatocellular malignancies is still not very clear, some studies have reported that the various intracellular regulatory mechanisms involved in *HOXA10* are closely related to the occurrence and development of various types of cancers (6,7). *HOXA10* is a member of the homeobox gene family and is overexpressed in cancer tissues. Studies have found that *HOXA10* is highly expressed in oral cancer (8), endometrial cancer (9), breast cancer (10), and other tissues, which is related to the grade and invasion of the corresponding tumors. On the one hand, in normal liver cells infected with the HBV virus, the *HOXA10* protein promotes the replication of the HBV viral genome (7). On the other hand, it exerts a carcinogenic effect by disturbing the epigenetic modification of the host cells (11). The *HOXA10* protein can promote the expression of oncogenes and inhibit the activation of tumor suppressor genes by changing the ribosomal histone modification of cells or via DNA methylation modification (12). Importantly, *HOXA10* can inhibit the proliferation of HCC cells through *HDAC1* and induce cell cycle arrest and apoptosis (6).

Recent study has reported that *HOXA10* is related to the expression of certain microRNA (miRNA) in liver cells (11). MiRNA is a type of endogenous non-coding small RNA with complex regulatory functions found in eukaryotic cells (13). It regulates the expression of target genes at the post-transcriptional level and participates in the regulation of

many cell biological processes (14). Studies have shown that a variety of miRNAs play important roles in the occurrence and development of HCC caused by HBV infection (15). Exosome *miR-142-3p* secreted by HBV-HCC cells can regulate ferroptosis and HCC progression in M1-type macrophages by targeting *SLC3A2* (16). *MiR-744-5p* has been shown to regulate the behavior of HCC cells by regulating the TGF- β 1 signaling pathway and epithelial-mesenchymal-transition (EMT) (17). Although the effects of *HOXA10* on the expression of miRNA in cells have been reported, the regulatory roles between *miR-135a* and *HOXA10* in terms of biological function remain unknown. It has been reported that *miR-135a* is up-regulated in HCC (18). Our previous study confirms inhibiting *miR-135a* expression could promote HCC cell apoptosis (19). Therefore, *miR-135a* might be significantly related to the occurrence and development of HCC. At present, studies have not reported on the effects of HBV on the changes in the overall expression profile of miRNAs in liver cells or the bioinformatics analysis of the cellular pathways involved in these miRNAs.

This study explored the mechanism and roles of *miR-135a* and *HOXA10* in HBV-induced HCC and provided a theoretical basis and new targets for further research on the mechanism and clinical treatment of HCC induced by HBV viral infection. We present the following article in accordance with the MDAR reporting checklist (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-22-2789/rc>).

Methods

Sample collection

The HCC tissues used in this study were obtained from 90 patients with HCC, including 80 males and 10 females, with an average age of 52.37 years. Nineteen cases were HBV negative, and 71 cases were HBV positive. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the ethics committee of Xiangshan First People's Hospital, Ningbo Fourth Hospital (No. 20190530-2), and all subjects voluntarily participated in this study and signed the written informed consent.

Cell culture and transfection

Human normal hepatocytes (LO2), hepatoma cells (Bel-

Highlight box

Key findings

- HBV promoted the proliferation and invasion and inhibited the apoptosis of HCC cells via the *miR-135a*/*HOXA10* pathway.

What is known and what is new?

- miRNAs regulate HCC cell proliferation, apoptosis, migration and invasion.
- This study explored the mechanism and roles of *miR-135a* and *HOXA10* in HBV-induced HCC.

What are the implications, and what should change now?

- This study provides a theoretical basis and new targets for further research on the mechanism and clinical treatment of HBV-induced HCC.

7402, Huh7, HepG2), and HBV-infected hepatoma cells (HepG2.2.15) were cultured in Dulbecco's Modified Eagle Medium (DMEM) medium containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 μ L/mL penicillin, and 0.1 mg/mL streptomycin in a 5% CO₂ incubator at 37 °C.

Transfection was performed when the cell confluence reached 70–80%, and Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) was used for transient transfection according to the manufacturer's instructions. After 6 h, the old medium was discarded and replaced by the fresh complete medium. After culturing for 24 h, the cells were used for *in vitro* experiments.

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The total RNAs of cells in each group were extracted using Trizol reagent, and complementary DNA (cDNA) was synthesized by reverse transcription according to the previous literature (20,21). The expression level of *miR-135a* was determined using the TaqMan miRNA reverse transcription kit (Life Technologies, USA). Using U6 as the internal parameter, the expression level of *miR-135a* in each group was calculated according to the $2^{-\Delta\Delta CT}$ method.

Dual luciferase experiment

The wild-type and mutant luciferase plasmids of *HOXA10* were constructed and co-transfected with a *miR-135a* mimics and inhibitor. Luciferase activity was detected using a dual luciferase assay kit (Promega, WI, USA). The luminous intensity of each well was measured with a microplate fluorometer (Thermo Scientific, USA), and the dual luciferase activity of each group was calculated. Transfection was performed in duplicate and repeated three times.

Cell Counting Kit-8 (CCK)-8 assay

1×10^3 to 3×10^3 cells were inoculated in 96-well plates and cultured overnight. The cells were treated according to the experimental requirements. After the cell culture reached the target time, the medium was discarded and replaced by the fresh medium containing 10% CCK-8 reagent (Meilun, Dalian, China). After incubation for 1–4 h, the absorbance value of optical density (OD450) was measured

using a multifunctional microplate reader (Thermo Scientific, USA).

Apoptosis assay

Cells from different groups were collected in a flow tube. Next, the cells were digested with 1 mL of 0.25% trypsin. The cell suspension was collected in a flow tube for 300 g centrifugation for 5 min and the supernatant was discarded. The pelleted cells were then resuspended in 300 μ L of binding buffer. Subsequently, 5 μ L Annexin V-fluorescein isothiocyanate (FITC) was added, mixed, and incubated in the dark for 10 min. Next, 5 μ L of propidium iodide (PI) solution was added and the cells were incubated in the dark for 5 min after mixing. Finally, the apoptosis rate was detected by flow cytometry within 1 h.

Transwell assay

First, 600 μ L of medium containing 10% FBS was added to the lower chamber of the transwell plate, and 5×10^4 cells were inoculated in the upper chamber and maintained in the incubator for 24 h. The liquid in the chamber was then sucked up and fixed in the hole pre-filled with about 800 μ L of methanol at room temperature for 30 min. Subsequently, the cells were stained with 800 μ L of Giemsa staining solution for 30 min at room temperature, and then gently rinsed several times with phosphate buffer saline (PBS) and observed under the microscope (Nikon TiU, Japan).

Western blotting assay

The total cell protein was extracted using protein lysate and the protein concentration was calculated as previously described (22–24). For Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) experiments, the same amount of protein solution was added to each well. After electrophoresis, the protein was transferred to a polyvinylidene fluoride (PVDF) membrane and blocked with 5% skim milk at room temperature for 2 h. The primary antibody was incubated overnight at 4 °C. After the membrane was washed three times with Tris Buffered Saline Tween (TBST), the secondary antibody was incubated for 1 h at room temperature. After the membrane was washed three times with TBST, enhanced chemiluminescence (ECL) luminescent solution was used to analyze the protein gray value. All antibodies were

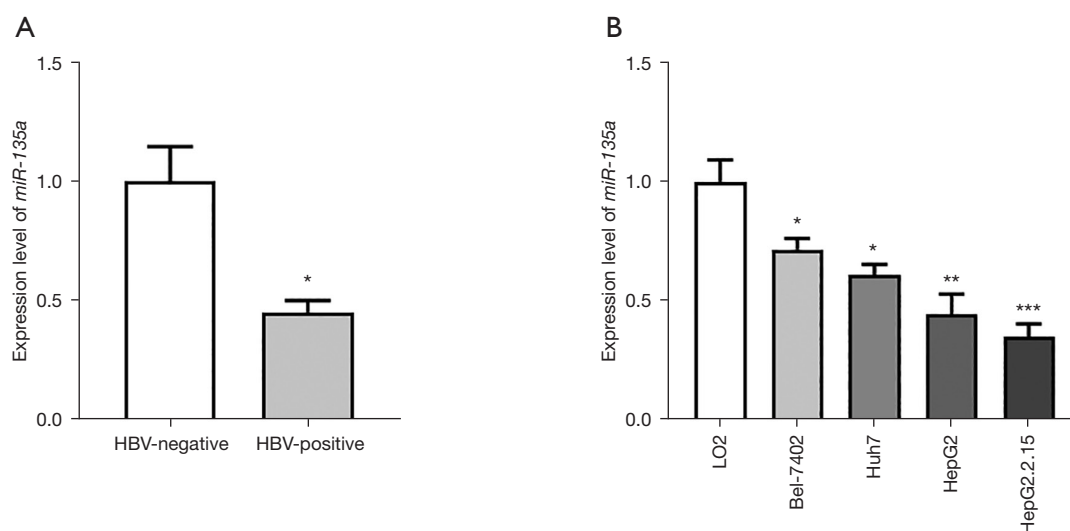


Figure 1 The expression of *miR-135a* in HCC tissues and cells. (A) The expression level of *miR-135a* in HBV-negative and HBV-positive liver tissues. (B) The expression levels of *miR-135a* in LO2, Bel-7402, Huh7, HepG2, and HepG2.2.15. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. HBV, hepatitis B virus; HCC, hepatocellular carcinoma.

purchased from Cell Signaling Technology, USA. The dilution ratio of the primary antibody was 1:1,000, and the dilution ratio of the secondary antibody was 1:4,000.

Statistical analysis

The data were analyzed using GraphPad Prism 7.0 software (GraphPad Software, USA). The data were expressed as means \pm standard deviation (SD). The comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by the post hoc test for multiple comparisons. $P < 0.05$ indicated a significant difference.

Results

miR-135a expression in HCC tissues and cells

HBV can seriously aggravate the prevalence of liver cancer patients, and it is very important to effectively study HCC disease under HBV infection. To explore the expression of *miR-135a* in HCC, we detected the expression level of *miR-135a* in HCC tissues and cells using qRT-PCR analysis. The results indicated that the expression level of *miR-135a* was significantly decreased in the tissues from the HBV-positive group, as compared with those from the HBV-negative group (Figure 1A). Also, the results from

subsequent experiments showed that the expression of *miR-135a* was significantly reduced in Bel-7402, Huh7, HepG2, and HepG2.2.15 cells compared to LO2 cells, while the expression level was the lowest in the HepG2.2.15 cells (Figure 1B). These results indicated that *miR-135a* was decreased in HCC tissues and cells, which suggested that *miR-135a* might be related to the occurrence and development of HCC.

The effects of *miR-135a* on the cell proliferation of HCC cells

The expression of *miR-135a* was significantly decreased in HepG2 and HepG2.2.15 cells. Thus, these HCC cells (HepG2 and HepG2.2.15) were selected for further experiments. These experiments were used to explore the effects of *miR-135a* on cell proliferation through CCK-8 assay and by detecting the expression of proliferation-related proteins. The CCK-8 assay revealed that the OD values of the *miR-135a* mimics + HepG2 group and the HepG2.2.15 + *miR-135a* mimics group were markedly reduced compared with the HepG2 and HepG2.2.15 group (Figure 2A). Moreover, the OD values of the HepG2.2.15 + *miR-135a* mimics group were the lowest (Figure 2A). These findings suggested that *miR-135a* significantly inhibited the proliferation of HCC cells, and the proliferation rate in HBV-infected cells was also inhibited (Figure 2A).

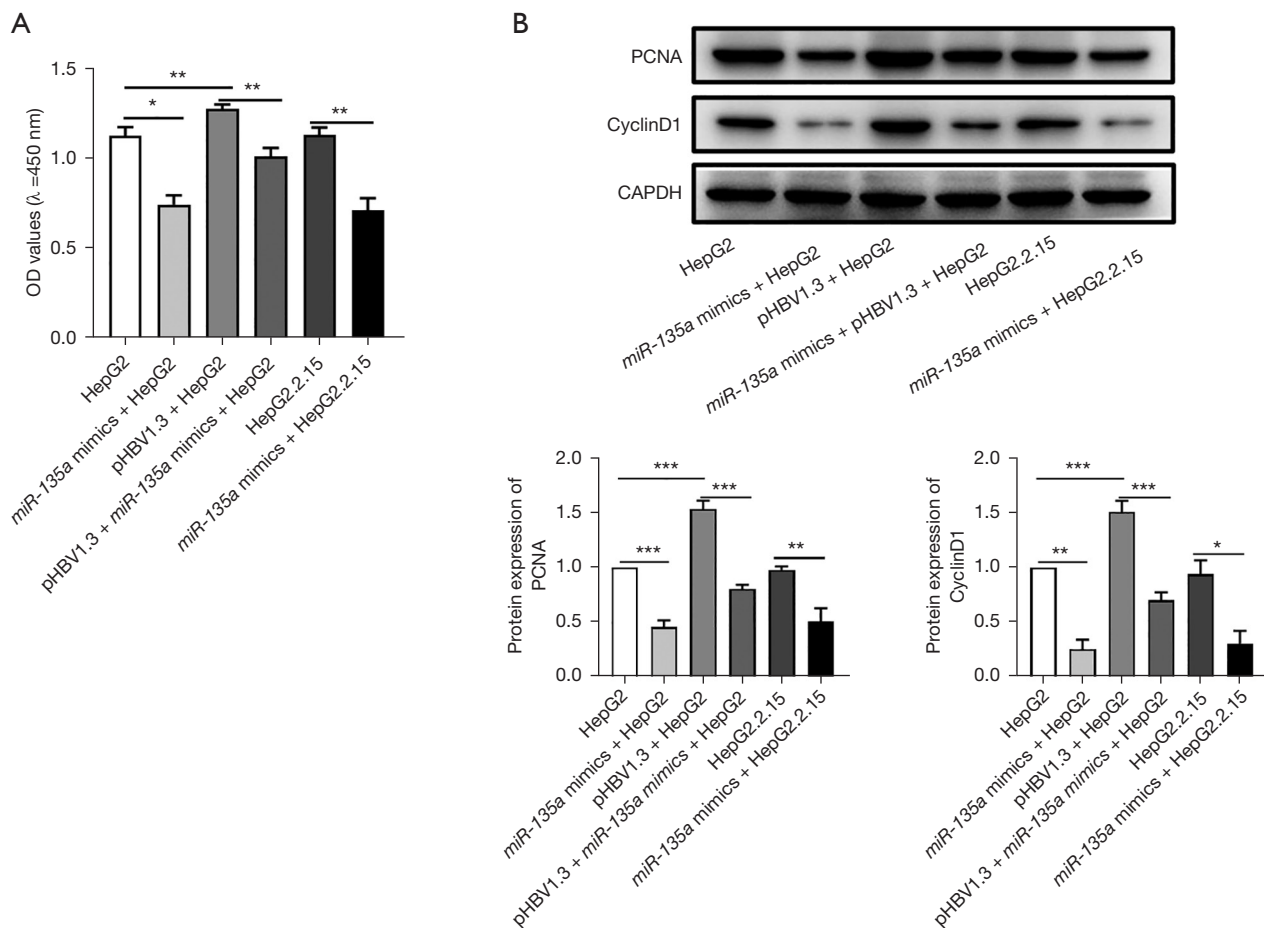


Figure 2 The effects of *miR-135a* on the cell proliferation of HCC cells. (A) A CCK-8 assay was used to detect the effects of *miR-135a* on cell proliferation of HCC cells. (B) A western blot assay was applied to measure the expression levels of proliferation-related proteins, *PCNA* and *CyclinD1*. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. HCC, hepatocellular carcinoma; CCK-8, Cell-Counting-Kit-8; OD, optical density; PCNA, proliferating cell nuclear antigen.

Also, the expression levels of proliferation-related proteins, proliferating cell nuclear antigen (PCNA) and CyclinD1, were significantly downregulated in the *miR-135a* mimics + HepG2 group and the HepG2.2.15 + *miR-135a* mimics group, as compared with the HepG2 and HepG2.2.15 group (Figure 2B). Furthermore, the expression levels of PCNA and CyclinD1 in the HepG2.2.15 + *miR-135a* mimics group were the lowest (Figure 2B). These results indicated that *miR-135a* inhibited HCC cell proliferation, especially after HBV infection.

The effects of *miR-135a* on the cell invasion of HCC cells

To further investigate the roles of *miR-135a* in the development of HCC, we determined the HCC cell

invasion after *miR-135a* transfection. Transwell assays were applied to detect the invasion ability of *miR-135a* on HCC cells (Figure 3). According to the experimental results, the number of invasive cells in the *miR-135a* mimics group was significantly decreased compared to the untransfected *miR-135a* group (Figure 3A-3F). Interestingly, compared with the pHBV1.3 + HepG2 group, the number of invasive cells in the pHBV1.3 + HepG2 + *miR-135a* mimics group was significantly reduced (Figure 3A-3F). Additionally, the number of invasive cells in the HepG2.2.15 + *miR-135a* mimics group was lower than that in the HepG2.2.15 group (Figure 3A-3F). Taken together, these results showed that the overexpression of *miR-135a* significantly inhibited the invasion ability of HCC cells, especially after HBV infection.

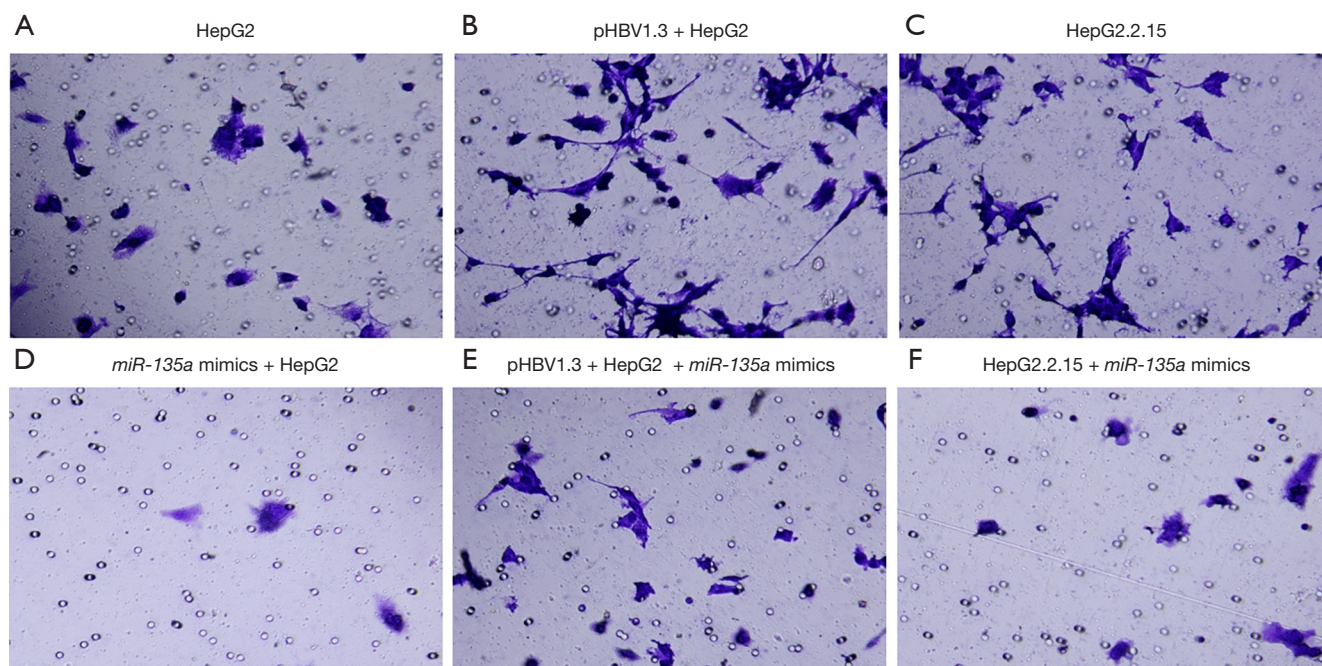


Figure 3 The effects of *miR-135a* on cell invasion of HCC cells. (A) HepG2. (B) pHBV1.3 + HepG2. (C) HepG2.2.15. (D) *miR-135a* mimics + HepG2. (E) pHBV1.3 + *miR-135a* mimics + HepG2. (F) HepG2.2.15 + *miR-135a* mimics. (200 \times , Giemsa staining). HCC, hepatocellular carcinoma; HBV, hepatitis B virus.

The effects of miR-135a on the apoptosis of HCC cells

We further detected the roles of *miR-135a* in the apoptosis of HCC cells to determine the functions of *miR-135a* in HCC. Through flow cytometry experiments, we found that the *miR-135a* mimics + HepG2 group and the HepG2.2.15 + *miR-135a* mimics group both increased the apoptosis rate of HCC cells, as compared to the HepG2 group (Figure 4A). Also, the apoptosis rate of the HepG2.2.15 + *miR-135a* mimics group was significantly higher than that of the HepG2.2.15 group (Figure 4A). These results suggested that *miR-135a* markedly increased the apoptotic rate of HCC cells, and the level of apoptosis was much higher under HBV infection (Figure 4A).

In addition, the results of western blotting experiments also showed that the expressions of pro-apoptotic proteins (Bax and Cleaved Caspase-3) were upregulated in HCC cells following *miR-135a* mimics transfection (Figure 4B). Meanwhile, the expression of the anti-apoptotic protein Bcl-2 was markedly downregulated in HCC cells transfected with *miR-135a* mimics (Figure 4B). These results suggested that the overexpression of *miR-135a* promoted the apoptosis of HCC cells under HBV infection.

HOXA10 was the target of miR-135a

To further investigate the mechanism of *miR-135a* in HCC cells, the target genes of *miR-135a* were further determined. Through the dual luciferase experiment, we found that the *miR-135a* mimics and the WT-*HOXA10*-3'UTR plasmid group had significantly decreased luciferase activity compared with the WT-*HOXA10*-3'UTR plasmid and mimics negative control (NC) co-transfection group (Figure 5A). However, compared with the MUT-*HOXA10*-3'UTR plasmid and mimics NC co-transfection group, the luciferase activity of the *miR-135a* mimics and the MUT-*HOXA10*-3'UTR plasmid group did not exhibit a notable change (Figure 5A).

HOXA10, as a key cancer gene, plays an important role in liver cancer. Therefore, we examined the expression level of *HOXA10* in HCC cells. The results showed that the expression level of *HOXA10* was significantly increased in HCC cells, especially in the HBV-transfected HepG2 cells, as compared with LO2 cells (Figure 5B). Also, the western blotting results showed that the expression level of *HOXA10* in HCC cells was significantly increased (Figure 5C). The above results suggested that *HOXA10* was significantly

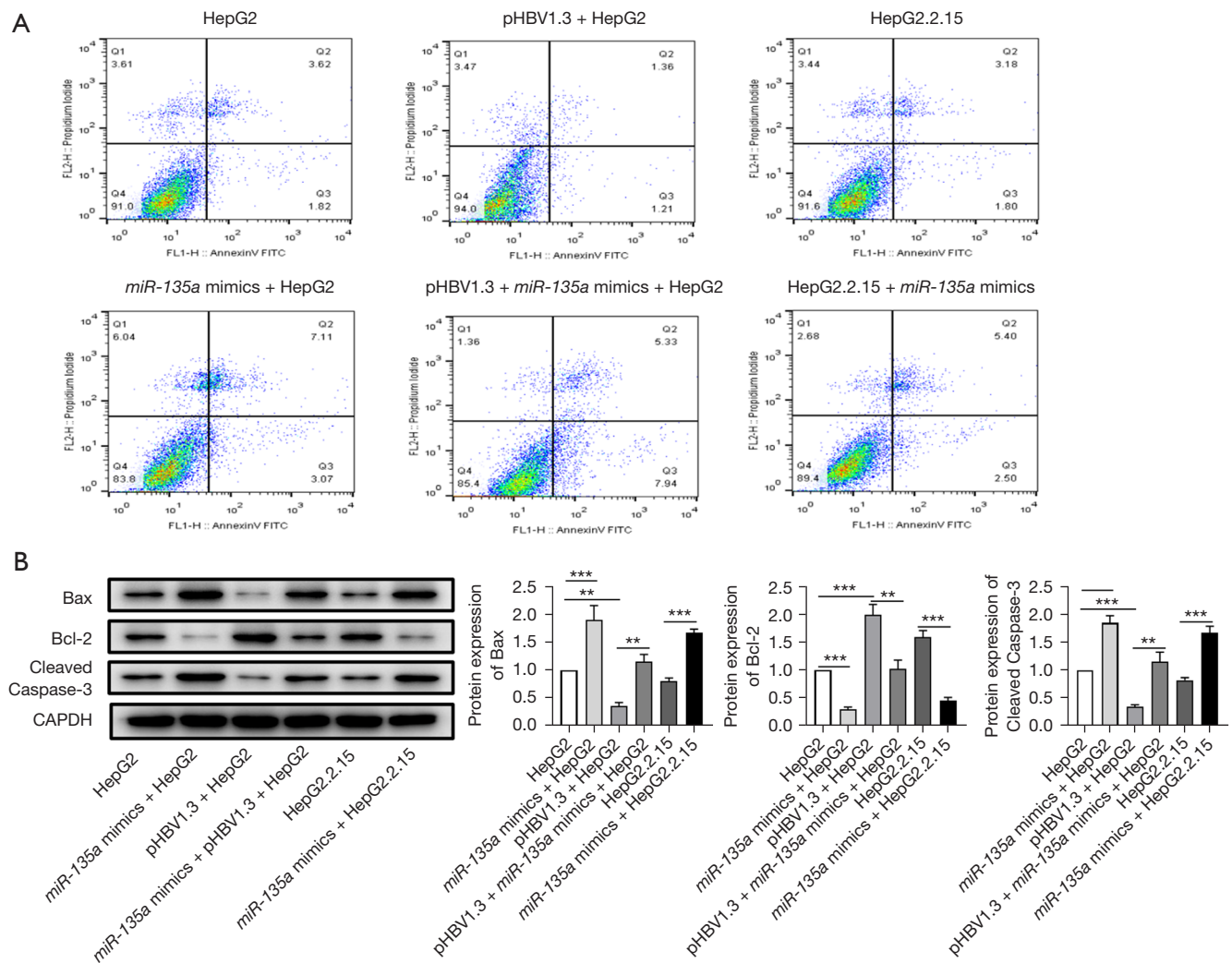


Figure 4 The effects of *miR-135a* on the cell apoptosis of HCC cells. (A) Flow cytometry was used to detect the effects of *miR-135a* on cell apoptosis of HCC cells. (B) The expression levels of apoptosis-related proteins, Bcl-2, Bax, and Cleaved Caspase-3. **, $P < 0.01$; ***, $P < 0.001$. HCC, hepatocellular carcinoma; HBV, hepatitis B virus; FITC, fluorescein isothiocyanate.

upregulated in HCC cells, especially after HBV infection, and thus, *HOXA10* might be a target of *miR-135a* in HCC cells.

The effects of *HOXA10* on cell proliferation in HCC cells

A CCK-8 assay was applied to detect the roles of *HOXA10* in HCC cells. The CCK-8 experiment showed that *miR-135a* mimics and si-*HOXA10* could inhibit the proliferation of HCC cells, and co-transfection of the *miR-135a* mimics and si-*HOXA10* could significantly reduce the OD values of HCC cells in the HepG2 + pHBV1.3 cell group (Figure 6A).

Similarly, in the HepG2.2.15 cell group, co-transfection of the *miR-135a* mimics and si-*HOXA10* also substantially inhibited the proliferation of HCC cells (Figure 6B).

The protein expression levels of proliferation-related proteins, PCNA and CyclinD1, were further measured using a western blot assay. The results indicated that in HepG2 cells transfected with pHBV1.3 or HepG2.2.15 cells, the *miR-135a* mimics + si-*HOXA10* considerably reduced the expression levels of PCNA and CyclinD1 (Figure 6C,6D). Together, these results showed that *miR-135a* might inhibit the proliferation of HCC cells through its target gene, *HOXA10*.

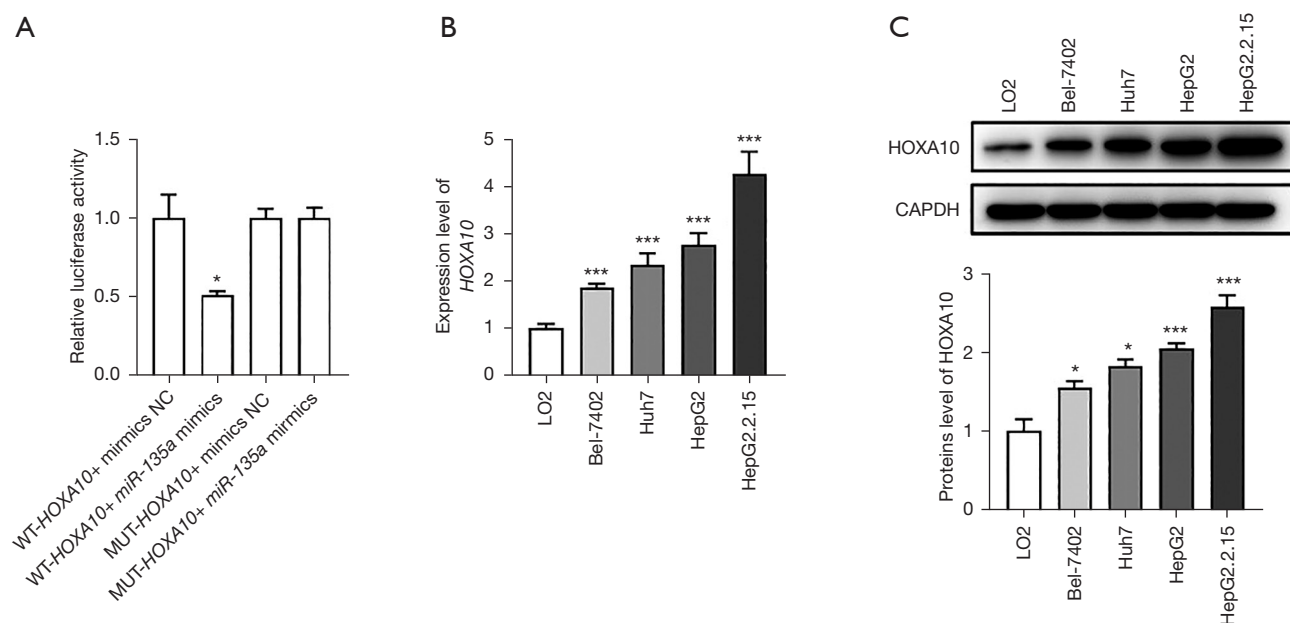


Figure 5 The relationship between *miR-135a* and *HOXA10*. (A) Double luciferase reporter gene detection was performed to detect the ratio of firefly/Renilla activity. (B) The mRNA expression of *HOXA10* in HCC cells. (C) The protein expression of *HOXA10* in HCC cells. *, $P < 0.05$; ***, $P < 0.001$. HCC, Hepatocellular Carcinoma; HBV, hepatitis B virus; WT, wild type; MUT, mutant type; NC, negative control.

The effects of *HOXA10* on cell invasion of HCC cells

Our results showed that *miR-135a* could inhibit the invasion of HCC cells. We then investigated the roles of *HOXA10* in HCC cell invasion. By silencing the target gene, *HOXA10*, we found that the number of invasive cells in *miR-135a* mimics + si-*HOXA10* was significantly reduced in the HepG2 + pHBV1.3 cell group (Figure 7). Similarly, in the HepG2.2.15 cell group, the number of invasive cells in *miR-135a* mimics + si-*HOXA10* was also markedly reduced (Figure 7). Therefore, these results suggested that *miR-135a* could significantly inhibit the invasion of HCC cells by silencing *HOXA10* in HBV-infected HCC cells.

The effect of *HOXA10* on the cell apoptosis of HCC cells

In the HepG2 + pHBV1.3 group, the co-transfection of *miR-135a* mimics and si-*HOXA10* significantly increased the apoptotic rate of HCC cells, and the same results were also obtained in the HepG2.2.15 group (Figure 8A, 8B). Furthermore, the western blot assay results showed that the *miR-135a* mimics and si-*HOXA10* co-transfection also upregulated the protein expression level of the pro-apoptotic proteins, Bax and Cleaved Caspase-3, and downregulated the protein expression of the anti-apoptotic

protein, Bcl-2, in the HepG2 + pHBV1.3 group (Figure 8C). In addition, the same results were also obtained in the HepG2.2.15 group (Figure 8D). The above results indicated that *miR-135a* could significantly promote the apoptosis of HCC cells by silencing *HOXA10* in HBV-infected HCC cells.

The effects of *miR-135a*/*HOXA10* on the activation of the MAPK pathway

The MAPK pathway is a classic inflammation pathway, and the inflammatory response is a protective mechanism for the body to resist the invasion of pathogenic microorganisms. Thus, the MAPK pathway might be related to the *miR-135a* and *HOXA10*-mediated regulatory roles in HCC cells and further experiments were performed to investigate this hypothesis. The western blot analysis results showed that, compared with the control group, the *miR-135a* mimics + si-*HOXA10* group significantly reduced the expression levels of phosphorylated proteins the p-p38, p-ERK, and p-JNK in HepG2 cells transfected with pHBV1.3 (Figure 9A). In HepG2.2.15 cells, the expression levels of p-p38, p-ERK, and p-JNK were also significantly inhibited via the co-transfection of *miR-135a* and si-*HOXA10* (Figure 9B).

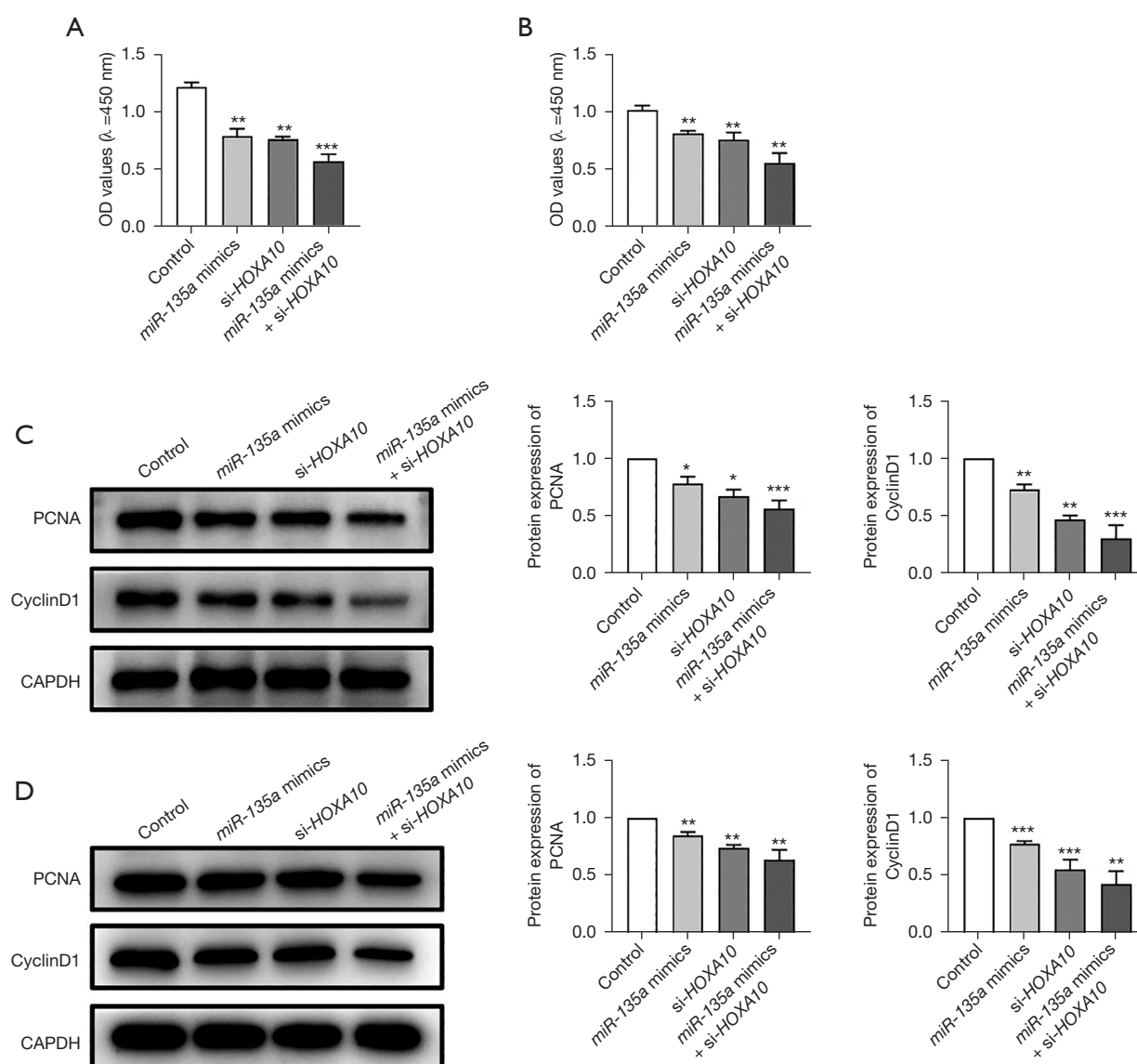


Figure 6 The effects of si-*HOXA10* on cell proliferation of HCC cells. (A,B) The effect of CCK-8 detection on the proliferation of the HepG2 + pHBV1.3 cell group (A) and the HepG2.2.15 cell group (B). (C,D) The expression levels of apoptosis-related proteins PCNA and CyclinD1 in the HepG2 + pHBV1.3 cell group (C) and the HepG2.2.15 cell group (D). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. HCC, hepatocellular carcinoma; HBV, hepatitis B virus; CCK-8, Cell-Counting-Kit-8; OD, optical density.

Therefore, *miR-135a*/si-*HOXA10* could significantly inhibit the activation of the MAPK pathway, which suggested that *miR-135a* might regulate the development of HCC by inhibiting the activation of the MAPK pathway.

Discussion

HCC has a high incidence and poor prognosis. Various

types of genes and signal pathways are dysregulated in the pathogenesis of HCC (25). Recent studies have shown that more than 50% of miRNAs are located in relevant oncogenomic regions, and their expression is frequently dysregulated in cancer, highlighting their key role in tumorigenesis (13,26). Currently, miRNA mimics and siRNA, as targeted molecular therapy programs, are favored by the medical community (27). MiRNAs have proved to

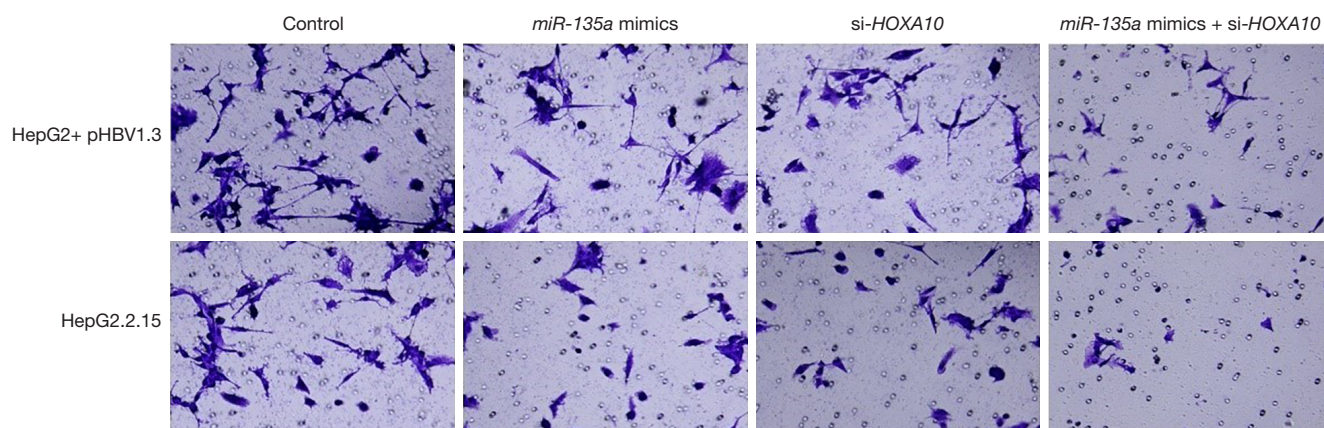


Figure 7 The effects of *miR-135a*, *si-HOXA10*, and *miR-135a* + *si-HOXA10* on cell invasion in the HepG2 + pHBV1.3 and HepG2.2.15 groups, respectively. (200×, Giemsa staining). HBV, hepatitis B virus.

be dysregulated in HBV-infected HCC. 37 miRNAs are differently expressed in HBV-related TCGA-HCC cohorts between TPX2 Microtubule Nucleation Factor (TPX2)^{low} and TPX2^{high} groups (28). *MiR-933* could affect cell growth and HBV DNA synthesis (29). *LINC01232* could sponge *miR-708-5p* to regulate HCC progression (30). *MiR-135a* has been confirmed to play an important role in the invasion and metastasis of a variety of tumors. For example, it promotes the growth and invasion of colorectal cancer through metastasis inhibitor 1 *in vitro* (31), and inhibits the proliferation of gastric cancer cells by targeting *JAK2* (32). However, there are few studies on *miR-135a* in HBV-infected liver tumors. Therefore, it is of practical significance to study the effect of *miR-135a* on HCC under HBV infection.

To explore the relationship between HBV and *miR-135a*, we used qRT-PCR to verify whether HBV could regulate the expression of *miR-135a*. The results showed that the expression of *miR-135a* was significantly down-regulated in HCC tissues and cells. This suggests that HBV may downregulate the expression of *miR-135a* through a particular mechanism. So, we attempted to determine how *miR-135a* is regulated by HBV. It is known that the biosynthesis of miRNAs is regulated by numerous aspects and levels, including the transcription level of miRNAs, RNA modification processes such as RNA methylation, and the localization of Ago proteins, etc. Among these, abnormal regulation of the miRNA transcription level is an important factor for the abnormal expression of miRNA. The abnormal regulation of *miR-135a* determines the fate of hepatoma cells to a certain extent. In our research, we

observed that *miR-135a* is lowly expressed in HCC tissues and cell lines, and this downregulation suppressed the proliferation of HCC cells.

Invasion ability and apoptosis level are important indicators reflecting the vitality of HCC cells (33). It is known that HCC cells exert a strong ability to quickly invade surrounding cells, resulting in cell pathology and death (34,35). MiRNA regulation of tumor cells is inseparable from its influence on the invasion ability of tumor cells. A study has shown that *miR-135a* promotes the migration and invasion of HCC cells by targeting fork head box O1 (36). In this study, we came to the opposite conclusion; *miR-135a* not only does not promote the invasion ability of HepG2 or HepG2.2.15 cells but also inhibits their invasion, which is the more interesting aspect. MiRNA is known to possess the ability of two-way regulation, both promoting and inhibiting, so this may happen. By detecting the apoptosis ability, we found that under HBV infection, *miR-135a* significantly promoted the apoptosis of HCC cells, and at the same time elevated the expression of related pro-apoptotic proteins and reduced the expression of related anti-apoptotic proteins. These results illustrated the inhibitory effect exerted by HBV on the apoptosis of HCC cells, one of which was achieved by regulating the expression of *miR-135a*.

HOXA10 is a human homeobox gene; it is a DNA-binding transcription factor with sequence-specific DNA-binding activity (37). It is widely involved in reproductive tract development, embryo implantation regulation, and cell-directed differentiation and proliferation (38). *HOXA10* plays an important role in embryonic development,

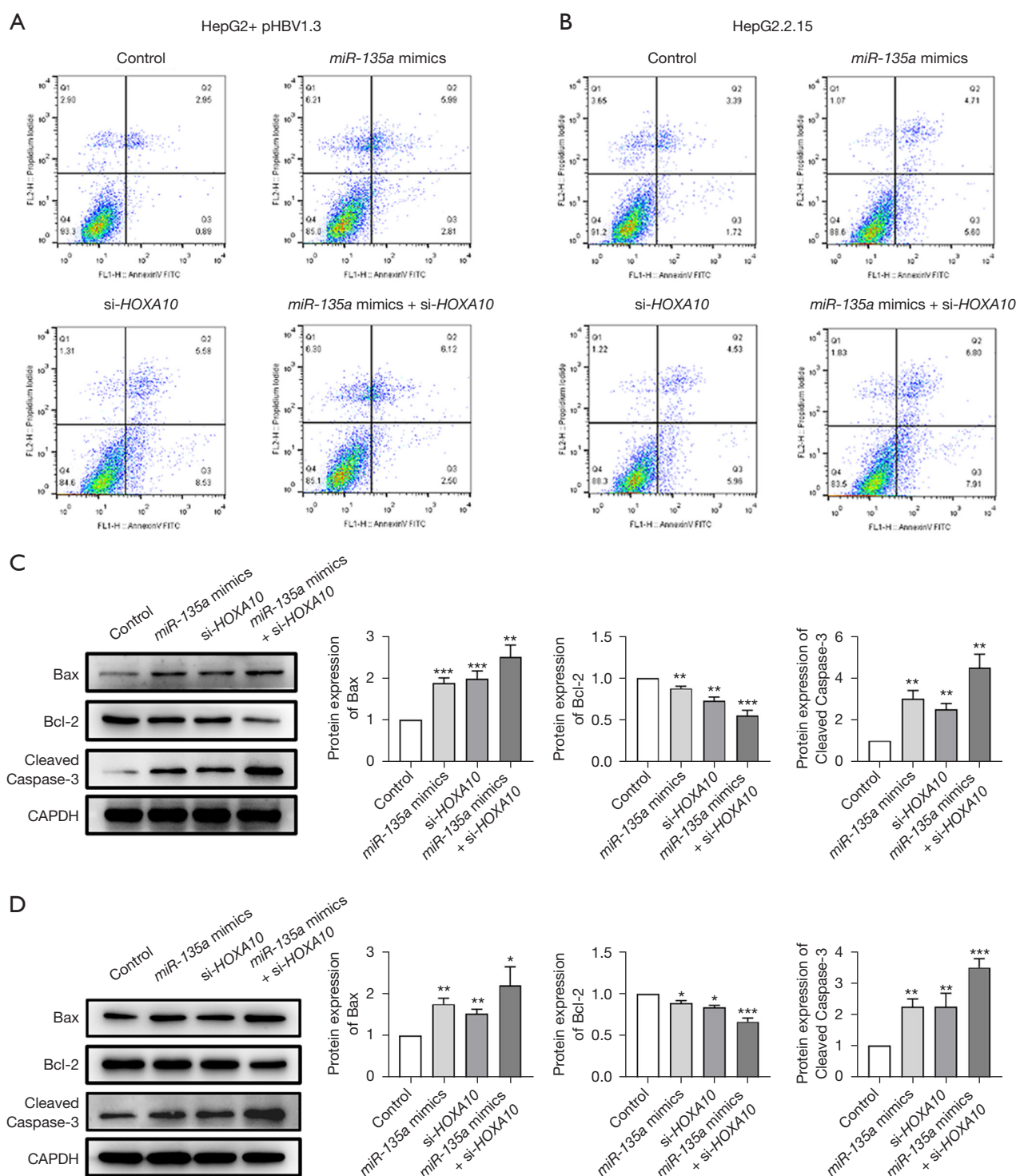


Figure 8 The effect of *si-HOXA10* on the cell apoptosis of HCC cells. (A,B) Flow cytometry was used to detect cell apoptosis in the HepG2 + pHBV1.3 group (A) and the HepG2.2.15 group (B). (C,D) The expression levels of apoptosis-related proteins (Bcl-2, Bax, and Cleaved Caspase-3) in the HepG2 + pHBV1.3 group (C) and the HepG2.2.15 group (D). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. HCC, hepatocellular carcinoma; HBV, hepatitis B virus; FITC, fluorescein isothiocyanate.

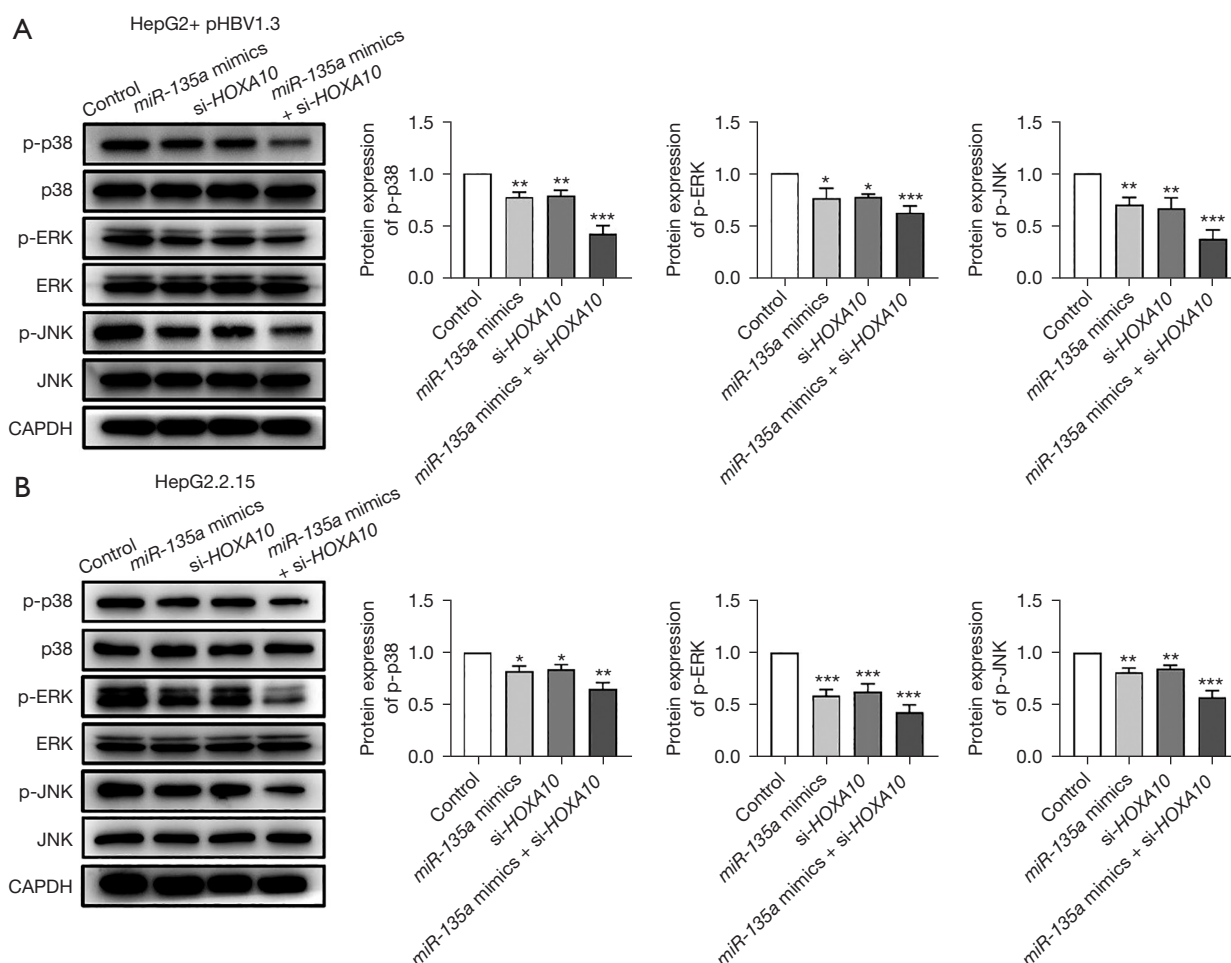


Figure 9 The effect si-*HOXA10* on the activation of the MAPK pathway. (A,B) The expression levels of p-p38, P38, p-ERK, ERK, p-JNK, and JNK in the HepG2 + pHBV1.3 group (A) and the HepG2.2.15 group (B). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

differentiation, cancer, and hematopoietic diseases. Studies have shown that *HOXA10* promotes gastric cancer metastasis by partially mediating epithelial-mesenchymal transition via regulation of the *TGFB2/Smad/METTL3* signaling axis (39). *LncHOXA10* drives the self-renewal and tumorigenesis of hepatic TICs through transcriptional activation of *HOXA10* (40). However, so far, the role of *HOXA10* in HBV infection has been rarely reported. Zhu *et al.* (41) used gene chip screening and found that *HOXA10* was highly expressed in HepG2.2.15 cell lines, which integrated the entire HBV genome.

The results of the experiments in this study confirmed that *HOXA10* was the target gene of *miR-135a*. Moreover, the high expression of *miR-135a* and silencing of *HOXA10* exerted a similar effect on HBV-infected HCC cells. It is

known that miRNA plays a role by regulating target mRNA, so *miR-135a* might exert a regulatory function on HCC cells by regulating the expression of *HOXA10*. Importantly, the high expression of *miR-135a* and silencing of *HOXA10* could significantly enhance the unilateral effects of both on HBV infection of HCC cells.

In addition, this study investigated the MAPK pathway regulation by *miR-135a/HOXA10*. MAPK is a classic inflammatory pathway that plays an important role in the occurrence and development of tumors. Studies have shown that isoquercitrin inhibits the progression of liver cancer *in vivo* and *in vitro* by regulating the MAPK signaling pathway (42). Interestingly, miRNAs and MAPK exhibit significant associations in the occurrence and development of tumor diseases, and jointly affect disease progression.

MiR-101 can affect the proliferation of hepatoma cells through the MAPK/ERK signaling pathway (43). In addition, *miR-338-3p* overexpression reduces the expression of *MACC1* in cervical cancer cells via the MAPK pathway, and significantly inhibits the proliferation of cervical cancer cells and induces apoptosis (44). Although miRNAs can participate in the development of liver cancer by regulating the MAPK pathway, it is still unknown whether *miR-135a* regulates the progression of liver cancer through the MAPK pathway. In this study, we found that *miR-135a* could significantly inhibit MAPK pathway activation.

Conclusions

In conclusion, HBV promoted the proliferation and invasion of HCC cells and inhibited the apoptosis by regulating *miR-135a/HOXA10* pathway. Importantly, *miR-135a* also promotes cell proliferation and inhibits MAPK pathway activation. This study provides a theoretical basis for understanding the mechanism of HBV infection of HCC cells.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-22-2789/rc>

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-22-2789/coif>). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the ethics

committee of Xiangshan First People's Hospital, Ningbo Fourth Hospital (No. 20190530-2), and all subjects voluntarily participated in this study and signed the written informed consent.

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