

miR-135b-5p Suppresses Androgen Receptor-Enhanced Hepatocellular Carcinoma Cell Proliferation via Regulating the HIF-2 α /c-Myc/P27 Signals in vitro

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Introduction: Hepatocellular carcinoma (HCC) accounts for more than 90% of liver cancers and is ranked as the fifth most common malignancy. Androgen receptor (AR) may promote the progression of HCC at an early stage of the disease. However, this study identified miR-135b-5p as an AR upstream regulator can suppress AR protein expression and inhibit HCC proliferation, consistent with the idea that AR expression is negatively correlated with HCC progression.

Methods: The target microRNAs were predicted using online databases (TargetScan, miRDB, and MicroCosm Targets). Cell proliferation ability was measured by MTT and colony formation assay. Western blot was performed to analyze the expression levels of AR, HIF-2 α , c-Myc, and p27, which are related to HCC proliferation. Chromatin immunoprecipitation (ChIP) assay and luciferase reporter assay were carried out to investigate the mechanism by which miR-135b-5p decreases AR expression.

Results: miR-135b-5p suppresses HCC cell proliferation and AR expression. Downregulation of AR expression by miR-135b-5p may in turn transcriptionally modulate HIF-2 α expression via direct binding of AR to the androgen response element (ARE) in the HIF-2 α promoter. Further dissection of the mechanism revealed that AR-modulated HIF-2 α could suppress c-Myc expression resulting in increased p27 expression that likely contributes to the suppression of proliferation in HCC cells.

Conclusion: miR-135b-5p suppresses HCC cell proliferation via targeting AR-modulated HIF-2 α /c-Myc/p27 signals, which may help to develop more effective therapies to prevent HCC progression.

Keywords: HCC, miR-135b-5p, AR, HIF-2 α , c-Myc, p27

Introduction

Hepatocellular carcinoma (HCC) accounts for more than 90% of liver cancers and is ranked as the fifth most common malignancy.¹ It is the second leading cause of cancer-induced death in males and the sixth in females with more than 700,000 deaths every year worldwide.² While targeted molecular therapy and surgical resection have experienced rapid progress recently, the advancement of long-term survival remains far from satisfactory. Moreover, patients who have received surgical resection often have a high recurrence, with a 5-year survival rate of only 25%.^{3,4} Therefore, new and better therapies are needed to defeat this deadly disease.

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Androgen receptor (AR), a member of nuclear hormone receptors, plays an important role in HCC progression.⁵ It has been reported that AR might promote HCC progression in its early developmental stages,^{6–9} and targeting AR can inhibit HBV- or carcinogen-induced HCC development at an early stage *in vitro* and *in vivo*.⁸ However, the molecular mechanisms leading to the activation of AR signaling during HCC progression remain to be further elucidated.

microRNAs (miRNAs) are a group of small, well-conserved, non-coding, endogenous RNA molecules containing around 18–25 nucleotides,¹⁰ which play key post-transcriptional regulatory roles in mRNA translation and degradation, predominantly in the 3'-untranslated region (3'-UTR).^{11,12} Recent studies have indicated that aberrantly expressed miRNAs might play crucial roles in a variety of biological processes, including cancer pathogenesis.^{13,14} Certain miRNAs have been shown to regulate tumor progression via binding to the AR 3'-UTR sequence.^{15–17} As the expression of miRNAs is associated with HCC progression and metastasis,^{18–20} and AR might promote HCC cell proliferation, it would be interesting to determine if miRNAs serve as AR upstream regulators to alter HCC cell proliferation. Bioinformatic analyses from literature and online databases (TargetScan, miRDB, and MicroCosm Targets) suggest that a subset of miRNAs, including miR-99b-5p, miR-101-3p, miR-135b-5p, miR-373-3p, and miR-375, may target the AR 3'-UTR sequence and simultaneously inhibit HCC progression. Our experiments identified miR-135b-5p as an AR upstream regulator, which suppresses HCC cell proliferation via downregulating the AR-mediated HIF-2 α /c-Myc/p27 signal pathway.

Materials and Methods

Materials

MISSION[®] Synthetic miR-135b-5p inhibitor was purchased from Sigma-Aldrich (St. Louis, MO, USA) and transfected according to the technical bulletin provided by the manufacturer. AR, HIF-2 α , c-Myc, and GAPDH antibodies for Western blotting were purchased from Santa Cruz Biotechnology. The p27 antibody for Western blotting was purchased from Cell Signaling. Anti-mouse/rabbit secondary antibodies for Western blotting were from Invitrogen.

In vitro Cell Culture/Maintenance

SK-hep1, HepG2, SNK, and Huh7 human HCC cell lines were purchased from the American Type Culture

Collection (ATCC, Manassas, VA, USA). HA22T human HCC cell line was purchased from the Food Industry Research and Development Institute in Taiwan (BCRC number: 60,168). All cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS), penicillin (25 U/mL), streptomycin (25 g/mL), and 1% (v/v) L-glutamine in a 5% (v/v) CO₂ humidified incubator at 37°C.

Lentiviral-Based Gene Delivery

The pWPI/pWPI-AR, PLVTHM plasmid, and pLVTHM plasmids containing the miRNAs, psAX2 packaging plasmid, and pMD2G envelope plasmid were transfected into 293T cells using the standard calcium phosphate transfection method. The lentivirus soups were collected after incubating for 48 h and concentrated by density gradient centrifugation before freezing at –80°C for later use.

Cell Proliferation Assay

Cells were seeded in 24-well plates (1x10⁴ cells/well) and cultured in a 5% (v/v) CO₂ humidified incubator at 37°C. The following day, the lentivirus soups of pWPI/pWPI-AR, PLVTHM, and PLVTHM-miR-135b-5p were added for 6 h, and the cells were continually cultured for 48 h. MTT agent was added, and OD (570 nm) was determined.

Colony Formation Assay

Cells were seeded in 6-well plates (1x10³ cells/well) with three repetitions. After 12-day incubation, these plates were washed with phosphate buffered saline (PBS) twice, fixed by methanol for 10 min and stained with 0.1% crystal violet solution within 10 min for further analysis.

RNA Extraction, Reverse Transcription, and Quantitative Real-Time PCR Analysis (qRT-PCR)

Total RNAs were isolated using TRIzol reagent (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. Reverse transcription was performed using 1 μ g of total RNA and Superscript III Reverse Transcriptase (Invitrogen, Grand Island, NY, USA). Quantitative real-time PCR (qRT-PCR) used a Bio-Rad CFX96 system with SYBR green to determine the mRNA expression level of the gene of interest. Expression levels were normalized to the expression of GAPDH

mRNA. The qRT-PCR protocol was as follows: 50°C for 2 min, 95°C for 8 min 30sec, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. The extension was 95°C for 1 min, 55°C for 1 min, and 55°C for 10 s.

The PureLink[®] miRNA kit was used to extract the miRNAs. Then, 2 µg of RNA was incubated with 1 U of poly (A) polymerase with 1 mM ATP in 1x RT buffer at 37°C for 15 min in a volume of 10 µL. Reverse transcription was performed with the addition of 50 µM RT anchor primer and annealing at 65°C for 5 min, then 4°C for 2 min. Finally, cDNA synthesis was performed at 42°C for 60 min after the addition of 2 µL 10 mM dNTPs, 2 µL 5x RT buffer, 1 µL reverse transcriptase, and ddH₂O to a total volume of 20 µL. The qRT-PCR protocol was as follows: 95°C for 2 min, followed by 45 cycles at 95°C for 15 s, and 60°C for 45 s using a Bio-Rad CFX96 system with SYBR green to determine the mRNA expression level of the gene of interest. Expression levels were normalized to the expression of 5s RNA and/or U6.

Western Blot Analysis

Cells were lysed in lysis buffer, and proteins (30 µg) were separated on 10–12% gel by SDS/PAGE before transferring onto PVDF membranes (Millipore, Billerica, MA, USA). After blocking, the membranes were incubated with appropriate dilutions of specific primary antibodies against GAPDH, AR (Santa Cruz Biotechnology, Dallas, TX, USA), HIF-2α, c-Myc, or p27 (Cell Signaling). The blots were incubated with HRP-conjugated secondary antibodies (Invitrogen, USA) and visualized using the ECL system (Thermo Fisher Scientific, Rochester, NY, USA).

Luciferase Reporter Assay

AR 3'-UTR fragments of 540 bp containing wild-type or mutant miRNA response elements (MREs) were cloned into the psiCHECK-2 vector (Promega, Madison, WI, USA) downstream of the Renilla luciferase ORF. Cells were plated in 24-well plates, and the cDNAs were transfected using Lipofectamine 3000 (Invitrogen, USA) according to the manufacturer's instructions. Luciferase activity was measured by the Dual-Luciferase Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Chromatin Immunoprecipitation Assay (ChIP)

Cells were crosslinked with 4% formaldehyde for 10 min, followed by cell collection and sonication with

a predetermined power to yield genomic DNA fragments of about 400 bp in length. Lysates were pre-cleared sequentially with normal rabbit IgG (sc-2027, Santa Cruz Biotechnology, Dallas, TX, USA) and protein A-agarose. Anti-AR antibody (2.0 µg) was added to the cell lysates, which were then incubated at 4°C overnight. For the negative control, IgG was used in the reaction. The target sequence within the human HIF-2α promoter was amplified using the following primers: Forward 5'-GGGCCGAGAAATTATCCCCA -3'; Reverse 5'-GTT TATGAGGTCGTCTGGGC-3'. PCR products were analyzed by agarose gel electrophoresis.

Statistical Analysis

Data are expressed as mean ± SD from at least three independent experiments. Statistical analyses involved Student's *t*-test with GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). *P* < 0.05 was considered statistically significant.

Results

miR-135b-5p Suppresses HCC Cell Proliferation

Since AR may promote HCC cell proliferation, an upstream regulator of AR may also be involved in this process. We identified a subset of miRNAs, including miR-99b-5p, miR-101-3p, miR-135b-5p, miR-373-3p, and miR-375, that are predicted to regulate AR expression while also being involved in HCC tumorigenesis. This hypothesis was directly tested by overexpressing these miRNAs in HCC cell lines. Cell proliferation assays showed that miR-135b-5p significantly suppressed HCC cell proliferation in SK-hep1 and HA22T cells (Figure 1A) and HepG2, SNK, and Huh7 cells (Figure 1B).

Importantly, a colony formation assay showed that miR-135b-5p significantly suppressed colony formation in HCC cell lines (Figure 1C and D), further supporting the role of miR-135b-5p as a suppressor of HCC progression.

miR-135b-5p Suppresses AR Protein Expression in HCC Cell Lines

To link the suppression of HCC cell proliferation by miR-135b-5p to the modulation of AR expression, we first examined the effect of miR-135b-5p on AR expression in HCC cell lines. Analysis of AR expression by Western blot revealed that miR-135b-5p could significantly suppress the expression of AR protein in SK-hep1 and

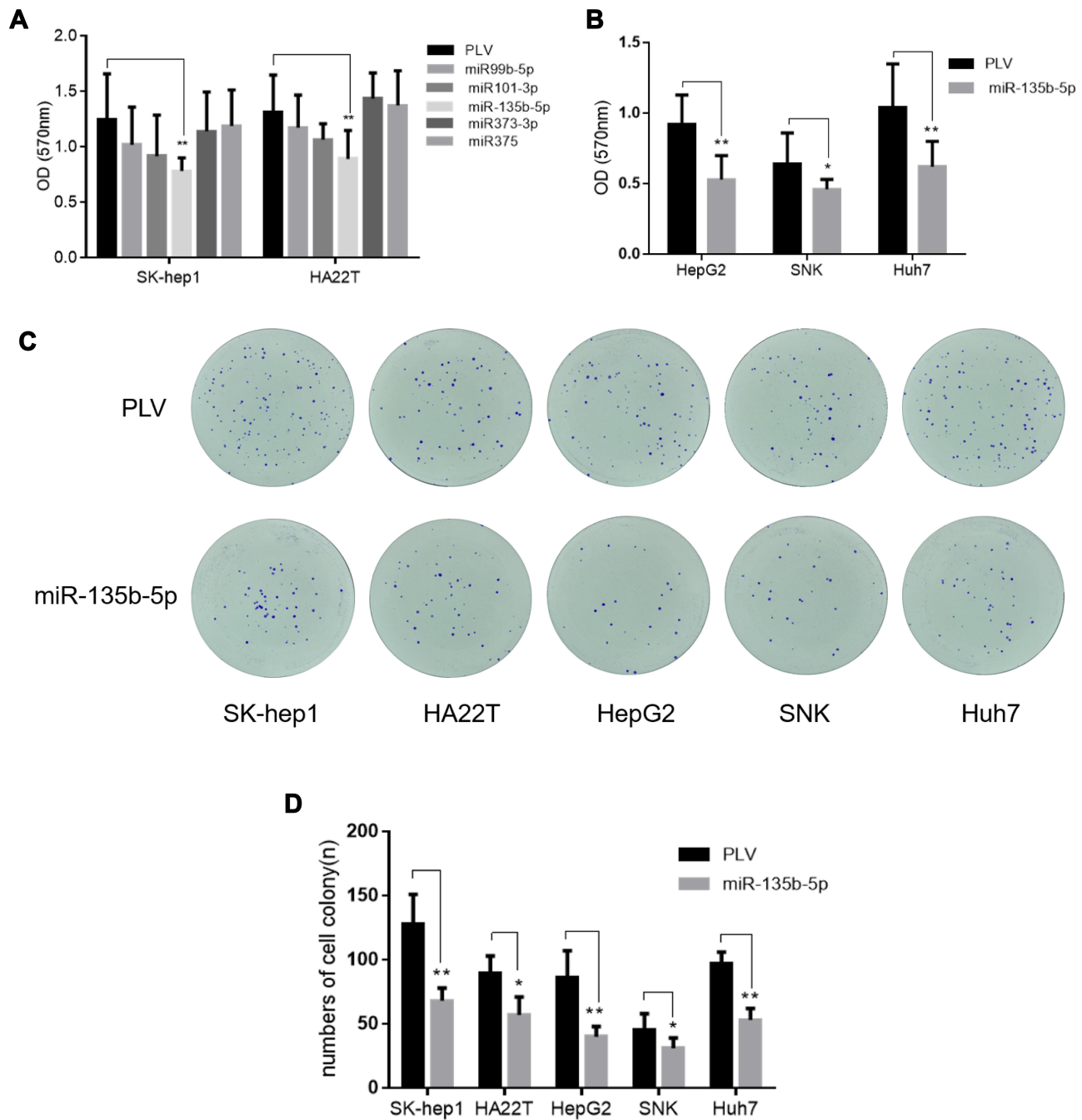


Figure 1 miR-135b-5p suppresses cell proliferation in HCC cells. **(A)** The effect of miR-99b, miR-101, miR-135b-5p, miR-373-3p, and miR-375 on proliferation of SK-hep1 and HA22T cells. The MTT cell proliferation assay revealed that miR-135b-5p significantly suppressed HCC cell proliferation in SK-hep1 and HA22T cells. **(B)** The effect of miR-135b-5p on proliferation of HepG2, SNK and Huh7. The MTT proliferation assay revealed that miR-135b-5p significantly suppressed HCC cell proliferation in HepG2, SNK and Huh7. **(C)** The effect of miR-135b-5p on colony formation of HCC. **(D)** The number of colonies was counted. The colony formation assay showed that miR-135b-5p significantly suppressed HCC cell colony formation in SK-hep1, HA22T, HepG2, SNK, and Huh7. **p* < 0.05, ***p* < 0.01.

HA22T cells (Figure 2A) and HepG2 and Huh7 cells (Figure 2B).

To determine whether miR-135b-5p might function via modulation of AR expression to suppress HCC cell proliferation, we applied the interruption approach. The results revealed that over-expression of AR significantly increased AR protein expression in SK-hep1 and HA22T cells, and

promoted their proliferation, while miR-135b-5p suppressed the expression of AR and decreased the proliferation of SK-hep1 and HA22T cells. However, in AR-overexpressing SK-hep1 and HA22T cells, miR-135b-5p did not suppress the expression of AR or decrease HCC cell proliferation (Figure 2C and D). This could be due to exogenous AR mRNA lacking the 3'-UTR, thus the miR-135b-5p target sequence.

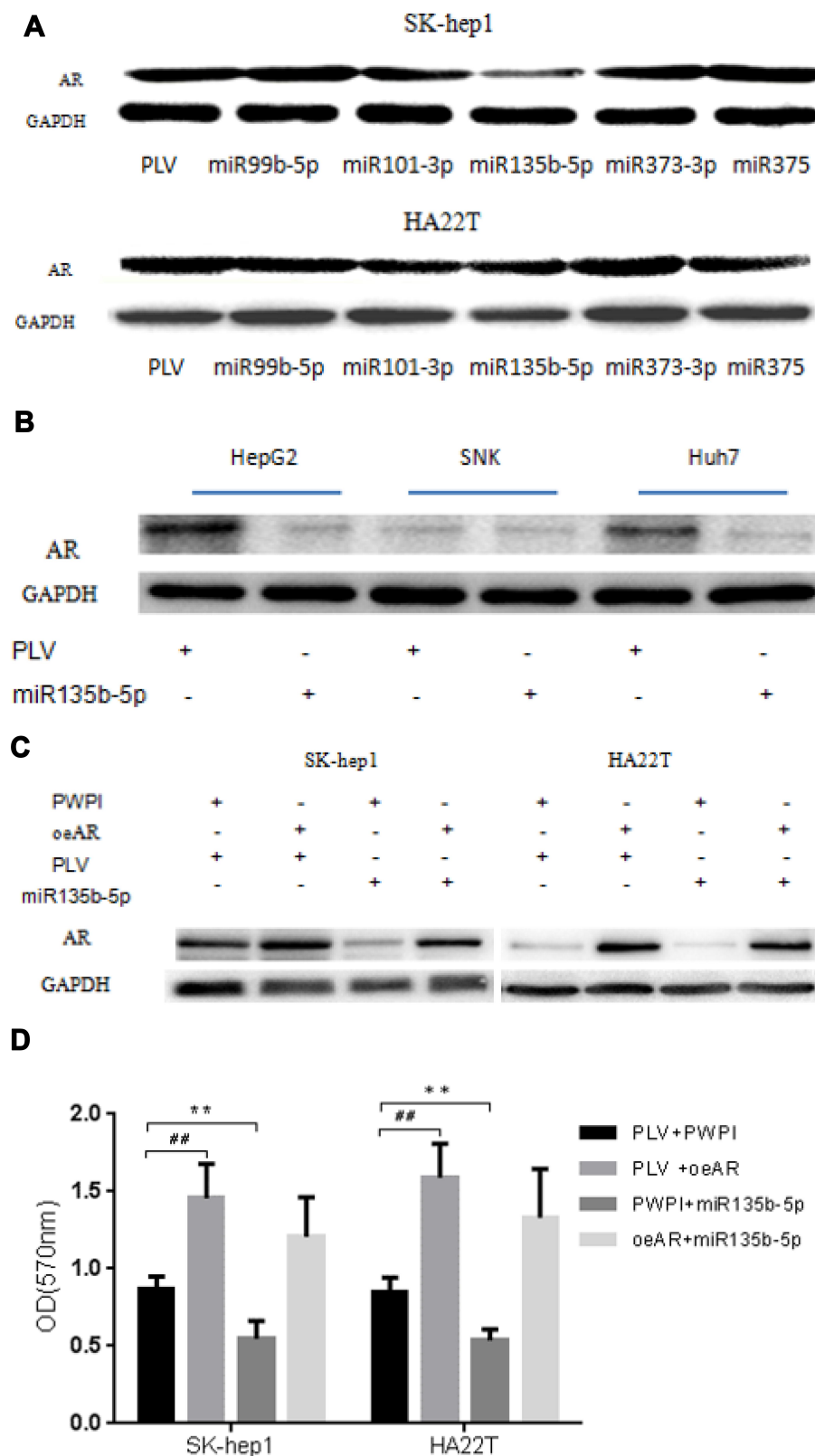


Figure 2 miR-135b-5p suppresses expression of AR protein in HCC cells. **(A)** The effect of miR-99b, miR-101, miR-135b-5p, miR-373, and miR-375 on expression of AR protein in SK-hep1 and HA22T cells. Analysis by Western blot assay revealed that miR-135b-5p could significantly suppress AR protein expression in SK-hep1 and HA22T cells. **(B)** The effect of miR-135b-3p on expression of AR protein in HepG2, SNK, and Huh7 cells. Analysis by Western blot assay revealed miR-135b-5p could significantly suppress AR protein expression in HepG2 and Huh7. Western blot assays were performed as described in Materials and Methods. **(C)** miR-135b-5p suppresses AR expression unless AR is overexpressed in SK-hep1 and HA22T cells. **(D)** miR-135b-5p suppresses HCC proliferation unless AR is overexpressed in SK-hep1 and HA22T cells. ***p* < 0.01, ###*p* < 0.01.

Together, the results suggest that miR-135b-5p may suppress HCC cell proliferation via modulation of AR expression.

Mechanism by Which miR-135b-5p Decreases AR Expression

To further dissect the mechanism by which miR-135b-5p decreases AR expression at a molecular level, we first identified a potential MRE that might match the seed sequence of miR-135b-5p in the 3'-UTR of AR gene. The MRE was mutated, and both wild-type and mutated MREs were cloned into the Renilla luciferase vector (Figure 3A). A luciferase reporter assay using SK-hep1 and HA22T cells revealed that miR-135b-5p decreased the relative luciferase activity with the wild-type AR 3'-UTR, but there was no effect on the relative luciferase activity with the mutated AR 3'-UTR (Figure 3B and C), suggesting that miR-135b-5p could directly target AR 3'-UTR to suppress AR expression.

Downregulation of AR Expression by miR-135b-5p May Alter HIF-2α/c-Myc/P27 Signals to Suppress HCC Cell Proliferation

To dissect the mechanism by which suppression of AR expression by miR-135b-5p might alter HCC cell proliferation, we focused on HIF-2α/c-Myc/p27 signals. Recent studies, including in renal cell carcinoma, have indicated that AR might alter HIF-2α/c-Myc/p27 signals to promote tumor progression.²¹

Overexpression of AR in SK-hep1 and HA22T cells increased the expression of HIF-2α and c-Myc but decreased expression of p27. Expression of miR-135b-5p in SK-hep1 and HA22T cells resulted in decreased expression of HIF-2α and c-Myc and increased expression of p27. However, in AR-overexpressing SK-hep1 and HA22T cells, miR-135b-5p failed to suppress the increased expression of HIF-2α and c-Myc or to increase the decreased expression of p27 caused by overexpression of AR (Figure 4A and B).

To further confirm that miR-135b-5p suppresses HCC cell proliferation through regulation of AR-mediated HIF-2α/c-Myc/p27 signals, we applied the interruption approach adding TC-S, an inhibitor of HIF-2α. The results revealed that TC-S could suppress the levels of HCC cell proliferation observed in AR-overexpressing SK-hep1 and HA22T cells (Figure 4C). Importantly, TC-S could reverse the AR-modulated expression of c-Myc and p27 in SK-hep1 and HA22T cells (Figure 4D).

Together, these results suggest that miR-135b-5p suppresses HCC cell proliferation via altering AR-modulated HIF-2α/c-Myc/p27 signals in SK-hep1 and HA22T cells.

Mechanism by Which AR Modulates HIF-2α Expression at a Molecular Level in SK-hep1

To further dissect the molecular mechanism by which AR can modulate HIF-2α expression in SK-hep1, bioinformatic analysis using Ensembl and PROMO 3.0 was

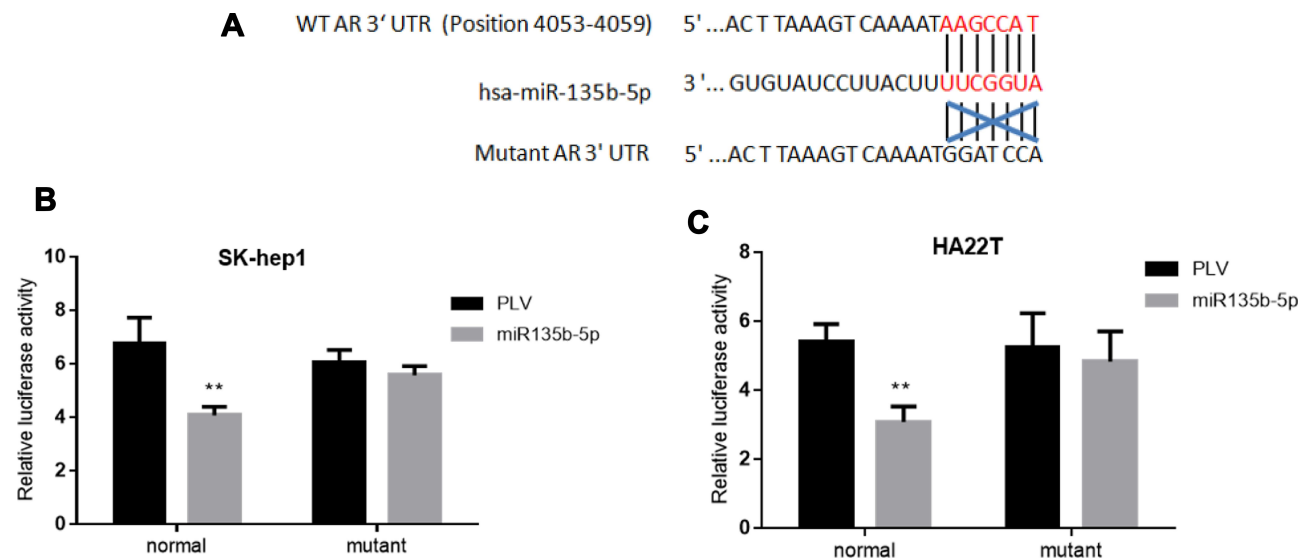


Figure 3 Luciferase reporter assay to determine miR-135b-5p binding to the 3'-UTR of AR. (A) AR 3'-UTRs of wild type or mutant MREs were cloned into the psiCHECK-2 vector downstream of the Renilla luciferase ORF. (B) Transfection of AR 3'-UTR constructs containing wild-type seed regions with pLKO.1-miR-135b-5p significantly decreased relative luciferase activity in SK-hep1 cells. (C) Transfection of AR 3'-UTR constructs containing wild-type seed regions with pLKO.1-miR-135b-5p significantly decreased relative luciferase activity in HA22T cells. **p < 0.01.

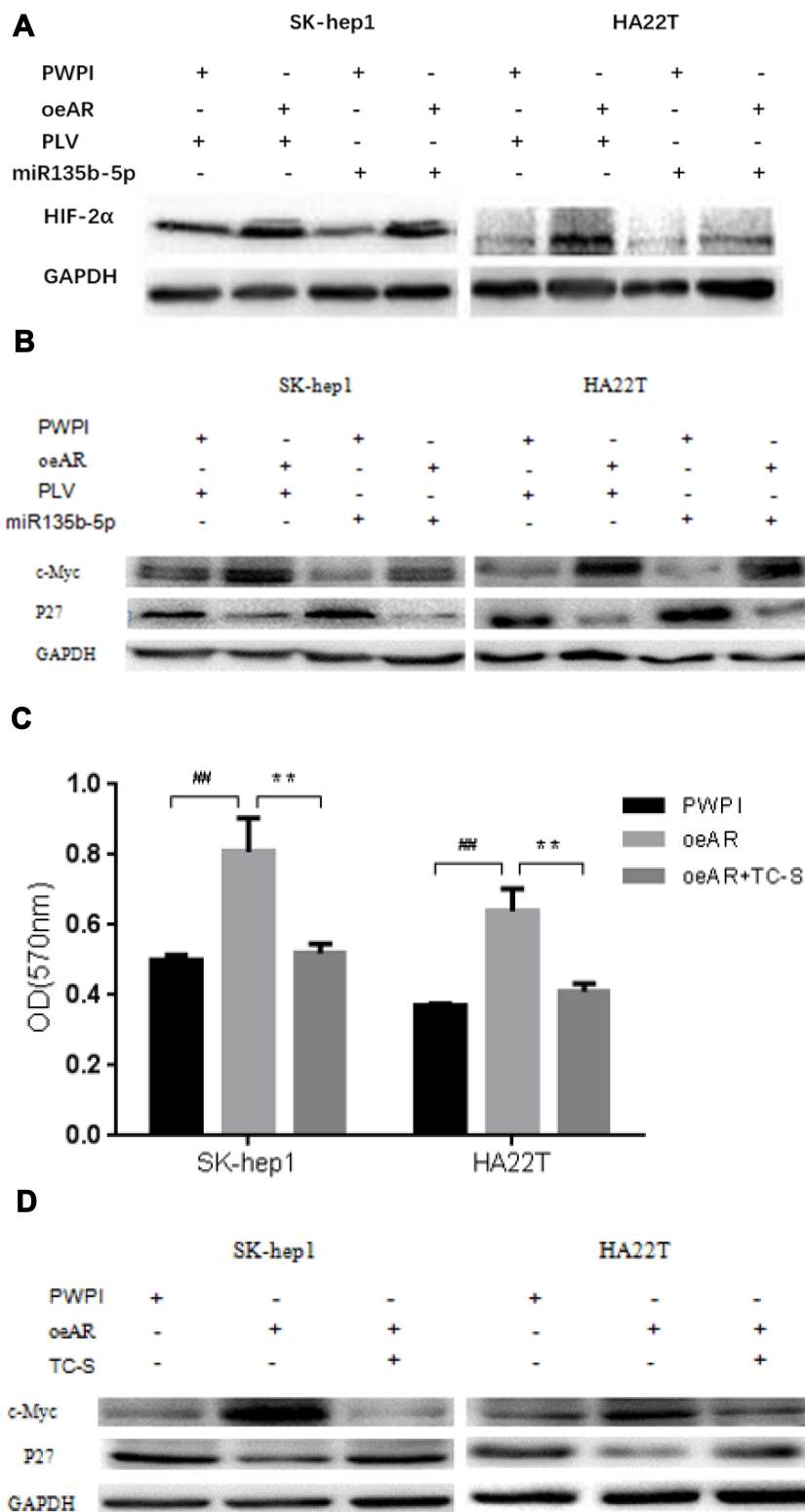


Figure 4 miR-135b-5p suppresses the expression of HIF-2 α and c-Myc protein but increases the expression of p27 protein in HCC cells SK-hep1 and HA22T. **(A)** Effect of overexpression of miR-135b-5p on HIF-2 α in SK-hep1 and HA22T cells. Western blot analysis revealed that miR-135b-5p suppressed the expression of HIF-2 α in SK-hep1 and HA22T cells, but was unable to suppress the expression of HIF-2 α in AR-overexpressing SK-hep1 and HA22T cells. **(B)** Effect of overexpression of miR-135b-5p on c-Myc and p27 in SK-hep1 and HA22T cells. Western blot analysis revealed that miR-135b-5p suppressed the expression of c-Myc and increased the expression of p27, however, in AR-overexpressing SK-hep1 and HA22T cells miR-135b-5p was unable to suppress the expression of c-Myc or increase the expression of p27. **(C)** TC-S suppresses the increase in HCC proliferation in AR-overexpressing SK-hep1 and HA22T cells. **(D)** TC-S suppresses the expression of c-Myc protein but promotes the expression of p27 protein in AR-overexpressing SK-hep1 and HA22T cells. ** $p < 0.01$, ## $p < 0.01$.

performed to search for potential androgen response elements (AREs) in the HIF-2 α promoter region. A potential ARE was found within 1.5 kb upstream of the transcriptional start site. Investigation by ChIP assay showed that AR could bind to the ARE located on the HIF-2 α 5' promoter (Figure 5A). A luciferase reporter assay using a construct containing the HIF-2 α 5' promoter sequence with either the wild-type or mutant ARE was performed in SK-hep1 cells. Increased luciferase activity was observed with the wild-type ARE but not with the mutant ARE (Figure 5B).

Together, these results suggest that AR can transcriptionally modulate HIF-2 α expression via direct binding to the ARE located in the HIF-2 α 5' promoter region in HCC SK-hep1 cells.

Discussion

Recent studies have indicated that targeting AR could suppress HBV-²² HCV-²³ and carcinogen-²⁴ induced HCC development at an early stage. Based on these discoveries, several clinical trials have been proposed to test the efficacy of anti-androgen therapy to suppress HCC progression. It was discovered that androgen deprivation

therapy (ADT) using anti-androgens could significantly suppress HCC progression.²⁵ However, Dai et al found that ADT failed to yield fruitful results.²⁶ Instead of using anti-androgens to prevent androgen binding to AR, Ma et al²⁷ and Wu et al²⁸ applied the AR degradation enhancer ASC-J9 to directly target AR to suppress HCC progression. The experiments reported in this manuscript further support the notion that AR can increase the proliferation of HCC promoting its progression. We have also provided detailed molecular mechanisms of this process describing the roles of miR-135b-5p and AR target gene HIF2 α .

Growing evidence indicates that the deregulation of miRNAs occurs in HCC progression²⁹ and recent studies have further developed the miRNA-based risk of HCC recurrence following liver resection.³⁰ Results of miRNA therapies in HCC showed that either reintroducing suppressor miRNAs or inhibiting oncogenic miRNAs suppress HCC progression in various HCC cell lines in vitro, animal models in vivo, and human clinical trials.³¹ Our work provides additional details, identifying a specific miRNA and its target gene, which are involved in the regulation of HCC proliferation. We first identified several miRNAs as potential AR upstream regulators including miR-99b-5p, miR-101-3p, miR-135b-5p, miR-373-3p, and miR-375. An MTT assay showed that the exogenous expression of miR-135b-5p was capable of suppressing cell proliferation in SK-hep1, HA22T, HepG2, SNK, and Huh7 HCC cell lines. The miRNAs miR-99b-5p and miR-101-3p also inhibited proliferation of the HCC cell lines SK-hep1 and HA22T, however, the level of inhibition was not statistically significant. In addition, miR-135b-5p also inhibited colony formation in SK-hep1, HA22T, HepG2, SNK, and Huh7 HCC lines. It is likely that the target of miR-135b-5p in HCC is AR since miR-135b-5p significantly suppressed the expression of AR in HCC cell lines. Furthermore, the luciferase reporter assay using SK-hep1 and HA22T cells showed that miR-135b-5p decreased the relative luciferase activity with the wild-type AR 3'-UTR but not with the mutant AR 3'-UTR. Moreover, miR-135b-5p failed to suppress AR expression and HCC cell proliferation in AR-overexpressing SK-hep1 and HA22T cells since exogenously expressed AR lacks the 3'-UTR sequence.

When characterizing the molecules involved in regulating HCC proliferation downstream of miR-135b-5p-regulated AR expression, we found that HIF-2 α /c-Myc/p27 signals likely play a significant role, similar to the

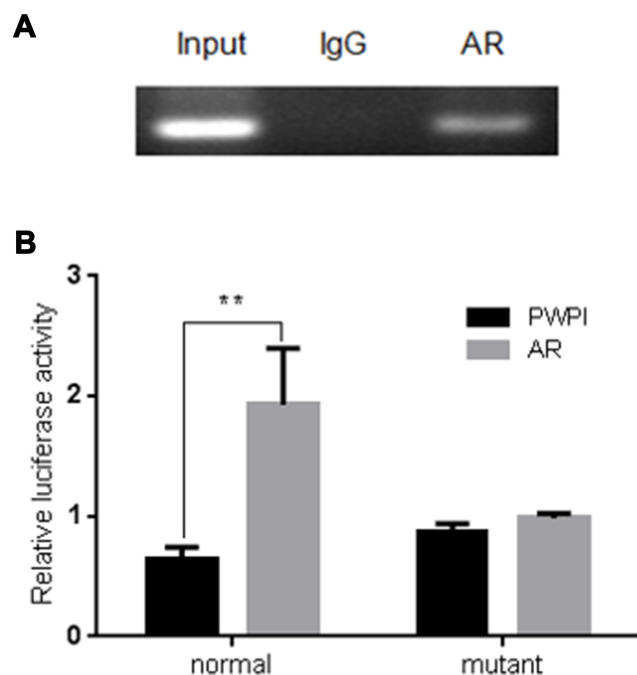


Figure 5 ChIP assay and luciferase reporter assay in SK-hep1 cells. (A) AR binds to the ARE located on HIF-2 α promoter in SK-hep1 cells. ChIP products were amplified by PCR. The ChIP assay revealed that AR could bind to the ARE located on the HIF-2 α 5' promoter. (B) Luciferase reporter assay using a construct containing the HIF-2 α 5' promoter sequence with either the wild-type or mutant ARE. AR increases relative luciferase activity with the wild-type sequence but not with the mutant ARE sequence in SK-hep1 cells. **p < 0.01.

findings in lung cancer and renal cell carcinoma (RCC).²¹ Overexpression of AR increased expression of HIF-2 α and c-Myc but decreased expression of p27 in HCC. The addition of miR-135b-5p suppressed the expression of HIF-2 α and c-Myc but increased the expression of p27. However, miR-135b-5p did not suppress the increased expression of HIF-2 α and c-Myc or increase the decreased expression of p27 in AR-overexpressing HCC cells. The HIF-2 α inhibitor TC-S suppressed the levels of HCC cell proliferation observed in AR-overexpressing SK-hep1 and HA22T cells and reversed the AR-modulated expression of c-Myc and p27. These results suggest that miR-135b-5p may suppress HCC proliferation via downregulation of AR-modulated HIF-2 α , which in turn suppresses c-Myc expression, and increases expression of p27 in HCC cells. The ChIP assay and luciferase reporter assay further confirmed the direct molecular relationship by which AR transcriptionally modulates HIF-2 α expression via its binding to the ARE located on the HIF-2 α 5' promoter in HCC. These experimental results provide a compelling narrative that miR-135b-5p suppresses androgen receptor-enhanced HCC in vitro via downregulating HIF-2 α /c-Myc/p27 signals, which may help to better understand the HCC mechanism and develop more effective therapies to combat malignant HCC in the clinic.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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Disclosure

The author reports no conflicts of interest in this work. Shi-xiang Bao and Chun-hua Wang contributed equally.

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