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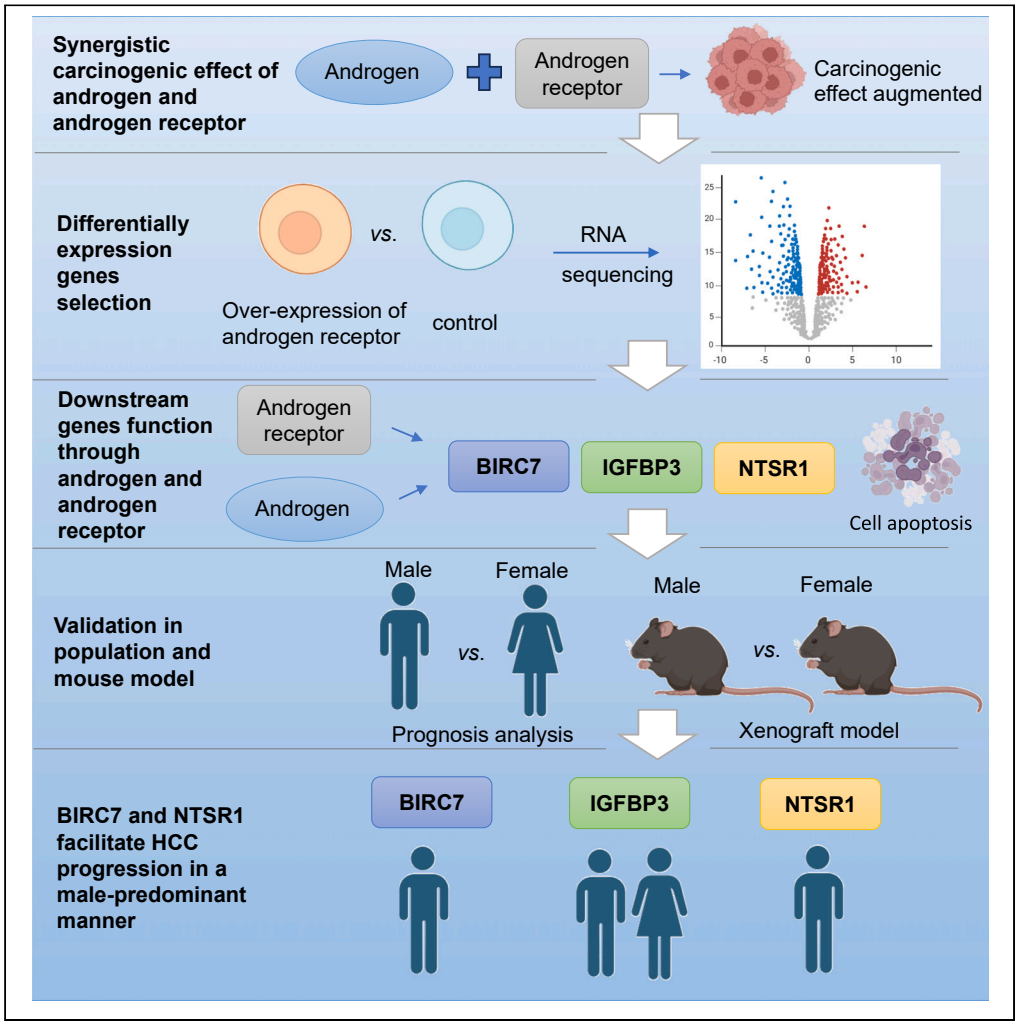

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Highlights

Androgen receptor promotes hepatocellular carcinoma through BIRC7, IGFBP3, and NTSR1

Androgen enhances the transcription of BIRC7, IGFBP3, and NTSR1

The effect of wild-type HBV X protein on androgen receptor was androgen-dependent

BIRC7 and NTSR1 might facilitate the development of HCC in a male-predominant way

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Androgen receptor-induced molecules and androgen contribute synergistically to male-predominance of hepatocellular carcinoma

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SUMMARY

We aimed to clarify the mechanisms of male predominance of hepatitis B virus (HBV) -related hepatocellular carcinoma (HCC). Androgen receptor (AR) facilitates HCC cell growth, which was augmented by androgen (dihydrotestosterone [DHT]) and attenuated by anti-androgen (flutamide). AR upregulated the expressions of BIRC7, IGFBP3, and NTSR1 via increasing their promoter activities, which were enhanced by DHT. Wild-type HBV X (WT-HBx) upregulated AR transcription, which depended on DHT; whereas the effect of C-terminal carboxy-truncated HBx on AR transcription was independent of DHT. BIRC7, IGFBP3, and NTSR1 increased the growth of HCC. High expression of BIRC7 and NTSR1 contributes to poor HCC outcomes in male patients, but not in female patients. Downregulation of NTSR1 inhibits tumor growth in male mice rather than in female mice. Conclusively, AR promotes HCC at least partially via upregulating BIRC7, IGFBP3, and NTSR1, which is enhanced by androgen and HBx. BIRC7 and NTSR1 facilitate HCC progression in a male-predominant manner.

INTRODUCTION

According to Global Cancer Statistics 2020, primary liver cancer (PLC) is the 2nd leading cause of cancer death in men (proportion, 10.5%) and the 6th (proportion, 5.7%) in women worldwide.[1] In China, 410,000 new cases of PLC were diagnosed, accounting for 45.27% of the world's new cases.[2] PLC is the 1st leading cause of cancer death in working males of 65 years or younger, while hepatocellular carcinoma (HCC) accounts for 93% of PLC in China.[3,4] HCC mostly occurs in males, with a male-to-female ratio of 2-3:1, especially in HBV-HCC, with a ratio of 3–4:1, develops faster, and causes a higher mortality in males than in females.[3–6] Approximately 85% of HCC cases in China are seropositive for hepatitis B surface antigen (HBsAg). hepatitis B virus X (HBx) protein plays an essential role in the occurrence and progression of HCC, especially the HBx mutants. Compared to wild-type HBx (WT-HBx), HBx combo mutations (A1762T/G1764A + C1653T + T1753C) and carboxylic acid terminal-truncated HBx (Ct-HBx) increase the risk of HCC.[7,8] Interestingly, hepatitis B virus (HBV)-related HCC is more male-predominant than HCC caused by other factors.[3–6]

The high incidence of HCC in males suggests that sex hormones (androgens and estrogens) may be involved in HCC development. Estrogen enhances the innate and adaptive immune response to viral infection in females, while androgens have negative regulatory effects on both innate and adaptive immunity.[9] In the absence of androgen, androgen receptor (AR) binds to heat shock proteins to form a complex in the cytoplasm. In the presence of androgen, AR is separated from the complex and binds to androgen as a dimer, which *trans*-locates to the nucleus to participate in transcriptional regulation. Hepatitis C virus (HCV) enhances vascular endothelial growth factor (VEGF) expression and facilitates tube formation in human coronary microvascular endothelial cells in the presence of AR.[10] Novel AR antagonists, such as abiraterone[11] and apalutamide,[12] are promising in treating castration-resistant prostate cancer, prolonging tumor-free survival and overall survival (OS). These findings provide a basis for investigating the effect of AR on HCC, a male-predominant cancer, and the potential effect of anti-androgen therapy for HCC treatment.

The role of AR in HCC remains controversial.[13] AR is pro-oncogenic by binding to the EZH2 promoter and inhibiting the expression of Wnt signal suppressor, leading to Wnt/β-catenin signaling activation, inducing cell proliferation and tumorigenesis in HCC. However, another study has demonstrated that AR inhibits HCC, enhancing cell adhesion and decreasing cell migration via modulating β1-integrin-AKT signaling.[14] The role of AR in creating the gender disparity in HCC, especially in HBV-HCC, remains largely unknown. Most researches on AR investigate the pathways through which AR alone promotes or inhibits HCC; however, the effect of AR alone cannot explain the high

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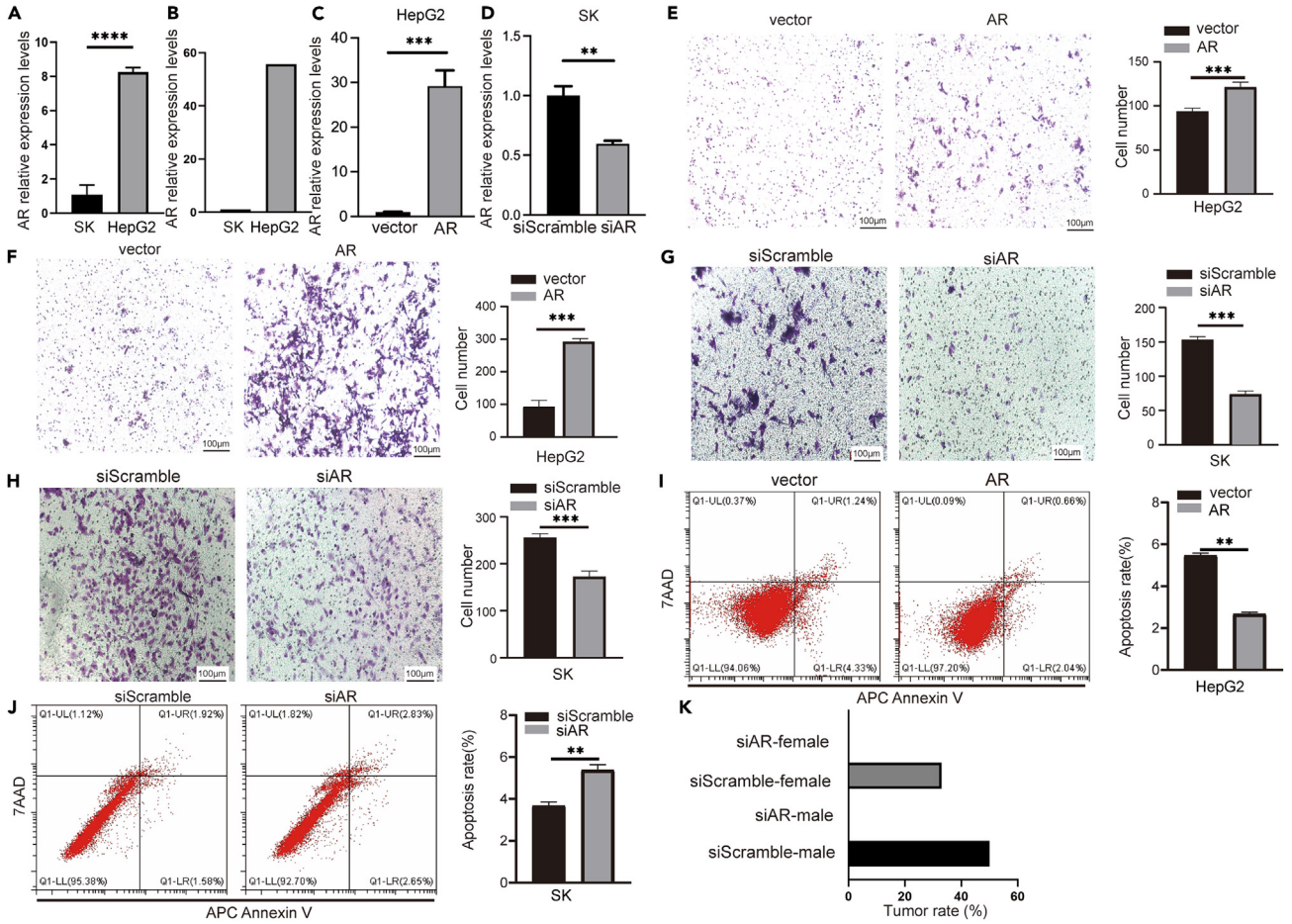

Figure 1. The effects of ectopic expression and knockdown of AR on cell migration, invasion, apoptosis, and tumor formation in HCC

(A) Expression of AR in SK-Hep-1 cells and HepG2 cells, analyzed by RT-qPCR.

(B) Expression of AR in SK-Hep-1 cells and HepG2 cells, analyzed by CCLE database.

(C) Expression of AR following ectopic expression in HepG2 cells, analyzed RT-qPCR.

(D) Expression of AR after knockdown in SK-Hep-1 cells, analyzed by RT-qPCR.

(E) The effect of ectopic expression of AR on cell migration in HepG2 cells. The scale bar represents 100 μm.

(F) The effect of ectopic expression of AR on cell invasion in HepG2 cells. The scale bar represents 100 μm.

(G) The effect of AR knockdown on cell migration in SK-Hep-1 cells. The scale bar represents 100 μm.

(H) The effect of AR knockdown on cell invasion in SK-Hep-1 cells. The scale bar represents 100 μm.

(I) The effect of ectopic expression of AR on cell apoptosis in HepG2 cells.

(J) The effect of AR knockdown on cell apoptosis in SK-Hep-1 cells.

(K) The effect of AR knockdown in SK-Hep-1 cells on tumor formation in female and male xenograft nude mice. Data represent means ± SD of three independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$, $^{****}p < 0.0001$. Unpaired t tests for (A) and (C–J). RT-qPCR, real-time RT-qPCR; AR, androgen receptor; SK, SK-Hep1; CCLE, Cancer Cell Line Encyclopedia; UL, up left; UR, up right; LL, low left; LR, low right.

incidence of HCC in males and poor outcomes. In this study, we explore the role of androgen and AR interaction on the development of HCC and identify AR target genes that contribute to the gender disparity in HCC, providing new option for HCC treatment.

RESULTS

AR significantly increased the growth of HCC

Firstly, the basal expression levels of AR in HCC cell lines were analyzed, including HepG2 and SK-Hep-1 (Figures 1A and 1B). HepG2 and SK-Hep-1 cell lines were derived from males without HBV infection. Based on the basal expression level of AR, ectopic expression of AR in HepG2 cells and downregulation of AR in SK-Hep-1 cells by siRNA were achieved (Figures 1C and 1D). The effects of AR on cell proliferation, migration, and apoptosis of HepG2 and SK-Hep-1 cells were evaluated in HepG2 cells and SK-Hep-1 cells. Transwell assays showed that cell migration and invasion were significantly increased by upregulation of AR (Figures 1E and 1F) and significantly decreased by downregulation of AR

(Figures 1G and 1H). Cell apoptosis assays indicated that overexpression of AR reduced the apoptotic proportion, while downregulation of AR increased the apoptotic proportion (Figures 1I and 1J). These findings suggest that AR promotes cell migration and invasion and inhibits apoptosis in HCC cells.

The effect of AR on the growth of SK-Hep-1 cells with or without AR knockdown were investigated in male and female nude mice. It was found that tumors of SK-Hep-1 cells with AR knockdown were not grown in nude mice of both genders three weeks after the subcutaneous (s.c.) transplantation; however, the tumor growth was more evident in male than in female nude mice transplanted with SK-Hep-1 without AR knockdown (Figure 1K). This result indicates that AR alone is not responsible for the gender disparity of HCC. Higher level of androgen in males might attribute to the gender disparity in HCC.

We collected tumor tissues and adjacent liver tissues from 108 HCC patients (93 males and 15 females; 41 patients with postoperative HCC recurrence and 67 patients without HCC recurrence) and evaluated the association of AR expression levels using RT-qPCR with gender disparity and postoperative recurrence in HCC. Information of human participants was listed in Table S1. Furthermore, we also analyzed the expression ratio of AR/ERa using The Cancer Genome Atlas (TCGA) liver database to investigate potentially protective effects of ERa. No statistically significant difference in AR expression level was evident between adjacent liver tissues and tumor tissues from female patients, but the expression ratio of AR/ERa in the tumors was significantly higher than that in adjacent liver tissues from female patients (Figure S1A); Interestingly, the level of AR expression in the tumors was significantly lower than that in adjacent liver tissues from male patients, while the expression ratio of AR/ERa in the tumors was significantly higher than that in adjacent liver tissues from male patients (Figure S1B). These indicate that ERa may have protective effects in both females and males. No difference in both AR expression and the expression ratio of AR/ERa in adjacent tissues between male and female patients was evident, neither in the tumor tissues (Figures S1C and S1D). Thus, AR expression and the expression ratio of AR/ERa in liver tissues or tumors are not related to gender. No difference in both the expression of AR and the expression ratio of AR/ERa was evident between recurrent and non-recurrent HCC in the total and male populations (Figures S1E and S1F). Thus, AR may not contribute to the high occurrence and poor outcomes of HCC in males alone.

The effect of androgen on the growth of HCC regulated by AR

We hypothesized that the gender disparity of HCC might be related to the higher levels of androgen in males. The synergistic effect of androgen and AR on HCC development was then investigated. As AR binds to androgen, we used dihydrotestosterone (DHT), a natural ligand of AR, to activate AR and then used the AR antagonist flutamide to block the activation of AR by DHT. Transwell assays revealed that DHT and AR overexpression promoted migration and invasion of HCC cells, but this enhancement was further improved when DHT and overexpression of AR worked together. The enhanced effect by DHT was neutralized by flutamide (Figures 2A and 2B).

Apoptosis experiments showed similar results: DHT decreased the apoptotic effect of AR on HCC, while the effect was neutralized when flutamide was added (Figure 2C). These findings suggest that DHT and AR enhance the migration and invasion and reduce apoptosis in HCC. The carcinogenic effect was augmented when they worked together, while AR antagonist flutamide neutralized these carcinogenic effects. These findings suggest that the carcinogenic effects of AR on HCC are augmented by androgen, and higher levels of androgen in males can explain the gender disparity in HCC.

In addition to investigate the synergistic effect of androgen and AR on HCC development, the effect of estrogen and AR on HCC development was also investigated. Transwell assays revealed that estradiol (E2, a kind of estrogen) inhibited migration and invasion of HCC cells, and also mitigated the enhancement of AR on liver cancer cell migration and invasion (Figures S2A and S2B). Apoptosis experiments showed similar results: E2 increased the apoptotic effect of AR on HCC, and also enhanced the inhibitory effect of AR on HCC cell (Figure S2C). These findings suggest that the carcinogenic effects of AR on HCC were mitigated by estrogen. Since this research aimed to focuses more on the mechanisms for the high incidence of HCC in males, the role of androgen and AR would be emphasized in subsequent studies.

Molecules and signaling pathways regulated by AR in HCC

HepG2-AR cells and HepG2-vector cells were subjected to mRNA sequencing analysis. The transcriptome sequencing data were analyzed by raw confidence analysis, revealing 451 differentially expressed genes (DEGs) (fold change > 2 and $p < 0.05$). Of the 451 DEGs, 359 were upregulated and 92 downregulated by AR, as shown in a volcano diagram (Figure S3A). The top ten DEGs (Table S2) were taken for functional annotation using the online gene annotation tool Metascape.[15] The 451 DEGs were subjected to Gene Ontology (GO) analysis of target biological processes to observe the alteration of target biological processes due to AR overexpression. GO analysis showed significant enrichment in small molecule biosynthesis processes, positive regulation of cell death, and vasculature development (Figure S3B). The GO analyses enriched 18 migration and invasion-related bioprocesses (Table S3) and 22 apoptosis-related signaling pathways (Table S4). Ten DEGs involved in the migration, invasion, apoptosis, and energy metabolism were selected for further study (Table S5). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis indicated that the 451 DEGs were involved in inflammation and cancer-related pathways, such as the mitogen-activated protein kinase (MAPK) signaling pathway, pathways in cancer, the cancer signaling pathway, the Hippo signaling pathway, and the p53 signaling pathway (Figure S3C). Most of pathways are related with AR signaling, such as MAPK signaling pathway,[15,16] Hippo signaling pathway,[17] and focal adhesion.[18]

Transcriptional regulation of BIRC7, IGFBP3, and NTSR1 by AR

Ten DEGs related to migration, invasion, apoptosis, and energy metabolism were selected from the top 100 DEGs in RNA sequencing (RNA-seq) and were subjected to RT-qPCR for validation. The expressions of BIRC7, IGFBP3. NTSR1, NDUFA4L2, AQP3, BMP6, DNAJC12, and

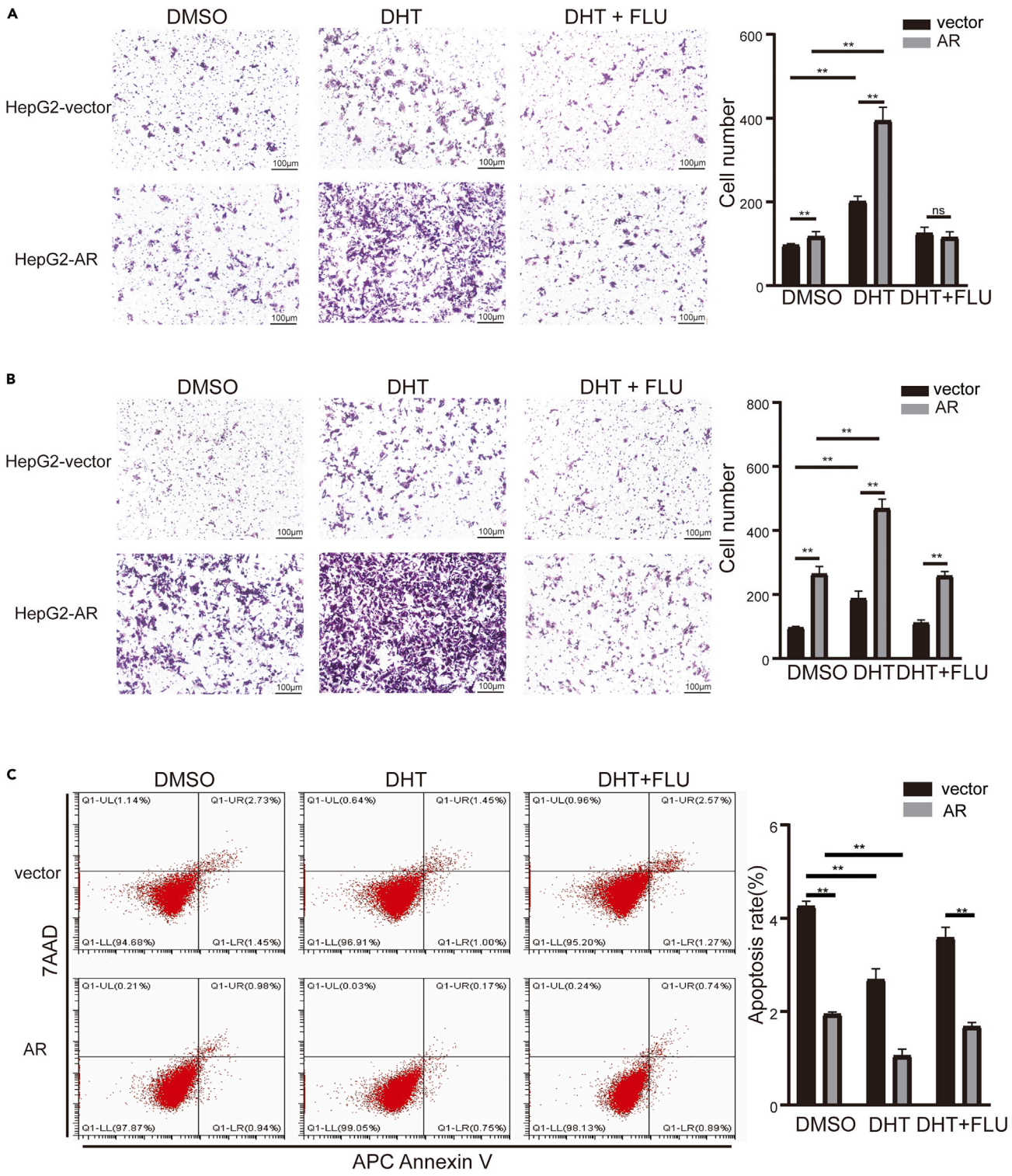

Figure 2. The effect of interaction of androgen with AR on cell migration, invasion, and apoptosis

(A) Cell migration of HepG2 transfected with AR with or deprived of androgen. The scale bar represents 100 μm.

(B) Cell invasion of HepG2 transfected with AR with or deprived of androgen. The scale bar represents 100 μm.

(C) Cell apoptosis of HepG2 transfected with AR with or deprived of androgen (left: 0.1% DMSO; middle: 100 nM DHT; right: 100 nM DHT + 50 μM flutamide). Data represent means ± SD of three independent experiments. $^{**}p < 0.01$. ns, not significant. Unpaired t tests for (A–C). DMSO, dimethyl sulfoxide; DHT, dihydrotestosterone; AR, androgen receptor; FLU, flutamide; 7AAD, 7-amino actinomycin D; APC, allophycocyanin; UL, up left; UR, up right; LL, low left; LR, low right.

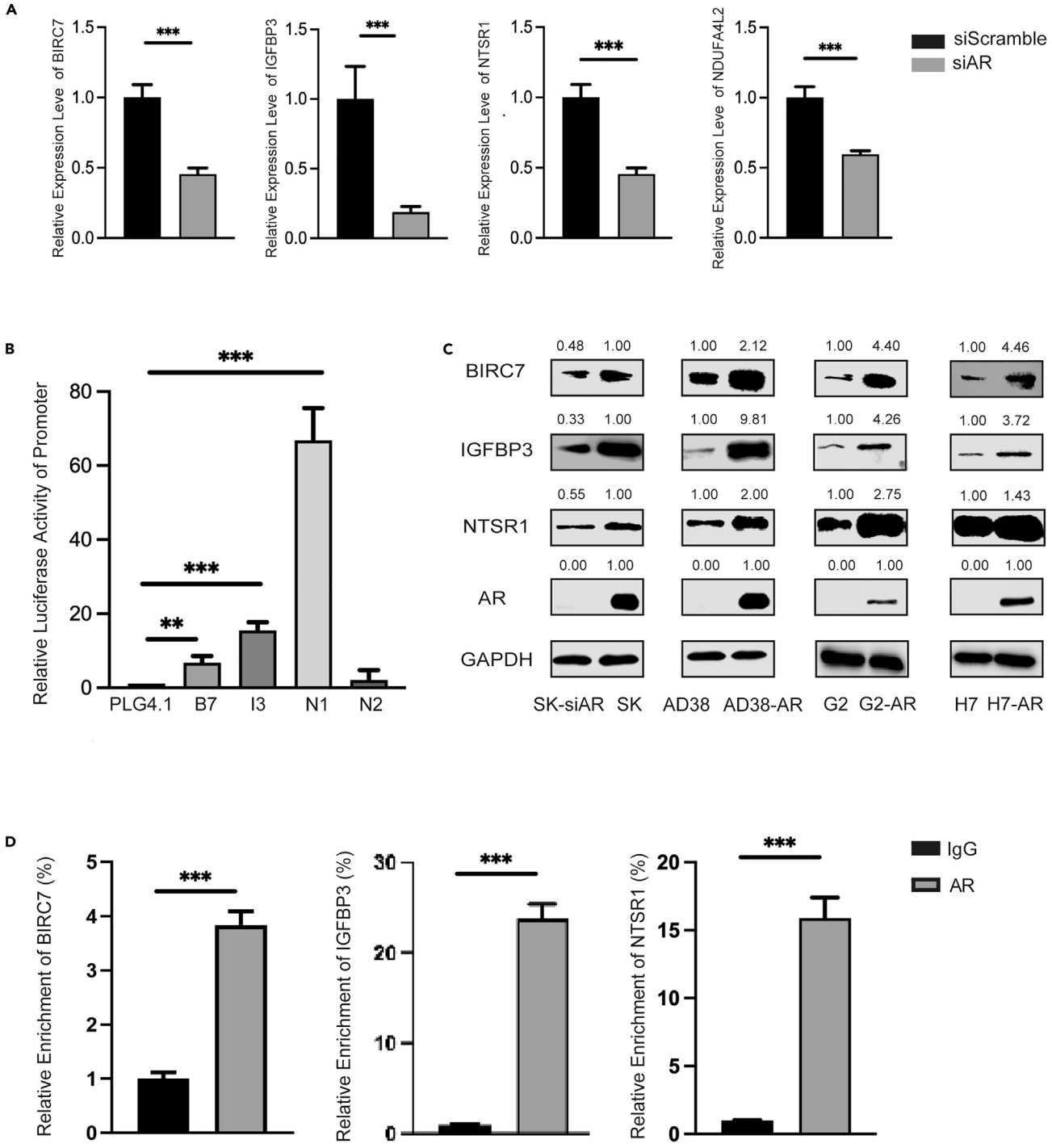

Figure 3. Expression of AR-regulated genes

(A) The RNA levels of BIRC7, IGFBP3, NTSR1, and NDUFA4L2 in AR-knocked down SK-Hep-1 cells and siScramble-SK-Hep-1 cells were determined by RT-qPCR, and values were normalized to GAPDH.

(B) The activity of the *BIRC7* promoter, the *IGFBP3* promoter, the *NTSR1* promoter, and the *NDUFA4L2* promoter, indicated by luciferase reporter, transcriptionally regulated by AR expressing construct.

(C) The protein levels of BIRC7, IGFBP3, and NTSR1 in SK-Hep-1 with AR knockdown (SK-siAR) and control SK-siScramble, HepG2 cells transfected with AR (HepG2-AR) and control HepG2-empty vector, Huh7 cells transfected with AR (Huh7-AR) and control Huh7-empty vector, and AD38 cells transfected with AR

Figure 3. *Continued*

(AD38-AR) and control AD38-empty vector were determined by western blot and corresponding statistical results were calculated by ImageJ software, GAPDH as a reference.

(D) The enrichment, measured by qPCR, of BIRC7, IGFBP3, and NTSR1 sequences in HepG2 cells transfected with AR using ChIP-PCR. Data represent means ± SD of three independent experiments. ***$p < 0.001$. Unpaired t tests for (A), (B), and (D). AR, androgen receptor; SK, SK-Hep-1; G2, HepG2; H7, Huh7; B7, BIRC7; I3, IGFBP3; N1, NTSR1; N2, NDUFA4L2.

FADS6 were significantly upregulated by AR in HepG2 cells, consistent with the RNA sequencing results (Figure S4A). The Cox regression analysis was used to analyze the effect of 8 genes expression on the OS in HCC using the TCGA Liver Hepatocellular Carcinoma (LIHC) database. It was found that high expressions of BIRC7, IGFBP3, and NTSR1 significantly shortened OS and high expression of NDUFA4L2 has a negative trend on OS (Figure S4B). Therefore, BIRC7, IGFBP3, NTSR1, and NDUFA4L2 were selected for further study. The expressions of BIRC7, IGFBP3, NTSR1, and NDUFA4L2 were also detected in SK-Hep-1 cells with or without knockdown AR. The expressions of BIRC7, IGFBP3, NTSR1, and NDUFA4L2 were downregulated in SK-Hep-1-siAR cells, consistent with the RNA sequencing results (Figure 3A). These data suggest that AR positively regulates the expressions of BIRC7, IGFBP3, NTSR1, and NDUFA4L2 genes.

A dual-luciferase reporter gene assay explored their regulatory relationship based on the positive correlation between AR and BIRC7, IGFBP3, NTSR1, and NDUFA4L2. HepG2 was transfected with a plasmid expressing AR and then transfected with plasmids containing the BIRC7 promoter, the IGFBP3 promoter, the NTSR1 promoter, and the NDUFA4L2 promoter, respectively. An empty vector (pGL4.10) served as the control. Figure 3B shows that the promoter activities of BIRC7, IGFBP3, and NTSR1 were significantly increased by AR, while the NDUFA4L2-promoter activity was not significantly changed compared with the control. These findings suggest that AR *trans*-activated the expressions of BIRC7, IGFBP3, and NTSR1 rather than NDUFA4L2. The correlation in the expression of AR and its target genes in more HCC cell lines including Huh7 and AD38 were tested. These results confirmed that AR upregulated the expressions of BIRC7, IGFBP3, and NTSR1 (Figure 3C).

To determine how AR upregulates target genes BIRC7, IGFBP3, and NTSR1, we identified their binding sites using chromatin immunoprecipitation (ChIP)-PCR. Potential AR binding sites in the promoter regions of BIRC7, IGFBP3, and NTSR1 were predicted using the online tool JASPAR (https://jaspar.genereg.net/)[19] (Table S6) and validated by PCR. HepG2-AR was treated with negative control IgG or AR antibodies to obtain purified DNA. The outcomes revealed a significant enrichment of BIRC7, IGFBP3, and NTSR1 in the AR-treated group (Figure 3D). These findings suggest that AR has binding sites with the promoter regions of target genes BIRC7, IGFBP3, and NTSR1 and regulates the expression of target genes through targeted binding.

The effects of BIRC7, IGFBP3, and NTSR1 on the progression of HCC

To study the oncogenic effects of the AR-targeted genes on HCC, the effects of the target genes on migration, invasion, and apoptosis were observed by knocking down the expressions of BIRC7, IGFBP3, and NTSR1. Transwell experiments showed that migration of HepG2 and SK-Hep-1 was not significantly altered when BIRC7 and IGFBP3 were knocked down; however, siNTSR1 significantly decreased the migration in HepG2 and SK-Hep-1 cells (Figures 4A and 4B). Thus, AR affects cell migration by regulating NTSR1 rather than BIRC7 and IGFBP3. Our subsequent invasion experiment showed that siBIRC7 had no significant effects on cell invasion, but siIGFBP3 and siNTSR1 reduced the invasion ability of HCC cells significantly (Figures 4C and 4D). AR affects cell invasion possibly by regulating IGFBP3 and NTSR1 rather than BIRC7. The apoptosis assay showed that the apoptotic proportion was significantly increased in the siBIRC7, siIGFBP3, and siNTSR1-transfected HepG2 and SK-Hep-1 compared to their controls (Figures 4E and 4F). AR affects apoptosis possibly by regulating BIRC7, IGFBP3, and NTSR1 expressions. In summary, AR affects cell migration possibly by upregulating the expression of NTSR1, affects cell invasion by upregulating the expression of IGFBP3 and NTSR1, and affects apoptosis by upregulating the expressions of BIRC7, IGFBP3, and NTSR1.

The rescue experiments were performed to investigate the downstream gene function through AR. Of the three kinds of phenotype experiments, knockdown of BIRC7, IGFBP3, or NTSR1 in HepG2 and SK-Hep-1 cells significantly increased the apoptotic proportion compared to their controls (Figures 4E and 4F). Therefore, we further conducted rescue experiments on apoptosis. Compared with HepG2-vector-siScramble, HepG2-vector-siBIRC7, HepG2-vector-siIGFBP3, and HepG2-vector-siNTSR1 significantly promoted apoptosis, while this phenotype was neutralized by upregulation of AR (Figure S5).

Transcriptional regulation of AR and target genes by DHT

To determine whether DHT enhances the transcription of BIRC7, IGFBP3, and NTSR1 upregulated by AR, the promoter activities of BIRC7, IGFBP3, and NTSR1 were subsequently measured. The promoter activities of BIRC7, IGFBP3, and NTSR1 were increased by 3- to 13-folds in the DHT group compared to the dimethyl sulfoxide (DMSO)-treated controls (Figures 5A–5C), indicating that androgen upregulates AR-regulated transcription of BIRC7, IGFBP3, and NTSR1 in HCC cells.

Rescue experiments were performed to confirm the regulation of DHT on AR targeted genes of cell activities. Of the three candidate target genes, NTSR1 affected migration, invasion, and apoptosis of HCC simultaneously *in vitro* (Figure 4). Therefore, we further conducted rescue experiments on NTSR1. Compared with SK-Hep-1-shScramble, SK-Hep-1-shNTSR1 significantly reduced the migration and invasion ability and promoted apoptosis, while these phenotypes were neutralized by DHT. Thus, NTSR1, the essential target gene upregulated by AR, plays a critical role in the male predominance of HCC (Figure S6).

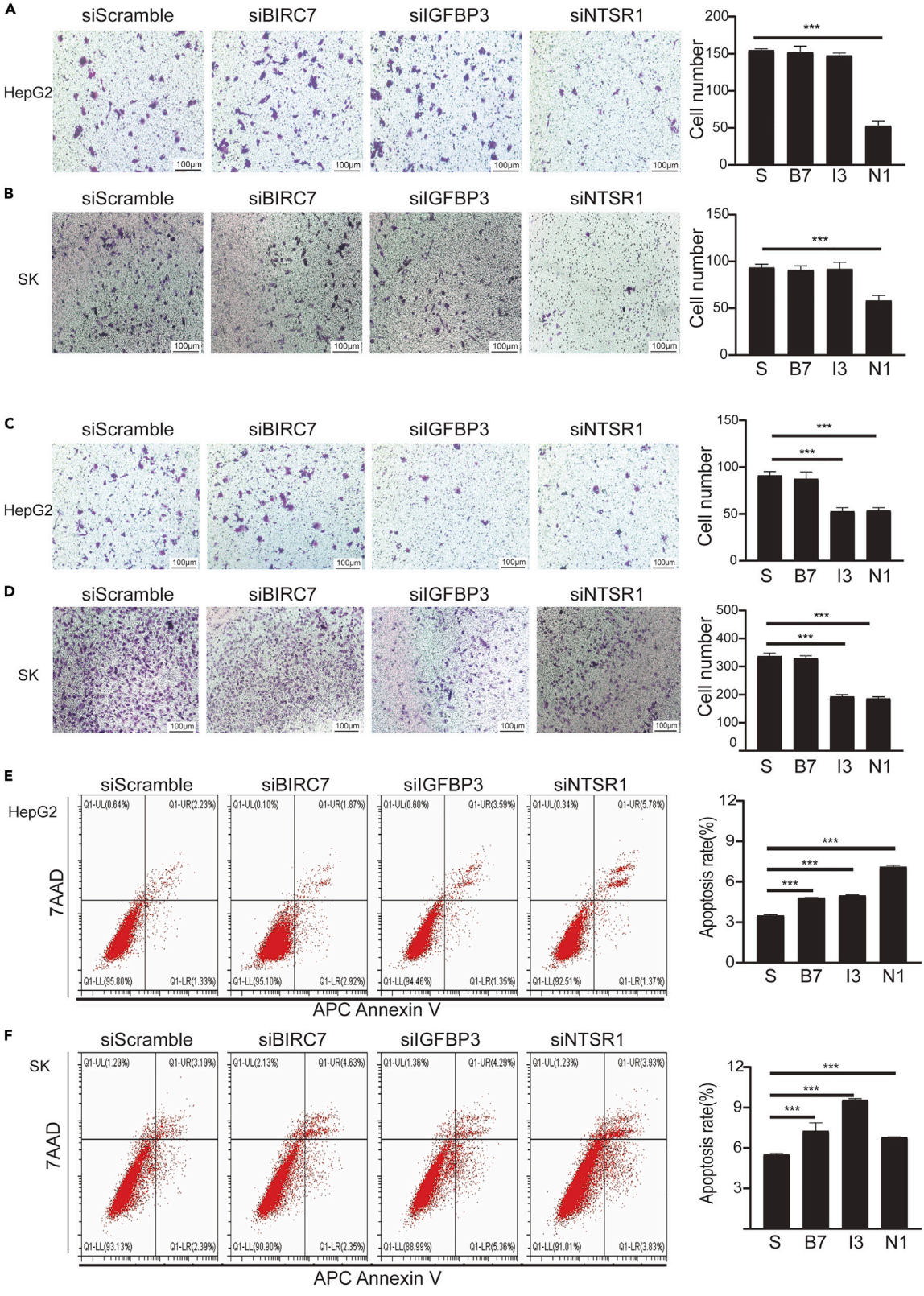
A
siScramble
siBIRC7
siIGFBP3
siNTSR1
HepG2
100μm
Cell number
200
150
100
50
0
***
S
B7
I3
N1
B
SK
C
D
E
7AAD
APC Annexin V
Q1-UL(0.64%)
Q1-UR(2.23%)
Q1-LL(95.80%)
Q1-LR(1.33%)
Q1-UL(0.10%)
Q1-UR(1.87%)
Q1-LL(95.10%)
Q1-LR(2.92%)
Q1-UL(0.60%)
Q1-UR(3.59%)
Q1-LL(94.46%)
Q1-LR(1.35%)
Q1-UL(0.34%)
Q1-UR(5.78%)
Q1-LL(92.51%)
Q1-LR(1.37%)
Apoptosis rate(%)
12
9
6
3
0
F
Q1-UL(1.29%)
Q1-UR(3.19%)
Q1-LL(93.13%)
Q1-LR(2.39%)
Q1-UL(2.13%)
Q1-UR(4.63%)
Q1-LL(90.90%)
Q1-LR(2.35%)
Q1-UL(1.36%)
Q1-UR(4.29%)
Q1-LL(88.99%)
Q1-LR(5.36%)
Q1-UL(1.23%)
Q1-UR(3.93%)
Q1-LL(91.01%)
Q1-LR(3.83%)

Figure 4. The effect of silencing BIRC7, IGFBP3, and NTSR1 on cell migration, invasion, and apoptosis of HCC cell lines

(A) Cell migration after silencing targeted genes using siRNAs in HepG2 cells. The scale bar represents 100 μm.

(B) Cell migration after silencing targeted genes using siRNAs in SK-Hep-1 cells. The scale bar represents 100 μm.

(C) Cell invasion after silencing targeted genes using siRNAs in HepG2 cells. The scale bar represents 100 μm.

(D) Cell invasion after silencing targeted genes using siRNAs in SK-Hep-1 cells. The scale bar represents 100 μm.

(E) Cell apoptosis after silencing targeted genes using siRNAs in HepG2 cells.

(F) Cell apoptosis after silencing targeted genes using siRNAs in SK-Hep-1 cells. Data represent means ± SD of three independent experiments. ***$p < 0.001$. Unpaired t tests for (A–F). S, siScramble; B7, siBIRC7; I3, siIGFBP3; N1, siNTSR1; UL, up left; UR, up right; LL, low left; LR, low right.

Effect of HBV X mutants on the expression of AR and downstream targeted genes

Clinical studies have reported that baseline serum HBV DNA titers are significantly higher in males than in females.[20] WT-HBx protein is a positive coregulator that augments AR-mediated transcriptional activity, and this regulation increases in an androgen-dependent manner.[21] As compared with WT-HBx, however, combo HBx mutations (e.g., A1762T/G1764A + C1653T + T1753C + T1674G) and C-terminal carboxy-truncated HBx protein (Ct-HBx) are related to a higher HCC incidence, especially Ct-HBx.[7,8] To further investigate the interaction between HBx proteins and AR, we constructed WT-HBx- and Ct-HBx-expressing plasmids to determine if these mutants enhance AR expression. The AR promoter was co-transfected with WT-HBx, Ct-HBx, or empty vector into HepG2 cells. Dual-luciferase reporter gene experiments showed that WT-HBx did not improve the promoter activity of AR compared to an empty vector. Interestingly, the AR promoter activity was significantly upregulated by Ct-HBx directly (Figure 5D).

We then investigated if androgen changes the effect of WT-HBx on the AR promoter activity. Figure 5E shows that compared with 0.1% DMSO, the effect of WT-HBx on the AR promoter activity was significantly enhanced by DHT (100 nM) stimulation. Thus, the effect of WT-HBx is androgen-dependent. However, Ct-HBx enhanced AR transcription, and this effect was independent of DHT. The effects of WT-HBx and HBx mutants on the expression of targeted genes were measured by re-assessing our previous GSE179125 data from the Gene Expression Omnibus database.[8] The expression of BIRC7 was significantly upregulated in HBx mutants, compared with WT-HBx (Figures 5F–5H).

The effect of NTSR1 knockdown on HCC growth in mouse model

Of the three target genes, only NTSR1 was essential for migration, invasion, and apoptosis of HCC *in vitro*. Therefore, NTSR1 was chosen for the experiments in nude mice. Stable transcripts of SK-Hep-1-shNTSR1 were constructed, as confirmed using RT-qPCR and western blot (Figure 6A). The mRNA expression level of VEGF in shScramble- and shNTSR1-transfected cells was also analyzed. The level of VEGF mRNA was lower in SK-Hep-1-shNTSR1 than in SK-Hep-1-shScramble control, suggesting that downregulation of NTSR1 decreased tumor angiogenesis (Figure 6B). *Ex vivo* experiments in mouse model were designed to evaluate if NTSR1 affected the growth of HCC cells and if this effect was related to androgen. Twenty 5-week-old BALB/c nude mice were randomly divided into two groups. One group was subcutaneously transplanted with shNTSR1-transfected SK-Hep-1 cells as the experimental group and another group subcutaneously transplanted with shScramble-transfected SK-Hep-1 cells as the control. Five mice in each group were injected with 50 μL flutamide or 50 μL vehicle DMSO. Flutamide was injected at 25 mg/kg/day intraperitoneally every other day as previously described.[22–24] It was found that tumor growth was significantly slower in the shNTSR1-DMSO group than with shScramble-DMSO, suggesting that downregulation of NTSR1 inhibits tumor growth. Tumor size was smaller in the shScramble-flutamide group than in the shScramble-DMSO, suggesting that flutamide inhibits tumor growth. However, shNTSR1 and flutamide did not have a synergistic effect (Figures 6C and 6D).

To further investigate whether NTSR1 are responsible for gender disparity in HCC prevalence, we performed *ex* vivo experiment in female and male mice. The result revealed that tumor growth was significantly slower in shNTSR1 group than in shScramble group in male mice, while tumor growth had no difference between shNTSR1 and shScramble group in female mice (Figure 6E). Tumor size showed similar results (Figure 6F). This suggests that knockdown of NTSR1 inhibited tumor growth in male mice, rather than in female mice, indicating NTSR1 are responsible for gender disparity in HCC prevalence.

Effects of BIRC7, IGFBP3, and NTSR1 on HCC prognosis

The Cox regression analysis was used to analyze the effect of BIRC7, IGFBP3, and NTSR1 expression on the OS and progression-free survival (PFS) in HCC using the TCGA LIHC database. Higher expressions of BIRC7, IGFBP3, and NTSR1 had a significant negative correlation with OS in males, with age-adjusted HR (95% CI) of 1.619 (1.007–2.063), 1.957 (1.241–3.085), and 2.132 (1.252–3.629), respectively (Figures 7A–7C). However, in females, only higher expression of IGFBP3 had a significant negative correlation with OS. Higher expression of NTSR1 had a strong positive correlation with PFS in females, with an age-adjusted HR (95% CI) of 0.428 (0.228–0.805) (Figure S7C). No associations of other genes' expression with PFS were identified (Figure S7). The expression of BIRC7 and NTSR1 significantly shortened OS in males rather than in females, indicating that BIRC7 and NTSR1 might play a role in the gender disparity of HCC progression. The prognosis was further analyzed according to HBV status. The result showed that higher expression of NTSR1 had a significant negative correlation with OS in males with HBV, with an age-adjusted HR (95% CI) of 2.388 (1.055–5.404) (Figure S8C). Higher expression of BIRC7 significantly shortened PFS in males with HBV rather than in males without HBV (Figure S9A). Interestingly, high expression of NTSR1 prolonged PFS in females without HBV rather than in females with HBV (Figure S9F). Thus, high expression of BIRC7 or NTSR1 shortened PFS or OS in males with HBV, but high expression of NTSR1 prolonged PFS in females without HBV. These indicate that the function of BIRC7 and NTSR1 may be related to HBV.

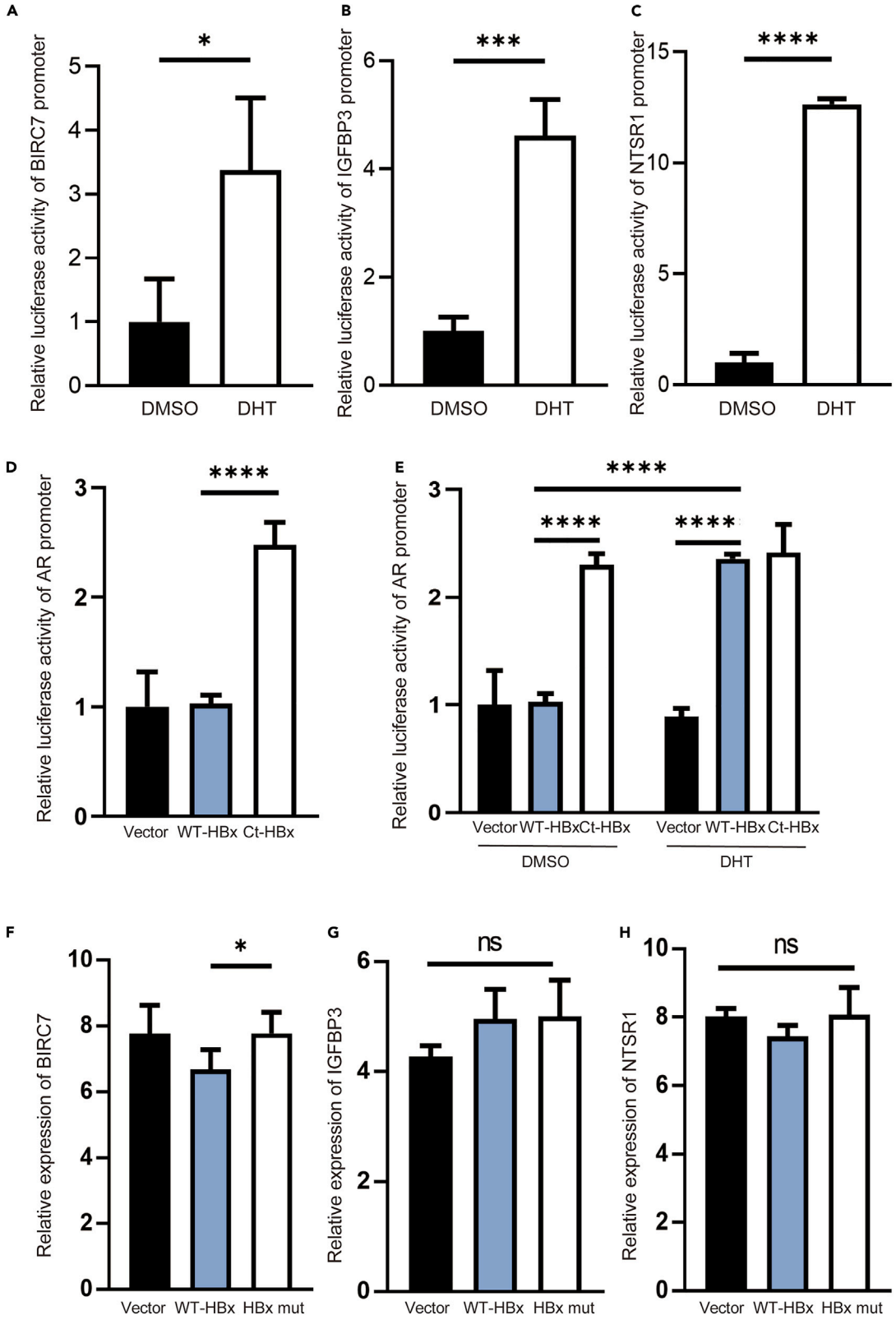
A
Relative luciferase activity of BIRC7 promoter
*
DMSO
DHT
B
Relative luciferase activity of IGFBP3 promoter
***
DMSO
DHT
C
Relative luciferase activity of NTSR1 promoter
****
DMSO
DHT
D
Relative luciferase activity of AR promoter
****
Vector
WT-HBx
Ct-HBx
E
Relative luciferase activity of AR promoter
****
****
****
Vector
WT-HBx
Ct-HBx
Vector
WT-HBx
Ct-HBx
DMSO
DHT
F
Relative expression of BIRC7
*
Vector
WT-HBx
HBx mut
G
Relative expression of IGFBP3
ns
Vector
WT-HBx
HBx mut
H
Relative expression of NTSR1
ns
Vector
WT-HBx
HBx mut

Figure 5. Transcriptional regulation of androgen on targeted genes and AR

(A) The effect of DHT on the transcription of BIRC7 *trans*-activated by AR.

(B) The effect of DHT on the transcription of IGFBP3 *trans*-activated by AR.

(C) The effect of DHT on the transcription of NTSR1 *trans*-activated by AR.

(D) The activity of the AR promoter was *trans*-activated by WT-HBx or CT-HBx.

(E) The effect of DHT and HBx (WT and mutant) on the AR promoter activity.

(F) The effect of the WT-HBx and HBx mutant on the expression of BIRC7.

(G) The effect of the WT-HBx and HBx mutant on the expression of IGFBP3.

(H) The effect of the WT-HBx and HBx mutant on the expression of NTSR1. Data represent means ± SD of three independent experiments. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$, ns, not significant. Unpaired t tests for (A–H). AR, androgen receptor; DHT, dihydrotestosterone; HBx, HBV X; WT, WT-HBx; CT, Ct-HBx; WT-HBx, wild-type HBx; Ct-HBx, C-terminal carboxy-truncated HBx; HBx mut, HBx mutants.

DISCUSSION

In this study, AR was proven to promote the migration and invasion of HCC cell lines and inhibit their apoptosis, while knockdown of AR inhibited tumor growth in mice, suggesting that AR facilitates HCC. AR expression in adjacent tissues or tumor tissues did not significantly differ between males and females, indicating that AR expression is unrelated to gender. This result is consistent with results from the sex-associated gene database. This database indicates that, although males exhibit slightly higher average levels of hepatic AR expression, the differences in AR expression between males and females are not statistically significant.[25] However, HCC is a male-predominant cancer, with a male-to-female ratio of 2–3:1, especially in HBV-HCC, with a ratio of 3–4:1.[26,27] Thus, AR alone might not be responsible for the gender disparity; the higher levels of androgens can explain the gender disparity of HCC. Epidemiological data on androgens and HCC showed that males in HCC groups had higher level of testosterone (a kind of androgen) than females. A prospective study of HCC in males found that elevated testosterone levels were associated with an increased risk of HCC.[28] A retrospective study of males with cirrhosis also suggested elevated levels of testosterone were predictive of HCC risk among male cirrhotic patients.[29] These results indicated the relationship between androgens and HCC. However, due to the lack of samples or data, we were unable to analyze the androgen levels in patients with HCC or recurrent HCC compared to healthy controls or patients with non-HCC liver disease, which might be useful to unravel androgen contribution to higher susceptibility to HCC in males. Therefore, more epidemiological and mechanistic studies are needed to confirm the relationship between androgens and HCC. Androgen causes translocation of the transcription factor AR into the nucleus to exert oncogenic effects. In this study, we found that DHT and AR promoted migration and invasion and inhibited apoptosis of HCC; furthermore, combined DHT and AR significantly enhanced their cancer-promoting capacity. This effect was attenuated by the anti-androgen flutamide, suggesting that the interaction between androgen and AR is critical for HCC development. Other studies also demonstrated that host androgen/AR signaling contributes to significant male-predominant liver metastasis, independent of primary cancer type, mouse strain, or tumor growth pattern.[30] Our findings, together with others, may explain why HCC is a male-predominant cancer, as androgen levels are much higher in males than in females. In addition to the effect of androgen, the effect of estrogen was also investigated. It was found that estrogen inhibited migration and invasion, and promoted apoptosis of HCC. It also mitigated the cancer-promoting capacity of AR on HCC cell activities. Other studies also found that estrogen could suppress tumor growth via regulating the polarization of macrophages.[31]

Our RNA-seq data indicate that overexpression of AR activates migration and invasion-related bioprocesses and apoptosis-related signaling pathways. BIRC7, IGFBP3, NTSR1, and NDUFA4L2 are the DEGs involved in the enriched pathways and related to the prognosis of HCC. The expression levels of BIRC7, IGFBP3, NTSR1, and NDUFA4L2 positively correlate with AR. Our dual-luciferase reporter gene assay showed that AR positively regulated BIRC7, IGFBP3, and NTSR1 expressions but did not regulate NDUFA4L2 expression directly. Our ChIP-PCR results demonstrated that AR regulated the expression of target genes by targeting and binding their promoter, indicating that AR *trans*-activate the expressions of BIRC7, IGFBP3, and NTSR1 directly. BIRC7, IGFBP3, and NTSR1 are newly identified target genes of AR. Our study reveals that AR affects cell migration possibly by regulating the expression of NTSR1; affect cell invasion by regulating the expression of IGFBP3 and NTSR1; and affect apoptosis by regulating the expression of BIRC7, IGFBP3, and NTSR1. Rescue experiments demonstrated that BIRC7, IGFBP3, and NTSR1 function through AR and confirmed the regulation of DHT on AR target genes cell activities. The effect of SK-shNTSR1 was neutralized by androgen (DHT), suggesting that NTSR1, the most critical target gene regulated by AR, is essential for the male predominance of HCC. The discovery of new target genes may provide new insights into the mechanisms of gender disparity in HCC.

Several lines of evidence suggest that HBV may interact with AR, and this interaction might be one of the reasons that HBV-HCC is substantially higher in males.[32,33] Our previous study has demonstrated that Ct-HBx is related to a significantly higher HCC occurrence in sleeping-beauty mouse model than WT-HBx.[8] In the present study, we designed a dual-luciferase assay to observe the interaction between HBx and AR. WT-HBx alone did not upregulate the transcription activity of AR, but WT-HBx plus androgen efficiently upregulated AR expression, which facilitates the male predominance of HBV-HCC. Interestingly, Ct-HBx regulated AR, which is independent of androgen. This phenomenon may be explained by the fact that Ct-HBx has more potent regulatory capacity than androgen on AR, indicating that Ct-HBx has more capacity to increase the aggressiveness of HCC than WT-HBx.[34] In the present study, the expression of BIRC7 was significantly upregulated in the HBx mutants compared with WT-HBx, suggesting that BIRC7 might be related to the male predominance observed in HCC caused by the HBV mutants. Overall, these findings suggest that the male predominance in HBV-HCC is partially attributed to the interaction of HBx plus androgen or the HBx mutants by inducing BIRC7.

We found that NTSR1 was associated with the malignant phenotype of HCC *in vitro*, and both the NTSR1 knockdown and anti-androgen flutamide significantly suppressed tumor growth *in vivo*. However, the combination of NTSR1 knockdown and flutamide treatment did not

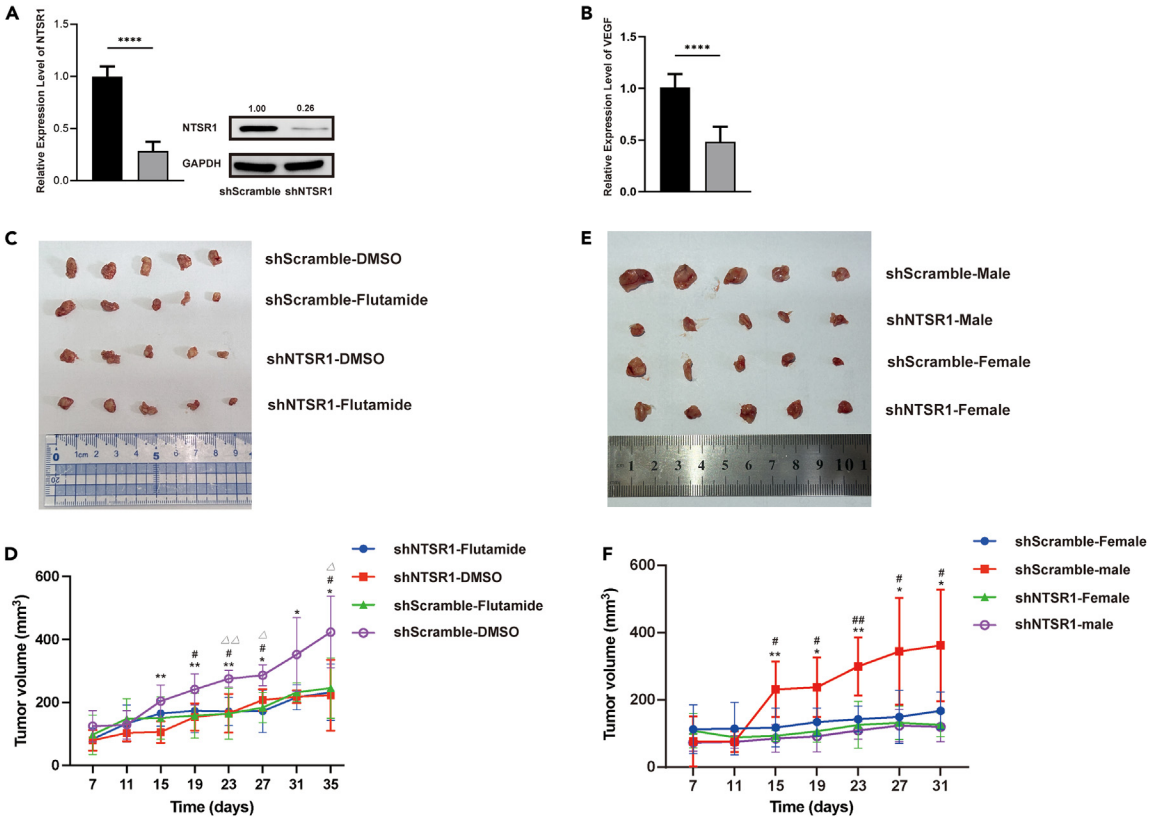

Figure 6. NTSR1 knockdown attenuated tumor growth in xenograft mouse model

(A) The mRNA expression level of NTSR1 in SK-Hep-1 cells with and without NTSR1 knockdown were determined by RT-qPCR and values were normalized to GAPDH (right) and the protein level of NTSR1 in SK-Hep-1 cells with and without NTSR1 knockdown were determined by western blot, GAPDH as a reference (left). Data represent means ± SD of three independent experiments. ****$p < 0.0001$ by unpaired t tests.

(B) The expression level of VEGF mRNA in SK-Hep-1 cells with and without NTSR1 knockdown were determined by RT-qPCR and values were normalized to GAPDH. Data represent means ± SD of three independent experiments. ****$p < 0.0001$ by unpaired t tests.

(C) Tumor sizes at the 35th day after tumor transplantation in male mice infected with control lentivirus or NTSR1-knockdown lentivirus and intraperitoneal injection with DMSO or flutamide.

(D) The growth curve of tumor volume of male mice infected with control lentivirus or NTSR1-knockdown lentivirus and intraperitoneal injection with DMSO or flutamide. Data represent means ± SD ($N = 5$). *shScramble-DMSO vs. shNTSR1-DMSO, $p < 0.05$, **$p < 0.01$; $^{\#}$shScramble-DMSO vs. shScramble-flutamide, $p < 0.05$, $^{\#\#}p < 0.01$; $^{\Delta}$shScramble-DMSO vs. shNTSR1-flutamide, $p < 0.05$, $\Delta\Delta$ $p < 0.01$. Unpaired t tests.

(E) Tumor sizes at the 31th day after tumor transplantation in male and female mice infected with control lentivirus or NTSR1-knockdown lentivirus.

(F) The growth curve of tumor volume in male and female mice infected with control lentivirus or NTSR1-knockdown lentivirus. Data represent means ± SD ($N = 5$). *shScramble-male vs. shNTSR1-male, $p < 0.05$, **$p < 0.01$; $^{\#}$shScramble-female vs. shScramble-male, $p < 0.05$, $^{\#\#}p < 0.01$. Unpaired t tests. shScramble-DMSO: infected with control lentivirus and intraperitoneal injection with DMSO; shScramble-flutamide: infected with control lentivirus and intraperitoneal injection with flutamide; shNTSR1-DMSO: infected with NTSR1-knockdown lentivirus and intraperitoneal injection with DMSO; shNTSR1-flutamide: infected with NTSR1 knockdown lentivirus and intraperitoneal injection with flutamide. DMSO, dimethyl sulfoxide.

have a synergistic effect, indicating that flutamide competitively inhibits androgens via binding AR, thus downregulating AR and AR-regulated BIRC7, IGFBP3, and NTSR1. Since the HCC-inhibiting capacity of siNTSR1 was stronger than siBIRC7 and siIGFBP3 *in vitro*, NTSR1 should be more carcinogenic than BIRC7 and IGFBP3 in males. More importantly, it was found that downregulation of NTSR1 inhibited tumor growth in male mice, rather than in female mice, indicating NTSR1 are responsible for gender disparity in HCC prevalence.

Our prognosis analysis revealed that high expression of IGFBP3 had a significant negative correlation with OS in males and females. High expression of BIRC7 significantly shortened OS in males rather than in females. Interestingly, high expression of NTSR1 shortened OS in males but prolonged PFS in females. Thus, AR, with androgen and its downstream molecules BIRC7 and NTSR1, should be responsible for the gender dimorphism of HCC. Furthermore, high expression of BIRC7 or NTSR1 shortened PFS or OS in males with HBV, but high expression of NTSR1 prolonged PFS in females without HBV.

In summary, AR promotes HCC development, and this oncogenic effect is enhanced by androgen. Furthermore, AR exhibits its pro-cancer properties by upregulating specific target genes—BIRC7, IGFBP3, and NTSR1—which could be augmented by androgen. The transcription of WT-HBx on AR is androgen-dependent, whereas Ct-HBx-enhanced AR transcription is androgen-independent. The HBx mutants upregulate

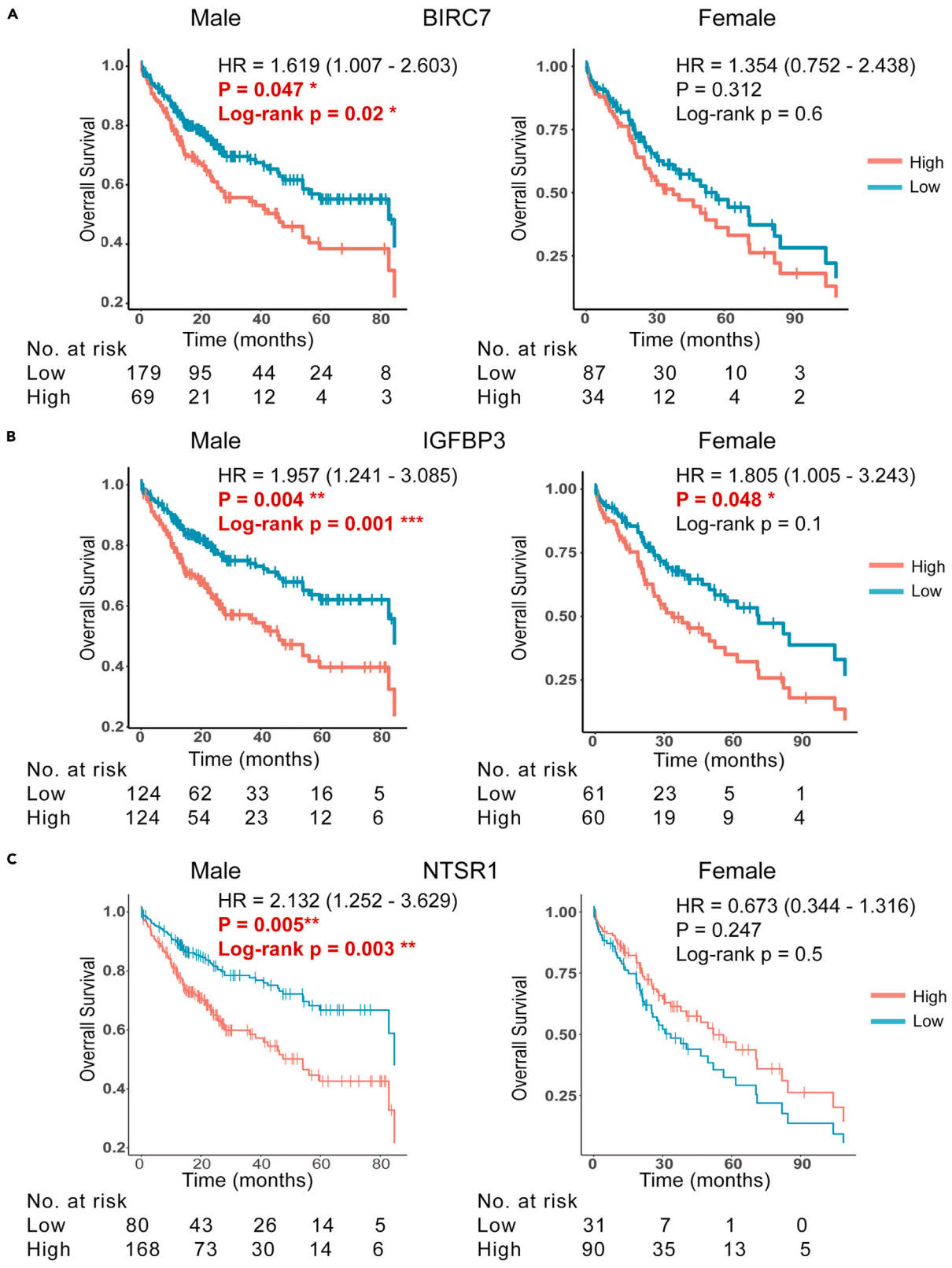
A
Male
BIRC7
Female
HR = 1.619 (1.007 - 2.603)
P = 0.047 *
Log-rank p = 0.02 *
HR = 1.354 (0.752 - 2.438)
P = 0.312
Log-rank p = 0.6
Overall Survival
Time (months)
High
Low
No. at risk
Low 179 95 44 24 8
High 69 21 12 4 3
Low 87 30 10 3
High 34 12 4 2
B
Male
IGFBP3
Female
HR = 1.957 (1.241 - 3.085)
P = 0.004 **
Log-rank p = 0.001 ***
HR = 1.805 (1.005 - 3.243)
P = 0.048 *
Log-rank p = 0.1
Low 124 62 33 16 5
High 124 54 23 12 6
Low 61 23 5 1
High 60 19 9 4
C
Male
NTSR1
Female
HR = 2.132 (1.252 - 3.629)
P = 0.005**
Log-rank p = 0.003 **
HR = 0.673 (0.344 - 1.316)
P = 0.247
Log-rank p = 0.5
Low 80 43 26 14 5
High 168 73 30 14 6
Low 31 7 1 0
High 90 35 13 5

Figure 7. The association of BIRC7, IGFBP3, and NTSR1 expression in tumor tissues with overall survival in males and females with HCC

(A) The association of BIRC7 expression with OS.

(B) The association of IGFBP3 expression with OS.

(C) The association of NTSR1 expression with OS. HR was adjusted for age. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ by Cox regression analysis. HR: hazard ratio; OS: overall survival.

the expression of BIRC7 compared with WT-HBx. Downregulation of NTSR1 inhibits tumor growth in male mice rather than in female mice. BIRC7 and NTSR1 play an essential role in the gender disparity of HCC. This study provides new evidence to explain the male predominance of HCC and offers new options for HCC control.

Limitations of the study

This study has some limitations. The AR expression was detected in a cohort; however, the sample size of this cohort was small. The survival analyses of BIRC7, IGFBP3, and NTSR1 were analyzed using a public database, which needs to be testified in a cohort. Although we found the difference between WT-HBx and Ct-HBx on AR, its mechanism could explore in depth. We also found that BIRC7 and NTSR1 work on gender disparity of HCC; however, the precise mechanism needs to be studied more in-depth. Additionally, we used a large number of experiments to prove that androgen contribution to higher susceptibility to HCC in males, but epidemiological investigation also indispensable. Future research is necessary to detect androgen levels in patients with HCC or recurrent HCC compared to healthy controls or patients with non-HCC liver disease.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110519.

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AUTHOR CONTRIBUTIONS

J.Z., R.P., L.F., and W.L. have contributed equally to this work and share first authorship. G.C. designed and supervised the study. S.C., R.W., Zheng Li, Y.S., Z.Z., and Zishuai Li took part in the cell experiments. J.Z., R.P., L.F., and W.L. conducted the statistical analyses and drafted the manuscript. G.C. interpreted the data and extensively revised the manuscript. All authors read and approved the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit GAPDH Polyclonal Antibody	Abcam	Cat#ab9485; RRID:AB_307275
Rabbit NTSR1 Polyclonal Antibody	Signalway Antibody	Cat#31251; RRID:AB_3107085
Rabbit IGFBP3 Polyclonal Antibody	Signalway Antibody	Cat#41888; RRID:AB_3107086
Rabbit BIRC7 Polyclonal Antibody	Signalway Antibody	Cat#31094; RRID:AB_3107087
Rabbit AR Polyclonal Antibody	Signalway Antibody	Cat#32572; RRID:AB_3107088
Biological samples		
Human adjacent and tumor liver tissues	Eastern Hepatobiliary Surgery Hospital (Shanghai, China)	N/A
Chemicals, peptides, and recombinant proteins		
Dulbecco's modified Eagle's medium	Baselmedia	Cat #L110KJ
fetal bovine serum	Gibco	Cat #10270106
penicillin/streptomycin	Bio-light	Cat #BL20131
4% paraformaldehyde solution	Bio-light	Cat #BL20263
crystal violet staining solution	Bio-light	Cat #BL31211
0.25% trypsin without EDTA	Gibco	Cat #15050057
Dihydrotestosterone	selleck	Cat #S4757
Flutamide	Sigma-Aldrich	Cat #F9397-5G
Estradiol	selleck	Cat #S1709
TRIzol	Invitrogen	Cat #15596018CN
Matrigel	Corning	Cat #356234
Critical commercial assays		
Lipofectamine™ 3000 Transfection Reagent	Thermo Fisher Scientific	Cat #L3000001
Lipofectamine™ RNAiMAX Transfection Reagent	Thermo Fisher Scientific	Cat #13778030
PrimeScript RT Master Mix (Perfect Real Time)	Takara	Cat #RR036A
TB Green™ Premix Ex Taq™ II	Takara	Cat #RR820A
Annexin V-PE/7-AAD Apoptosis Kit	Multi Sciences	Cat #AT104
Deposited data		
RNA-seq data	This paper	PRJNA1024056
cDNA microarray assay	Gene Expression Omnibus	GSE179125
Experimental models: Cell lines		
HepG2	Chinese Academy of Sciences (Shanghai, China)	CSTR:19375.09.3101HUMTCHu72
SK-Hep-1	Chinese Academy of Sciences (Shanghai, China)	CSTR:19375.09.3101HUMTCHu109
Huh7	Chinese Academy of Sciences (Shanghai, China)	CSTR:19375.09.3101HUMTCHu182
AD38	Obtained from Dr. Zhenghong Yuan (Fudan University, Shanghai, China)	N/A
Experimental models: Organisms/strains		
BALB/c mice	Jihui Laboratory Animal Care Corporation	N/A
Oligonucleotides		
siRNA targeting sequence: BIRC7: GCTCCGGTCAAAAGGAAGA	This paper	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
siRNA targeting sequence: IGFBP3: GCTACAGCATGCAGAGCAA	This paper	N/A
siRNA targeting sequence: NTSR1: ACACCATCATCGCCAACAA	This paper	N/A
siRNA targeting sequence: AR: CTGGCGATCCTTCACCAAT	This paper	N/A
Primers for AR, BIRC7, IGFBP3, NTSR1, NDUFA4L2, GAPDH, VEGF, AQP3, BMP6, DNAJC12, CITED1, FADS6, and NTN4 see Table S7	This paper	N/A
Recombinant DNA		
Plasmid: AR-overexpressing lentivirus	This paper	N/A
Plasmid: BIRC7 promoter	This paper	N/A
Plasmid: IGFBP3 promoter	This paper	N/A
Plasmid: NTSR1promoter	This paper	N/A
Plasmid: NDUFA4L2 promoter	This paper	N/A
Software and algorithms		
R	R Core Team	https://www.r-project.org/
GraphPad Prism version 5.0	GraphPad	https://www.graphpad.com
Image J	Image J	https://imagej.nih.gov/ij/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Guangwen Cao (gcao@smmu.edu.cn).

Materials availability

New plasmids generated in this study can be obtained by contacting the lead author.

Data and code availability

- The transcriptional profiling data are available in NCBI: PRJNA1024056. This paper analyzes existing, publicly available data (GSE179125). These accession numbers for the datasets are listed in the key resources table.
- This study did not generate any original code.
- Any additional information for reanalyzing the data in this study is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

A total of 64 5-week-old BALB/c nude mice (22 females and 42 males) were purchased from Jihui Laboratory Animal Care Corporation (Shanghai, China). The mice were housed with free access to water and food. All animal studies followed the ethical review of animal welfare (GB/T 35823-2018), and the ethical approval number is 81520108021.

Cell culture

Human HCC cell lines HepG2, SK-Hep-1, and Huh7 were purchased from the Chinese Academy of Sciences (Shanghai, China). Human HCC cell line AD38 was kindly donated by Dr. Zhenghong Yuan (Fudan University, Shanghai, China). All cell cultures were tested negative for mycoplasma contamination every three months. HCC cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (BasalMedia, China) supplemented with 10% fetal bovine serum (FBS; Gibco, South America) and 1% penicillin/streptomycin (Bio-light, China). The cells were cultured in a 5% CO_2 atmosphere at 37°C.

Patients and samples

In total, matched adjacent and tumor samples from 108 patients (93 men and 15 women) who underwent surgical resection and were pathologically diagnosed with HCC at the Eastern Hepatobiliary Surgery Hospital (Shanghai, China) were included in this study.[35] Patients did not receive any antitumor therapy before surgery. Of those, 41 recurred after surgery. The study protocol conformed to the ethical guidelines of the 2000 Declaration of Helsinki and was approved by the ethics committees of the Second Military Medical University and the Eastern Hepatobiliary Surgery Hospital, and the ethical approval number is 81520108021, and all participants provided written informed consent.

METHOD DETAILS

Cell invasion, migration, and apoptosis assay

Cell invasion and migration were measured using Transwell inserts (Corning, USA). Matrigel (Corning, USA) (100 μL) was added vertically to the bottom center of the upper chamber and incubated at 37°C for 5 h, and the residual liquid was discarded for invasion experiments, whereas migration experiments did not require Matrigel (Corning, USA). DMEM (100 μL) containing 0.1% bovine albumin (BSA) was used to cultivate 4×10^5 cells in the upper chamber. DMEM containing 10% FBS (500 μL) was filled in the lower chamber. After incubation for 24 h, the upper chamber was transferred to 4% paraformaldehyde solution (Bio-light, China) (500 μL) and incubated at room temperature for 15 min. The upper chamber was then stained with crystal violet staining solution (Bio-light, China) for 20 min, followed by drying with cotton swabs and photographed under a microscope.

The apoptosis assay was performed using flow cytometry. Cells were harvested using 0.25% trypsin without EDTA (Gibco, USA), then added to the binding buffer (BD Pharmingen, USA) to resuspend them. These cells were stained with Annexin V-PE/7-AAD Apoptosis Kit (Multi Sciences, China) according to the manufacturer's instructions.

RNA sequencing and analysis

Ectopic expression of AR in HepG2 cells was achieved, and an empty vector served as the control. Total RNA was isolated from HepG2-AR and HepG2-vector control cells using TRIzol (Invitrogen), reversely transcribed to cDNA, and then amplified by PCR. The quality of constructed libraries was checked using an Agilent 2100 Bioanalyzer and sequenced on an Illumina HiSeqTM 2500 sequencer. The read number of the transcripts (protein coding) in each sample was obtained using eXpress software. The data were normalized using the estimateSizeFactors function of the DESeq (2012) R package, and the p-values and fold changes were calculated using the *nbinomTest*. Differentially expressed genes (DEGs) ($p < 0.05$ and fold change > 2) were selected for volcano plotting, Gene Ontology (GO) analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis to screen for significantly enriched pathways. The mRNA sequencing data are available in the Sequence Read Archive database under the accession number SUB13883745.

Dual-luciferase reporter gene assay

To determine the transcription factors involved in AR regulating the expression of target genes BIRC7, IGFBP3, NTSR1, and NDUFA4L2, the promoter fragments (2000 bp upstream of the transcription start site) of BIRC7, IGFBP3, NTSR1, and NDUFA4L2 were synthesized and cloned into pGL4.10 plasmid by Obio Technology (Shanghai, China), respectively. AR was co-transfected with each of the four plasmids in HCC cell lines using the Lip3000 kit. The transfected cells were then incubated at 37°C for 48 h. The luciferase activity was measured after lysed the cells using the passive lysis buffer.

Quantitative real-time reverse-transcription PCR

TRIzol reagent (Invitrogen) extracted total RNA from tissue samples and HCC cells. The transcript abundance of AR, BIRC7, IGFBP3, NTSR1, and NDUFA4L2 was quantified using quantitative real-time reverse-transcription PCR (qRT-PCR) with PrimeScript RT Master Mix (Takara) and TB Green Premix Ex Taq II (Takara). All primers for qRT-PCR assays are listed in Table S7.

Western Blot

Whole cell proteins were extracted by solubilizing in Cell lysis buffer. Proteins were separated by 10%-12% SDS-PAGE. After addition of protein uploading buffer, proteins were transferred to a methanol-fixed polyvinylidene difluoride membrane (PVDF). Membranes were blocked with 5% skimmed milk at room temperature (RT) for 1 h. Membranes were then incubated with the specific primary antibody overnight at 4°C and the secondary antibody at RT for 2 h. At the end of the antibody incubation, the membranes were washed three times with TBST for 10 min each. The blots were imaged using Odyssey® XF. Western blot analyses were performed in triplicate. GAPDH was used as the loading control. ImageJ software was used to quantify the signal strength of the bands. The antibodies are listed in the key resources table.

Chromatin immunoprecipitation assay

According to the manufacturer's instructions, a chromatin immunoprecipitation (ChIP) assay was performed using a ChIP-IT Express Enzymatic Kit (Active Motif, CA). We added 1/10 volume of freshly prepared cell fixative as required to fix the cells before interrupting with sonication. The purified DNA was obtained and subjected to PCR. The enrichment value was expressed as a percentage of input. Primers are listed in Table S7.

Xenograft mouse model

To study the effect of AR in males and females, 12 male and 12 female BALB/c nude mice (5-week-old) were purchased from Jihui Laboratory Animal Care Corporation (Shanghai, China). 100 μL of PBS and Matrigel (1:1) (Corning) containing 1×10^7 SK-Hep-1 cells were subcutaneously injected into the right flank. Seven days after injection, 12 mice were randomly assigned to each group, the siRNAs against AR or the siRNA with scrambled sequence were injected into the tumor bodies twice a week at a concentration of 5 nmol in 100 μL PBS.

To determine if androgen inhibitors would enhance the effect of shNTSR1 in HCC, 20 male BALB/c nude mice (5-week-old) were purchased from Jihui Laboratory Animal Care Corporation (Shanghai, China). Ten mice were randomly assigned to each group; 100 μL of PBS and Matrigel (1:1) (Corning) containing 1×10^7 SK-Hep-1 cells expressing shNTSR1 or infected with lentivirus negative control were subcutaneously injected into the right flank. To determine the function of androgen inhibitors, the anti-androgen flutamide (25 mg/kg/day, suspended in 50 μL DMSO, Sigma-Aldrich) or vehicle (50 μL DMSO) was intraperitoneally injected every other day in five of the ten mice in each group.[22–24]

To investigate whether NTSR1 are responsible for sex differences in HCC prevalence, 10 male and 10 female BALB/c nude mice (5-week-old) were purchased from Jihui Laboratory Animal Care Corporation (Shanghai, China). 5 mice were randomly assigned to each group; 100 μL of PBS and Matrigel (1:1) (Corning) containing 1×10^7 SK-Hep-1 cells expressing shNTSR1 or infected with lentivirus negative control were subcutaneously injected into the right flank.

Tumor growth was measured every four days. Tumor volume was calculated as $V = \text{length} \times \text{width}^2/2$. The Ethics Committee of the Second Military Medical University approved all animal experimental procedures.

QUANTIFICATION AND STATISTICAL ANALYSIS

Survival analysis was conducted after adjusting the age and gender factor using Cox regression analysis. The Student's *t*-test was used for differences in continuous variables. The analyses of the data were performed with GraphPad Prism version 5.0. For all analyses, a two-sided $p < 0.05$ was considered statistically significant. Further statistical details, including n values, are provided in the figure legends.