Androgen receptor suppresses vasculogenic mimicry in hepatocellular carcinoma via circRNA7/miRNA7-5p/VE-cadherin/Notch4 signalling

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Abstract
Androgen receptor (AR) can suppress hepatocellular carcinoma (HCC) invasion and metastasis at an advanced stage. Vasculogenic mimicry (VM), a new vascularization pattern by which tumour tissues nourish themselves, is correlated with tumour progression and metastasis. Here, we investigated the effect of AR on the formation of VM and its mechanism in HCC. The results suggested that AR could down-regulate circular RNA (circRNA) 7, up-regulate micro RNA (miRNA) 7-5p, and suppress the formation of VM in HCC. Small hairpin circR7 (ShcircR7) could reverse the impact on VM and expression of VE-cadherin and Notch4 increased by small interfering AR (shAR) in HCC, while inhibition of miR-7-5p blocked the formation of VM and expression of VE-cadherin and Notch4 decreased by AR overexpression (oeAR) in HCC. Mechanism dissection demonstrated that AR could directly target the circRNA7 host gene promoter to suppress circRNA7, and miR-7-5p might directly target the VE-cadherin and Notch4 3′UTR to suppress their expression in HCC. In addition, knockdown of Notch4 and/or VE-cadherin revealed that shVE-cadherin or shNotch4 alone could partially reverse the formation of HCC VM, while shVE-cadherin and shNotch4 together could completely suppress the formation of HCC VM. Those results indicate that AR could suppress the formation of HCC VM by down-regulating circRNA7/miRNA7-5p/VE-Cadherin/Notch4 signals in HCC, which will help in the design of novel therapies against HCC.

KEYWORDS
androgen receptor, circRNA7, hepatocellular carcinoma, miRNA7-5p, Notch4, vasculogenic mimicry, VE-Cadherin
1 | INTRODUCTION

Hepatocellular carcinoma (HCC), which accounts for more than 90% of liver cancers, is ranked as the fourth most common malignancy. It is also the fourth leading cause of cancer-induced death, with more than 780,000 deaths every year. While molecular targeting chemotherapy and surgical resection have recently experienced rapid progress, the advancement of long-term survival remains far from satisfactory. Moreover, patients receiving surgical resection often have a high rate of recurrence, with a five-year survival only in near 25% of cases. Currently, detailed mechanisms for HCC initiation and progression remain unclear.

Androgen receptor (AR) is a nuclear receptor that plays a key role in influencing HCC progression. Some reports have indicated that AR can promote HCC development at earlier stages, and that inhibition of AR suppressed hepatitis B virus (HBV)- or carcinogen-induced HCC progression from early stages in vitro and in vivo. Inhibiting AR can increase HCC metastasis in advanced stages; therefore, AR likely inhibits HCC metastasis in advanced stages. This might account for the conflicted outcomes of clinical trials using different anti-androgens to treat HCC. Ma et al reported that AR could suppress HCC metastasis in HCC cell lines and in advanced stage HCC in L-ARKO mice. AR together with Sorafenib might suppress HCC late-stage progression. However, the molecular mechanisms underlying how activated AR signals suppress HCC late-stage progression remain to be elucidated.

Vascularization plays a crucial role in tumour metastasis. The progression of cancer depends on tumour vascularization, as new vessel formation ensures a sufficient supply of oxygen, nutrients and growth factors to the growing tumour and facilitates tumour dissemination. Recent studies have revealed a vasculogenic mimicry (VM) pattern, a new pattern where tumour tissues nourish themselves. The pattern includes vessels lined exclusively with tumour cells mimicking the presence and function of endothelial cells, as one means for blood supply to tumours. VM has been identified in many aggressive metastatic tumours, showing that some tumour cells are able to form highly patterned vascular channels in vitro. These channels consist of a basement membrane that stains positive with the VM marker periodic acid-Schiff (PAS) in the absence of CD31-positive endothelial cells. Maniotis et al reported that tumour cells, rather than endothelial cells, can form blood vessels in highly aggressive uveal melanomas, and the phenomenon of tumour vascularization was described as vascular mimicry. As that early description, VM has been found in prostatic carcinoma, breast carcinoma, ovarian carcinoma, lung cancer and pancreatic carcinoma. Recent studies have indicated that VM is also associated with metastasis and invasion, and is implicated in poor prognosis of hepatocellular carcinoma and gastrointestinal stromal tumours. However, whether AR can influence VM formation in HCC is still unclear. In this study, we investigated the effect of AR on the formation of VM and its mechanism in HCC.

2 | MATERIALS AND METHODS

2.1 | Materials

AR, VE-cadherin and GAPDH antibodies for Western blotting were purchased from Santa Cruz Biotechnology. Notch4 antibody for Western blotting analysis was purchased from Biorbyt. Anti-mouse/rabbit secondary antibodies for Western blotting were from Invitrogen.

2.2 | In vitro cell culture/maintenance

The SKhep1 human HCC cell line was purchased from the American Type Culture Collection (ATCC). The HA22T human HCC cell line was purchased from the Food Industry Research and Development Institute in Taiwan (BCRC number: 60168). All cell lines were cultured in DMEM (Invitrogen), supplemented with 10% foetal bovine serum (v/v), penicillin (25 U/mL), streptomycin (25 g/mL) and 1% L-glutamine. All cell lines were cultured in an atmosphere containing 5% (v/v) CO₂ at 37°C.

2.3 | VM formation assay and quantification

SK and HA22T cells that overexpressed AR or were transfected with knockdown vectors were trypsinized, then resuspended in serum-free DMEM at a concentration of about 5 × 10⁵/mL. Human microvascular endothelial cells were cultured and used as a positive control. A total of 50 µL of Matrigel (BD Bioscience) were plated in 96-well plates and incubated at 37°C for 1 hour. Then, 100 µL of resuspended SK or HA22T cells was loaded on the Matrigel. Three replicate wells were used for each condition. Following incubation for 4-6 hours at 37°C, 100 µL of 10% formalin was gently added to each well for 10 minutes to fix VM. Each well was analysed under a microscope with 10× phase contrast. VM numbers in each field were imaged and 3-5 fields in each well were randomly selected to calculate the average value.

2.4 | RNA extraction, miRNA extraction, reverse transcription and quantitative real-time PCR analysis

Total RNA was extracted from HCC cells using Trizol reagent (Invitrogen). A total of 1 µg of extracted RNA were reverse transcribed into cDNA using Superscript III transcriptase (Invitrogen). Quantitative real-time PCR (qRT-PCR) was used to determine the mRNA expression levels of target genes. Expression levels were normalized to the expression of GAPDH RNA.

miRNAs were extracted using the PureLink® miRNA kit. Briefly, 2 µg of RNA was processed for poly A polymerase addition by adding 1 mmol/L ATP and 1 U of polymerase in 1x RT buffer for 15 minutes at 37°C in a total of 10 µL. Reverse transcription
was performed by annealing at 65°C for 5 minutes, followed by 4°C for 2 minutes after adding 50 µm of the RT anchor primer. Finally, cDNA was synthesized at 42°C for 1 hour after adding 2 µL 10 mmol/L dNTP, 2 µL 5x RT buffer, 1 µL reverse transcriptase and ddH₂O to a total 20 µL. QRT-PCR was used to determine miRNA expression levels. Expression levels were normalized to the expression of 5s RNA and/or U6.

2.5 | Western blot analysis

Cells were harvested and lysed in lysis buffer. Protein concentrations were measured, followed by heating under reducing conditions. A total of 30 µg of protein were loaded on SDS-PAGE gels and then transferred onto PVDF membranes. After blocking, they were blotted with the following antibodies: GAPDH, AR, Notch4 and VE-cadherin.

2.6 | Lentiviral-based gene delivery

293T cells were transfected with pWPI-AR, PLV-THM plasmid, psAX2 packaging plasmid, and pMD2G envelope plasmid to generate lentivirus expressing AR. The lentivirus was collected and frozen at −80°C for later use.

2.7 | Luciferase assay

Fragments (540 bp) of VE-cadherin and Notch4 3’UTR containing mutant or wild-type miRNA-responsive elements were cloned into the psiCheck2 construct (Promega) downstream of the Renilla luciferase ORF. Fragments (2600 bp) of circRNA7 promoter containing mutant or wild-type miRNA-responsive elements were cloned into the pGL3 construct (Promega) downstream of the Renilla luciferase ORF. SK cells were plated in 24-well plates for 24 hours; then, the DNA was transfected into SK cells with Lipofectamine 3000 (Invitrogen) according to the manufacturer’s instructions. Luciferase activity was assayed by dual-luciferase assay (Promega), according to the manufacturer’s protocol.

2.8 | Chromatin immunoprecipitation assay (ChIP)

SK cells were crosslinked with 1% formaldehyde for 10 minutes, followed by quenching with glycine. Sonication was performed to obtain genomic DNA fragments of approximately 200-400 bp. Sonicated DNA was immunoprecipitated with 2.0 µg anti-AR antibody (Santa Cruz Biotechnology) at 4°C overnight. IgG was used in the reaction as a negative control. A target sequence within the human circRNA7 promoter was detected by PCR with specific primers (Table S1).

2.9 | Statistical analysis

Quantitative data were expressed as mean ± standard deviation (SD) from at least three independent experiments. Statistical analyses were performed using the Student’s t test with GraphPad Prism 10 (GraphPad software, Inc). Statistical significance among the control and treated groups was analysed by ANOVA, and *P < .05 was regarded as the threshold value for statistical significance.

3 | RESULTS

3.1 | AR suppresses VM formation in SK and HA22T cells

Recent studies indicated that AR might suppress HCC metastasis in vitro and in vivo, and might particularly suppress HCC late-stage progression. VM is a type of vascularization which ensures an adequate supply of oxygen, nutrients and growth factors to the growing tumour and facilitates tumour dissemination. It has also been shown to contribute to tumour metastasis. To test whether AR suppresses HCC metastasis through regulating VM formation, we manipulated AR expression in the VM formation process. We found that overexpression of AR (oeAR) could suppress significantly HCC VM formation in both SK and HA22T cells. Consistent with this finding, reducing AR expression with shAR in HCC cells significantly increased VM formation (Figure 1).

3.2 | AR suppresses the expression of VE-cadherin and Notch4 in HCC

Many factors, including EphA2, Notch4, VE-cadherin, SLPI, TWIST, VEGF, vimentin and HIF-1α, are related to the formation of VM in many cancers. Recent studies indicated that Notch4, VE-Cadherin, TWIST and VEGF participated in the formation of VM in HCC. Therefore, we focused on the potential role of AR in regulating the expression of VM-related factors in HCC cells. Through qRT-PCR, we found that oeAR inhibited gene expression of Notch4 and VE-cadherin, while shAR increased the expression of both genes in SK cells (Figure 2A). To further confirm the effect of AR on the expression of VE-cadherin and Notch4 in SK and HA22T cells, we determined their protein levels under the same conditions. Similar to changes in mRNA expression, oeAR inhibited protein expression of Notch4 and VE-cadherin, while shAR increased expression of both proteins in SK and HA22T cells (Figure 2B). These results suggest that AR can suppress the formation of HCC VM by inhibiting the expression of Notch4 and VE-cadherin.
AR suppresses the formation of HCC VM by inhibiting expression of Notch4 and VE-cadherin

As AR can suppress the formation of VM in SK and HA22T, and can also suppress the expression of VE-cadherin and Notch4 in HCC, it is likely that AR may suppress the formation of HCC VM by inhibiting the expression of Notch4 and VE-cadherin. To directly test this theory, we knocked down Notch4 and VE-cadherin expression with shVE-cadherin and shNotch4, and observed the effects on the HCC VM formation following shAR. This blocking experiment revealed that shVE-cadherin and shNotch4 significantly decreased the expression of VE-cadherin and Notch4 increased by shAR in SK and HA22T cells. Although shVE-cadherin or shNotch4 alone could partially reverse the formation VM increased by shAR in SK and HA22T cells, combined use of shVE-cadherin and shNotch4 completely reversed the effects in SK and HA22T cells (Figure 3A,B). These results show that AR suppresses the formation of HCC VM by inhibiting the expression of Notch4 and VE-cadherin in SK and HA22T cells.

AR increases the expression of miR-7-5p in SK cells

To determine the molecular mechanisms underlying AR suppression of VM formation in HCC cells through inhibiting the expression of VE-cadherin and Notch4, we utilized miRNAs. As miRNAs can target multiple genes, it is possible that a single miRNA can target both VE-cadherin and Notch4. Through online databases (TargetScan, miRDB and MicroCosm Targets), we identified a subset of miRNAs that can do so, including miRNA185-5p, miRNA328-3p, miRNA423-5p, miRNA542-3p, miRNA7-5p, miRNA128-3p and miRNA125-3p. Further qRT-PCR analysis of these miRNA under the impact of AR in SK cells led to the identification of miRNA-7-5p, which is increased in response to oeAR, while reduced by AR knockdown through shRNA (Figure 4A). To further confirm that AR suppressed the formation of VM via miR-7-5p/VE-Cadherin/Notch4 signals in HCC, we investigated the effect of miR-7-5p inhibition on VM formation and expression of VE-cadherin and Notch4 in SK and HA22T cells. The results revealed that inhibition of miR-7-5p rescued VM formation blocked by AR (Figure 4B,C) in SK and HA22T cells. miR-7-5p inhibition also reversed expression of VE-cadherin and Notch4 that were down-regulated by AR in SK and HA22T cells (Figure 4D). These results further confirmed that AR suppressed the formation of VM via miR-7-5p/VE-Cadherin/Notch4 signals in HCC.

miR-7-5p regulation of VE-cadherin and Notch4 expression

To further dissect the mechanisms by which miR-7-5p decreased VE-cadherin and Notch4 expression at a molecular level, we used the luciferase reporter assay to determine whether miR-7-5p directly targeted the 3’UTR of VE-cadherin and Notch4 mRNA to suppress their expression. We first identified potential miRNA responsive elements that matched the seed sequence of miR-7-5p in the 3’UTR of...
the VE-cadherin and Notch4 genes. We then mutated this miRNA-responsive element and cloned both wild-type and mutated miRNA responsive elements into the Renilla luciferase vector. Luciferase assay results revealed that miR-7-5p decreased the levels of VE-cadherin and Notch4 containing the wild-type 3′UTR, but not of the mutated constructs (Figure 5A,B), suggesting miR-7-5p might directly target the 3′UTR of VE-cadherin and Notch4 to suppress their expression.

3.6 | AR inhibits the expression of circRNA7

To determine the molecular mechanism responsible for how AR regulated miR-7-5p expression in HCC cells, we focused on the potential role of circRNA7, which has been reported to be able to sponge over 50 miRNA-7-5p in its sequence. We found that oeAR could significantly suppress the expression of circRNA7, while shAR had the opposite effect in SK cells (Figure 6A). The results suggested that AR increased the expression of the miR-7-5p through inhibiting circRNA7 expression in HCC cells. To further confirm that AR suppressed the formation of VM via circRNA7/miR-7-5p/VE-cadherin/Notch4 signals in HCC, we investigated the effect of shcircRNA7 on VM formation and expression of miR-7-5p, VE-cadherin, and Notch4 in HCC cell lines. The results revealed that shcircRNA7 increased the expression of miR-7-5p that had been decreased by shAR (Figure 6B) and decreased formation of VM that had been increased by shAR (Figure 6C,D). It also reversed the expression of VE-cadherin and Notch4 that had been up-regulated by shAR in SK and HA22T cells (Figure 6E). Thus, these results suggest that AR may regulate circRNA7 expression to impact miRNA-7-5p expression as well as its downstream effect on VE-cadherin and Notch4 expression and VM formation.

3.7 | AR regulates the expression of circRNA7 in HCC cells

To further dissect the mechanism by which AR decreases circRNA7 expression at a molecular level, we used the luciferase reporter assay to determine whether AR directly targeted the circRNA7 host gene promoter to suppress its expression. We first identified five potential AR targets in the promoter (Figure 7A) and found one potential AR responsive element that matched the seed sequence of AR in the promoter of the circRNA7 host gene by ChIP assay (Figure 7B). We then mutated this AR responsive element and cloned both the wild-type and mutated AR responsive element into a pGL3 reporter construct (Figure 7C). The results revealed that AR could decrease the expression of circRNA7 containing the wild-type host gene promoter, but not the mutated constructs (Figure 7D), suggesting that AR might directly target the circRNA7 host gene promoter to suppress its expression.

4 | DISCUSSION

HCC, a primary neoplasm of the liver, is a common primary cancer with multifaceted molecular pathogenesis pathways. HCC has
become the fourth most prevalent malignant tumour worldwide, and the fourth leading cause of cancer-related deaths. Most importantly, the incidence of HCC is increasing. Invasion and metastasis are two fundamental properties which determine the prognosis of HCC patients. Currently, there are no available effective treatments. Therefore, understanding the mechanisms that contribute to HCC progression is an urgent issue for enhancing patient survival.

**FIGURE 3** AR suppresses the formation of HCC VM by inhibiting expression of Notch4 and VE-cadherin. (A) shVE-cadherin inhibits the expression of VE-cadherin increased by shAR in SK and HA22T cells. (B) shNotch4 inhibits the expression of Notch4 increased by shAR in SK and HA22T cells. (C) shVE-cadherin and shNotch4 suppresses VM formation increased by shAR in SK and HA22T cells. (D) Quantification of VM formation in SK and HA22T cells in Figure 3C; *P < .05, **P < .01, compared with shAR + PLV + PLV group; ##P < .01, compared with PLKO + PLV + PLV group.
FIGURE 4  AR increases the expression of miR-7-5p in SK cells. (A) Relative normalized expression of miRNAs by qRT-PCR analysis in SK cells. oeAR significantly up-regulates expression of miR-7-5p and shAR down-regulates the expression of miR-7-5p in SK cells. (B) The effect of shmiR-7-5p on VM formation. The number of VM formations were counted and compared in SK and HA22T cells. (C) Quantification of VM formation in SK and HA22T cells. (D) The effect of shmiR-7-5p on expression of VE-cadherin and Notch4 in SK and HA22T. Western blotting was performed as described in Materials and Methods. **P < .01 compared with PWPI + PLV group. ***P < .01 compared with oeAR + PLV group.
Recent studies indicated that targeting AR could suppress HBV-, HCV- and carcinogen-induced HCC development at early stages. Several clinical trials based on these discoveries have been proposed to test their efficacy to suppress HCC progression. However, whether AR can influence VM formation in HCC was still unclear. Therefore, we investigated the effect of AR on the formation of VM in HCC. The results revealed that AR can significantly inhibit the formation of HCC VM in vitro, suggesting that AR may inhibit HCC metastasis progression by blocking VM formation. In addition, oeAR down-regulated gene expression of Notch4 and VE-cadherin in SK and HA22T cells, and shAR increased protein expression in both SK and HA22T cells. These results suggest that AR can suppress the formation of HCC VM by inhibiting the expression of Notch4 and VE-cadherin. To further confirm these results, we knocked down Notch4 and VE-cadherin and observed the effects of shAR on the formation of HCC VM. Results revealed shVE-cadherin or shNotch4 alone could partially reverse the formation of HCC VM that had been increased by shAR shVE-cadherin and shNotch4 together completely reversed the formation of HCC VM that had been increased by shAR. These results confirmed that AR suppressed the formation of HCC VM via inhibiting the expression of Notch4 and VE-cadherin.

miRNAs are a class of endogenous non-coding RNAs that are approximately 22 nucleotides long and can bind target specific bases of mRNA, leading to their degradation or inhibition of mRNA translation. Circular RNAs (circRNAs) are another class of non-coding RNAs that interact with miRNAs through miRNA response elements. Thus, they can act as competitive endogenous RNAs through binding and sponging of miRNAs, thereby blocking the inhibition of miRNAs to up-regulate the expression of target genes. Indeed, we identified that this relationship is likely the molecular mechanism mediating the effect of AR on HCC VM formation. We found AR down-regulated the expression of circRNA7, up-regulated the expression of miR-7-5p and suppressed VM formation in HCC. ShcircRNA7 blocked VM formation and the expression of VE-cadherin and Notch4 that had been increased by shAR in HCC, and shmiR-7-5p rescued VM formation and expression of VE-cadherin and Notch4 that had been decreased by oeAR in HCC. Altogether, these results indicate that AR suppresses the formation of VM via circRNA7/miRNA7-5p/VE-Cadherin/Notch4 signals in HCC.

To further dissect the mechanism by which miRNA-7-5p decreased VE-cadherin and Notch4 at a molecular level, we performed luciferase reporter assays to determine the binding of miRNA-7-5p to the 3′UTR of VE-cadherin and Notch4 mRNA. The results revealed that miRNA-7-5p directly targets the 3′UTR of VE-cadherin and Notch4 to suppress their expression, and that AR can directly target the promoter of circRNA7 to suppress its expression in HCC. In conclusion, AR can inhibit the formation of HCC VM via down-regulation of AR-circRNA7/miRNA7-5p/VE-Cadherin/Notch4 signals, and these mechanisms may contribute to develop new strategies towards defeating HCC.
FIGURE 6  AR inhibits the expression of circRNA7 in SK cells. (A) Relative normalized expression of circRNA7 by qRT-PCR analysis in SK cells. oeAR significantly suppressed expression of circRNA7 and shAR increased expression of circRNA7 in SK cells. (B) The effect of shcircRNA7 on gene expression of miR-7-5p in SK cells. (C) The effect of shcircRNA7 on VM formation in SK and HA22T cells. (D) Quantification of VM formation. (E) The effect of shcircRNA7 on expression of VE-cadherin and Notch4 in SK and HA22T cells. **P < .01 compared with PLKO + PLV group. ***P < .01 compared with shAR + PLV group.
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CONFLICT OF INTEREST
The authors confirm that there are no conflicts of interest.

AUTHOR CONTRIBUTION
Shixiang Bao: Conceptualization (equal); Methodology (lead); Project administration (equal); Writing-original draft (equal). Shuai Jin: Data curation (lead); Methodology (equal); Software (lead). Chunhua Wang: Methodology (equal); Writing-original draft (equal). Peipei Tu: Data curation (supporting); Methodology (equal). Kongwang Hu: Conceptualization (equal); Writing-review & editing (equal). Jingtao Lu: Conceptualization (equal); Project administration (lead); Resources (lead); Supervision (lead); Writing-review & editing (equal).

ETHICAL APPROVAL
This study does not contain any experiments with human participants or animals performed by any of the authors.

DATA AVAILABILITY STATEMENT
All data generated or analysed during this study are available from the corresponding author upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.