# miR-29a-5p regulates the malignant biological process of liver cancer cells through ARID2 regulation of EMT

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## Abstract

**Background.** Liver cancer, the vast majority of cases being hepatocellular carcinoma (HCC), is now the most malignant tumor in the world. Recurrence and metastasis remain the major obstacles on the way to the successful treatment of HCC. In recent years, the vital function of microRNAs (miRNAs) in human health and disease have been demonstrated. Large amounts of evidence demonstrate that miRNAs play an important role in the occurrence and progression of HCC.

**Objectives.** To find new targets for improving the early diagnosis, treatment and clinical prognosis of liver cancer.

**Materials and methods.** We used quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to analyze the expression of miR-29a-5p. A cell counting kit-8 (CCK-8) assay was used to measure the proliferation of liver cancer cells. Wound healing and transwell assays were used to detect migration and invasion in vitro. Western blot was used to detect the expression of the related protein.

**Results.** The miR-29a-5p was identified as a tumor-related miRNA. It is upregulated in HCC. The overexpression of miR-29a-5p contributes to the proliferation, invasion and metastasis of HCC cells. Furthermore, the downregulation of miR-29a-5p inhibited the growth, migration and invasion of HCC cells in vitro. Subsequently, we used bioinformatics methods to predict that AT-rich interaction domain 2 (*ARID2*) is the downstream target gene of miR-29a-5p. The downregulation of ARID2 could reverse the tumor suppressive effect caused by the knockdown of miR-29a-5p. Similarly, the epithelial–mesenchymal transition (EMT)-related protein epithelial marker E-cadherin expression increased and the mesenchymal marker Vimentin decreased when we downregulated the expression of miR-29a-5p. Interestingly, the knockdown of ARID2 could reverse this phenomenon.

**Conclusions.** Our study demonstrated that miRNA-29a-5p was overexpressed in HCC cells. It promotes the progression of HCC by targeting ARID2 in an EMT manner.

**Key words:** liver cancer, metastasis, proliferation, invasion, miR-29a-5p

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# Background

Hepatocellular carcinoma (HCC) is the 2nd leading cause of cancer-related deaths globally.[1,2] The 5-year overall survival rate of HCC patients was less than 10%[3] Despite the extensive application of early diagnosis techniques and continuous improvement in treatment strategies such as surgical intervention and targeted chemoradiotherapy,[4,5] HCC recurrence and metastasis remain the main challenges leading to poor prognosis.[6] Therefore, more precise targets for diagnosing and treating liver cancer are urgently required to improve the early detection rate and clinical treatment effect of liver cancer.

MicroRNAs (miRNAs) are small noncoding RNAs containing approx. 22 nucleotides. In recent decades, many researchers have focused on the critical role of miRNAs in human health and disease, as miRNA regulation is a common pathway for epigenetic regulation. The miRNAs exert post-transcriptional or translational control on the expression of various genes involved in cancer progression.[7,8] They can regulate the migration, proliferation, apoptosis, and other malignant biological behaviors of tumor cells in various ways. Several studies have demonstrated that miRNAs are overexpressed in many malignant tumors, including liver cancer. Tumor occurrence and progression often coincide with abnormal miRNA expression. They function as both tumor suppressors and oncogenes, and are critical in tumor development and progression.[9–13] The miRNAs have also been crucial in cancer development and epithelial–mesenchymal transition (EMT).[14]

Epithelial–mesenchymal transition is a unique morphological transformation process; its main mechanism is to transform inactive epithelial cells into active mesenchymal cells, enhancing cancer cell metastasis and invasion.[15] It can also affect the invasive ability, metastasis and chemoresistance of malignant tumors.[16–18] According to mounting data, miRNAs play an important role in the aggressiveness of cancer cells, including EMT-related cancer metastasis.[19]

Previous studies have found that miRNA-29a-5p is closely related to the postoperative recurrence of liver cancer,[20] but the precise mechanism remains unknown. In this study, we attempted to delve deeper into this specific mechanism.

# Objectives

Due to the significant threat liver cancer poses to human health and the important role of miRNAs in tumors, we conducted this study to find a new mechanism by which miRNAs regulate the progression and development of liver cancer. We explored the miRNA-29a-5p role in liver cancer and its specific mechanism of regulating the biological behavior of liver cancer. It is anticipated that our research will yield new ideas for liver cancer prevention and treatment, thereby improving the treatment and prognosis of liver cancer patients.

# Materials and methods

## Cell lines and culture

The human normal liver cell line (LO2) and the liver cancer cell lines (Huh-7, LM-3, MHCC97-H, and HepG2) were purchased from the Institute of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in high-glucose complete Dulbecco's modified Eagle's medium (DMEM) (containing 10% fetal bovine serum (FBS)) at 37°C, 5% $CO_2$.

## RNA extraction and polymerase chain reaction

The TRIzol reagent was utilized to separate total RNA (Invitrogen; Thermo Fisher Scientific, Waltham, USA), and miRNA was collected using Biospin miRNA Extraction Kit (GeneCopoeia, Carlsbad, USA). Reverse transcription of the miRNA into cDNA was performed, and quantitative real-time polymerase chain reaction (qRT-PCR) was carried out utilizing All-in-One™ miRNA qRT-PCR Detection System (GeneCopoeia, Carlsbad, USA). Quantitative reverse transcription-polymerase chain reaction was performed using SYBR® Premix Ex Taq™ (Takara, Dalian, China) and PrimeScript™ RT reagent kit (Takara). The miR-29a-5p was normalized to U6, and AT-rich interaction domain 2 (ARID2) was normalized to GAPDH. The quantitative expression levels were measured using the $2^{-\Delta\Delta Ct}$ method.

## Cell transfection

Oligonucleotides for hsa-miR-29a-5p mimics and inhibitors were obtained from Genebiogist (Shanghai, China). Small interfering RNA for ARID2 knockdown and blank control siRNA were obtained from GenePharma (Shanghai, China). A day before the transfection, cells were seeded into 6-well plates. When the cell density reached 70–80%, using Lipofectamine 2000 (Invitrogen) at a final dose of 100 nM, cells underwent transfection with oligonucleotides according to the manufacturer's specifications. After 48 h, quantitative reverse transcription-polymerase chain reaction was used to determine the miR-29a-5p transfection effectiveness, and the harvested cells were utilized for subsequent functional and mechanism validation experiments.

## Cell proliferation assay

Cell proliferation capacity was assessed using the Cell Counting Kit-8 (CCK-8; Dojindo (FBS), Kumamoto, Japan).

The cells in each treatment group were seeded into 96-well plates, with $4\times10^3$ cells in each well and 3 replicates in each group. Then, they were cultured at 37°C in 5% $CO_2$, CCK-8 reagent was added at 0 h, 24 h, 48 h, and 72 h, and the absorbance value was measured at a wavelength of 450 nm.

## Wound healing assay

Each treatment group cell was cultured at 37°C in 6-well plates until the density exceeded 95%. Then, we used a 10-microliter pipette tip for cell scratching in the 6-well plate to create an artificial wound. After gently washing each well 3 times with phosphate-buffered saline (PBS), the cells were cultured in serum-free high-glucose DMEM for 24 h. The wound area was visualized and images were captured at 0 h and 24 h.

## Cell migration and invasion assays

We tested the ability of cells to invade and migrate with transwell chambers (Corning, Corning, USA). Briefly, cells in each treatment group were trypsin-digested and suspended in serum-free high-glucose DMEM. Before seeding cells from each treatment group into an upper chamber, we added diluted basement Matrigel (BD Biosciences, Franklin Lakes, USA) to each chamber and allowed it to polymerize for 3 h at 37°C. A total of 200 µL of cell suspension (total number of $4\times10^4$ cells), resuspended in a serum-free medium, was placed in the top section of the chamber, and 600 µL of high-glucose DMEM supplemented with 20% FBS was placed in the lower section of the chamber. Later, cells were cultivated for 24 h and cells were fixed and stained with 4% paraformaldehyde for 15 min at room temperature and 0.1% crystal violet for 20 min at room temperature, respectively. Finally, cells were counted with a microscope in 5 random fields, and images were captured.

## Western blotting

Total protein was extracted from each group of cells using phenylmethanesulfonyl fluoride and RIPA lysis buffer (Beyotime, Shanghai, China). Briefly, cells were gently washed twice with cold PBS, scraped and lysed for 30 min on ice with lysis buffer. After lysis completion, centrifugation was performed at 4°C at 12,000 rpm for 15 min, and the supernatant was collected for later use. To determine protein concentration, we employed a BCA protein detection kit (Beyotime). The proteins were separated on a 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, St. Louis, USA), and blocked with QuickBlock™ Blocking Buffer (Beyotime) for 30 min at room temperature. Subsequently, the membranes were treated overnight at 4°C with primary antibodies. Then, the membrane was washed 3 times with Tris-buffered saline with Tween (TBST) before being treated with a secondary antibody for 1 h at room temperature. Next, the membrane was washed 3 times with TBST. Protein bands were visible using enhanced chemiluminescence (ECL; Cell Signaling Technology, Danvers, USA).

## Statistical analyses

All observations in this study were made in triplicate. The results were scrutinized using GraphPad Prism v. 8 (GraphPad Software, San Diego, USA) software. For repeated measurements, the general linear model was used to compare the statistical difference among groups. Before conducting one-way analysis of variance (ANOVA) and t-test, the normality and homogeneity of variance of data or variables were tested. If the data conformed to a normal distribution and had homogeneity of variance, the one-way ANOVA and t-test were used to compare the differences between different treatment groups. The value of $p < 0.05$ was considered significantly different.

# Results

## miR-29a-5p is highly expressed in HCC cells and was successfully knocked down and upregulated

To detect the differential expression of miR-29a-5p in HCC, we examined 4 HCC cell lines, including Huh-7, LM-3, MHCC97-H, HepG2, and the human normal liver cell line (LO2). Figure 1A illustrates that miR-29a-5p was present at lower levels in LO2 cells than in all HCC cell lines. The Huh-7 was selected for the subsequent experiments; hsa-miR-29a-5p mimics and inhibitors were used to upregulate and knock down miR-29a-5p, respectively (Fig. 1B).

## miR-29a-5p enhances the proliferation, migration and invasion of HCC cells

The CCK-8, wound healing and transwell assay data confirmed that miR-29a-5p knockdown significantly inhibited cell proliferation (Fig. 2A). Additionally, we discovered that miR-29a-5p knockdown HCC cells had less ability to migrate and invade than control cells (Fig. 2B,C). These results were reversed when miR-29a-5p was upregulated.

## *ARID2* is a target gene of miR-29a-5p

Subsequently, we utilized an online prediction database (TargetScan, https://www.targetscan.org/vert_80/; starBase, http://starbase.sysu.edu.cn/) to predict candidate targets for miR-29a-5p (Fig. 3). As expected, miR-29a-5p silencing significantly upregulated ARID2 expression; similarly, ARID2 was downregulated when miR-29a-5p was overexpressed (Fig. 4).

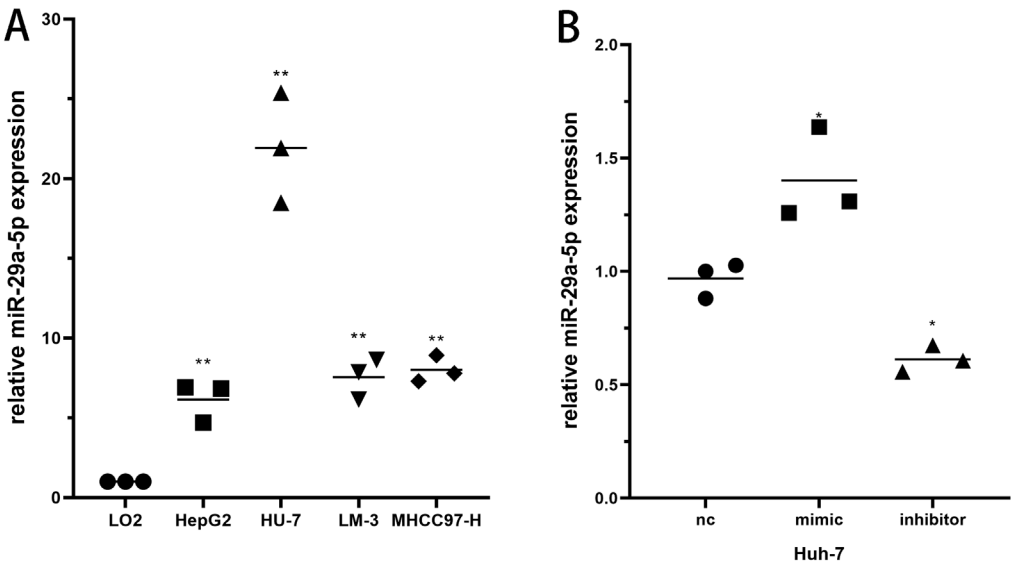


Fig. 1. A. miR-29a-5p expression levels in 4 hepatocellular carcinoma (HCC) cell lines were higher than in the human normal liver cell line (LO2) (3 distinct repetitions, one-way analysis of variance (ANOVA), HepG2: $p = 0.0207$; HU-7: $p < 0.0001$; LM-3: $p = 0.0044$; MHCC97-H: $p = 0.0028$; ** denotes $p < 0.01$); B. miR-29a-5p was knocked down and upregulated in Huh-7 cells (3 distinct repetitions, one-way ANOVA, mimic: $p = 0.0138$; inhibitor: $p = 0.0314$; * denotes $p < 0.05$)

### miR-29a-5p regulates EMT through ARID2 and affects HCC cell proliferation, metastasis and invasion

The miR-29a-5p inhibitor and ARID2 siRNA were co-transfected into Huh-7 cells to examine whether ARID2 participated in the changes in the proliferation and migration ability of HCC cells induced by miR-29a-5p (Fig. 5A). As expected, the effects of miR-29a-5p downregulation on cell proliferation and migration were reversed by the treatment with ARID2 siRNA co-transfection (Fig. 5B,C). Simultaneously, after co-transfection with ARID2 siRNA, western blot analysis revealed that E-cadherin expression was lowered, whereas Vimentin expression increased, which suggested that the EMT ability of Huh-7 cell was enhanced (Fig. 5D,E).

## Discussion

Primary liver cancer is the 4$^{th}$ most common carcinoma in China, posing a serious threat to the Chinese people's health and life.[21] Liver cancer metastasis includes 5 typical steps: local invasion of adjacent tissues, intravascular invasion, circulatory system survival, extravasation, and abnormal colonization of the liver or distant organs (extrahepatic colonization). With the increased research into liver cancer and the advancement of medical technology, liver cancer now has a precise multidisciplinary treatment model. Currently, regorafenib, sorafenib and immune checkpoint inhibitors are all molecularly targeted medications that have improved the prognosis of most patients.[22–25] Despite this, most liver cancer patients have extremely poor prognoses due to the interaction of multiple factors.[26] Most patients lose the opportunity for surgery due to the extremely high recurrence rate of liver cancer and the discovery of distant metastases at the initial diagnosis.[27] Therefore, finding a specific molecular marker for early disease prediction and treatment is particularly important.

The abnormal expression of oncogenes often regulates tumorigenesis and cancer progression. Understanding the oncogene function is crucial for preventing and treating carcinoma. Many malignant biological processes, such as cell proliferation and apoptosis, depend on miRNA regulation and autophagy. Mounting evidence suggests that under certain conditions miRNAs act as oncogenes and antioncogenes in HCC cells and regulate malignant biological behaviors such as apoptosis, invasiveness, proliferation, and metastatic abilities.[28] Many miRNAs, such as miR-519a and miR-1468 inhibit apoptosis and improve cell proliferation in liver cancer cells.

Interestingly, other miRNAs, including miR-1296 and miR-542-3p, inhibited the EMT, decreasing the HCC cell metastatic capacity. The miR-29a is a conserved miRNA regulating multiple coordinated post-transcriptional programs, thereby participating in diverse biological processes.[29] Previous studies have indicated that the overexpression of miR-29a inhibits leukemia cell line growth. However, several other studies have discovered that the increased miR-29a levels in mice promote leukemia progression, suggesting that miRNAs may be a double-edged sword in cancer.[30] Similarly, miR-29a regulates glioblastoma disease progression; in glioma patients, low miR-29a expression predicts higher tumor aggressiveness and a worse prognosis.[31] Numerous studies have examined the link between miRNA-29a-5p and early recurrence of HCC after surgical resection.[20] However, the biological function of miRNA-29a-5p in HCC has not been thoroughly described. In this study, miRNA-29a-5p was overexpressed in HCC cells. In vitro functional experiments further indicated that the downregulation of miRNA-29a-5p inhibited HCC cell invasion, migration and proliferation.

The ARID2 is a chromatin-remodeling complex subunit important in biological processes occurring in various

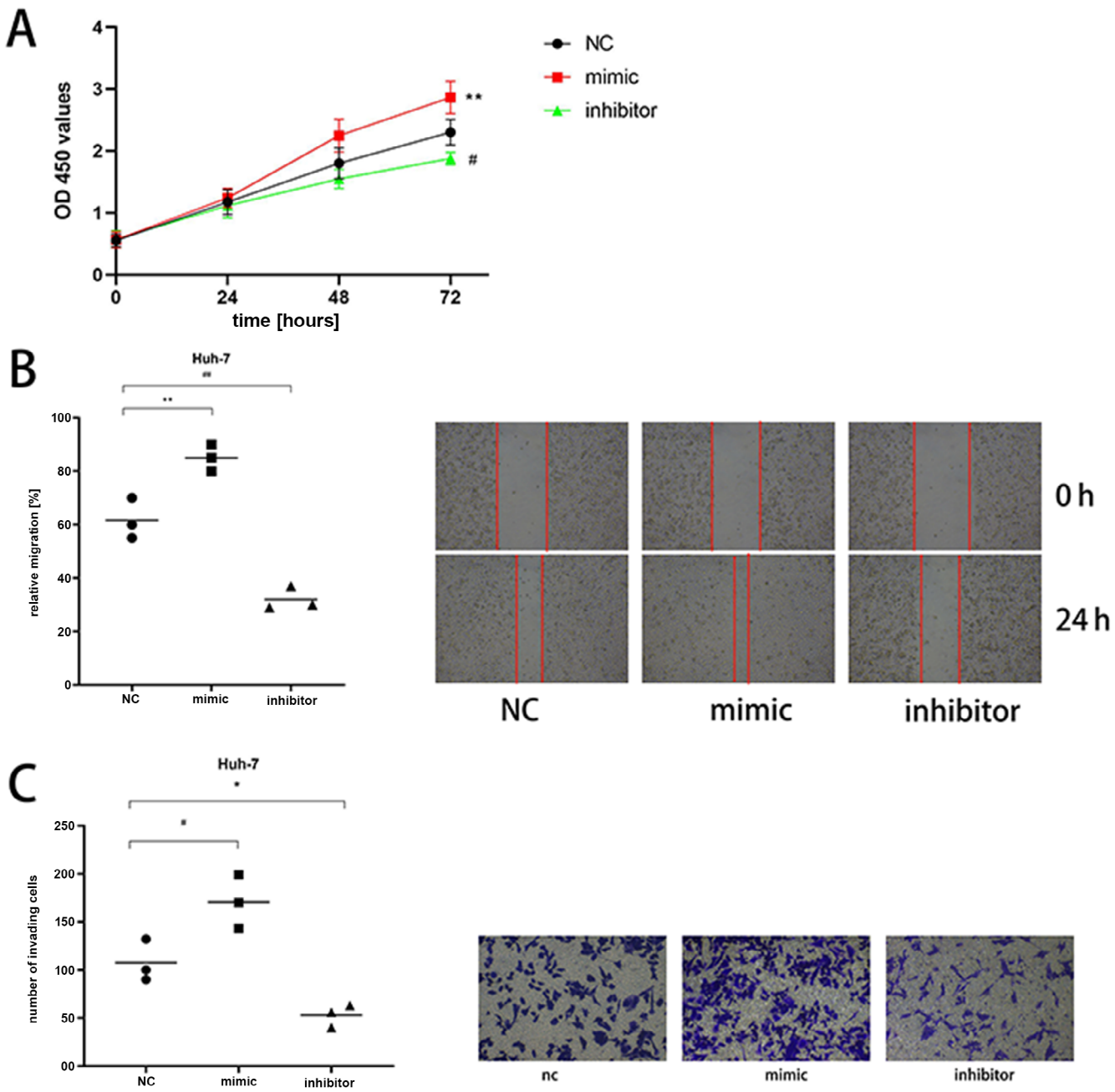


Fig. 2. miR-29a-5p controls the malignant biological behavior of hepatocellular carcinoma (HCC) cells. A. The cell counting kit-8 (CCK-8) detection revealed that HCC cell proliferation could be regulated via miR-29a-5p (Greenhouse–Geisser test, degrees of freedom (df) = 1.608, F = 1115.927, $p < 0.0001$, $\eta^2 = 0.995$, ** denotes $p < 0.01$; B,C. miR-29a-5p was shown to influence the ability of HCC cells to migrate and invade using transwell detection and wound healing assays (3 distinct repetitions, one-way analysis of variance (ANOVA); B. mimic: $p = 0.0055$; inhibitor: $p = 0.0016$; C. mimic: $p = 0.0205$; inhibitor: $p = 0.0460$; * and # denote $p < 0.05$; ** and ## denote $p < 0.01$)

OD – optical density; NC – negative control.

| | Predicted consequential pairing of target region (top) and miRNA (bottom) | Site type | Context++ score | Context++ score percentile | Weighted context++ score | Conserved branch length | $P_{CT}$ |
|---|---|---|---|---|---|---|---|
| Position 2430-2437 of ARID2 3' UTR | 5' ...GAUAAUGUCUUCUCAAAAUCAGA... | 8mer | -0.12 | 86 | 0.00 | 0 | N/A |
| hsa-miR-29a-5p | 3' GACUUGUGGUUUUUCUUUAGUCA | | | | | | |

Fig. 3. The complementary pairing sequences between miR-29a-5p and 3'-UTR of AT-rich interaction domain 2 (ARID2)

types of cells, including transcriptional regulation,[32] cell cycle modulation,[33,34] embryonic development,[35] and DNA damage repair.[36] The ARID2 mutations occur in most cancers, and this mutation usually results in partial or complete inactivation of the ARID2 protein.[37–39] The chromatin-remodeling complex can perform epigenetic regulation.[40] Similarly, ARID2 sometimes participates in DNA and histone regulation in epigenetic

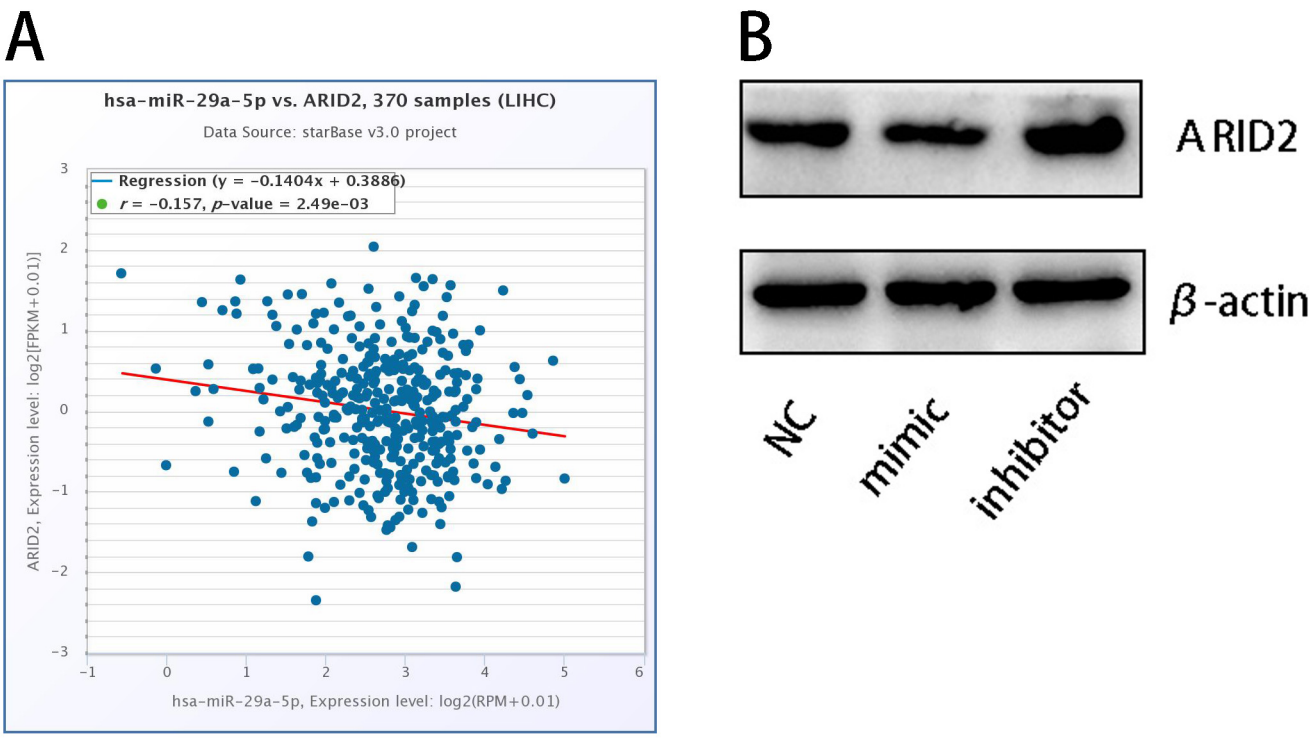


Fig. 4. A. Online prediction of the interaction between miR-29a-5p and AT-rich interaction domain 2 (ARID2); B. miR-29a-5p inhibitors or analogs were transfected into Huh-7 cells; mir-29a-5p negatively regulates ARID2 expression

NC – negative control.

regulation. Numerous studies have reported that ARID2 may become an antioncogene by regulating epigenetics, but its specific regulatory mechanism in tumors remains unknown.

The EMT regulates several malignant tumor behaviors, including proliferation, invasion and metastasis. In many cases, tumor migration and invasion are closely related to EMT, which refers to a specific morphologic transformation process in which an epithelial phenotype transforms into a mesenchymal phenotype, improving the ability of the cells to metastasize and invade. The key features of EMT are the reduced expression of cell adhesion molecules (such as E-cadherin), the transition from a cytokeratin to a Vimentin cytoskeleton, and the morphological properties of mesenchymal cells. Since EMT is a critical biological mechanism that enhances invasion and metastasis, identifying key molecules involved in this phenomenon is critical for studying the mechanism of tumor cell metastasis. Most notably, previous research has established that miRNAs play a critical role in EMT remodeling. Understanding the relationship between EMT-related miRNAs and cancer progression will benefit our clinical and basic research.

## Limitations

We investigated the molecular mechanism of miRNA-29a-5p regulating the invasion and metastasis of liver cancer cells in vitro. However, in vivo functional experiments are required in the future to more comprehensively demonstrate the regulatory role of miRNA-29a-5p in the invasion and metastasis of liver cancer. We should also collect clinical specimens for validation to combine our research with clinical trials. Another point is that the sample size used in our previous work is modest, and we may encounter difficulties in choosing the statistical analysis method. In the future experimental design, we may need to increase the sample size.

## Conclusions

Our study demonstrated that miRNA-29a-5p was overexpressed in HCC cells. In vitro experiments further indicated that the downregulation of miRNA-29a-5p attenuated the ability of HCC cells to invade, migrate and proliferate. The miRNA-29a-5p promotes HCC progression by targeting ARID2 through EMT. Our study proved that miRNA-29a-5p could potentially be used as a biomarker and therapeutic target for HCC. Due to the regulatory role of miRNA-29a-5p in HCC, it is expected that miRNA can be used as an early diagnosis and prognostic indicator of HCC. The miRNA-29a-5p–ARID–EMT axis can regulate the metastasis and proliferation of HCC, and it is hoped that a new therapeutic strategy for HCC can be found through this pathway.

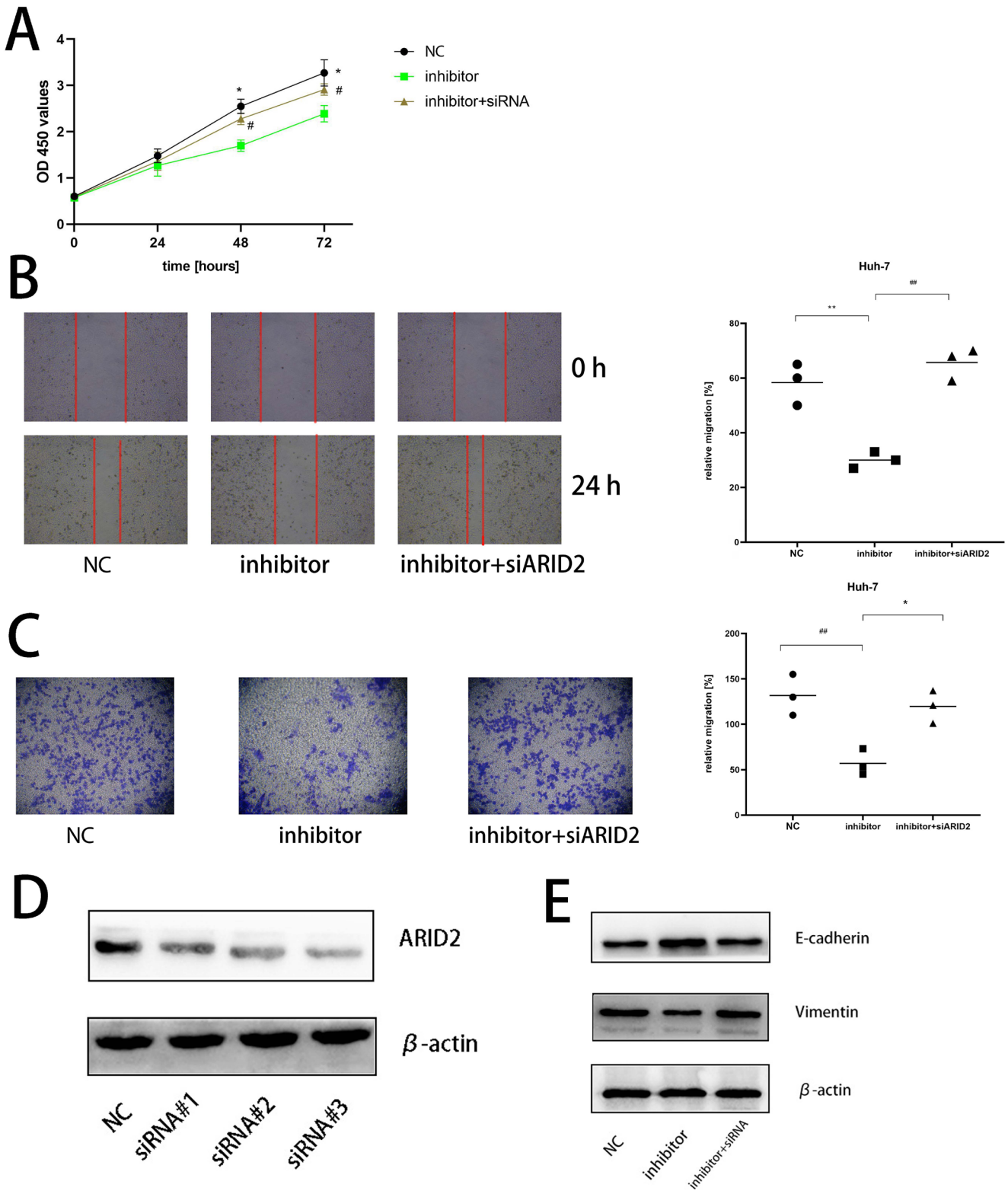


**Fig. 5.** A. miR-29a-5p knockdown inhibited the Huh-7 cells proliferation, while AT-rich interaction domain 2 (ARID2) silencing reversed its inhibition (Greenhouse–Geisser test, degrees of freedom (df) = 2.157, F = 555.135, $p < 0.0001$, $\eta^2 = 0.989$; * and # denote $p < 0.05$); B,C. miR-29a-5p knockdown inhibits the ability to invade and migrate, while *ARID2* gene silencing reverses them (3 distinct repetitions, one-way analysis of variance (ANOVA), B. negative control (NC): $p = 0.0020$; inhibitor+siARID2: $p = 0.0006$; C. NC: $p = 0.0054$; (inhibitor+siARID2) $p = 0.0124$; * denotes $p < 0.05$; ** and ## denote $p < 0.01$. D. Western blot analysis revealed that ARID2 was successfully silenced; E. After co-transfection with ARID2 siRNA, the epithelial–mesenchymal transition (EMT) ability of Huh-7 cells was enhanced

OD – optical density.

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