Abstract. There is little information on the role of microRNA (miR)-922 in the malignant behavior of liver cancer. The present study investigated the regulation of miR-922 expression levels by cAMP response element binding protein 1 (CREB1) in liver cancer tissue, its role in regulating malignant behavior and its potential targets in liver cancer. miR-922 expression in liver cancer cells and tissue was determined by reverse transcription-quantitative PCR. The binding of CREB1 to the promoter region of mir-922 was tested by chromatin immunoprecipitation-PCR. The predicted AT-rich interactive domain 2 (ARID2) and fidgetin, microtubule severing factor targets of miR-922 were characterized by dual luciferase reporter assay. The effects of altered ARID2 expression levels on miR-922-enhanced malignant behavior of liver cancer cells were tested. CREB1 bound to the promoter region of miR-922. Elevated miR-922 transcripts were inversely associated with ARID2 expression in liver cancer tissue and cells. miR-922 inhibited ARID2-regulated luciferase expression and was present in the miR/argonaute RISC catalytic component 2 complex. ARID2 significantly decreased malignant behavior of liver cancer MHCC97L cells. Similarly, ARID2 over-expression inhibited growth of xenograft liver cancer tumors and decreased miR-922, Bcl-2, proliferating cell nuclear antigen, cyclin D1, MMP3 and MMP9 expression and serum VEGF and TNF-α levels, but enhanced Bax expression levels in tumors. ARID2 over-expression abrogated malignant behavior promoted by miR-922 over-expression and enhanced miR-922-decreased malignant behavior of liver cancer cells. CREB induced miR-922 transcription, which targeted ARID2 to enhance malignant behavior of liver cancer cells, indicating that the CREB1/miR-922/ARID2 axis may be a potential target for liver cancer treatment.

Introduction

Liver cancer is a common type of neoplasia and has the second highest rate of cancer-associated mortality in the world (1). More than 90% of primary liver cancer cases are hepatocellular carcinoma (HCC). In China, the 5-year survival of HCC is only 12.1% (2). Etiologically, hepatitis B and C virus infection-associated fibrosis and cirrhosis are the most common risk factors for the development of liver cancer (3). Although therapeutic approaches, such as surgical resection, chemotherapy and immunotherapy, have advanced, liver cancer remains a major threat (4) because many patients with liver cancer are diagnosed at advanced stage and prone to development of multi-drug resistance. Hence, novel therapeutic targets for intervention and more reliable biomarkers for early diagnosis of liver cancer are urgently needed (4).

MicroRNAs (miRNAs or miRs), ~23 nucleotides in length, are key members of the non-coding RNA family (5). miRNAs control expression of their target mRNAs principally by binding to the 3'-untranslated region (3'-UTR) to inhibit the translation of mRNA and decrease its half-life. miR-922 is a tumor-promoting gene that promotes the development and progression of tumors (6,7). miR-922 expression levels are decreased in breast cancer (8). By contrast, miR-922 expression is significantly upregulated in hepatocellular carcinoma (HCC) tissue and promotes proliferation of HCC cells by targeting cylindromatosis (CYLD) to enhance c-Myc and cyclin D1 expression levels and inhibit retinoblastoma (Rb) phosphorylation (8). However, the role of miR-922 in regulating the malignant behavior of liver cancer cells is still unclear.

cAMP response element binding protein 1 (CREBI) is a member of the leucine zipper transcription factor family and enhances HCC progression by promoting angiogenesis.
and resistance to apoptosis (9). Upregulated CREB1 expression levels and phosphorylation have been detected in HCC tissue (10). Furthermore, CREB1 also mediates hepatitis B virus X protein-cortactin interactions to promote malignant behavior of HCC cells (11). AT-rich interactive domain 2 (ARID2) is a tumor suppressor and member of the switch/sucrose non-fermentable chromatin remodeling complex (12). ARID2 knockout disrupts DNA damage responses by inhibiting recruitment of xeroderma pigmentosum complementation group G (12). To the best of our knowledge, however, there is no information on whether miR-922 targets and regulates ARID2 expression and its effect on malignant behavior of liver cancer cells.

The present study validated miR-922 expression levels in 10 pairs of liver cancer and adjacent tissue, liver cancer cells and non-tumor hepatocytes. The aim was to determine how CREB1 regulates miR-922 expression levels in liver cancer cells. The impact of ARID2, a potential target of miR-922, on the malignant behaviors of liver cancer cells was also investigated both in vitro and in vivo.

Materials and methods

Clinical samples. The present study was approved by the Ethics Committee of the Third Xiangya Hospital of Central South University and all patients provided written informed consent. Liver cancer and matched adjacent (distance, >3 cm) non-tumor hepatic tissue (10 pairs) were collected from patients with liver cancer during surgical treatment between September 2018 and September 2019 in The Third Xiangya Hospital of Central South University, China (Table I). All samples were collected with signed informed consent. Patients were diagnosed based on the practice guidelines of the American Association for the Study of Liver Diseases (13). Liver specimens were evaluated by pathologists and clinical stage was determined according to the Barcelona Clinic Liver Cancer classification (14). Exclusion criteria were as follows: i) patients ≤18 or ≥70 years of age or without full civil capacity; ii) history of another organ malignancy or systemic immune disease; iii) history of tumor hepatic radiotherapy or chemotherapy, biological, immune or traditional Chinese medicine therapy; iv) incomplete postoperative follow-up data and iv) history of another organ malignancy or systemic immune disease.

Cell culture and transfection. Human liver cancer HepG2, MHCC97H, MHCC97L and non-tumor-hepatic THLE-2 cells were purchased from the Cell Bank of China (Shanghai, China). THLE-2 cells were cultured in RPMI-1640; other cells were cultured in DMEM supplemented with 10% fetal bovine serum (all Thermo Fisher Scientific, Inc.) at 37°C in 5% CO2. The authenticity of all cell lines was tested by STR (Shanghai Yibe Applied Biotechnology Co., Ltd. and Guangzhou Cellcook Biotech Co., Ltd.).

HepG2 and MHCC97L cells were transfected with control plasmid (pcDNA3.1-NC) or plasmid for the expression of ARID2 [pcDNA3.1-ARID2 (OE), GeneCopoeia, Inc.] using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.) and treated with 1 mg/ml G418 for 3 weeks to generate control HepG2/MHCC97L-overexpression (OE)-negative-control (NC) or ARID stable expressing HepG2/MHCC97L-ARID2-OE cells. HepG2/MHCC97L-, HepG2/MHCC97L-NC and HepG2/MHCC97L-ARID2-OE cells were transfected with control scramble miRNA, miR-922 mimics or miR-922 inhibitor (Guangzhou RiboBio Co., Ltd.) for 48 h at 37°C. In addition, HepG2/MHCC97L cells were transduced with control lentivirus or lentivirus for expression of ARID-specific short hairpin (sh)RNA [pGPU6/GFP-ARID2 (sh)] in the presence of 4 µg/ml puromycin for 4 days to generate control HepG2/MHCC97LshRNA-NC and ARID stably silencing HepG2/MHCC97L-ARID2sh cells. Subsequent experiments were performed 48 h after transfection.

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from individual groups of cells (THLE-2, HepG2, MHCC97H and MHCC97L) using TRIzol® reagent and reverse transcribed into cDNA using a PrimeScript RT reagent kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer’s protocol. The relative levels of miRNA and mRNA transcripts to control U6 and GAPDH were quantified by RT-qPCR in duplicate using SYBR Premix Ex Taq (TaKaRa Biotechnology Co., Ltd.) and specific primers (Table II). Thermocycling conditions were as follows: Initial denaturation at 94°C for 10 min, followed by 40 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 10 sec and final extension at 72°C for 8 min. The data were analyzed by 2-ΔΔCq method (15).

Cell proliferation assay. The HepG2 and MHCC97L cell proliferation was determined by Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc.) according to the manufacturer’s protocol. The absorbance at 450 nm was measured using a microplate reader following incubation for 1-2 h.

Wound healing assay. After serum-starved HepG2 and MHCC97L cells in each group reached 100% confluence, they were wounded with a plastic tip. The cells were cultured for 24 h at 37°C. Wound width was measured at 0 and 24 h under a light microscope (magnification, x100).

Transwell invasion assay. The HepG2 and MHCC97L cells (1x105 cells/well) were starved overnight, then cultured in the upper chamber pre-coated (37°C for 30 min) with Matrigel (BD Biosciences) of 24-well Transwell plates (pore size, 8 µm; Corning, Inc.). The bottom chamber was filled with complete medium supplemented with 10% FBS (Thermo Fisher Scientific, Inc.). The cells were cultured for 48 h at 37°C, fixed with 10% glutaraldehyde for 4°C for 30 min and stained with 1% crystal violet for 20 min at room temperature, followed by photoimaging under a light microscope (Olympus Corporation) at 100x magnification.

Colony formation assay. Each group of cells (300 cells/well) was cultured in 6-well plates for 2 weeks at 37°C. The formed cell colonies were fixed with 4% formaldehyde for 15 min at room temperature and stained with 1% crystal violet for 20 min at room temperature, followed by counting in a blinded manner.

Chromatin immunoprecipitation (ChIP). Potential binding of CREB1 to the promoter region of miR-299 was determined by ChIP assay as previously described (16). Briefly,
MHCC97L and HepG2 cells were transfected with scramble control or CREB1-specific shRNA (Table II) for 48 h and the efficacy of CREB1 silencing was verified by western blotting as aforementioned. The control and CREB1-silenced cells were cross-linked with 1% formaldehyde for 10 min at room temperature and sonicated on ice to generate ~500-bp DNA fragments. Following centrifugation (14,000 x g; 10 min; 4˚C), the obtained soluble chromatin samples were reacted with anti-CREB 1 (1:10; cat. no. ab31387; Abcam), anti-H3K27me3 (1:10; cat. no. ab6002; Abcam), anti-H3K27AC (1:10; cat. no. ab4729; Abcam) or control IgG (1:10; cat. no. ab171870; Abcam) overnight at 4˚C. The immunocomplex was precipitated by Protein A Agarose/Salmon Sperm DNA (50% Slurry) beads and eluted. DNA fragments were analyzed by qPCR, as aforementioned, using specific primers (Table II).

Luciferase reporter gene assay. Hsa-miR-922 mimics and hsa-miR-922 inhibitor were designed and synthesized by Shanghai GenePharma Co., Ltd. as follows: Mimics forward, 5’-GCA GCA GAG AAU AGG ACU ACG UC-3’ and reverse, 5’-CGU AGU CCU AUU CUC UGC UGC UU-3’; inhibitor forward, 5’-GAC GUA GUC CUA UUC UCU GCU GC-3’ and reverse, 5’-UUC UCC GAA CGU GUC ACG UTT-3’. Reporter plasmid pmirGLO Dual-Luciferase miRNA Target Expression Vector was obtained from Promega Corporation. Transfection was performed using Lipofectamine® 2000 (cat. no. 11668030; Thermo Fisher Scientific, Inc.) and Dual-Glo® Luciferase Assay system (cat. no. E2920; Promega Corporation). Luciferase activity was normalized to that of Renilla luciferase.

In situ hybridization. Digoxigenin (Dig)-labelled probe (5’-GCAGCAGGAUAGGCCUAGGC-3’) for miR-922 was designed and synthesized by BersinBi. The distribution of

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<th>P-value</th>
<th>ARID2 Mean ± SD</th>
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miR, microRNA; ARID2, AT-rich interactive domain 2; AFP, α fetoprotein; HBsAg, hepatitis B surface antigen.
miR-922 transcripts in liver cancer tissue was determined by in situ hybridization using an In Situ Hybridization kit (Wuhan Boster Biological Technology Ltd.), according to the manufacturer's protocol. Briefly, liver cancer and adjacent non-cancer tissue sections (thickness, 4 µm) were dewaxed, rehydrated and treated with 3% H2O2 for 10 min at room temperature to inactivate endogenous enzymes. The sections were digested with pepsin in 3% citric acid at 37˚C for 30 min and fixed with 4% paraformaldehyde for 5 min at room temperature. After being washed, sections were treated with pre-hybridization solution at 38‑42˚C for 2‑4 h and hybridized in the presence or absence (negative control) of the probe at 38‑42˚C overnight. The sections were washed with 2X saline‑sodium citrate and reacted with biotinylated mouse anti‑Dig (1:2,000; cat. no. D15041; Bellancom) at 37˚C for 60 min. After being washed, the sections were incubated with streptavidin biotin‑peroxidase complex for 60 min at room temperature and reacted with biotinylated peroxidase for 20 min at 37˚C, then visualized with 3,3’‑diaminobenzidine (DAB) at room temperature for 10 min and counterstained with hematoxylin. The sections were examined under a light microscope (magnification, x400).

Western blotting. The HepG2 and MHCC97L cells were harvested and lysed in RIPA lysis buffer (cat. no. P0013B; Beyotime Institute of Biotechnology), followed by centrifugation (9,000 x g; 4˚C; 10 min). After determining the protein concentrations using a BCA kit (Abcam), cell lysates (50 µg/lane) were separated by SDS‑PAGE on 12% gels and transferred onto polyvinylidene fluoride membranes (Amersham Pharmacia Biotech; Cytiva). The immunoblot membranes were blocked with 5% fat‑free dry milk in TBST (0.1% Tween‑20) buffer at 37˚C for 2 h and incubated with primary antibodies overnight at 4˚C (CREB2, 1:1,000, cat. no. #82342, Cell Signaling Technology, Inc.; FIGN, 1:1,000, cat. no. ab122238, Abcam). After being washed, the membranes were reacted at room temperature for 1 h with peroxidase‑conjugated secondary antibodies (1:10,000; cat. no. ab6721; Abcam). The signals were visualized using enhanced chemiluminescence reagents (GE Healthcare) and quantified by densitometric analysis using ImageJ software (version 1.48; National Institutes of Health).

RNA immunoprecipitation (RIP). A Magna RIP RNA‑Binding Protein Immunoprecipitation kit (cat. no. 17‑701; Merck Sharp & Dohme) was used for RIP assay according to the manufacturer’s instructions. Briefly, miR‑922‑expressing cells were fixed with 2% formaldehyde for 5 min at room temperature, lysed and sonicated, followed by centrifugation at 12,000 x g
and 4˚C for 1 min. The supernatants were collected and incubated with ARID2 antibody (1:1,000, cat. no. #82342; Cell Signaling Technology, Inc.) and FIGN antibody (1:1,000, cat. no. ab122238, Abcam); overnight at 4˚C. The immunocomplex was precipitated using protein A/G Dynabeads; after being washed, the immunocomplex was digested with proteinase K. Finally, RNA was extracted with TRIzol and the relative levels of miR-922 in the immunocomplex were determined by RT-qPCR, as aforementioned, using specific primers (forward, 5'-ATGCGGTTTTTCCTCTCC-3' and reverse, 5'-TGACGTAGTCTATTTCTGCG-3').

Cell apoptosis analysis. The number of apoptotic HepG2 and MHCC97L cells was determined by flow cytometry using an Annexin V-FITC Apoptosis Detection kit (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. Briefly, cells were stained with Annexin V-FITC and PI in the dark. After being washed, cells were analyzed by flow cytometry using an Attune NxT flow cytometer (BD Biosciences) and FlowJo™ software (version 10.7; BD Biosciences).

Bioinformatics analysis. Genecards (genecards.org/) and Jaspar (jaspar.genereg.net/) were used to predict transcription factor binding to the miR-922 promoter region. In order to determine the potential role of CREB1 expression in liver cancer, CREB1 expression levels were searched in liver cancer (n=371) and non-tumor tissue samples (n=50) of The Cancer Genome Atlas (TCGA) database (portal.gdc.cancer.gov). The association between CREB1 protein expression levels and the overall survival (OS) of 360 patients with liver cancer was analyzed in The Human Protein Atlas (proteomalas.org/).

Tumor xenograft. Animal experiments were approved by the Ethical Committee for Animal Research of The Third Xiangya Hospital of Central South University. A total of 25 male Balb/c nude mice (age, 8 weeks; weight, 18-20 g) were obtained from Charles River Laboratories, Inc. and housed in a specific pathogen-free room (temperature, 26˚C; humidity, 50%; 10/14-h cycling) with free access to autoclaved food and water. Individual mice were injected subcutaneously with 1x10⁷ MHCC97H, MHCC97H-ARID2-NC, MHCC97H-ARID2-sh, MHCC97H-NC-OE or MHCC97H-ARID2-OE cells (n=5/group). After 4 weeks, mice were anesthetized via inhalation of 2% pentobarbital sodium (30 mg/kg body weight). The mice were checked for deep anesthesia, including muscle relaxation, cardiovascular depression and complete muscle relaxation, and euthanized by cervical dislocation. Tumors were dissected and images were captured. Tumor volume and weight were measured.

ELISA. The levels of serum VEGF and TNF-α in mice were measured by ELISA using VEGF (cat. no. SEA143Mu; Uscnks, Inc.) and TNF-α ELISA kits (cat. no. RAAF129R, Biovendor, Inc.). The samples were tested in triplicate and the minimum detectable concentration for VEGF and TNF-α was 10 pg/ml.

Immunohistochemistry. The expression levels of Bax, Bcl-2, PCNA, Cyclin D1, MMP3, MMP9 and ARID2 in tumor tissue from mice were analyzed by immunohistochemistry. Briefly, tissues were fixed with 4% formaldehyde for 24 h at room temperature and paraffin-embedded at 54˚C for 4 h. The tissue sections (4 µm) were deparaffinized, rehydrated and subjected to antigen retrieval in sodium citrate buffer in a microwave. After being washed, the sections were incubated overnight at 4˚C with primary antibodies against Bax (1:200; cat. no. ab32503; Abcam), Bcl-2 (1:500; cat. no. ab32124; Abcam), PCNA (1:200; cat. no. ab92729; Abcam), Cyclin D1 (1:400; cat. no. ab16663; Abcam), MMP3 (1:200; cat. no. ab227755; Abcam), MMP9 (1:200; cat. no. ab19906; Abcam) and ARID2 (1:400; cat. no. #82342; Cell Signaling Technology, Inc.) for 1 min. The slides were incubated in moist chambers overnight at 4˚C. The sections were subjected to antigen retrieval in sodium citrate buffer and counterstained with hematoxylin for 10 sec at room temperature. Images were captured using a light microscope (magnification, x400) and analyzed using Image Pro-Plus (version 6.0; Media Cybernetics, Inc.).

Statistical analysis. Data are expressed as mean ± SD (n=3). All experiments were repeated three times. Statistical analysis was performed using SPSS 22.0 (IBM Corp). Paired student's t-test and one-way ANOVA followed by post hoc Tukey's test were used for comparisons between two or multiple groups, respectively. The survival data were estimated by the Kaplan-Meier method and analyzed by log-rank test. P<0.05 was considered to indicate a statistically significant difference.

Results

Elevated miR-922 transcripts in liver cancer tissue. In order to reveal the function of miRNAs in liver cancer progression, differentially expressed miRNAs in liver cancer tissue were screened; miR-922 expression levels were significantly elevated in liver cancer tissue. Expression levels of miR-922 were assessed in 10 pairs of liver cancer and matched adjacent tissue (Table I). miR-922 expression levels in liver cancer tissue were notably higher than in matched adjacent tissue (Fig. 1A, P<0.05). Similarly, miR-922 expression increased in HepG2, MHCC97H and MHCC97L cells, relative to non-tumor hepatic THLE-2 cells (Fig. 1B). Hence, upregulated miR-922 expression occurred in liver cancer tissue and may participate in the pathogenesis of liver cancer.

miR-922 enhances malignant behavior of liver cancer cells. In order to determine the effect of altered miR-922 expression on malignant behavior of liver cancer cells, the regulatory role of miR-922 in the proliferation, clonogenicity, wound healing, invasion and apoptosis of HepG2 and MHCC97L cells was assessed in vitro. In comparison with control HepG2/MHCC97L and HepG2-NC/MHCC97L-NC, miR-922 over-expression significantly increased proliferation, clonogenicity, would healing and invasion of HepG2 and MHCC97L cells, but decreased the number of apoptotic HepG2/MHCC97L cells (Figs. 1C-G and S1). By contrast, transfection with miR-922 inhibitor exhibited opposite effects on malignant behavior in HepG2 and MHCC97L cells. miR-922 mimics or inhibitor
was introduced into HepG2 and MHCC97L to overexpress or inhibit the expression of miR-922, respectively. The efficacy of overexpression or inhibition of miR-922 was confirmed by RT-qPCR (Fig. S5).

**CREB1 promotes miR-922 transcription.** Bioinformatics analysis was performed to predict the potential binding of transcription factors to the promoter region of miR-922 using the GeneCard database; results predicted binding of CREB1 to the promoter region of miR-922. Accordingly, it was speculated that CREB1 may enhance expression levels of miR-922 in liver cancer cells. CREB1 is a key transcription factor that promotes the development and progression of tumors (17). In order to determine whether CREB1 could regulate miR-922 transcription, the potential binding of CREB1 to the miR-922 promoter region was assessed by ChIP assay. Anti-CREB1 antibody precipitated chromatins containing the miR-922 promoter region, indicating that CREB1 bound to the miR-922 promoter region (Fig. 2A). Similarly, luciferase reporter assay indicated that co-transfection with the plasmid for CREB1
expression significantly increased miR-922 promoter activity in 293T cells (Fig. 2B). Furthermore, CREB1 silencing significantly decreased enrichment of H3K27Ac but elevated that of H3K27me3 in 293T cells (Fig. 2C and D). In situ hybridization indicated that the expression levels of miR-922 in liver cancer tissue were higher than in adjacent non-tumor tissue (Fig. 2E). Similarly, miR-922 expression levels in liver cancer tissue were significantly higher than in non-tumor liver tissue.

Figure 2. CREB1 stimulates miR-922 transcription in liver cancer. (A) ChIP-PCR indicated that CREB1 bound to the miR-922 promoter region. (B) Luciferase assay demonstrated that induction of CREB1 expression enhanced miR-922 promoter-controlled luciferase expression in 293T cells. ChIP-PCR analyzed enrichment of (C) H3K27Ac histone acetyltransferase and (D) H3K27me3 histone methyltransferase on different fragments of the CREB1 promoter region. (E) In situ hybridization demonstrated increased expression levels of miR-922 in liver cancer tissue compared with adjacent non-tumor tissue. (F) Immunohistochemistry showed increased CREB1 expression levels in liver cancer tissue compared with adjacent non-tumor tissue. (G) miR-922 expression levels are increased in LIHC tissue in The Cancer Genome Atlas database. (H) Higher levels of CREB1 expression were associated with a shorter overall survival of patients with liver cancer in The Human Protein Atlas database. *P<0.05, **P<0.01, ***P<0.001. CREB1, cAMP response element binding protein 1; miR, microRNA; ChIP, chromatin immunoprecipitation; NC, negative control; sh, short hairpin; C, cancer tissue; P, para-carcinoma tissue; ns, not significant; LIHC, liver hepatocellular carcinoma.
tissue in TCGA database (Fig. 2G). Consistently, immunohistochemistry revealed significantly higher levels of CREB1 in liver cancer tissue compared with non-tumor tissue (Fig. 2F). Higher levels of CREB1 expression were significantly associated with a shorter period of OS in patients with liver cancer in The Human Protein Atlas database (P<0.001; Fig. 2H). Together, these data indicated that higher levels of CREB1 enhanced miR-922 expression levels, promoting progression and poor prognosis of liver cancer.

miR-922 targets ARID2 in liver cancer cells. Potential targeted genes of miR-922 were studied via bioinformatics analysis. Among the predicted targeted genes of miR-922, miR-922 may target FIGN and ARID2 (data not shown). Given that FIGN and ARID2 regulate the development of malignant tumors (12,18), their expression levels were analyzed in 10 pairs of liver cancer tissue by RT-qPCR. FIGN mRNA transcripts notably increased, whereas ARID2 mRNA transcripts were decreased in liver cancer tissue compared with non-tumor tissue (Fig. 3A, P<0.05). A similar pattern of FIGN and ARID2 protein expression was observed in these tissues by western blotting (Fig. 3B). Moreover, upregulated FIGN and down-regulated ARID2 expression levels were detected in HepG2, MHCC97H and MHCC97L and non-tumor hepatic THLE-2 cells (Fig. 3C). In order to clarify the targeting association, WT or MT 3’UTR of FIGN and ARID2 was cloned into luciferase reporter vector to generate WT or MT FIGN and ARID2 luciferase reporter plasmids, respectively. Luciferase reporter gene assay was performed in MHCC97L cells following transfection with miR-922 mimics and plasmid. The presence of miR-922 in miRNA/AGO2 complex was determined by RNA immunoprecipitation using anti-AGO2 antibody. (A) FIGN and ARID2 mRNA transcripts in liver cancer tissue. FIGN and ARID2 protein expression levels in (B) liver cancer tissue and (C) HepG2, MHCC97H and MHCC97L and non-tumor hepatic THLE-2 cells. (D) Luciferase activity. (E) Representative images of agarose gel electrophoresis of PCR products. Data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01, ***P<0.001. C, cancer tissue; P, para-carcinoma tissue; ARID2, AT-rich interactive domain 2; miR, microRNA; FIGN, fidgetin, microtubule severing factor; WT, wild-type; MT, mutant; NC, negative control; ns, not significant.
into the luciferase reporter plasmid to generate WT or MT FIGN and ARID2 luciferase reporter plasmid, respectively. Following co-transfection, dual luciferase reporter assay indicated that co-transfection with miR-922 mimics significantly decreased ARID2-regulated, but not MT-ARID2-regulated, luciferase activity in MHCC97L cells (P<0.01); WT-FIGN or MT-FIGN-regulated luciferase activity was not affected in MHCC97L cells (Fig. 3D). These data suggest that miR-922 may target ARID2 to decrease its expression in liver cancer.

RIP assays using anti-AGO2 detected miR-922 in HepG2 and MHCC97L cells (Fig. 3E), indicating that miR-922 existed in the miR-922/AGO2 complex. These data suggest that miR-922 may target ARID2 and decrease its expression to modulate malignant behavior of liver cancer cells.

Altered ARID2 expression modulates malignant behavior of liver cancer cells. ARID2 is a tumor suppressor and serves as a genetic modulator during the progression of several types of cancers, including liver cancer (12). The regulatory role of ARID2 in the malignant behavior of HepG2 and MHCC97L cells was investigated by CCK-8, colony formation, wound healing, apoptosis and Transwell invasion assays in vitro. The ARID2 cDNA sequence was cloned into pcDNA3.1 vector for gene over-expression and three ARID2 specific shRNA were cloned into pGUP6 vector for stable gene-silence. The protein level of ARID2 in HEK293 was determined after transduction with either ARID2-pcDNA3.1 or sh-ARID2 plasmid. As shown in figure, the ARID2 expression was significantly increased in ARID2-pcDNA3.1 group and was dramatically reduced in sh-ARID2 group compared to the control group. Among three ARID2 specific shRNA, sh-ARID2-2 achieved most robust knockdown efficiency, which then was used in following experiment (Fig. S6). In comparison with the control NC-OE and NC-sh cells, ARID2 over-expression significantly decreased proliferation, clonogenicity, wound healing and invasion, but increased the number of apoptotic HepG2 and MHCC97L cells (Figs. 4 and S2). By contrast, ARID2 silencing exhibited opposite effects on the malignant behavior of HepG2 and MHCC97L cells.

Similarly, ARID2 over-expression in MHCC97L cells significantly decreased tumor volume and weight in vivo, whereas ARID2 silencing in MHCC97L cells did not significantly alter tumor volume and weight in mice (Fig. 5A-C). Furthermore, ARID2 over-expression significantly decreased miR-922 expression levels, whereas ARID2 silencing increased its expression in xenografts (Fig. 5D), suggesting that ARID2 may regulate
miR-922 expression. In addition, ARID2 over-expression significantly decreased serum levels of VEGF and TNF-α, whereas ARID2 silencing elevated the serum levels of VEGF and TNF-α in tumor-bearing mice (Fig. 5E and F). Immunohistochemistry revealed that ARID2 over-expression increased ARID2 and Bax expression levels, but decreased those of cyclin D1, PCNA, Bcl-2, MMP3 and MMP9 in liver cancer xenografts (Figs. 5G and S3). By contrast, ARID2 silencing exhibited opposite effects on expression levels in liver cancer xenografts (Figs. 5G and S3). Collectively, these data demonstrated that ARID2 mitigated malignant behavior of liver cancer cells.

Altered ARID2 expression modulates miR-922 inhibitor-decreased malignant behavior of liver cancer cells. Finally, it was determined whether altered ARID2 expression modulates miR-922 inhibitor-decreased malignant behavior of liver cancer cells. ARID2 over-expression enhanced miR-922 inhibitor-decreased proliferation, clonogenicity, invasion and wound healing of HepG2 cells and increased the number of apoptotic HepG2 cells (Fig. 6). By contrast, ARID2 silencing mitigated miR-922 inhibitor-decreased malignant behavior of HepG2 cells (Fig. 6). Similar effects of altered ARID2 expression on miR-922 inhibitor-decreased malignant behavior were...
observed in MHCC97L cells (Fig. S4). These data indicated that miR‑922‑regulated ARID2 expression was key for control of malignant behavior of liver cancer cells.

Discussion

A previous study verified that miRNAs regulate hepatocarcinogenesis (19). miR‑221/222, miR‑34a and miR‑224 serve as oncogenes to promote tumor cell growth (20,21), while miR‑122 and miR‑375 suppress tumor progression by inhibiting liver cancer cell invasion and metastasis (17,22). Previous studies have shown that miR‑922 expression is upregulated in liver cancer (23) and miR‑922 promotes the proliferation of liver cancer cells by targeting CYLD to enhance c‑Myc and cyclin D1 expression levels and inhibit Rb phosphorylation (7). However, the molecular mechanisms underlying the oncogenic role of miR‑922 are still unclear.

The present study found significantly elevated levels of miR‑922 transcripts in liver cancer tissue compared with non-tumor adjacent tissue, consistent with a previous study (7). In addition, miR‑922 expression levels were upregulated in liver cancer cell lines compared with non-tumor hepatocytes.
Induction of miR-922 over-expression enhanced malignant behavior, rapid proliferation, strong clonogenicity, lower numbers of apoptotic cells, potent wound healing and invasion ability in liver cancer cells. All these data indicate that miR-922 may serve as an oncogenic factor to promote malignant behavior of liver cancer cells.

Multiple factors regulate the transcription of miRNAs, including genetic abnormality, epigenetic regulation and transcription factors (24). CREB1, a leucine zipper-type transcription factor, regulates numerous types of malignancy (25). A previous study demonstrated that CREB1 transcriptionally regulates a number of miRNAs (17). The present data indicated that CREB1 bound to the miR-922 promoter region and stimulated its transcription. Inhibition of CREB1 decreased H3K27 acetylation upstream of the miR-922 promoter region but enhanced repressive H3K27 trimethylation. These results suggested that CREB1 may serve as a transcription factor to induce miR-922 expression in liver cancer cells. Future studies should investigate the potential role of other transcription factors in regulating miR-922 expression in liver cancer. In addition, CREB1 was upregulated in liver cancer tissue and positively associated with miR-922 expression levels. Increased CREB1 expression levels were associated inversely with OS of patients with liver cancer. Hence, high levels of CREB1 and miR-922 may be valuable for the prognosis of liver cancer.

In order to identify the targeted genes of miR-922 and the underlying mechanisms, potential targeted genes of miR-922 were predicted; miR-922 targeted ARID2. ARID2 is a tumor suppressor and subunit of SWitch/sucrose non-fermentable complex B (26). Given that ARID2 mutations are associated with the development of HCC (27-29), targeting ARID2 by miR-922 may promote malignant behavior of HCC. ARID2 over-expression eliminated malignant behavior of liver cancer cells whereas ARID2 silencing had opposite effects, consistent with a previous report (30). In addition, ARID2 over-expression significantly mitigated or abrogated miR-922-promoted malignant behavior of liver cancer cells. ARID2 over-expression also enhanced Bax expression levels, but decreased those of Bcl-2, PCNA, cyclin D1, MMP3 and MMP9 in liver cancer tumors, which explained why ARID2 over-expression inhibited growth of implanted liver cancer xenografts in mice. Previous studies have shown that ARID2 is targeted by miR-376c-3p, miR-208-3p and miR-155 in HCC (30-32). Accordingly, the present findings extended previous observations and indicate that the miR-922/ARID2 axis is key for regulating malignant behavior of liver cancer cells (33) and that miR-922 may collaborate with other miRNAs to attenuate ARID2 expression levels, which promotes development and progression of liver cancer. Further investigation is required to determine whether miR-922 directly interacts with ARID2 mRNA in liver cancer cells.

Together, the present data indicated significantly upregulated miR-922 expression levels in liver cancer tissue and cells; its elevated expression was associated with CREB1 expression levels in liver cancer tissue. Both in vitro and in vivo experiments demonstrated that miR-922 enhanced malignancy of liver cancer by promoting tumor growth and cell proliferation, wound healing and invasion. Mechanistically, these findings may provide novel insights into the CREB1/miR-922/ARID2 interaction network in liver cancer progression. Therefore, miR-922 may be a valuable diagnostic and prognostic biomarker for liver cancer; targeting the CREB1/miR-922/ARID2 axis may represent a new therapeutic strategy for intervention of liver cancer. The present study had limitations, including limited sample size of patients with HCC with chronic hepatitis B, but not with other risk factors, such as hepatitis B core and alcoholic liver disease. Therefore, further studies with a larger population of patients with liver cancer with diverse risk factors are warranted to validate the role of the CREB1/ARID2/miR-922 axis in the progression of liver cancer.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

XL and ZL designed the study. XL, HZ, PZ, HYZ, LC and JG performed the experiments. XL and YY collected the data. HZ, PZ, YY, LC and JG analyzed the data. XL, HZ, PZ, YY, HYZ, LC and JG interpreted the data. XL and ZL drafted the manuscript. HYZ and PZ revised the manuscript. All authors read and approved the final manuscript. All authors confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Third Xiangya Hospital of Central South University (approval no. 2020-S362). All participants provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


