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Original Article

miR-590-5p promotes liver cancer growth and chemotherapy resistance through directly targeting FOXO1

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Abstract: miR-590-5p functions as an onco-miR or an anti-onco-miR in various types of cancers. However, the exact role of miR-590-5p in liver cancer remains to be elucidated. In the present study, we explored the predictive role of miR-590-5p expression in liver cancer patients. In addition, CCK-8 assay, colony formation assay, and analysis of xenograft tumors were performed to investigate the biological effects of miR-590-5p in liver cancer. A direct target of miR-590-5p was identified based on a luciferase assay and further molecular experiments. Our results demonstrated that miR-590-5p was upregulated in malignant tissues of liver cancer patients and in liver cancer cell lines. miR-590-5p expression was found to be inversely correlated with disease-free survival of liver cancer patients. Furthermore, both *in vitro* and *in vivo* experiments showed that miR-590-5p knockdown inhibited the growth of HepG2 and Bel-7404 tumor cells by promoting apoptosis and cell cycle arrest. We also demonstrated that increasing of miR-590-5p in 5-Fu resistant patients and liver cancer cells, and knockdown of miR-590-5p enhances chemosensitivity to 5-Fu in liver cancer. FOXO1 was identified as a direct and necessary target of miR-590-5p during regulating liver cancer growth. Taken together, our findings provide insights into the role of miR-590-5p in liver cancer. Moreover, it is suggested that miR-590-5p can serve as a novel therapeutic target and predictive biomarker for liver cancer.

Keywords: Liver cancer, miR-590-5p, FOXO1, apoptosis, cell cycle, chemotherapy resistance

Introduction

Liver cancer is the fifth most common cancer affecting men, the ninth most common cancer among women, and the second most common cause of cancer-related mortality for men and women combined [1, 2]. Recent years have witnessed significant advances in the diagnosis and treatment of liver cancer. However, liver cancer patients continue to have very poor long-term prognoses [1, 2]. Preferred treatments for hepatocellular carcinoma (HCC) include surgical tumor resection, which has five-year survival rates ranging from 60% to 70%, and transplantation, which has four-year survival rates ranging from 70% to 80%. However, only a minority of patients are suitable candidates for surgery, and disease recurrence is common [3]. Non-surgical liver-directed therapies, such as chemotherapy and radiation have been used as treatments. However, many patients, especially those in the advanced stages of the disease, develop disease recurrence [4]. Thus, there is an urgent need to develop novel therapeutic targets for improving the prognoses of liver cancer patients.

FOXO family proteins, which include the Forkhead box O (FOXO) transcription factors FOXO1, FOXO3a, FOXO4, and FOXO6 have been increasingly recognized as an important family of proteins that modulate the expression of important genes involved in apoptosis, cell cycle progression, autophagy, and DNA damage repair [5, 6]. FOXO1 is the most abundant isoform expressed in insulin-responsive tissues such as the liver, and is known to regulate the expression of genes involved in apoptosis, cell

cycle, metabolism, stress response, and differentiation [5, 6]. Deletion of Aurora A leads to FOXO1 upregulation, which induces cell cycle arrest in HCC cells [7]. Increased nuclear localization of FOXO1 by trifluoperazine treatment *in vivo* was demonstrated to effectively suppress angiogenesis and tumor growth and downregulate the expression of VEGF, Bcl-2, and PCNA in HCC [8].

MicroRNAs (miRNAs) are a family of ~19-22-bp non-coding RNAs that regulate gene expression by binding to their target mRNAs and inducing mRNA cleavage or translational inhibition. Accumulating evidence has suggested the importance of miR-590 in cancer progression. miR-590-5p was reported to be upregulated as a tumor oncogene in human cervical cancer [9], colorectal cancer [10], lung adenocarcinoma [11], and gastric cancer [12]. On the other hand, miR-590-5p has also been demonstrated to exert an anti-tumor role in colorectal cancer [13, 14] and breast cancer [15]. However, the expression patterns and biological functions of miR-590-5p in liver cancer remain unclear.

The present study aims to determine the role of miR-590-5p in liver cancer. The potential predictive role of miR-590-5p in disease-free survival of liver cancer patients was analyzed using a clinical database. *In vivo* and *in vitro* experiments were performed to investigate the biological function of miR-590-5p in liver cancer. Luciferase assays and other molecular experiments were conducted to elucidate the mechanisms underlying miR-590-5p mediated regulation of liver cancer progression. Our study identified miR-590-5p as a novel therapeutic and predictive target for liver cancer.

Materials and methods

Clinical samples and analysis

Human liver cancer tissues and adjacent normal tissues (34 pairs) were obtained with informed consent under a general waiver by the Academic Medical Center Institutional Review Board for the proper secondary use of human material and were obtained from the People's Hospital of Sichuan from Jan 2017 to Sep 2017. Experiments described were approved by the Ethics Committee of Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital (Chengdu, China). The tumor grade were identified according to clinical diagnosis. The potential correlation between miR-590 family and disease-free survival was analyzed by the clinical database Kaplan-Meier Plotter (http://kmplot.com).

The plasma from 40 liver cancer patients were collected before 5-Fu treatment. The tumor size were measured by enhanced CT at the beginning of 5-Fu treatment and 6 weeks post 5-Fu treatment. The responses of liver cancer patients to 5-Fu chemotherapy was divided into 5-Fu sensitive (5-Fu-S) and 5-Fu resistant (5-Fu-R) following to the rules of RECIST1.1. The patients of complete remission and partial remission were defined as 5-Fu-S and of progressive disease were defined as 5-Fu-resistant.

Cell culture and treatment

Liver cancer cell lines, HepG2, SNU398, SMMC7721, Bel-7404, SK-Hep-1 and normal liver cell line LO2 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in culture medium in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FBS (Gibco) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin, Gibco). All the cells were maintained at 37°C with 5% CO_2 and humidified atmosphere. The chemotherapy resistant SNU-398 (SNU-398-R) cells were selected by adding 5-fluorouracil (5-Fu, Sigma, MA, USA) (from 0.1 μM to 2.0 μM progressively).

The lentivirus-based miR-590-5p overexpression system (lenti-miR-590-5p), knockdown system (lenti-anti-miR-590-5p) and the miRNA-negative control (lenti-NC) were purchased from GenePharma (Shanghai, China). The lentivirus were used to infect HepG2, Bel-7404 and SNU-398 cells with 20 MOI and the stably infected cells were selected by adding 2 μg/ml puromycin. siRNA targeting FOXO1 (siFOXO1) and negative control (siNC) were purchased from RiboBio Company (Guangzhou, China) and used to transfect cells with riboFECT CP transfection agent (RiboBio, Guangzhou, China), following the instructions of manufacturers.

Cell proliferation and colony formation assay

For the cell proliferation assay, human liver cancer cells were infected with lenti-miR-590-5p, lenti-anti-miR590-5p or lenti-NC, and

cultured (1,500 cells/well) in a 96-well plate. The cell proliferation was evaluated every 24 h using a cell counting kit-8 (CCK-8 Beyotime, Beijing, China) following the manufacturer's protocol. For colony formation assay, human liver cancer cells were infected with lenti-miR-590-5p, lenti-anti-miR590-5p or lenti-NC, and seeded into a 6-well plated (1,000 cells/well). Cells were cultured for 10-14 days, and then the cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature and stained with crystal violet for 10 minutes at room temperature. After washing with running water, the numbers of colonies were counted. All the experiments were performed in triplicate.

RNA extraction and real-time PCR analysis

Human liver cancer cells were harvested for total RNA extraction using TRizol (Invitrogen; Thermo Fisher Scientifc, Inc.), according to the manufacturer's protocol. The miRNA of plasma were extracted by miRNeasy kit (QIAGEN, Germany). The reverse transcription-real-time polymerase chain reaction (real time-qPCR) was used to analyze the expression of miR-590-5p with primers from RiboBio (Guangzhou, China), and U6 was selected as internal reference. Reverse transcription was performed on the isolated total RNA using a Reverse Transcription kit (Takara Bio, Inc., Otsu, Japan), and qPCR was performed using a Real Time PCR kit (Takara Bio, Inc). Reverse transcription was performed using 1 μg total RNA in 2 μl water at 65°C for 5 min, 30°C for 10 min, 42°C for 10-30 min and 2°C for 3 min. The qPCR conditions were as follows: Denaturation at 94°C for 2 min, amplification for 30 cycles at 94°C for 0.5 min, annealing at 58°C for 0.5 min and extension at 72°C for 1 min, followed by a terminal elongation step at 72°C for 10 min. The RT-qPCR analysis was performed on a Bio-Rad CFX96 thermal cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Data was analyzed with the $2^{-\Delta\Delta Ct}$ method. The sequences were not supplied due to the rules of the company. The same experiments were performed three times.

Flow cytometry analysis

Human liver cancer cells were infected with lenti-anti-miR590-5p or miR-NC, and cultured for 48 h. Then, the cells were harvested and stained with FITC-Annexin V and propidium iodide (Keygen, Nanjing, China) following the instructions of manufacturers. For cell cycle analysis, the harvested cells were stained with propidium iodide (Keygen, Nanjing, China). FACS assay was performed to evaluate cell apoptosis and cycle using flow cytometry. Data were analyzed using CellQuest software (BD Biosciences, San Jose, CA, USA). All the experiments were performed in triplicate.

Western blotting

The proteins were extracted in ice-cold RIPA lysis buffer (Beyotime) containing 1% protease inhibitor cocktail (Pierce, Rockford, IL, USA), then centrifuged at 12,000 rpm for 20 min at 4°C. After protein concentration measurement by BCA assay kit (Beyotime), 20 μg proteins were performed for western blotting as previous study indicated [16]. Primary antibodies against Caspase 3, Caspase 8, Caspase 9, Cyclin D1, Cyclin B1, PCNA, FOXO1, p-FOXO1, p21, Bim, Bcl-2 (Cell Signaling Technology, Inc., Danvers, MA, USA) were added for detection and all blots were probed with antibodies against GAPDH (Cell Signaling Technology, Inc., Danvers, MA, USA) as loading control. ECL kit from Millipore was purchased for band exposure. The density of each band was measured by Image J software (National, Institute of Health, Bethesda, MD, USA).

Animal study

HepG2 or Bel-7404 cells stable infection miR-NC or anti-miR590-5p (5×10^6 cells in 100 μl of DMEM) were injected subcutaneously into the flanks of nude mice (5 weeks old, male; Vital River Laboratories, Beijing China). Tumor volumes were measured at 10 days post cell injection and every five days. Tumor weights were measured immediately after sacrificing and tumor samples were harvested for RNA extraction and embedding in 4% paraffin for TUNEL assay analysis. All of the procedures involving animals conformed to the guidelines of the Institutional Animal Care and Use Committee of Sichuan Academy of Medical Sciences and Sichuan Provincial People's Hospital (Chengdu, China). A Terminal deoxynucleotidyl transferase-deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed using a DeadEndTM Fluorometric TUNEL system (Promega Corporation, Madison, WI, USA), according to the manufacturer's protocol.

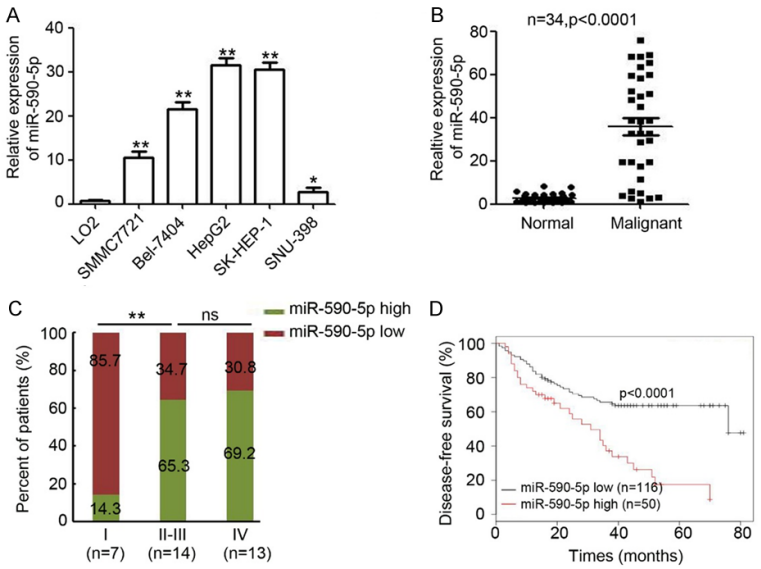

Figure 1. miR-590-5p is overexpressed in liver cancer and correlates with shorter survival in liver cancer patients. The total RNA of liver cancer cell lines (SMMC7721, Bel-7404, HepG2, SK-HEP-1 and SNU-398) and normal liver cell LO2 were extracted for real-time PCR analysis of miR-590-5p expression (A). U6 was used as internal control (n = 4, *, P < 0.05; **, P < 0.01, compared with LO2 cell). (B) Real-time PCR analysis of miR-590-5p expression in 34 paired liver malignant tissues and forward normal liver tissues. U6 was used as internal control. (C) Percentage of patients with high and low expression of miR-590-5p according to tumor grade (**P < 0.01; ns, no significant difference). (D) Kaplan-Meier curve showing disease-free survival in patients with liver cancer (percentage), stratified by miR-590-5p expression (high and low expression). The log-rank test was used to compare differences between groups (n = 166).

Luciferase assay

To construct the FOXO1 luciferase report vector, the wild-type (WT) or mutant (Mut) FOXO1 gene was cloned downstream of the luciferase gene in the pLUC luciferase vector. HEK-293T cells were seeded in 96-well plates. After 24 h incubation, cells were transfected with pLUC-FOXO1 3'-UTR or pLUC-FOXO1 3'-UTR mutant and lenti-NC or lenti-miR-590-5p. Forty-eight hours after transfection, the cells were assayed by both firefly and renilla luciferase using the dual luciferase assay system (Promega) according to manufacturer's instructions. All transfection experiments were conducted in triplicate and repeated 3 times independently. The relative luciferase activity were analyzed.

Immunofluorescence staining

Immunofluorescence staining was performed as previous study indicated [16]. A total of 2 × 10^4 HepG2 cells that were stably infected with lenti-NC or lenti-anti-miR590-5p were plated in EZ-slide (Merck Millipore) with 0.5 ml DMEM medium containing 10% FBS. Twenty-four hours later, 4% paraformaldehyde was employed to fix the cells at room temperature for 10 min. Then, the cells were blocked with goat serum and incubated with primary antibodies against FOXO1 (Cell Signaling Technology, Inc., Danvers, MA, USA) for 1.5 h at 37°C, followed directly by either fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (1:200, Santa Cruz) for 1.5 h at 37°C. Nuclei were counterstained with DAPI (Roche, Basel, Switzerland). All specimens were evaluated using an Olympus BX600 microscope and SPOT Flex camera.

Statistical analysis

Numerical continuous data were presented as the mean ± standard deviation and Student's t-test (two-tailed) and one-way ANOVA were performed to analyze statistical differences using SPSS 19.0 software (SPSS, Inc., Chicago, IL, USA). The log-rank test was used to compare differences of disease-free survival between miR-590-5p high and low expression patients by GraphPad Prism 5. P < 0.05 indicates a statistically significant.

Results

miR-590-5p expression predicts poor survival in liver cancer patients

To determine the potential role of miR-590-5p in liver cancer, the expression of miR-590-5p in liver cancer cell lines were investigated. Our results suggested that miR-590-5p expression was dramatically upregulated in all liver cancer cells (**Figure 1A**). Furthermore, real-time PCR analysis was performed to measure miR-590-5p levels in malignant liver and adjacent normal tissues. Results showed that miR-590-5p was significantly upregulated in malignant tis-

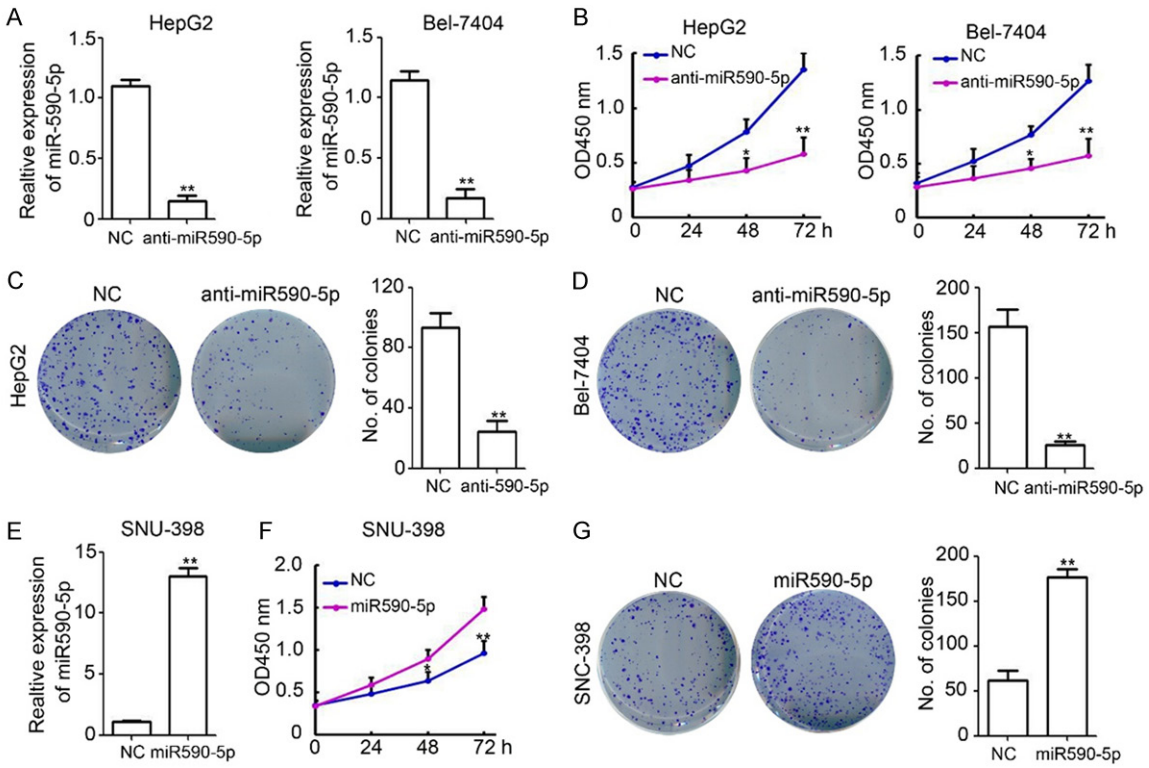

Figure 2. miR-590-5p promotes liver cancer cell growth *in vitro*. HepG2 and Bel-7404 cells were infected with lentivirus-based miR-NC (NC) or anti-miR590-5p. A. Real-time PCR analysis of miR-590-5p expression in HepG2 and Bel-7404 cells. U6 was used as internal control (n = 4; **, P < 0.01, compares with NC group). B. CCK-8 analysis of cell viability in HepG2 and Bel-7404 cells that infected with lentivirus-based miR-NC (NC) or anti-miR590-5p (n = 5; *, P < 0.05; **, P < 0.01, compares with NC group). C, D. Colony formation analysis of HepG2 and Bel-7404 cells (n = 5; **, P < 0.01, compares with NC group). E. Real-time PCR analysis of miR-590-5p expression in SNU398 cells that infected with lentivirus-based miR-NC (NC) or miR-590-5p. U6 was used as internal control (n = 4; **, P < 0.01, compares with NC group). F. CCK-8 Analysis of cell viability in SNU398 cells that infected with lentivirus-based miR-NC (NC) or miR-590-5p (n = 5; *, P < 0.05; **, P < 0.01, compares with NC group). G. Colony formation analysis of SNU398 cells that infected with lentivirus-based miR-NC (NC) or miR-590-5p (n = 5; **, P < 0.01, compares with NC group).

sues compared to that in normal liver tissues (normal: 2.88 ± 0.33 vs. malignant: 35.77 ± 4.03, **Figure 1B**). Further analysis indicated that miR-590-5p expression was lower in grade I tumors than in grade II-III tumors (**Figure 1C**). On the other hand, we observed no significant differences in miR-590-5p expression between grade II-III and grade IV tumors (**Figure 1C**). The clinical database Kaplan-Meier Plotter (http://kmplot.com) was used to investigate the potential predictive role of miR-590-5p in liver cancer. As shown in **Figure 1D**, the expression levels of both and miR-590-5p were inversely correlated with the disease-free survival of liver cancer patients. Taken together, these results indicated that miR-590-5p expression is upregulated in liver cancer tissues and that miR-590-5p levels predicted poor disease-free survival of liver cancer patients.

miR-590-5p deletion inhibits liver cancer growth in vitro

To investigate the functional role of miR-590-5p in the liver, lentivirus-based miR-590-5p deletion system (lenti-anti-miR590-5p) was used to infect HepG2 and Bel-7404 cells to stimulate miR-590-5p overexpression. Real-time PCR results indicated that that miR-590-5p effectively downregulated miR-590-5p expression in both HepG2 and Bel-7404 cells (**Figure 2A**). miR-590-5p-deficient HepG2 and Bel-7404 cells showed significantly reduced cell viability than miR-NC (negative control)-transfected cells (**Figure 2B**). Results of colony formation assay also confirmed the anti-tumor effects of miR-590-5p knockdown in HepG2 (**Figure 2C**) and Bel-7404 (**Figure 2D**) cells. In contrast, results of the CCK-8 assay (**Figure 2F**)

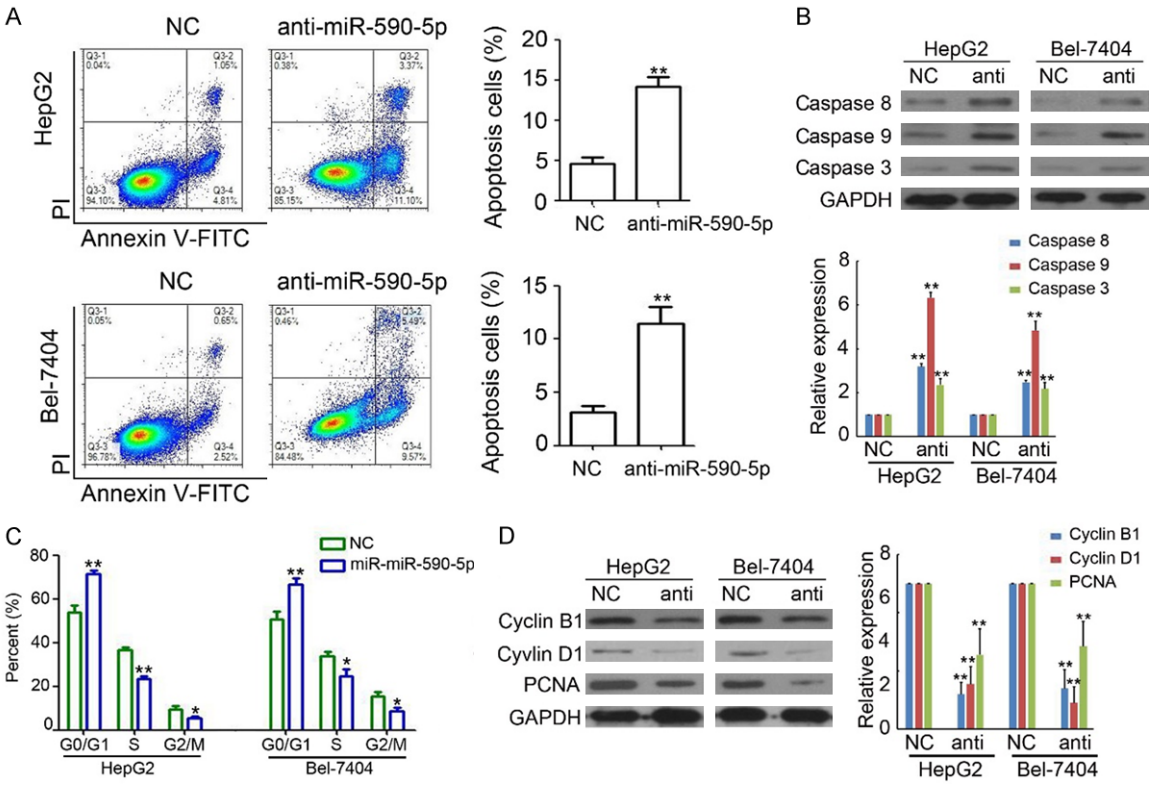

Figure 3. Knockdown of miR-590-5p promotes apoptosis and cell cycle arrest in liver cancer. A. HepG2 and Bel-7404 cells were infected with lentivirus-based miR-NC (NC) or anti-miR590-5p and collected for analysis of cell apoptosis by Annexin V and PI staining (n = 3; **, P < 0.01, compares with NC group). B. Western blotting analysis of apoptosis-related protein (Caspase 3, Caspase 8 and Caspase 9) expression in HepG2 and Bel-7404 cells that infected with lentivirus-based miR-NC (NC) or anti-miR590-5p (anti). GAPDH was used as a loading control. The relative expression of each band was qualified and analyzed (n = 3; **, P < 0.01, compares with NC group). C. PI staining and flow cytometry for cell cycle analysis in HepG2 and Bel-7404 (n = 5; *, P < 0.05; **, P < 0.01, compares with NC group). D. Western blotting analysis of cell cycle-related protein (Cyclin B1, Cyclin D1 and PCNA) expression in HepG2 and Bel-7404 cells. GAPDH was used as a loading control. The relative expression of each band was qualified and analyzed (n = 3; **, P < 0.01, compares with NC group).

and colony formation assay (**Figure 2G**) revealed that ectopic expression of miR-590-5p (**Figure 2E**) promoted SNC-398 cell growth. Our findings demonstrated the role of miR-590-5p in promoting liver cancer growth.

miR-590-5p knockdown promotes apoptosis and cell cycle arrest in liver cancer

To determine the potential mechanisms underlying miR-590-5p-mediated regulation of liver cancer growth, we assessed apoptosis and cell cycle in HepG2 and Bel-7404 cells. HepG2 and Bel-7404 cells treated with an anti-miR590-5p showed higher rates of apoptosis (HepG2 cells: NC 4.6 ± 0.8% vs. anti-miR-590-5p 14.1 ± 1.2%; Bel-7404 cells: NC 3.1 ± 0.6% vs. anti-miR590-5p 11.5 ± 1.5%, **Figure 3A**). Western blot analysis suggested that miR-590-5p knockdown of HepG2 and Bel-7404 cells significantly upregulated the expression of the apoptosis-related proteins caspase 3, caspase 8, and caspase 9 (**Figure 3B**). Then, we assessed the proportions of cells in the different cell cycle phases through PI staining and flow cytometry. Results showed that miR-590-5p knockdown in both HepG2 and Bel-7404 cells increased the proportion of cells in the G0/G1 phase and decreased the proportion of cells in the S phase (**Figure 3C**). The cell cycle checkpoint proteins Cyclin B1, Cyclin D1, and PCNA were dramatically downregulated in miR-590-5p-deficient HepG2 and Bel-7404 cells (**Figure 3D**). The above results suggested that miR-590-5p inhibits apoptosis and cell cycle progression, thereby promoting cell proliferation.

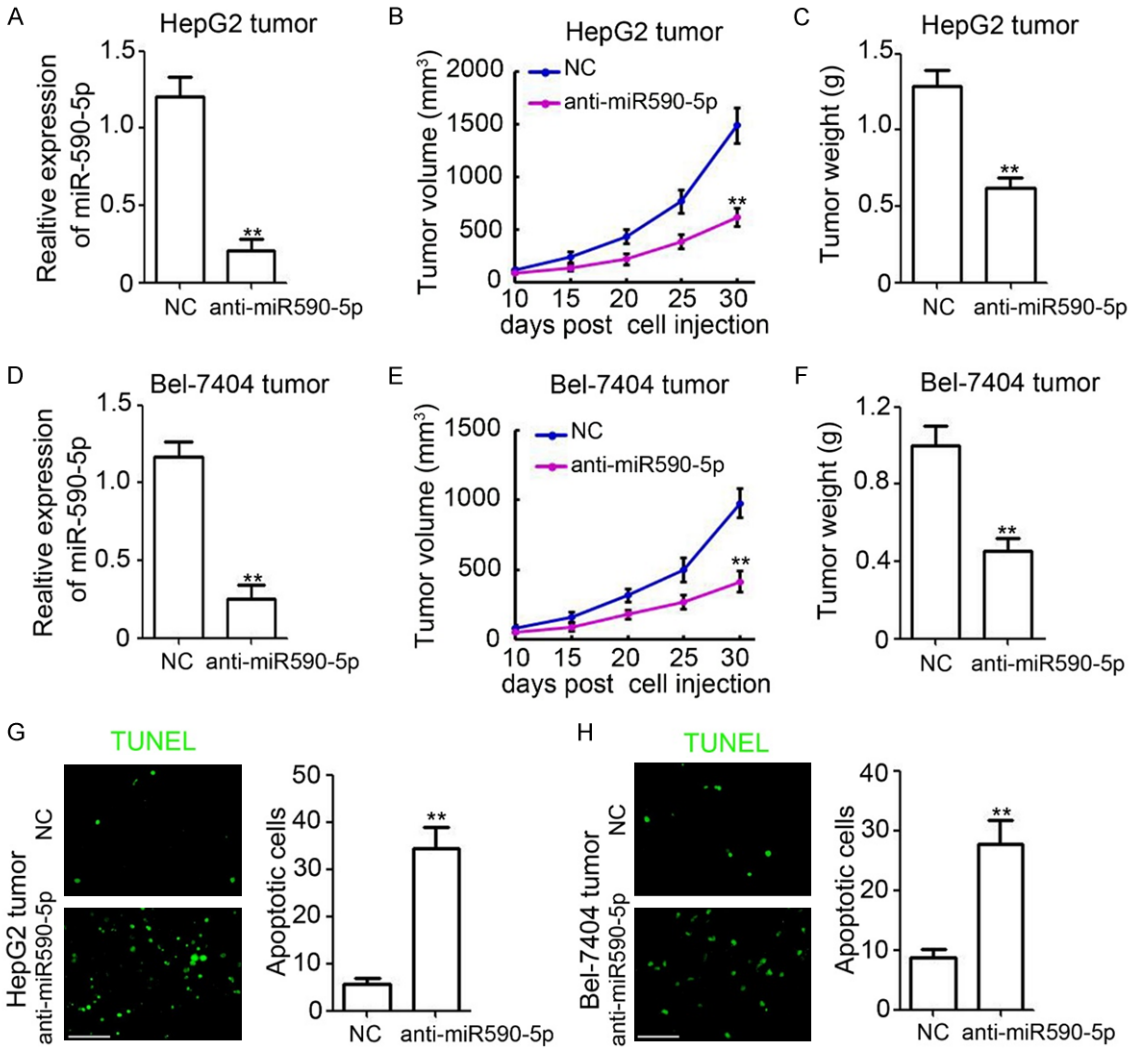

Figure 4. Knockdown of miR-590-5p impairs liver cancer tumor growth *in vivo*. The HepG2 cells that stably infected with lentivirus-based miR-NC (NC) or anti-miR590-5p were injected into mice. The HepG2 tumor tissues were collected for real-time PCR analysis of miR-590-5p expression (A). U6 was used as internal control. (n = 4; **, P < 0.01, compares with NC group). (B) Tumor volume after injection of 5 × 10^6 HepG2 cells that stably infected with lentivirus-based miR-NC (NC) or anti-miR590-5p cells into nude mice (n = 5; **P < 0.01, compared with miR-NC group). (C) End-stage weight after injection of 5 × 10^6 HepG2 cells that stably infected with lentivirus-based miR-NC (NC) or anti-miR590-5p cells into nude mice (n = 5; **P < 0.01, compared with miR-NC group). (D) Real-time PCR analysis of miR-590-5p expression in Bel-7404 tumors. U6 was used as internal control (n = 4; **, P < 0.01, compares with NC group). (E) Tumor volume after injection of 5 × 10^6 Bel-7404 cells that stably infected with lentivirus-based miR-NC (NC) or anti-miR590-5p cells into nude mice (n = 5; **P < 0.01, compared with miR-NC group). (F) End-stage weight after injection of 5 × 10^6 Bel-7404 cells that stably infected with lentivirus-based miR-NC (NC) or anti-miR590-5p cells into nude mice (n = 5; **P < 0.01, compared with miRNC group). (G, H) TUNEL analysis of apoptosis cells in miRNC and anti-miR590-5p stably infected HepG2 (G) and Bel-7404 (H) xenograft tumors, (n = 3; **P < 0.01, compared with miR-NC group; scale bar = 100 μm).

miR-590-5p knockdown inhibits the growth of liver xenograft tumor in vivo

To validate the above results *in vivo*, HepG2 and Bel-7404 cells stably infected with miR-NC and anti-miR590-5p were used to establish xenograft tumor models in nude mice. Real-time PCR results showed that HepG2 tumor cells stably infected with anti-miR590-5p showed lower miR-590-5p expression than those HepG2 tumor cells that were stably infected with miR-NC (**Figure 4A**). *In vivo* results suggested that miR-590-5p knockdown in HepG2 cells markedly reduced xenograft tumor growth (**Figure 4B**, **4C**). Tumor volume decreased by 58.7% (miR-NC: 1487.2 ± 168.3

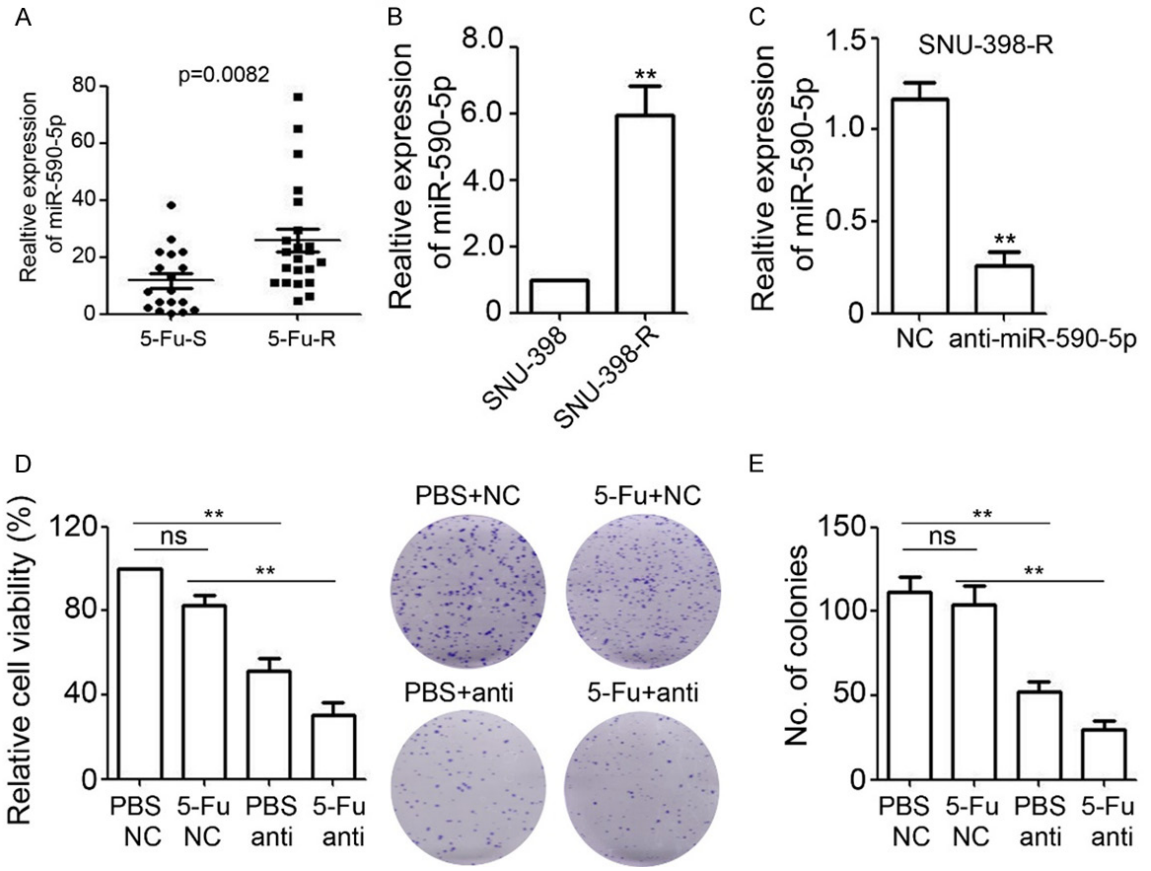

Figure 5. Knockdown of miR-590-5p enhances chemosensitivity to 5-Fu in liver cancer. A. Real-time PCR analysis of miR-590-5p expression in plasma from 5-Fu resistant (5-Fu-R) and 5-Fu sensitive (5-Fu-S) liver cancer patients (n = 40). B. Real-time PCR analysis of miR-590-5p expression in SNU-398 and SNU-398 resistance (SNU-398-R cells, n = 4; **, P < 0.01, compares with SNU-398 group). C. Real-time PCR analysis of miR-590-5p expression in SNU-398-R cells that infected with lentivirus-based miR-NC (NC) or anti-miR590-5p. U6 was used as internal control (n = 4; **, P < 0.01, compares with SNU-398 group). D. CCK-8 analysis of cell viability in SNU-398-R cells that infected with lentivirus-based miR-NC (NC) or anti-miR590-5p (anti) and treatment with 5-Fu (0.5 μM) or PBS (n = 5; **, P < 0.01; ns, no significant difference). E. Colony formation analysis of SNU-398-R cells that infected with lentivirus-based miR-NC (NC) or anti-miR590-5p (anti) and treatment with 5-Fu (0.5 μM) or PBS (n = 5; **, P < 0.01; ns, no significant difference).

mm^3 vs. anti-miR590-5p: 613.8 ± 83.4 mm^3), and tumor weight decreased by 51.9% (miR-NC: 1.29 ± 0.10 g vs. anti-miR590-5p: 0.62 ± 0.06 g). Furthermore, miR-590-5p knockdown in Bel-7404 cells also confirmed the anti-tumor effects of the anti-miR590-5p in liver cancer (**Figure 4D-F**). In Bel-7404 xenograft tumors, miR-590-5p knockdown decreased the tumor volume by 57.7% (miR-NC: 975.4 ± 106.5 mm^3 vs. anti-miR590-5p: 412.7 ± 75.4 mm^3) and decreased tumor weight by 55% (miR-NC: 1.00 ± 0.10 g vs. anti-miR590-5p: 0.45 ± 0.07 g). Results of TUNEL staining indicated significantly higher apoptotic indices following stable knockdown of miR-590-5p, in both HepG2 and Bel-7404 xenograft tumors (**Figure 4G**, **4H**). Taken together, the above results suggested that miR-590-5p knockdown inhibits liver cancer growth *in vivo*.

Knockdown of miR-590-5p enhances chemosensitivity to 5-Fu in liver cancer

To investigate the potential role of miR-590-5p in chemotherapy resistance of liver cancer, the expression of plasma miR-590-5p in 5-Fu resistant and sensitive patients were determined. The results suggested that plasma miR-590-5p expression was much higher in 5-Fu resistant patients than in 5-Fu sensitive patients (**Figure 5A**). The further results also suggested that miR-590-5p expression was significant upregulated in SNU-398-R cells (**Figure 5B**) and infected with lenti-anti-miR590-5p efficiently down-

regulated the expression of miR-590-5p in SNU-398-R cells (**Figure 5C**). Furthermore, knockdown of miR-590-5p not only suppressed the growth and colony formation of SNU-398-R cells, but also enhanced chemosensitivity to 5-Fu in SNU-398-R cells (**Figure 5D**, **5E**). The above results demonstrated that upregulation of miR-590-5p in 5-Fu resistant patients and knockdown of miR-590-5p enhanced chemosensitivity to 5-Fu in liver cancer.

FOXO1 is a direct target of miR-590-5p in liver cancer

miRDB and TargetScan were used to identify the putative targets of miR-590-5p during cell growth in liver cancer cells. Bioinformatics analysis predicted FOXO1 as a direct target of miR-590-5p (**Figure 6A**). Real-time PCR results showed that miR-590-5p expression was higher in lenti-miR-590-5p-infected 293T cells than in lenti-NC-infected 293T cells (**Figure 6B**). After transfection with luciferase-FOXO1-3'UTR, luciferase expression in miR-590-transfected 293T cells was significantly downregulated (**Figure 6C**). Meanwhile, after transfection with the luciferase-FOXO1-3'UTR mutant, similar luciferase levels were observed between miR-NC- and miR-590-transfected 293T cells (**Figure 6C**). Western blot analysis revealed that both FOXO1 and p-FOXO1 were upregulated in miR-590-5p-knockdown HepG2 and Bel-7404 cells (**Figure 6D**). Immunofluorescence staining showed a larger number of FOXO1-positive HepG2 cells after stable transfection with the lenti-anti-miR590-5p, relative to those transfected with miR-NC (**Figure 6E**). Marked upregulation of FOXO1 and p-FOXO1 expression was detected in HepG2 and Bel-7404 tumors in the miR-590 knockdown group (**Figure 6F**, **6G**). Knockdown of miR-590-5p promoted pro-apoptosis protein Bim expression and inhibited Bcl-2 expression, accompanied with upregulation of p21 in liver cancer cells (**Figure 6H**). Next, in order to investigate whether FOXO1 plays a crucial role in promoting miR-590-mediated cancer cell growth, HepG2 and Bel-7404 cells were transfected with siRNAs targeting FOXO1. As shown in **Figure 6I**, siRNA-FOXO1 effectively inhibited anti-miR590-5p-induced FOXO1 and p-FOXO1 upregulation in both HepG2 and Bel-7404 cells. Furthermore, results of CCK-8 assay demonstrated that reduced cell viability caused by miR-590-5p knockdown was blocked by siRNA-FOXO1 transfection in HepG2 (**Figure 6J**) and Bel-7404 (**Figure 6K**) cells. The above results suggested that FOXO1 is a direct target of miR-590-5p and plays a crucial role in promoting miR-590-5p-mediated cancer cell growth.

Discussion

miRNAs represent a class of snRNAs that play a central role in gene silencing, and function as a part of large gene regulatory networks. In the present study, we demonstrated that miR-590-5p is upregulated in liver cancer and that miR-590-5p levels are inversely correlated with disease-free survival of liver cancer patients. Furthermore, *in vitro* and *in vivo* results verified the anti-tumor effects of miR-590-5p knockdown in liver cancer, which promote apoptosis, cell cycle arrest and chemotherapy sensitivity. miR-590-5p knockdown experiments showed that FOXO1 is a direct target of miR-590-5p and plays a crucial role in inhibiting cancer cell growth.

miR-590 expression is dysregulated in various cancers. Previous studies have demonstrated that miR-590-5p is upregulated in human cervical cancer tissues [9], lung adenocarcinoma tissues, and metastatic lymph nodes [11]. miR-590-5p was also identified as a hypoxia-sensitive miRNA that is upregulated in colon cancer cells under hypoxia [10]. By contrast, miR-590-5p expression was found to be downregulated in breast cancer patients [15]. Our study is the first to demonstrate that miR-590-5p is upregulated not only in malignant tissues from liver cancer patients but also in plasma from chemotherapy resistant liver cancer patients. Further analysis confirmed the inverse correlation between miR-590-5p expression and tumor progression in liver cancer patients. Notably, our results highlighted the predictive role of miR-590-5p expression in poor disease-free survival of liver cancer patients. Thus, miR-590-5p can be used as a biomarker for predicting the prognosis and chemotherapy responses of liver cancer patients.

The role of miR-590 in tumors has been debated in previous studies. miR-590-3p overexpression has been demonstrated to promote cell proliferation and metastasis by targeting the Hippo pathway, and predict poor clinical outcomes in colorectal cancer patients [17]. Meanwhile, miR-590-3p functions as a sup-

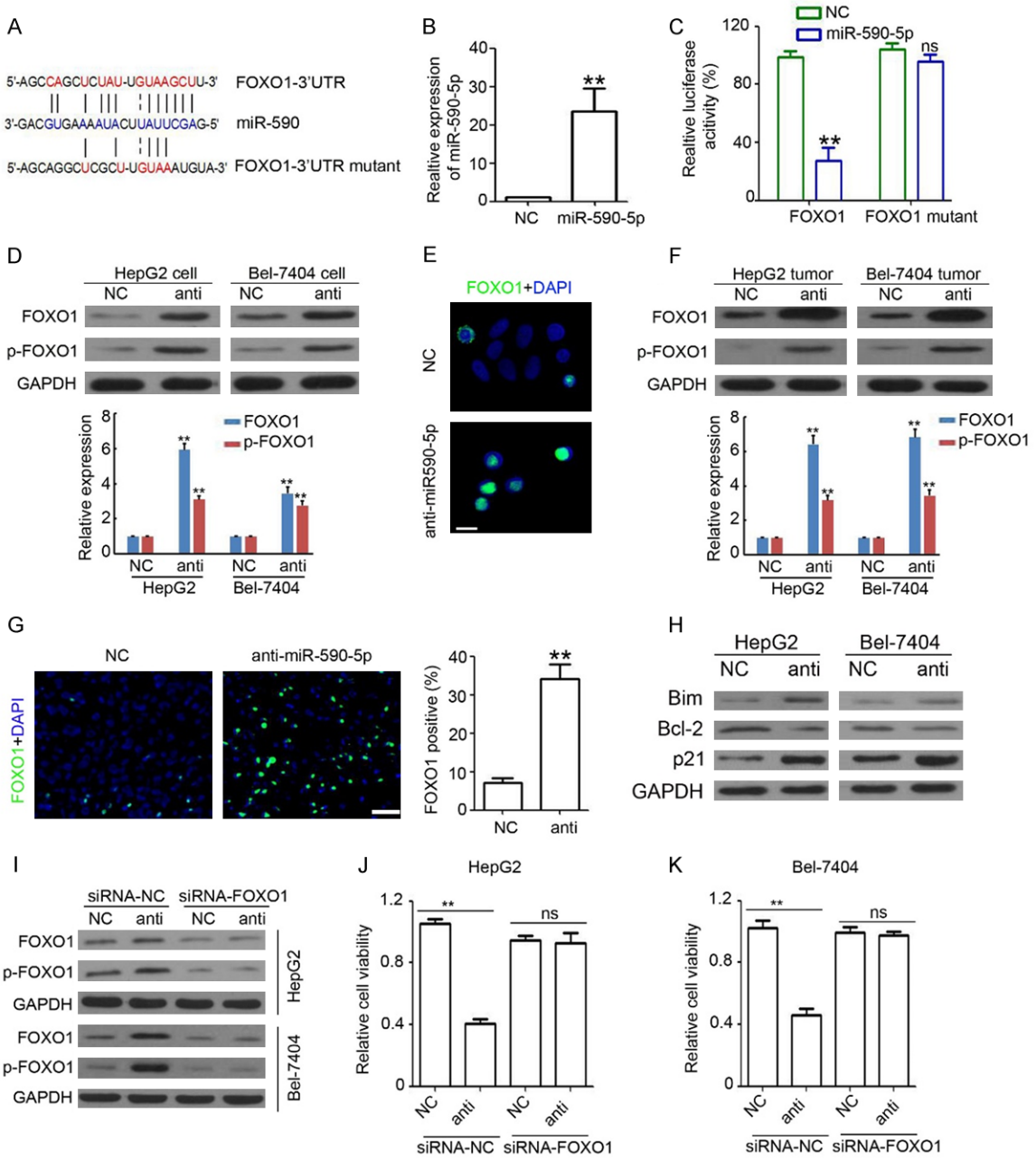

Figure 6. miR-590-5p directly targets FOXO1. A. FOXO1-3'UTR contains one predicted miR-590-5p binding site. The figure shows predicted duplex formations between FOXO1-3'UTR and miR-590-5p. The sites of target mutagenesis are indicated in blue. B. Real-time PCR analysis of miR-590-5p in 293T cells after enforced expression of miR-590-5p in 293T cells. C. Relative repression of luciferase expression was standardized to a transfection control. **, P < 0.01; ns, no significant difference, compared to miR-NC (NC). D. Western blotting analysis of FOXO1 and p-FOXO1 expression in HepG2 and Bel-7404 cells that stably infected with lentivirus-based miR-NC (NC) or anti-miR590-5p (anti). GAPDH was used as loading control. Relative expression analysis of FOXO1 and p-FOXO1 expression. (n = 3, **, P < 0.01, compared with miR-NC transfected group). E. Immunofluorescence staining of FOXO1 expression and location in HepG2 cells that stably infected with lentivirus-based miR-NC (NC) or anti-miR590-5p. The cell nucleic was stained by DAPI. Scale bar = 20 μm. F. Western blotting analysis of FOXO1 and p-FOXO1 expression in HepG2 and Bel-7404 tumors that stably infected with lentivirus-based miR-NC (NC) or anti-miR590-5p (anti). GAPDH was used as loading control. Relative expression analysis of FOXO1 and p-FOXO1 expression (n = 3, **, P < 0.01, compared with miR-NC transfected group.). G. Immunofluorescence staining of FOXO1 expression in HepG2 tumors. The cell nucleic was stained by DAPI. Scale bar = 100 μm. H. Western blotting analysis of Bim, Bcl-2, p21 expression in HepG2 and Bel-7404 cells that stably infected with lentivirus-based miR-NC (NC) or anti-miR590-5p (anti). GAPDH was used as loading control. I. Western blotting analysis of FOXO1 and p-FOXO1 expression in HepG2-NC, HepG2-

anti-miR590-5p, Bel-7404-NC and Bel-7404-anti-miR590-5p cells that transfected with siRNA-NC or siRNA-FOXO1. GAPDH was used as loading control. J, K. The relative cell viability of HepG2-NC, HepG2-anti-miR590-5p, Bel-7404-NC and Bel-7404-anti-miR590-5p cells that transfected with siRNA-NC or siRNA-FOXO1 for 48 hours (n = 5, **, P < 0.01; ns, no significant difference).

pressor of metastasis by targeting ZEB1 and ZEB2 in glioblastoma multiforme [18]. miR-590-5p functions as an anti-onco-miR that inhibits colorectal cancer angiogenesis and metastasis by regulating the NF90/VEGFA signaling axis [14]. However, subsequent studies identified miR-590-5p as an onco-miRNA in human cervical cancer [9], colorectal cancer [10], lung adenocarcinoma [11], and gastric cancer [12]. Our findings demonstrated the positive role of miR-590-5p in liver cancer, as evidenced by the observed tumor growth inhibition under miR-590-5p knockdown conditions both *in vitro* and *in vivo*. The role of miR-590-5p as an onco-miR in liver cancer was consistent with the results reported by Jiang *et al.* [19]. However, further studies should be conducted to establish the role of miR-590-5p as an onco-miR in liver cancer.

The transcription factor FOXO1 is a key effector of PI3K/Akt signaling and is known to regulate various biological processes, such as cell cycle regulation, cell differentiation, tumorigenesis, and oxidative stress responses [20-22]. FOXO1 levels are higher in HCC patients with better prognosis than in patients with poor prognosis [23, 24]. Inhibition of FOXO1 by miR-196a was demonstrated to promote human liver cancer cell growth [25]. Downregulation of FOXO1 expression by the secretory miR-27a was found to promote liver cancer cell proliferation by upregulating the expression of the cell cycle regulator cyclin D1 [26]. Luciferase assay and molecular analysis suggested that FOXO1 is a direct and necessary target of miR-590-5p for regulating liver cancer growth, accompanied with regulation of downstream targets of FOXO1 by miR-590-5p in liver cancer cells. Previous studies have demonstrated that FOXO1 overexpression upregulates the expression of caspase family proteins and downregulates the expression of cyclin family proteins in miR-590-5p-deficient liver cancer cells [27-29].

Previous miRNA expression profile analysis indicated that miR-590-5p was upregulated in both chemotherapy resistance and chemoradiation therapy resistance patients [30, 31]. However, no study investigated the potential role of miR-590-5p in chemotherapy resistance. In our study, we first demonstrated the upregulation of miR-590-5p in 5-Fu resistant liver cancer patients and cells. Notably, knockdown of miR-590-5p enhanced chemosensitivity to 5-Fu in liver cancer. This may contributed to anti-miR590-5p-mediated upregulation of FOXO1 expression, which was involved in chemotherapy resistance of liver cancer cells and breast cancer cells [32, 33].

Taken together, our results demonstrated that miR-590-5p upregulation is inversely correlated with disease-free survival and poor chemotherapy responses of liver cancer patients. MiR-590-5p promotes tumor cell growth and 5-Fu resistant by directly targeting FOXO1 in liver cancer. Our findings improved our understanding of the role of miR-590-5p in liver cancer and highlighted miR-590-5p as a novel therapeutic target and predictive biomarker for liver cancer.

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Disclosure of conflict of interest

None.

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