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Original Article

# The miR-491-3p/Sp3/ABCB1 axis attenuates multidrug resistance of hepatocellular carcinoma

Yang Zhao [a, b], Xinming Qi [a, b], Jing Chen [a, b], Wenxin Wei [c], Cunzhi Yu [a, b], Hong Yan [a, b], Mengfan Pu [a, b], Yu Li [a, b], Lingling Miao [a, b], Chunzhu Li [a, b, *], Jin Ren [a, b, **]

[a] Center for Drug Safety Evaluation and Research, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China
[b] Center for Drug Safety Evaluation and Research, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, University of Chinese Academy of Sciences, Beijing 100049, China
[c] Department of Hepatic Surgery, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China

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ABSTRACT

As one of main obstacles in the treatment and prognosis of hepatocellular carcinoma (HCC), multidrug resistance (MDR) is usually associated with the overexpression of the drug efflux pump P-glycoprotein (P-gp/ABCB1) which is responsible for reducing the intracellular concentration of chemotherapeutic agents. In current work, we discovered the novel role of miR-491-3p in ABCB1-mediated multidrug resistance in HCC and revealed the underlying mechanism in which miR-491-3p downregulated the expression of ABCB1 and its transcription factor Sp3 by directly targeting their 3′-UTR. Moreover, overexpressing ABCB1 or Sp3 reversed the sensitivity to chemotherapeutics in Hep3B cells induced by miR-491-3p, confirming miR-491-3p/Sp3/ABCB1 regulatory loop plays an important role in enhancing the drugs sensitivity of HCC. Meanwhile, the discovery of that the expression level of miR-491-3p was inversely correlated with that of ABCB1 and Sp3 in HCC cell lines and clinical samples pointed out the possibility of miR-491-3p in clinical use. In summary, our results reveal a pivotal role of miR-491-3p in the regulation of MDR in HCC, and suggest the potential application of miR-491-3p as a therapeutic strategy for modulating MDR in cancer cells.



## Introduction

Hepatocellular carcinoma (HCC) is the fifth solid tumor worldwide and causes about half a million deaths every year [1,2]. Although anti-cancer drugs against HCC have been used in clinic for decades, the efficient treatments of HCC still lack mostly due to the resistance of cancer cells to chemotherapy, also called multidrug resistance (MDR). MDR is mediated by high expression of adenosine triphosphate (ATP)-binding cassette (ABC) transporter family members that increase the efflux of chemotherapeutic agents out of cancer cells [3–5]. P-glycoprotein (P-gp), a 170-kDa transmembrane glycoprotein encoded by the ABCB1 gene on human chromosome 7p21, is one of the best studied ABC transporters in drug resistance [6,7]. In addition, P-gp is considered to contribute to drug resistance in various types of cancer, and changes in its expression or function could contribute to MDR [6–10]. Thus, reducing P-gp expression might assist in overcoming MDR in cancer chemotherapy.

MicroRNAs (miRNAs) are a class of endogenous, single-stranded, small non-coding RNAs that are 18–25 nucleotides in length and function as negative regulators of gene expression [11]. MiRNAs can trigger either mRNA degradation or translational repression by binding to the 3′ untranslated region (3′-UTR) of specific mRNAs with perfect or near-perfect complementary, respectively [12,13]. Emerging evidence shows that miRNAs play a relevant role in the initiation, development and progression of tumors [14–16]. In recent years, miRNAs have been reported to be involved in the modulation of MDR in various cancers [17,18]. For instance, Wu et al. demonstrated that let-7g and let-7i enhanced cellular

*abbreviations:* HCC, hepatocellular carcinoma; MDR, multidrug resistance; 3′-UTR, 3′ untranslated region; NC, negative control oligonucleotides; Ctrl, control; wt, wild-type; mt, mutant.

* Corresponding author. Center for Drug Safety Evaluation and Research, Shanghai Institute of Materia Medica, 501 Haike Road, Shanghai 201203, China.
** Corresponding author. Center for Drug Safety Evaluation and Research, Shanghai Institute of Materia Medica, 501 Haike Road, Shanghai 201203, China.
*E-mail addresses:* czli@cdser.simm.ac.cn (C. Li), jren@cdser.simm.ac.cn (J. Ren).

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sensitivity to drugs by directly targeting ABCC10 [19]. Moreover, miR-181a was found to restore sensitivity to mitoxantone-resistant cells by targeting breast cancer resistance protein (BCRP/ABCG2) [20]. It was also reported that hyper-methylation of miR-129-5p island played important roles in the development of gastric cancer chemo-resistance by targeting ABC transporters [21]. Therefore, miRNA targeting drug transporters has been proven to be effective in cancer therapy.

In this study, we obtained a list of candidate miRNAs that potentially targeted the 3′-UTR of ABCB1 via using bioinformatics algorithms TargetScan and Microcosm, and then miR-491-3p was discovered to remarkably reduce P-gp expression in hepatoma cell lines. Moreover, miR-491-3p was significantly downregulated in HCC clinical samples. To better understand the regulatory network between miRNA and ABCB1, we also focused on the transcription factors of ABCB1, and found Sp3 might be another target gene of miR-491-3p. Thereby, our results suggested that miR-491-3p downregulated ABCB1 via a dual inhibitory pathway: (i) directly targeting the ABCB1 3′-UTR (post-transcriptional inhibition), and (ii) indirectly targeting the promoter of ABCB1 via its transcription factor, Sp3 (transcriptional inhibition), resulting in increased sensitivity of hepatoma cells to chemotherapeutic drugs.

## Materials and methods

### *Cell culture and tissue samples*

Three human hepatocellular carcinoma cell lines Hep3B, BEL-7402 and SMMC-7721 were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. Two immortalized human liver epithelial cells lines THLE-2 and THLE-3 were obtained from American Type Culture Collection (ATCC, USA). Hep3B cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Hyclone, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma, USA) and antibiotics (penicillin and streptomycin, 50 units/mL each) (Invitrogen). BEL-7402 and SMMC-7721 were cultured in RPMI-1640 medium (Gibco, USA) containing 10% FBS and antibiotics (penicillin and streptomycin, 50 units/mL each). THLE-2 and THLE-3 cells were cultured in BMEM medium (Lonza/Clonetics) supplemented with 5 ng/mL EGF, 70 ng/mL Phosphoethanolamine and 10% FBS. All cells were cultured at 37 °C in a humidified air atmosphere containing 5% $CO_2$.

Human hepatocellular carcinoma tissues were obtained from Eastern Hospital of Hepatobiliary Surgery (Shanghai, China). This study was approved by the Ethics Committee of Eastern Hospital of Hepatobiliary Surgery.

### *Cell transfection*

MiRNA mimics, inhibitors and their negative control oligonucleotides (NC) were purchased from GenePharm (Shanghai, China). siRNAs targeting Sp3 were obtained from RiboBio Co. Ltd (Guangzhou, Guangdong, China). ABCB1 and Sp3 overexpression plasmids were purchased from Sino Biological Inc (Beijing, China). After seeded in 6-well plates at $2 \times 10^5$ cells/well, cells were cultured for 16 h, and then transfected with the oligonucleotides or plasmids using Lipofectamine 2000 reagent and OPTI-MEM I reduced serum medium (Invitrogen) according to the manufacturer's instructions.

### *Drug sensitivity assay*

Hep3B or SMMC-7721 cells at the densities of $2 \times 10^5$ cells/well in 6-well plates were cultured for 16 h and then transfected with 50 nM miR-491-3p/NC mimics or inhibitors, respectively. $5 \times 10^3$ cells were reseeded in 96-well plates after 72 h transfection, and treated with medium containing doxorubicin (DOX) or vinblastin (VBL) for 48 h. Finally, 10 μl of CCK-8 (Cell Counting Kit-8) was added into each well, and incubated for a further 2 h. The absorbance of each sample was measured at 450 nm by the microplate reader.

### *RNA extraction and real-time qRT–PCR*

Total RNA extracted from cells or tissues by UNIQ-10/Trizol total RNA extraction kit (Sangon, Shanghai, China) was reverse-transcribed into cDNA using the PrimeScript RT Reagent Kit (Takara, Otus, Shiga, Japan). qRT–PCR was performed to detect ABCB1 and Sp3 mRNA using the QuantiNova SYBR Green PCR kit (Qiagen, Valencia, CA, USA). ABCB1 and Sp3 mRNA expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels. Primer sequences were listed as follows: ABCB1 (human) sense 5′–CCCATCATTGCAATAGCAGG-3′, antisense 5′-TGTTCAAACTTCTGCTCCTGA-3′, Sp3 (human) sense 5′-ATGACTGCAGGCATTAATGCCG-3′, antisense 5′-TGTCTCTTCAGAAACAGGCGAC-3′, GAPDH (human) sense 5′-GGTGGTCTCCTCTGACTTCAACA-3′, antisense 5′-GTTGCTGTAGCCAAATTCGTTGT-3′.

MiRNA was isolated using the *mir*Vana™ miRNA isolation kit (Ambion, Austin, TX). MicroRNA cDNAs were synthesized using the Taqman® MicroRNA Reverse Transcription Kit (Invitrogen). Taqman® MiRNA Assays (Invitrogen) were used to amplify the expression of miR-491-3p (cat. no. 4427974, ID 002360) and RNU6B (cat. no. 4427975, ID 001093) according to the manufacturer's instructions. Amplification and detection of specific products were performed with Rotor-Gene Q 2plex HRM System (Qiagen, Valencia, CA, USA).

### *Western blot analysis*

Cells or tissues were solubilized in radio immunoprecipitation assay (RIPA) lysis buffer (Beyotime, China) containing 1:1000 phenylmethanesulfonyl fluoride (Beyotime, China). Equal amounts of proteins were separated by 8% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated with antibodies against P-gp (1:1000; Abcam, USA), Sp3 (1:1000; Santa Cruz Biotechnology, USA), and β-actin (1:1000, Santa Cruz Biotechnology, USA). Antibody binding was assessed by incubation with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., USA). Chemiluminescence was detected using an ECL Plus immunoblotting detection system (Millipore, Billerica, MA, USA).

### *Luciferase assay*

The cDNA fragment corresponding to the 3′-UTR of ABCB1 or Sp3 mRNA was inserted into the downstream region of the luciferase gene in the psiCHECK™-2 Vector (Promega, USA). Bases mutant lacking the seed sequence of miR-491-3p binding sites in 3′-UTR of ABCB1 or Sp3 mRNA was created using KOD Plus Mutagenesis kit (TOYOBO, Japan) according to the manufacturers' protocol. Seventy-two hours after transfection, cells were lysed with 1 × passive lysis buffer and both of firefly and *Renilla* luciferase activities were measured with the Dual-Luciferase Reporter Assay System (Promega, USA). Luciferase activity values were normalized to firefly luciferase values.

The promoter region (−790 to +63-bp) of the ABCB1 gene was amplified and cloned into pGL3-basic vector. Cell extracts were prepared 48 h after transfection, and then the luciferase activity was determined. Luciferase activity values were normalized to *Renilla* luciferase values.

### *Flow cytometry*

Fluorescene intensity of intracellular DOX was measured by flow cytometry. Briefly, $2 \times 10^5$ cells were seeded in 6-well plates and cultured overnight at 37 °C. Then the cells were transfected with 50 nM miR-491-3p/NC mimics or inhibitors. After 72 h, the cells were incubated in medium containing DOX (15 μM) for 3 h. At the end of incubation, cells were washed thrice with ice-cold PBS and the fluorescence intensity of DOX in the cells was measured using FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

### *CRISPR/Cas9-mediated miR-491-3p knockdown*

CRISPR/Cas9-mediated nucleotide deletion was performed as previously described [22]. Two sgRNAs (5′-caccgagtgttctactcctcatgga-3′; 5′-caccgggttggagtcatatcctgc-3′) were cloned into px330-mCherry and px330-GFP vectors, respectively. The plasmids were co-transfected into SMMC-7721 cells, and positively transfected cells were isolated using flow cytometry and used in the related experiments.

### *Statistical analysis*

Data are presented as mean ± SD (standard deviation) of at least three independent experiments. Student's *t*-test (two-tailed) or a one-way analysis of variance was employed to analyze data unless otherwise mentioned. Spearman's correlation test was used to analyze the relationship between the expression of miR-491-3p and its targets in HCC tumor samples. All statistical analyses were performed using SPSS v.11.5 software (SPSS, Chicago, IL, USA). A *P*-value of less than 0.05 was considered statistically significant.

## Results

### *Identification of candidate miRNAs inhibiting P-gp expression in Hep3B cells*

Given the crucial role of P-gp in MDR and the possibility of miRNA targeting drug transporters in cancer therapy, we predicted candidate miRNAs potentially binding to the 3′-UTR of human ABCB1 (P-gp) using the bioinformatics algorithms Targetscan and MicroCosm Targets. MiRNAs at the top of prediction list were selected to identify which one can efficiently affect P-gp expression in hepatocarcinoma. In the screening assay, miRNAs mimics (50 nM) were separately transfected into Hep3B cells, and then the