

# MicroRNA-140-3p enhances the sensitivity of hepatocellular carcinoma cells to sorafenib by targeting pregnenolone X receptor

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**Background:** Pregnane X receptor (PXR), which is a member of the nuclear receptor protein family (nuclear receptor subfamily 1 group I member 2 [NR 1I2]), mediates the drug-resistance in the hepatocellular carcinoma (HCC) via enhancing the expression of drug-resistance-related genes which accelerate the clearance of antitumor drugs, eg, sorafenib. However, there are few reports on miRNA targeting PXR participating in the epigenetic regulation of PXR in HCC cells.

**Materials and methods:** TargetScan 7.2, an online method, was used to predict the miRNAs potentially targeting PXR. The expression of PXR and PXR downstream genes was detected by quantitative real-time PCR (qPCR) and Western blot. The clearance of sorafenib in HCC cells was monitored by liquid chromatograph-mass spectrometer/mass spectrometer (LC-MS/MS). The effects of miRNA on sorafenib's efficacy were examined by in vitro methods, eg, MTT, and in vivo methods, eg, subcutaneous or intrahepatic tumor model.

**Results:** By virtual screening, we identified that miR-140-3p possibly targets PXR and then confirmed that the overexpression of miR-140-3p via lentiviral particles inhibited the expression of PXR in HCC cells. The downregulation of PXR's expression by miR-140-3p led to the reduction of PXR downstream genes' expression, which finally resulted in the decelerating clearance of sorafenib in HCC cells and enhanced the sensitivity of HCC cells to sorafenib. The effect of miR-140-3p could not modulate the expression of mutated PXR and the effect of miR-140-3p could also be inhibited by miR-140-3p's inhibitor. Moreover, miR-140-3p enhanced the anti-tumor effect of sorafenib in both the subcutaneous and intrahepatic HCC tumor models.

**Conclusion:** Our study suggests that targeting PXR by miR-140-3p is a promising strategy for enhancing sorafenib's efficacy during HCC treatment.

**Keywords:** hepatocellular carcinoma, microRNA, pregnenolone X receptor, sorafenib

## Introduction

Pregnane X receptor (PXR) is a member of the nuclear receptors (NRs) protein family (classified as NR1I2).<sup>1,2</sup> In mammal cells, PXR could function as a transcription factor that could be activated by endogenous or exogenous ligands, in turn leading to the transcription of downstream genes, eg, CYP/CYP450 (CYP450) or ATP-binding cassette (ABC), which often participate in drug-resistance or drug-metabolisms.<sup>3,4</sup> Previously, PXR was considered as one of the key regulators mediating the expression of phase I, II or III drug metabolism enzymes (DMEs) to accelerate the clearance of exogenous drugs to protect the human body and liver organ from drug-induced injuries.<sup>5-7</sup> Recently, PXR has also been considered to play an important role in the resistance of antitumor drugs.<sup>8,9</sup> Our recent study indicated that sorafenib, an oral

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molecular-targeted antitumor agent, enhances its own clearance in hepatocellular carcinoma (HCC) cells via enhancing the activation of PXR during treatment.<sup>11</sup> Although current researches are focusing on PXR as a potential target to overcome drug-resistance of HCC treatment, the approaches targeting PXR have been poorly developed.

MicroRNAs (miRNAs or miRs) are a series of RNAs which repress the targeted messenger RNAs (mRNAs) via recognizing and interacting their 3'-untranslated regions (3'-UTR), and miRs have critical roles in regulating proliferation, metastasis or apoptosis of human cancer cells.<sup>12,13</sup> Many miRs have been applied for regulating cancers' progress via targeting cancer-related signaling pathways.<sup>14,15</sup> In the present work, we identified miRNAs potentially targeting PXR. By screening a series of miRNAs via online tools, PXR would be a target of miR-140-3p. Transfection of miR-140-3p decreased the expression of PXR or its downstream genes and enhanced the sensitivity of HCC cells to sorafenib.

## Materials and methods

### Plasmids and reagents

The expression vector of PXR with a mutation of miR-140-3p target sequences in 3'-UTR was constructed and purchased from Vigene Corporation, Jinan City, Shandong Province, People's Republic of China. For cell-based experiments, the mimic miR-140-3p (Cat. no 4464070) and miR-140-3p inhibitor (Cat. no 4464088) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). MHCC97-H (a highly metastatic cell line of HCC) and HepG2 (a cell line of HCC) cell lines were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, People's Republic of China), the culture collection center of the Chinese government. Cells were preserved in our laboratory; maintaining conditions are described in our previous work.<sup>16,17</sup> Control miRNA (random molecular agent), was a gift from Dr Mingyang Li in the General Hospital of Chinese PLA. For animal experiments, a vector with miR-140-3p was constructed and purchased from Vigene Corporation. Sequences of miR-140-3p were prepared into lentivirus particles. The antitumor agent sorafenib (Cat. no S7397) was purchased from Selleck Corporation, Houston, TX, USA. For cell culture experiments, sorafenib were carefully dissolved in dimethyl sulfoxide (DMSO) and the concentrations of sorafenib in DMSO were 10, 3, 1, 0.3, 0.1, 0.03 or 0.01 mmol/L. For subcutaneous tumor experiments, sorafenib (4 mg) was dissolved by a mixture of DMSO (15  $\mu$ L), PEG400 (60  $\mu$ L) and Tween80 (40  $\mu$ L). Then, the solution was carefully added with physiological saline to a

total volume 2 mL. This sorafenib-containing solution was named as Sor-Sol. Rifampicin (Cat. no HY-B0272), which is a typical agonist of PXR, was purchased from MedChem Express (MCE) (Monmouth Junction, NJ, USA). Rifampicin was dissolved in DMSO and the indicated concentrations of rifampicin in DMSO were 10, 3, 1, 0.3, 0.1, 0.03 or 0.01 mmol/L.

### Luciferase reporter gene assay

MHCC97-H or HepG2 cells, which were transfected with control miRNA or miR-140-3p, were seeded in 24-well plates (Corning Incorporated, Corning, NY, USA), and were co-transfected with luciferase reporters (DR3-Luc, ER6-Luc, XREM-Luc or PXRE-Luc).<sup>18</sup> After being cultured for 24 hours, cells were harvested and analyzed for luciferase activities, as described by Ma et al.<sup>19</sup> For luciferase experiments, the concentrations of rifampicin on HCC cells were 10, 3, 1, 0.3, 0.1, 0.03 or 0.01  $\mu$ mol/L diluted from rifampicin solution (1:1,000).

### RNA extraction and qPCR experiments

MHCC97-H or HepG2 cells were treated with the indicated concentrations of rifampicin (10, 3, 1, 0.3, 0.1, 0.03 or 0.01  $\mu$ mol/L) for 24 hours. The cells were then harvested and the total RNA samples were extracted and qPCR experiments were performed following protocols provided by the manufacturer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). The expression of drug-resistance-related genes within MHCC97-H cells was examined by qPCR with primers listed in Table 1.

### Antibodies and Western blot

Antibodies against PXR (Cat. no ab85451), CYP3A4 (Cat. no ab155029), P-glycoprotein (Cat. no ab235954) and  $\beta$ -actin (Cat. no ab205) were obtained from Abcam (Cambridge, MA, USA). Cells, which were transfected with control miRNA,

**Table 1** Primers used in qPCR experiments

Targets	Primers
PXR	Forward primer: 5'-AGAGCGGCATGAAGAAGGAGATG-3' Reverse primer: 5'-GAAATGGGAGAAGGTAGTGTCAGG-3'
CYP3A4	Forward primer: 5'-CTAGCACATCATTGGACTG-3' Reverse primer: 5'-ACAGAGCTTTGTGGGACT-3'
P-gp (MDR-1)	Forward primer: 5'-CCATAGCTCGCGCCCTTGTAGAG-3' Reverse primer: 5'-CGGTGAGCAATCACAATGCAG-3'
$\beta$ -actin	Forward primer: 5'-CTCCATCCTGGCCTCGCTGT-3' Reverse primer: 5'-GCTGTACCTTACCCTTCC-3'

**Abbreviations:** PXR, pregnane X receptor; qPCR, quantitative real-time PCR.

miR-140-3p mimic, PXR<sup>Mut</sup> or miR-140-3p inhibitor, were harvested to be extracted for Western blot experiments, which were performed following the standard protocol of Western blot experiments.

## Cell culture and proliferation analysis

HCC cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA, USA) with 0.5%–1% fetal bovine serum (FBS) (Thermo Fisher Scientific) in a sterile incubator maintained at 37°C under 5% CO<sub>2</sub> condition. For drug antitumor, cells were seeded in 24-well plate (Corning Incorporated) and incubated by the indicated concentrations (10, 3, 1, 0.3, 0.1, 0.03 or 0.01 μmol/L) of sorafenib diluted by sorafenib DMSO solution (final DMSO concentration was 1%). Then, cells were incubated by sorafenib for 48 hours and harvested for MTT experiments. The inhibition rates of sorafenib on HCC cells were calculated by the methods described by Feng et al.<sup>20</sup>

## Subcutaneous HCC tumor model

Animal experiments were permitted by the Institutional Animal Care and Use Committee of the 302nd Hospital, People's Liberation Army of China. All animal studies were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 and associated guidelines. For subcutaneous tumor experiments,<sup>21,22</sup> nude mice (severe combined immune deficiency mice) aged 4–6 weeks were purchased from Si-Bei-Fu Biotechnology Corporation, Beijing, People's Republic of China. MHCC97-H or HepG2 cells which were cultured in DMEM adding 10% FBS, were injected into nude mice (5×10<sup>6</sup> cells per inoculation point). After 2–4 weeks' growth, the tumoral volume reached almost 1,000–1,200 mm<sup>3</sup>.

## Clearance of sorafenib in HCC cells

To examine the clearance of sorafenib in HCC cells, MHCC97-H or HepG2 cells were transfected with miR-140-3p or PXR expression plasmids, and were treated with 1 μmol/L (the IC<sub>50</sub> concentration of sorafenib on MHCC97-H cells) for 12 hours. For cultured MHCC97-H cells, cells were harvested after sorafenib treatment at the indicated time points and then harvested for liquid chromatograph-mass spectrometer/mass spectrometer (LC-MS/MS) examination.<sup>23</sup> For the subcutaneous tumor model, MHCC97-H or HepG2 cells which were infected with miR-140-3p or PXR, were seeded into nude mice to form subcutaneous tumors. Sor-Sol was injected into subcutaneous tumors (20 μL per tumor) in

the subcutaneous tumors of the nude mice formed by HCC cells. Cells or tumor tissues were harvested and the sorafenib was extracted by acetonitrile (ACN) at the indicated time points. The amount of sorafenib in tumor tissues was measured by LC-MS/MS.<sup>11,23</sup>

## In vivo tumor growth

MHCC97-H cells, which were transfected with plasmids, were injected into the nude mice to form subcutaneous tumors or into the liver organs via hepatic portal vein injection.<sup>24</sup> Five days after injection, the nude mice were orally administered 2 mg/kg sorafenib treatment every 2 days. After 10 × treatments for 21 days, the mice were collected. For the subcutaneous tumor model, tumors were harvested and the tumor weights or volumes were examined following the methods described in the references.<sup>21,22</sup> For the intrahepatic growth model, mice were harvested and the liver organs with nodules formed by MHCC97-H were collected for taking photographs. Photographs were quantitatively analyzed to determine the total amount of nodules by ImageJ software following the methods described by Xie et al.<sup>24</sup> The inhibition rate of each group was calculated as [control group relative nodule area (percentages of nodules to total area of liver organs, %) – administration group relative nodule area]/control group relative nodule area × 100%.

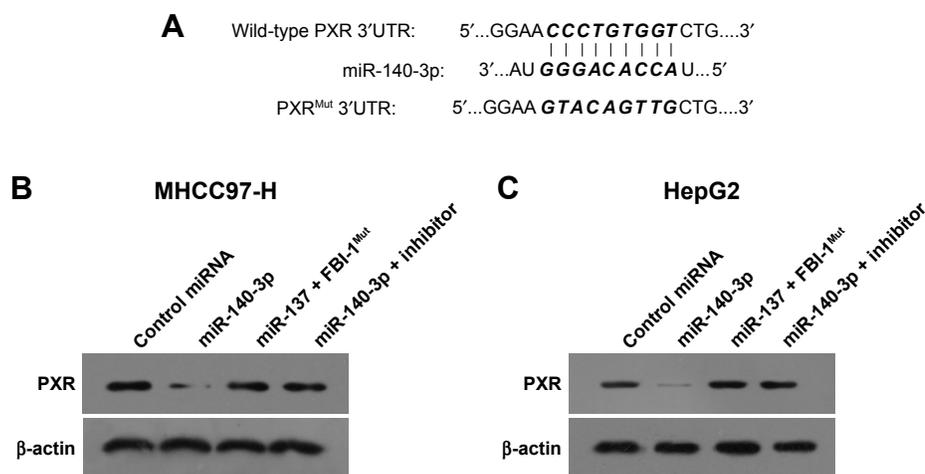
## Statistical analysis

Statistical analysis was performed by Bonferroni's correction with or without two-way ANOVA, SPSS Software (IBM Corporation, Armonk, NY, USA). The IC<sub>50</sub> values half-life (t<sub>1/2</sub> value) values were calculated by an Origin software (Version No 6.1, OriginLab Corporation, Northampton, MA, USA). A *P*-value of <0.05 was considered statistically significant.

## Results

### Transfection of miR-140-3p decreased PXR expression by targeting PXR mRNA's 3'-UTR

In order to predict the miR potentially targeting PXR, online tools TargetScan and MiRanda database were used, and it was found that PXR was a potential target of miR-140-3p: the italicized font in Figure 1A indicates the miR-140-3p targeted sequence within PXR mRNA's 3'-UTR (Figure 1). Next, MHCC97-H cells and HepG2 cells were transfected with control miRNA, miR-140-3p mimic, miR-140-3p mimic+ PXR<sup>Mut</sup> or miR-140-3p mimic+ inhibitor for 48 hours and harvested for Western blot. We found that compared



**Figure 1** miR-140-3p targets PXR's 3'-UTR.

**Notes:** (A) The bold, italicized type indicates the miR-140-3p binding sites or mutated site within PXR's 3'-UTR. (B) MHCC97-H or (C) HepG2 cells, which were transfected with control miRNA, miR-140-3p mimic, miR-140-3p mimic + PXR<sup>Mut</sup> or miR-140-3p mimic + inhibitor, were harvested for Western blot analysis. The protein level of PXR or  $\beta$ -actin was examined by their antibodies.

**Abbreviations:** miR, microRNA; PXR, pregnane X receptor; Mut, mutation; 3'-UTR, 3'-untranslated regions.

with control miRNA, miR-140-3p significantly decreased the expression of PXR (Figure 1B and C) but not PXR<sup>Mut</sup> which had a mutation in the miR-140-3p-binding site (shown in Figure 1A–C); whereas transfection of miR-140-3p's inhibitor could block the effect of miR-140-3p on PXR's expression in MHCC97-H cells (Figure 1B) or HepG2 cells (Figure 1C). Therefore, miR-140-3p could repress the expression of PXR in HCC cells by directly targeting PXR mRNA's 3'-UTR.

### MiR-140-3p inhibits PXR pathway's activation

To further identify the effect of miR-140-3p on PXR, luciferase experiments were performed. As shown in Table 2, PXR's agonist rifampicin could induce the transcription factor activity of PXR, whereas miR-140-3p blocked the activity of PXR induced by rifampicin. Moreover, transfection of PXR<sup>Mut</sup> or miR-140-3p's inhibitor decreased the

effect of miR-140-3p on PXR's transcription factor activity. Accordingly, miR-140-3p inhibited the effect of rifampicin on the mRNA or protein expression of PXR downstream genes, *CYP3A4* and *MDR-1*. Transfection of PXR<sup>Mut</sup> or miR-140-3p's inhibitor inhibited the effect of miR-140-3p (Table 3 and Figure 2). Therefore, miR-140-3p inhibits PXR pathway activation.

### MiR-140-3p decelerates the clearance of sorafenib in HCC cells

Since PXR, *CYP3A4* and *MDR-1* are related to the clearance of sorafenib in HCC cells, we then aimed to study the correlation of miR-140-3p and the clearance of sorafenib in HCC cells. We performed sorafenib treatment on HCC cells in vitro or injected Sor-Sol in vivo in HCC tumors and detected the clearance of sorafenib under different conditions. As shown in Table 4, miR-140-3p decelerated the clearance of sorafenib in cultured HCC cells or subcutaneous HCC

**Table 2** The EC<sub>50</sub> values of rifampicin inducing luciferase reporters' activation in each group

Cell lines	Luciferase reporters	Control miRNA	miR-140-3p	miR-140-3p + PXR <sup>Mut</sup>	miR-140-3p + inhibitor
		EC <sub>50</sub> value of rifampicin ( $\mu$ mol/L)			
MHCC97-H	DR-3	1.80 $\pm$ 0.14	–	1.96 $\pm$ 0.10	2.39 $\pm$ 0.17
	ER-6	2.07 $\pm$ 0.07	–	2.17 $\pm$ 0.52	3.21 $\pm$ 0.76
	XREM-Luc	1.08 $\pm$ 0.15	–	1.77 $\pm$ 0.19	1.59 $\pm$ 0.08
	PXRE-Luc	1.98 $\pm$ 0.11	–	2.71 $\pm$ 0.41	2.96 $\pm$ 0.31
HepG2	DR-3	2.29 $\pm$ 0.52	–	1.91 $\pm$ 0.26	2.23 $\pm$ 0.76
	ER-6	2.95 $\pm$ 0.15	–	2.73 $\pm$ 0.30	3.84 $\pm$ 0.67
	XREM-Luc	1.29 $\pm$ 0.08	–	1.50 $\pm$ 0.25	1.69 $\pm$ 0.44
	PXRE-Luc	1.76 $\pm$ 0.55	–	2.15 $\pm$ 0.35	1.99 $\pm$ 0.42

**Note:** Data are presented as mean $\pm$ SD.

**Abbreviations:** miR, microRNA; EC<sub>50</sub>, concentration for 50% of maximal effect; PXR, pregnane X receptor.

**Table 3** The EC<sub>50</sub> values of rifampicin inducing the expression of PXR downstream gene *CYP3A4* or *MDR-1* in each group

Cell lines	Genes	Control miRNA	miR-140-3p	miR-140-3p + PXR <sup>Mut</sup>	miR-140-3p + inhibitor
MHCC97-H	<i>CYP3A4</i>	1.34±0.57	–	1.90±0.50	1.61±0.46
	<i>MDR-1</i>	1.81±0.29	–	1.40±0.22	1.92±0.40
HepG2	<i>CYP3A4</i>	2.01±0.38	–	2.52±0.53	2.41±0.39
	<i>MDR-1</i>	1.89±0.77	–	2.36±0.65	1.96±0.13

**Note:** Data are presented as mean±SD.

**Abbreviations:** miR, microRNA; PXR, pregnane X receptor.

tumors, the half-life ( $t_{1/2}$  value) of sorafenib in cultured HCC cells correspondingly increased. PXR<sup>Mut</sup> or miR-140-3p's inhibitor inhibited the effect of miR-140-3p on the clearance of sorafenib. Therefore, our in vitro and in vivo data suggest that miR-140-3p decelerated the clearance of sorafenib in HCC cells.

### MiR-140-3p enhances the sensitivity of HCC cells to sorafenib

To determine whether miR-140-3p enhances the in vitro antitumor effect of sorafenib on HCC cells, MTT assays were performed. As shown in Table 5, miR-140-3p enhanced the sensitivity of MHCC97-H and HepG2 cells to sorafenib, and the IC<sub>50</sub> value of sorafenib on MHCC97-H or HepG2 cells were decreased.

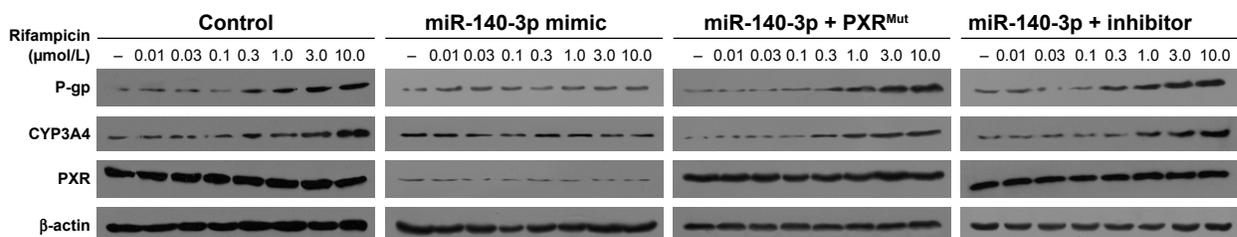
Next, the effect of miR-140-3p and sorafenib co-administration on subcutaneous HCC tumors was determined. As shown in Figure 3, treatment of sorafenib could significantly inhibit the subcutaneous growth of HCC cells in nude mice. Infection of miR-140-3p could enhance the antitumor effect of sorafenib on MHCC97-H cells' subcutaneous growth and PXR<sup>Mut</sup> expressing cells/tumors could not be affected by miR-140-3p (Figure 3D and E and Table 6).

To mimic the in vivo growth of MHCC97-H cells in mouse liver, MHCC97-H cells were injected into nude mice via the hepatic portal vein to form tumor nodules on their livers (Figure 4). We firstly established an oral

sorafenib administration method for this intrahepatic HCC tumor model (Figure 4C and Table 6). Then, we performed miR-140-3p and sorafenib co-administration. As shown in Figure 4, MHCC97-H cells could form multi-nodules in the livers via hepatic portal vein injection (Figure 4). The results showed that treatment of sorafenib could significantly inhibit the intrahepatic growth of MHCC97-H cells in the livers of nude mice (Figure 4 and Table 6). Infection of miR-140-3p in MHCC97-H cells could enhance the antitumor effect of sorafenib on MHCC97-H cells' intrahepatic growth (Figure 4 and Table 6), whereas transfection of PXR<sup>Mut</sup> in MHCC97-H cells could almost block the effect of to miR-140-3p on sorafenib's antitumor activation. Therefore, miR-140-3p could enhance the sensitivity of HCC cells to sorafenib by targeting PXR.

### Discussion

Advanced HCC is one of the foremost cancers in People's Republic of China nowadays.<sup>25–27</sup> Due to the limitation of current diagnostic and therapeutic strategies, most patients could not be treated by the radical ablation.<sup>28,29</sup> Due to the fact that advanced HCC is insensitive to chemotherapy<sup>30</sup> and radiotherapy,<sup>31</sup> the prognosis or clinical outcome of patients with advanced HCC is poor. Although some kinds of local ablation therapies, eg, radiofrequency ablation (RFA)<sup>32</sup> or transarterial chemoembolization (TACE),<sup>33</sup> could decelerate the progress of HCC to some extent, the rapid and aggressive



**Figure 2** Transfection of miR-140-3p inhibited the expression of PXR downstream genes.

**Notes:** MHCC97-H cells, which were transfected with control miRNA, miR-140-3p mimic, miR-140-3p mimic + PXR<sup>Mut</sup> or miR-140-3p mimic + inhibitor, were treated with the indicated concentrations of rifampicin for 48 hours. Then, cells were harvested for Western blot analysis. The protein level of PXR, CYP3A4, P-gp or β-actin was examined by their antibodies.

**Abbreviations:** miR, microRNA; PXR, pregnane X receptor; CYP3A4, CYP/CYP450 3A4; P-gp, P-glycoprotein; Mut, mutation.

**Table 4** The clearance (half-life/ $t_{1/2}$  value) of Sor in HCC cells

Cell line	Models	Control miRNA	miR-140-3p	miR-140-3p + PXR <sup>Mut</sup>	miR-140-3p + inhibitor
		Half-life ( $t_{1/2}$ value) of Sor (hours)			
MHCC97-H	Cultured cells	12.72±2.25	21.6±5.30	11.28±2.96	14.73±5.87
	Subcutaneous tumor	21.07±6.55	35.44±8.97	22.49±5.68	24.01±4.67
HepG2	Cultured cells	17.34±6.17	25.21±5.57	18.11±3.44	18.88±7.62
	Subcutaneous tumor	28.82±6.91	39.86±4.07	26.42±7.87	29.58±6.78

**Note:** Data are presented as mean±SD.

**Abbreviations:** miR, microRNA; HCC, hepatocellular carcinoma; Sor, sorafenib; PXR, pregnane X receptor.

recurrence of HCC after RFA or TACE treatment affect the application of these kinds of local ablation therapies. Therefore, molecular-targeted agents play a central role in advanced HCC treatment. Sorafenib is the only choice for advanced HCC treatment.<sup>34–36</sup> Although the application of sorafenib enhances the survival of HCC patients, sorafenib-resistance has become one of the major obstacles in improving its efficacy.<sup>37–39</sup> Thus, it is urgent to develop new strategies to overcome sorafenib-resistance.

Previous work has often focused on the antitumor drug-resistance by regulating multi-drug resistance genes, such as ABCs, eg, MDR-1/ABCB1 (ABC Subfamily B Member 1).<sup>40–49</sup> However, the role of PXR, which is the transcription factor of ABCs and mediates the transcription of ABCs in antitumor agents-resistance, is not very clear. PXR expressed in the liver organ or intestine organ could mediate drug clearance in general; however, the tumor-specific expression of PXR could inhibit the therapeutic efficacy of anticancer agents by accelerating these drugs during treatment.<sup>9–11</sup> As a central regulator of drug metabolism or clearance, PXR alters the absorption and clearance of xenobiotics (eg, antitumor drugs), leading to the reduced efficacy of the treatments.<sup>9–11</sup> In our previous work, we showed that the endogenous PXR is a master regulator of sorafenib-resistance in HCC cells.<sup>11</sup> Sorafenib can bind to and activate PXR and facilitate the expression of PXR downstream genes' expression.<sup>11</sup> By examining the clinical specimens, we found that a high level of PXR is correlated with poor prognosis of advanced HCC patients receiving sorafenib treatment. However, the regulatory mechanism of endogenous PXR mRNA level is not clear.

MiR is an important component of gene expression regulation.<sup>50–56</sup> MiRs are important regulators of HCC proliferation and metastasis.<sup>57–63</sup> In the present work, we firstly identified miR-140-3p as a potential miRNA target for PXR. Then, the regulatory effect of miR-140-3p on PXR was identified. Results showed that overexpression of miR-140-3p via lentiviral vector decreased the expression of PXR and PXR downstream genes in HCC cells; whereas overexpression of PXR with mutated miR-140-3p target sequence or transfection of miR-140-3p's inhibitor blocked the effect of miR-140-3p on PXR's expression. Moreover, miR-140-3p also enhanced the sensitivity of HCC cells to sorafenib. The effect of sorafenib on HCC cells were examined by in vitro methods or in vivo experiments. Our data illustrated that miR-140-3p administration was a promising strategy to enhance the antitumor effect of sorafenib on HCC cells. Interestingly, miR-140-3p could decelerate the clearance of sorafenib in cultured HCC cells or HCC subcutaneous tumor by decreased PXR's expression. This result could extend our knowledge of miRs regulating sorafenib clearance and provide useful methods in metabolism-related drug-resistance researches.

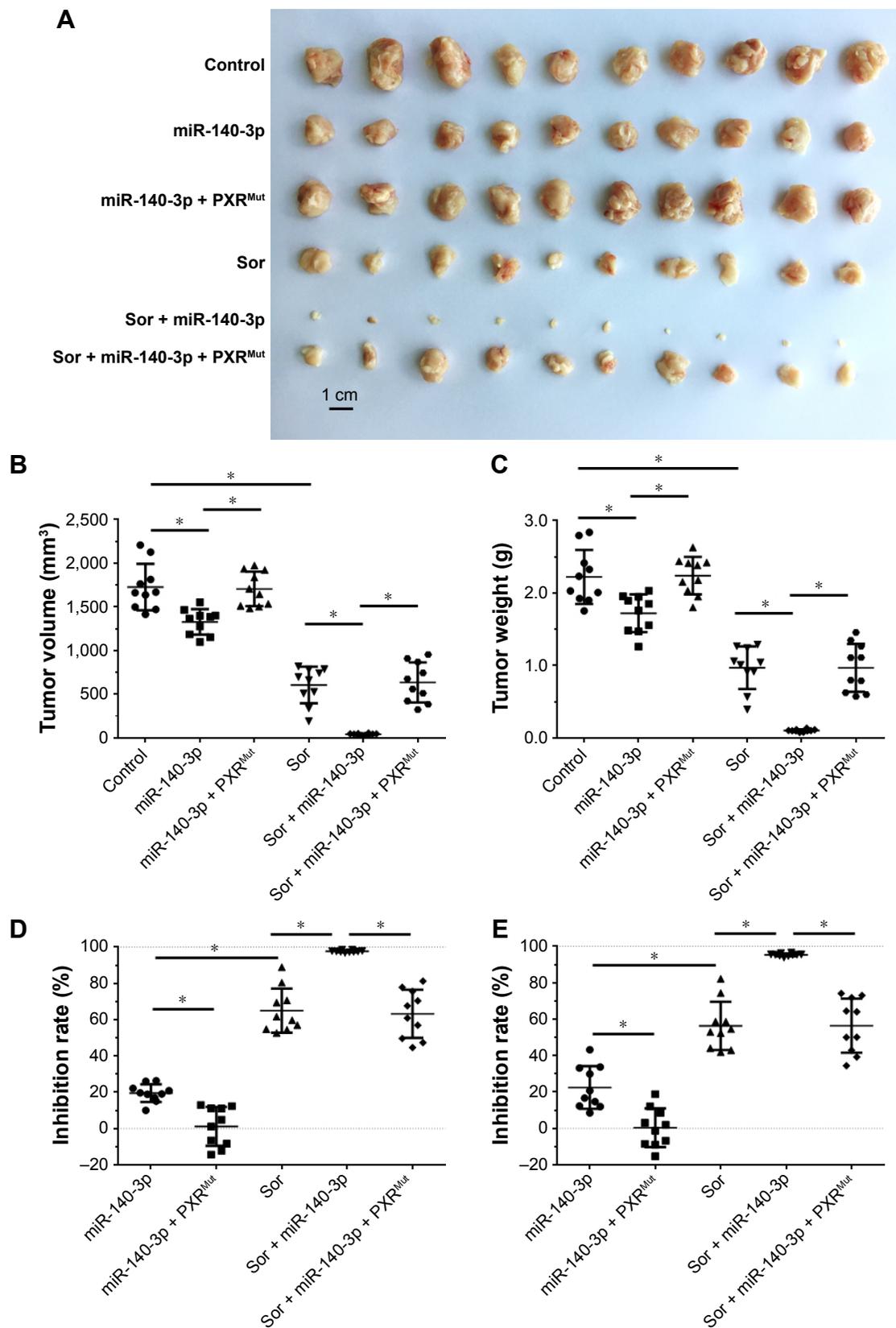
Moreover, this work reported on miR-140-3p targeting PXR and such reports of miRs targeting PXR are rare. Beside PXR, researchers often focus on the miRs targeting ABCs.<sup>64,65</sup> Awortwe et al and Rao et al reported that miR-655-3p or miR-148a could modulate the drug-resistance by targeting ABCs, eg, MRP3 or ABCG2.<sup>65,66</sup> Following on from the paper by Sharma et al that concluded that miR-18a-5p is a negative regulator and research by Vachirayonstien et al that stated that miR-30c-1-3p could be a suppressor of

**Table 5** The antitumor effect (IC<sub>50</sub> values) of Sor on HCC cells in each group

Cell line	Control miRNA	miR-140-3p	miR-140-3p + PXR <sup>Mut</sup>	miR-140-3p + inhibitor
	IC <sub>50</sub> values of Sor (μmol/L)			
MHCC97-H	1.04±0.54	0.18±0.05	1.25±0.33	1.01±0.29
HepG2	1.39±0.44	0.13±0.01	1.74±0.47	1.40±0.26

**Note:** Data are presented as mean±SD.

**Abbreviations:** miR, microRNA; HCC, hepatocellular carcinoma; Sor, sorafenib; PXR, pregnane X receptor.



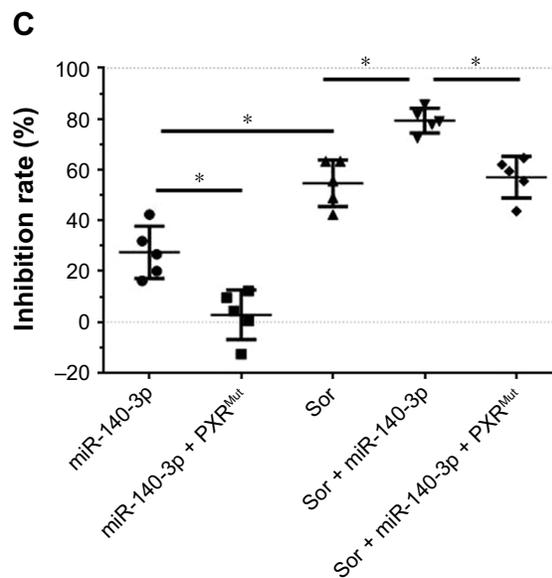
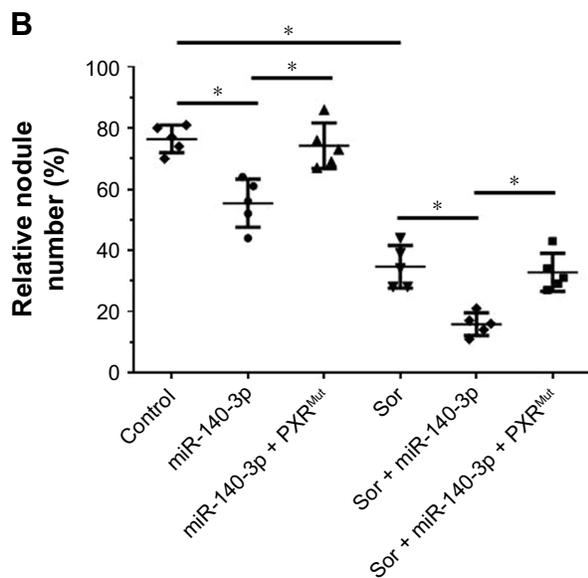
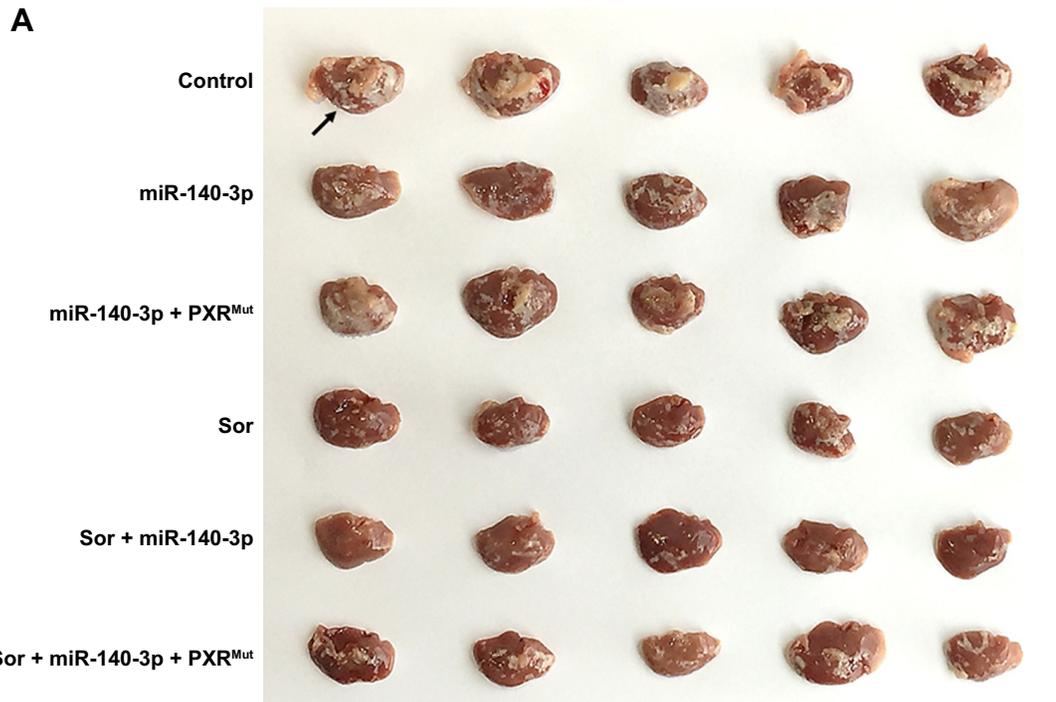
**Figure 3** Transfection of miR-140-3p enhanced the antitumor effect of Sor on MHCC97-H cells' subcutaneous growth. **Notes:** MHCC97-H cells, which were transfected with control miRNA, miR-140-3p mimic or miR-140-3p mimic+ PXR<sup>Mut</sup>, were seeded into nude mice to form subcutaneous tumors. The mice received 2 mg/kg sorafenib by oral administration. The results are shown as (A) photographs, (B) tumor volumes or (C) tumor weights. (D, E) The inhibition rate calculated by tumor volumes (D) or tumor weights (E) is shown. \*P<0.05. **Abbreviations:** miR, microRNA; PXR, pregnane X receptor; Mut, mutation; Sor, sorafenib.

**Table 6** The antitumor effect (inhibition rate) of Sor on HCC cells in each group

Models	miR-140-3p	miR-140-3p + PXR <sup>Mut</sup>	Sor	Sor + miR-140-3p	Sor + miR-140-3p + PXR <sup>Mut</sup>
<b>Inhibition rate (%)</b>					
Tumor volume	19.36±6.18	1.22±8.44	64.97±9.16	97.66±0.55	63.34±10.27
Tumor weight	21.16±6.54	0.59±7.84	65.53±9.07	97.71±0.51	62.46±10.84
Nodule area	23.33±7.68	1.57±7.69	62.12±6.58	80.05±4.18	64.89±5.61

**Note:** Data are presented as mean±SD.

**Abbreviations:** miR, microRNA; HCC, hepatocellular carcinoma; Sor, sorafenib; PXR, pregnane X receptor.



**Figure 4** Transfection of miR-140-3p enhanced the antitumor effect of Sor on MHCC97-H cells' intrahepatic growth.

**Notes:** MHCC97-H cells, which were transfected with control miRNA, miR-140-3p mimic or miR-140-3p mimic + PXR<sup>Mut</sup>, were seeded into the livers of nude mice via hepatic portal vein injection. The mice received 2 mg/kg sorafenib by oral administration. The results are shown as (A) photographs, (B) area of nodule in liver organs formed by MHCC97-H cells or (C) inhibition rates. \*P<0.05; the black arrow indicates the nodules in the liver organ.

**Abbreviations:** miR, microRNA; PXR, pregnane X receptor; Mut, mutation; Sor, sorafenib.

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PXR, our results extend the knowledge of miRs on the PXR signaling pathway.<sup>67,68</sup>

## Conclusion

This work for the first time reported that PXR is a target of miR-140-3p. MiR-140-3p suppresses the PXR pathway and enhances the sensitivity of HCC cells to sorafenib. Downregulation of PXR by miR-140-3p may be a promising strategy for enhancing sorafenib-based HCC treatment.

## Author contributions

All authors made substantial contributions to the design and conception; acquisition, analysis or interpretation of data. All authors took part in either drafting or revising the manuscript. At the same time, all authors gave final approval of the version to be published; and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

## Disclosure

The author reports no conflicts of interest in this work.

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