

MicroRNA-223 promotes hepatocellular carcinoma cell resistance to sorafenib by targeting *FBW7*

XIAOFENG TANG^{1*}, WEIGANG YANG^{1,2*}, ZHEYUE SHU¹, XIAODONG SHEN¹, WEICHEN ZHANG¹, CHAO CEN¹, LINPING CAO¹, MIN ZHANG¹, SHUSEN ZHENG^{1,3,4} and JUN YU^{1,3,4}

¹Division of Hepatobiliary and Pancreatic Surgery, Department of Surgery, First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, Zhejiang 310003;

²Department of General Surgery, Huzhou Hospital of Traditional Chinese Medicine, Huzhou, Zhejiang 313000;

³Key Laboratory of Combined Multi-Organ Transplantation, Ministry of Public Health;

⁴Key Laboratory of The Diagnosis and Treatment of Organ Transplantation, CAMS, Hangzhou, Zhejiang 310003, P.R. China

Received April 3, 2018; Accepted October 30, 2018

DOI: 10.3892/or.2018.6908

Abstract. Hepatocellular carcinoma (HCC) is a globally prevalent malignancy associated with a poor patient prognosis. We investigated the relationship between microRNA-223 (miR-223) expression and the sensitivity of HCC cells to sorafenib treatment. miR-223 expression was determined in HCC cell lines with differential sorafenib sensitivity using reverse transcription-quantitative PCR. miR-223 inhibitor, miR-223 mimic, and F-box and WD repeat domain-containing 7 (*FBW7*) short interfering RNAs (siRNAs) were transfected into the HCC cells to regulate the expression levels of miR-223 and *FBW7*. Cell proliferation was evaluated using an ethynyl deoxyuridine (EdU) incorporation assay and Cell Counting Kit-8. *FBW7* protein expression levels were observed using western blotting. miR-223 expression was increased in the HCC cells with sorafenib resistance. HCC cells with miR-223 knockdown had significantly increased sorafenib sensitivity, but the miR-223 mimic had the opposite effect. The TargetScan web server predicted that *FBW7* is a target of miR-223, which was confirmed by western blotting. Furthermore, *FBW7* siRNA transfection increased HCC cell resistance to sorafenib in an obvious manner, and entirely

eliminated the effect of the miR-223 inhibitor on enhancing sorafenib sensitivity. To conclude, miR-223 expression is upregulated in sorafenib-resistant HCC cells, and miR-223 knockdown significantly enhances HCC cell sensitivity to sorafenib by increasing expression of the target gene, *FBW7*, suggesting that miR-223 may be a new therapeutic target for overcoming sorafenib resistance.

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies worldwide, and most new HCC cases are found in Asia, with about half in China alone (1). The long-term survival rate of patients with HCC remains low, and HCC is the fifth most common cause of cancer-related mortality worldwide (2). Differing from the Western hemisphere, where alcohol abuse is the main factor in HCC development, the major risk factor in China is the high prevalence of viral hepatitis B infection (3). Given the inconspicuous symptoms and lack of screening at the early stages of HCC, a portion of patients with HCC present with macrovascular invasion and intra/extrahepatic spread at the time of diagnosis. Over past decades, understanding of the molecular mechanisms of HCC has advanced significantly, and there has been a robust increase in clinical trial activity, improving the long-term survival outcomes of patients with advanced HCC. Compared with yttrium-90 radiation therapy, transarterial bland embolization/transarterial chemoembolization and ablation (or a combination thereof), sorafenib is the only efficacious strategy for prolonging life in patients with advanced HCC (4).

Sorafenib is an oral multi-targeting tyrosine kinase inhibitor (TKI) that suppresses Fms-like tyrosine kinase 3, vascular endothelial growth factor (VEGF) receptors, platelet-derived growth factor (PDGF) receptors, and the RAF serine/threonine kinases (5). Several random clinical trials of sorafenib have reported consistent improvement in overall survival for patients with advanced HCC. Llovet *et al* first reported that the median survival and time to radiologic progression for

Correspondence to: Dr Jun Yu or Dr Shusen Zheng, Division of Hepatobiliary and Pancreatic Surgery, Department of Surgery, First Affiliated Hospital, School of Medicine, Zhejiang University, 79 Qingchun Road, Hangzhou, Zhejiang 310003, P.R. China
E-mail: dryujun@zju.edu.cn
E-mail: shusenzheng@zju.edu.cn

*Contributed equally

Key words: hepatocellular carcinoma, sorafenib, drug resistance, miR-223, *FBW7*

patients treated with sorafenib were nearly 3 months longer than for those given placebo (6). Vilgrain *et al* demonstrated that patients with advanced HCC who received continuous oral sorafenib (400 mg twice daily) had a median overall survival of up to 9.9 months (7). However, sorafenib can cause serious adverse effects, and drug resistance develops frequently. The negative sorafenib responses have been associated with the regulation of multiple intracellular signaling pathways.

MicroRNAs (miRNAs), tiny non-coding RNA molecules, play an important role in regulating multiple signaling pathways and play differential roles in terms of sorafenib response, including resistance (8). In humans, >1,800 distinct miRNAs have been identified to date, which account for ~5% of the transcribed genome and which modulate 30-80% of genes (9). The silencing mechanism depends on the extent of complementarity between the miRNA and mRNA target, resulting in either degradation or inhibition of the mRNA target at the translational level (10,11). The present study was designed to reveal the function of miR-223 and its mRNA target F-box and WD repeat domain-containing 7 (*FBW7*) on promoting HCC resistance to sorafenib.

Materials and methods

Cell lines, chemicals and antibodies. Three human HCC cell lines (Huh7, SNU387 and SNU449) were purchased from the Cell Bank of the Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The Huh7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and the SNU449 and SNU387 cells were cultured in RPMI-1640 complete medium (Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator at 37°C and 5% CO₂. All media were supplemented with 10% fetal bovine serum (FBS; Gibco™; Thermo Fisher Scientific, Inc.) and 100 U/ml mixture of streptomycin and penicillin. The chemicals and antibodies used were sorafenib and diamidinophenylindole (DAPI) (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), anti-*FBW7* antibody (cat. no. ab105752; Abcam, Cambridge, MA, USA), anti-glyceraldehyde-3-phosphate dehydrogenase antibody (GAPDH; cat. no. 5174), goat anti-rabbit horseradish peroxidase (HRP) antibody (cat. no. 7074) and goat anti-mouse HRP antibody (cat. no. 7056; Cell Signaling Technology, Inc., Beverly, MA, USA).

Small interfering RNA (siRNA) transfection. The gene-targeting siRNAs or a scramble control were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). Untreated Huh7, SNU387 and SNU449 cells were plated in 6-well plates at 1×10⁵ cells/well and supplemented with 2 ml corresponding media. Then, 50 nm siRNA and 50 μl Invitrogen™ Lipofectamine 2000 transfection reagent (Thermo Fisher Scientific, Inc.) were added to the plated cells when the cells were 20-30% confluent, following the manufacturer's protocol. The cells were collected for subsequent experiments after 48-72 h of transfection.

Cell proliferation assay. All siRNA-transfected HCC cells were plated at 3×10³ cells/well in 100 μl medium for 24 h.

After sorafenib or phosphate-buffered saline (PBS; control) treatment, cell viability was detected using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) in a microplate reader (ELx800; BioTek Instruments, Inc., Winooski, VT, USA). The optical density at 450 nm was recorded to calculate the median inhibitory concentration (IC₅₀).

Ethynyl deoxyuridine (EdU) incorporation assay. HCC cell proliferation ability was detected using a Click-iT EdU Imaging kit (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Briefly, 50 μM EdU/well was added to HCC cell monolayers that were 50-70% confluent and incubated for 2 h. After washing three times with PBS, the cells were fixed with 4% paraformaldehyde. Then, Apollo fluorescent dye solution (Invitrogen; Thermo Fisher Scientific, Inc.) was added and incubated for 30 min, and the cell proliferation rate was visualized and calculated under a fluorescence microscope (Olympus Corp., Tokyo, Japan).

Quantitative real-time PCR (RT-PCR). Total RNA was isolated from the HCC cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Real-time PCR was conducted using a SYBR Premix Ex Taq kit (Takara Bio, Inc., Otsu, Japan) in a Roche LightCycler system (Roche, Basel, Switzerland). All reactions were performed in triplicate. The primers for the target genes were as follows: miR-223 mimic forward primer, 5'-UGUCAGUUUGUCAAAUACCCCA-3' and reverse primer, 5'-GGGUAUUUGACAAACUGACAUU-3'; miR-223 inhibitor, 5'-UGGGGUAUUUGACAAACUGACA-3'; *FBW7* forward primer, 5'-CACTCAAAGTGTGGAATGCAGAGAC-3' and reverse primer, 5'-GCATCTCGAGAACCGCTACAA-3'; GAPDH forward primer, 5'-UGACCUCAACUACAUGGUUTT-3' and reverse primer, 5'-AACCAUGUAGUUGAGGUCATT-3'.

Western blotting. Radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors was used to extract the total proteins from the HCC cells. Then, a bicinchoninic acid (BCA) kit (Thermo Fisher Scientific, Inc.) was used to quantify the protein concentration. Samples (10 μl) containing 20-50 g protein were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 120 V, and then electrophoretically transferred to 0.45-μm polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Bedford, MA, USA) at 350 mA for 1 h. The membranes were blocked with a 5% skim milk and 0.05% Tween-20 mixture. The membranes were incubated with the corresponding primary antibody (dilution 1:1,000) at 4°C for overnight and subsequently incubated with the secondary HRP-conjugated antibody (dilution 1:2,000) at room temperature for 1 h. The target protein expression levels were visualized with enhanced chemiluminescence (GE Healthcare, Piscataway, NJ, USA) in the western blotting detection system Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis. All experimental data are reported as the mean ± SD (n=3). The two-tailed Student t-test and Fisher

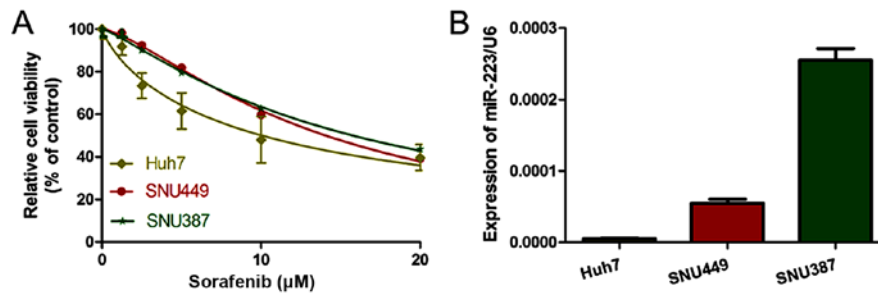


Figure 1. Effect of sorafenib treatment on HCC cells. (A) Relative cell viability of Huh7, SNU449 and SNU387 cells in the presence of 0, 10 and 20 μ M sorafenib after 48 h. (B) miR-223 expression levels in the HCC cell lines. HCC, hepatocellular carcinoma.

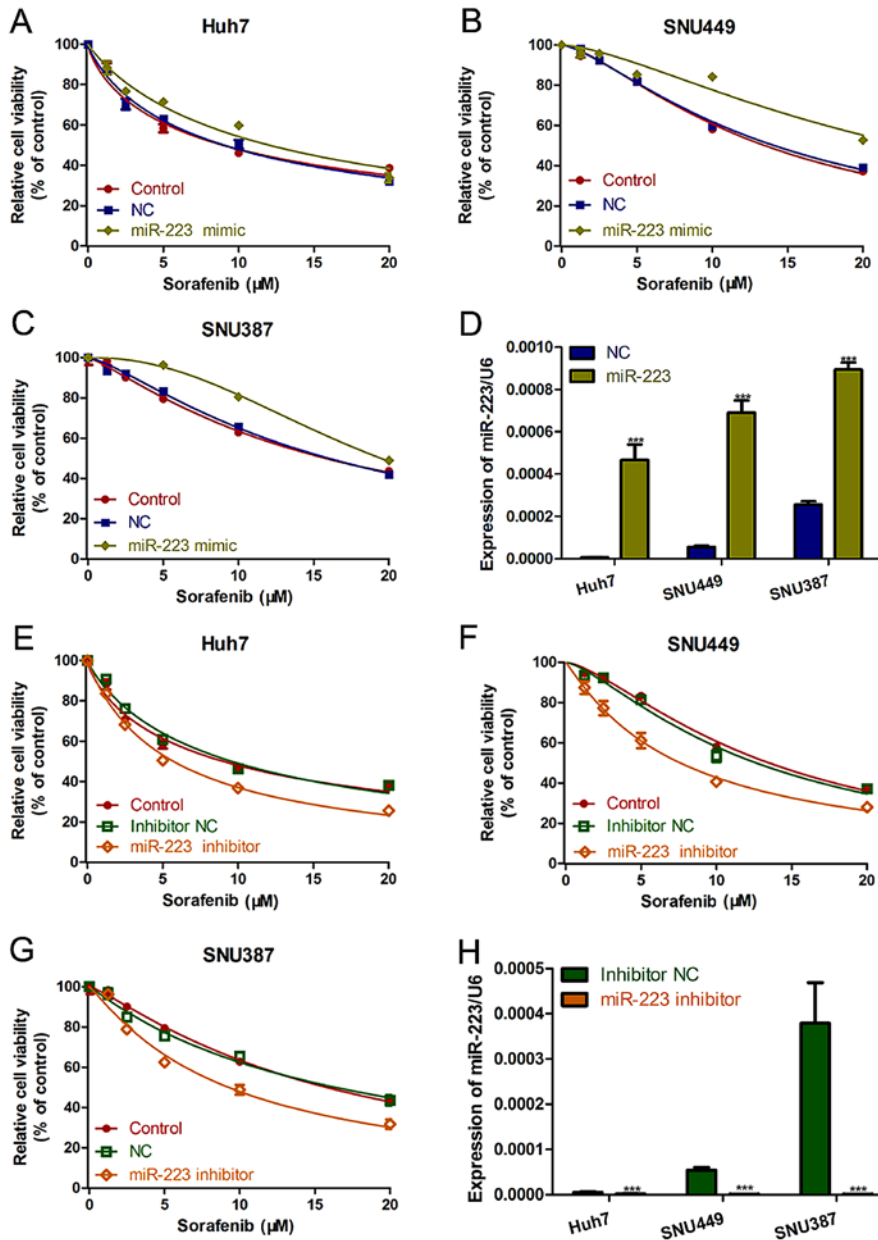


Figure 2. Effect of miR-223 mimic and miR-223 inhibitor transfection on HCC cell viability. (A-D) miR-223 overexpression increased the viability of Huh7 (A), SNU449 (B) and SNU387 cells (C) following sorafenib treatment. (E-H) miR-223 knockdown decreased the viability of Huh7 (E), SNU449 (F) and SNU387 cells (G) following sorafenib therapy. NC, negative control. (D and H) miR-223 mimic or miR-223 inhibitor increased or decreased miR-223 expression, respectively, in the HCC cells. *** $P < 0.001$ vs. NC. HCC, hepatocellular carcinoma.

exact test were used to analyze differences between groups. Statistical analysis was conducted using SPSS 19.0 software

(SPSS, Inc., Chicago, IL, USA). All statistical results with a P -value < 0.05 were considered statistically significant.

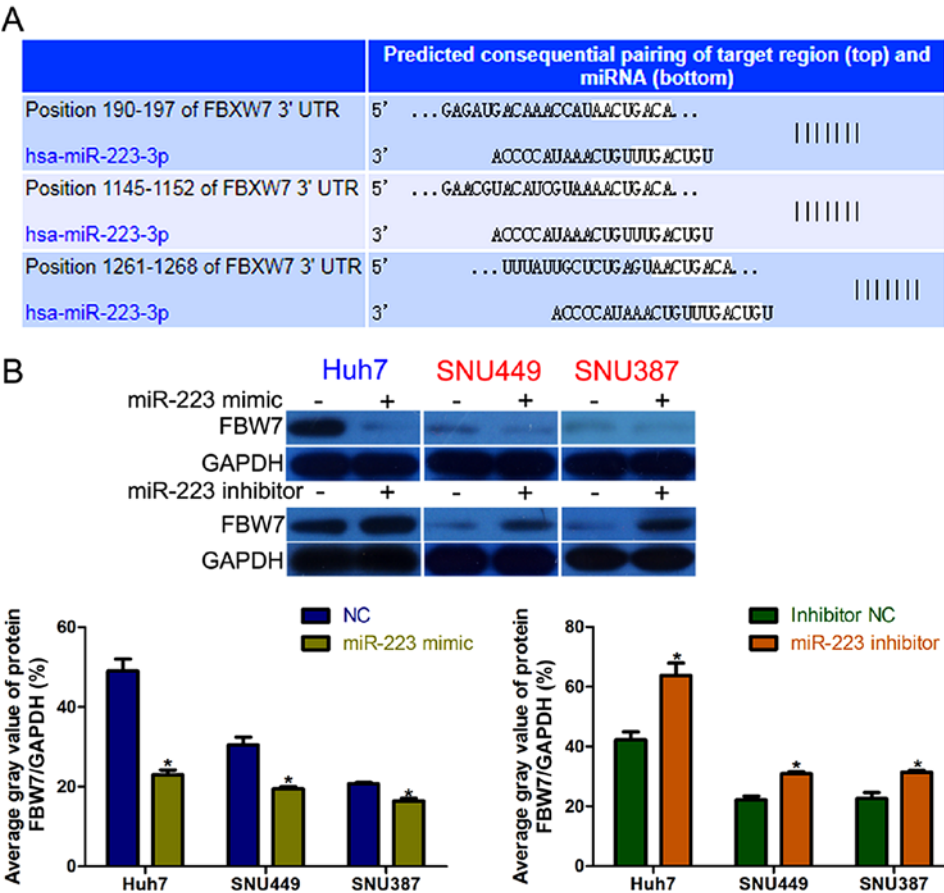


Figure 3. Prediction of the target gene of miR-223 and effect of the miR-223 mimic and miR-223 inhibitor on *FBW7* expression. (A) TargetScan was used to predict *FBW7* as a potential target of miR-223. (B) miR-223 mimic inhibited *FBW7* expression and miR-223 inhibitor transfection upregulated *FBW7* expression in HCC cells. HCC, hepatocellular carcinoma. *P<0.05 vs. NC.

Table I. IC₅₀ values of sorafenib treatment in HCC cell lines.

Cell lines	Huh7	SNU449	SNU387
IC ₅₀ (μM)	8.749±0.876	13.4±1.05	15.72±1.58

HCC, hepatocellular carcinoma.

Results

High miR-223 expression is correlated with sorafenib resistance in HCC cells. We detected altered cell viability of the three HCC cell lines (SNU387, SNU449 and Huh7) in the presence of sorafenib after 48 h. The sensitivity of the HCC cell lines to sorafenib, from high to low, was Huh7, SNU449 and SNU387 (Fig. 1A). The IC₅₀ of sorafenib was 8.749±0.876, 13.4±1.05 and 15.72±1.58 μM in the Huh7, SNU449 and SNU387 cells, respectively (Table I). Notably, miR-223 expression levels in the HCC cell lines followed a similar trend to that of the sorafenib IC₅₀ (Fig. 1B). This result suggests that miR-223 potentially correlates with sorafenib resistance.

miR-223 knockdown increases HCC cell sensitivity to sorafenib. To investigate the link between miR-223 and sorafenib resistance, miR-223 mimic or miR-223 inhibitor were

packaged in lentivirus and transfected into the HCC cell lines to induce miR-223 overexpression or knockdown, respectively. The cell proliferation assay showed that, in all three HCC cell lines, miR-223 upregulation increased cell viability in the presence of sorafenib (Fig. 2A-C). On the contrary, miR-223 knockdown significantly increased the therapeutic effect of sorafenib on the HCC cells (Fig. 2E-G). qRT-PCR was used to determine the expression of miR-223 with miR-223 mimic or miR-223 inhibitor in HCC cells (Fig. 2D and H).

FBW7 is a direct and functional target of miR-223 in HCC. The TargetScan web server was used to explore the mechanism by which miR-223 exerts its function, and identified *FBW7* as a potential target of miR-223 in HCC cells (Fig. 3A). High miR-223 expression inhibited *FBW7* expression in an obvious manner, and the opposite effect was observed in miR-223-knockdown HCC cells (Fig. 3B).

FBW7 increases HCC cell sensitivity to sorafenib. We detected the *FBW7* expression level in the HCC cell lines and found that Huh7 cells had higher *FBW7* expression than that noted in the SNU449 and SNU387 cells (Fig. 4A). *FBW7* expression was significantly inhibited in the surviving HCC cells after a 24-h sorafenib treatment (Fig. 4B). *FBW7* siRNA was transfected into HCC cells to decrease *FBW7* expression (Fig. 4E). After sorafenib treatment, HCC cells

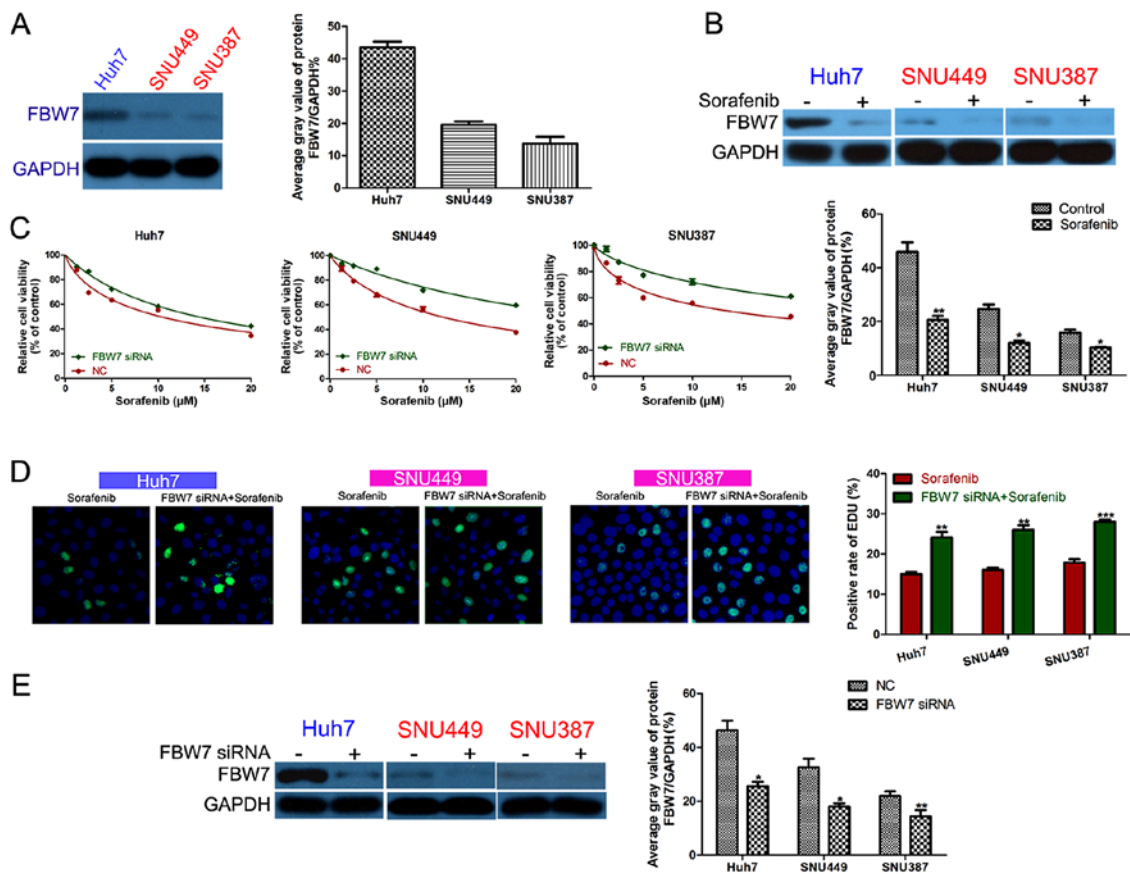


Figure 4. Effect of *FBW7* siRNA transfection on HCC cell viability. (A and B) *FBW7* expression levels in Huh7, SNU449 and SNU387 cells without (A) and with (B) sorafenib treatment. (C) After sorafenib treatment, the viability of HCC cells with *FBW7* knockdown was increased compared to the control group. * $P < 0.05$, ** $P < 0.01$ vs. control. (D) HCC cells with *FBW7* knockdown had a higher EdU-positive rate than the control group in the presence of sorafenib. ** $P < 0.01$, *** $P < 0.001$ vs. sorafenib. (E) *FBW7* expression in the siRNA-transfected HCC cells. * $P < 0.05$, ** $P < 0.01$ vs. NC.

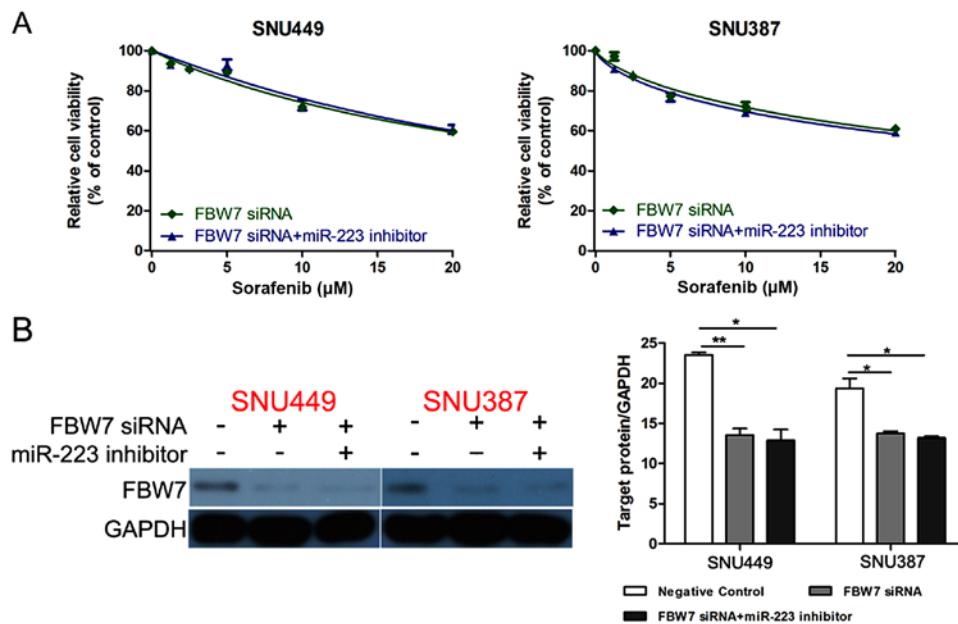


Figure 5. Effect of *FBW7* siRNA and miR-223 inhibitor transfection on HCC cells. (A) Cell viability did not differ between the SNU449 and SNU387 cells transfected with *FBW7* siRNA and with *FBW7* siRNA+miR-223 inhibitor. (B) *FBW7* siRNA decreased *FBW7* expression effectively regardless of miR-223 inhibitor transfection or lack thereof. * $P < 0.05$, ** $P < 0.01$.

with *FBW7* knockdown had inhibited viability compared to the control group (Fig. 4C). Furthermore, *FBW7*

knockdown decreased HCC cell proliferation in the presence of sorafenib (Fig. 4D).

FBW7 reverses the effect of miR-223 in promoting sorafenib resistance. *FBW7* siRNA was transfected into SNU449 and SNU387 cells together with miR-223 inhibitor to investigate whether *FBW7* knockdown could reverse the effect of miR-223 inhibitor in promoting sorafenib sensitivity. As expected, the cell viability was not different between the cells transfected with *FBW7* siRNA and *FBW7* siRNA+miR-223 inhibitor (Fig. 5A). Western blotting confirmed that the *FBW7* siRNA could eliminate the effect of miR-223 inhibitor on increasing *FBW7* expression (Fig. 5B).

Discussion

Despite significant advances in the management and treatment of patients with HCC over the last decades, the prognosis remains poor. Unfortunately, HCC is very resistant to cytotoxic and targeted therapies, even against the multikinase inhibitor, sorafenib, the first and only approved systemic therapy that improves the overall survival in patients with advanced HCC (6); the gradually increasing rate of sorafenib resistance has significantly limited its therapeutic benefit. The reason for this limited effect and for the failure of all targeted agents, including sorafenib, against HCC, varies, and includes the molecular complexity of the tumor and the presence of primary and acquired drug resistance mechanisms (12,13). In most instances, the HCC cells that initially respond well to anticancer drugs gradually display a loss of response and acquire resistance during treatment, subsequently leading to HCC recurrence (14). Recently, sorafenib resistance has often been referred to as a 'hot' term used to describe the impaired efficacy of sorafenib, especially for patients with advanced HCC. A large body of mechanisms are involved in the acquired resistance to sorafenib, such as the phosphatidylinositol 3-kinase (PI3K)-AKT pathway, epithelial-mesenchymal transition, epigenetic regulation, and autophagy (12,15). There is an urgent need to understand the underlying mechanism and identify new, promising chemotherapeutic therapies.

A glance at the molecular mechanistic aspect reveals the regulation of various signaling pathways potentially modulated by miRNAs (16,17). Deregulation, i.e., either downregulation or upregulation, of several miRNAs has been reported in a series of *in vivo*, *in vitro*, and patient studies, demonstrating that it may be responsible for the response to sorafenib. Here, we explored the relationship between miR-223 expression and sorafenib resistance in HCC. Previously, miR-223 was considered a potential diagnostic and prognostic biomarker of various malignancies, including osteosarcoma (18), Barrett's esophagus (19), and esophageal squamous cell carcinoma (20). Moreover, Han *et al* revealed that miR-223 regulates the insulin-like growth factor 1 receptor (IGF1R)/PI3K/AKT signaling pathway to reverse epidermal growth factor receptor (EGFR) TKI resistance (21). Our results revealed that miR-223 expression levels correlate with HCC cell sensitivity to sorafenib. Treating HCC cells with miR-223 inhibitor increased their sensitivity to sorafenib in an obvious manner. These data demonstrated that miR-223 is a suitable predictive biomarker of HCC cell resistance to sorafenib. To further assess the function of miR-223, we used TargetScan to predict the miR-223 target genes and determined that *FBW7* is a

functional target of miR-223 in HCC cells. miR-223 mimic markedly downregulated *FBW7*, and miR-223 inhibitor had the opposite effect on *FBW7* expression. Furthermore, *FBW7* siRNA entirely eliminated the effect of the miR-223 inhibitor on increasing HCC cell sensitivity to sorafenib. These results strongly suggest that miR-223 regulates HCC cell resistance to sorafenib by targeting *FBW7*.

Notably, a growing number of studies have observed that *FBW7* is also involved in regulating drug resistance (22,23). Several groups have shown that the loss of *FBW7* led to elevated expression of the c-Jun, c-Myc, and Notch-1 oncoproteins, all of which can promote cell growth, although they can also provoke apoptosis as a side-effect. In the present research, we transfected HCC cells with *FBW7* siRNA, consistent with previously published research (24). The results confirmed that *FBW7* knockdown significantly inhibited HCC cell sensitivity to sorafenib. These results show an intimate relationship between drug resistance and miR-223/*FBW7* genetic status, and these observations imply that the miRNA pathway can modulate *FBW7* expression and activity directly, demonstrating that targeting miR-223/*FBW7* may open a new therapeutic window for drug administration (25).

In conclusion, miR-223 expression is upregulated in HCC cells with sorafenib resistance. miR-223 knockdown significantly enhances HCC cell sensitivity to sorafenib by increasing the expression of the target gene, *FBW7*, suggesting that miR-223 may be a new therapeutic target for overcoming sorafenib resistance.

Acknowledgements

Not applicable.

Funding

The present study was supported by Zhejiang Natural Science Foundation (grant nos. LY16H160068 and LY15H160060).

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

JY, SZ and XT conceived the research idea; WY, ZS and XS performed the experiments; WZ, CC, LC and MZ analyzed the data; SZ wrote the manuscript. All authors drafted, read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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