ACTA ABP BIOCHIMICA POLONICA

Vol. 70, No 2/2023
239–246
https://doi.org/10.18388/abp.2020_5657

Regular paper

MiR-375 attenuates sorafenib resistance of hepatocellular carcinoma cells by inhibiting cell autophagy

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Objective: Sorafenib is the first-line treatment for hepatocellular carcinoma (HCC), but its efficacy is limited by the drug resistance of HCC cells. MiR-375 has been shown to be an inhibitor of autophagy that contributes to sorafenib resistance of HCC cells. In this context, this study probed into the unaddressed molecular target of miR-375 in inhibiting the autophagy of HCC cells under sorafenib treatment. Methods: Western blotting and qRT-PCR (quantitative reverse transcription-polymerase chain reaction) have been applied to measure the expressions of miR-375 and SIRT5 in parental HCC cells (HepG2 and Huh7) and sorafenib-resistant HCC cells (HepG2/so and Huh7/so). HepG2/so cells were accordingly transfected with miR-375 mimic, miR-375 inhibitor, sh-SIRT5, pcDNA3.1-SIRT5 or negative control. Expressions of p62, LC3I and LC3II in HCC cells have been measured by Western blotting. Viability and apoptosis of HCC cells have been assessed by CCK-8 (cell counting kit 8) and flow cytometry respectively. Bioinformatics techniques and dual-luciferase reporter assay have been used to predict and verify the targeting relationship between miR-375 and SIRT5. Results: MiR-375 was under-expressed and SIRT5 was over-expressed in HCC cells. An autophagy inhibitor impaired the survival of HepG2/so cells transfected with miR-375 inhibitor. An autophagy activator enhanced the drug resistance of HepG2/so cells transfected with miR-375 mimic. MiR-375 suppressed the drug resistance of HepG2/so cells by inhibiting autophagy. SIRT5 enhanced the drug resistance of HepG2/so cells by promoting autophagy and it could be targeted by miR-375. Conclusion: MiR-375 suppresses autophagy to attenuate the sorafenib resistance of HCC cells by regulating SIRT5. The findings of this study may provide new therapeutic targets for treating HCC.

Keywords: MiR-375, SIRT5, autophagy, drug resistance, Sorafenib, hepatocellular carcinomacell

Received: 14 April, 2021; revised: 18 October, 2021; accepted: 01 February, 2023; available on-line: 17 April, 2023

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Acknowledgements of Financial Support: This research was funded by the grant from the National Natural Science Foundation of China (Grant No. 81602601).
Abbreviations: ATG2A, autophagy-related gene 2A; BCA, bicinchoninic acid; CCK-8, cell counting kit 8; DMEM, Dulbecco's modified Eagle medium; FITC, fluorescein isothiocyanate; HCC, hepatocellular carcinoma; PVDF, polyvinylidenefluoride; PI, propidium iodide; PBS, phosphate-buffered saline; PBST, PBS Tween 20; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RAP, rapamycin; 3-MA, 3-Methyladenine

INTRODUCTION

Liver cancer belongs to the commonest deadly cancers worldwide. The death rate of liver cancer rose from 2012 to 2016 and the incidence rate continues to increase (Siegel *et al.*, 2019). Hepatocellular carcinoma (HCC) accounts for the vast majority of primary liver cancers, which can be caused by cirrhosis, infection of hepatitis B or C virus, alcohol abuse and other risk factors (Villanueva, 2019). Sorafenib is a multikinase inhibitor that has been considered the first treatment option and standard therapy for advanced-stage HCC for over a decade (Marisi *et al.*, 2018). Sorafenib targets multiple tyrosine kinases and therefore impairs proliferation, migration and angiogenesis and activates apoptosis in HCC (Brunetti *et al.*, 2019). However, a considerable portion of patients with HCC are insensitive to sorafenib, resulting in an unsatisfactory overall efficacy of this widely used drug (Cheng *et al.*, 2020). HCC cells developing sorafenib resistance exhibit significant mesenchymal phenotypes and stemness features (Xia *et al.*, 2020). Therefore, exploration of the mechanisms of sorafenib resistance is important for prolonging the survival of patients with HCC.

Autophagy is a metabolic process that is unavoidably altered in cancers and it can be well manipulated to improve the clinical outcomes of cancer patients (Levy *et al.*, 2017). Autophagy prevents cell damage and improves survival in response to energy or nutrient shortage and various cytotoxic insults (Dikic & Elazar, 2018). An autophagy inhibitor, chloroquine, overcame the resistance of liver cancer cells to drugs targeting hepatocyte growth factor-activated MET kinase which stimulated liver carcinogenesis and tumor metastasis (Huang *et al.*, 2019). Autophagy is also suggested to be an important participant in mediating sorafenib resistance of HCC. For instance, depletion of METTL3, a primary m^6 A methyltransferase, enhanced the sorafenib resistance of HCC cells by activating autophagy-associated pathways through destabilization of FOXO3 mRNA (Lin *et al.*, 2020). CD24 activated autophagy by regulating PP2A/AKT/mTOR signaling pathway and therefore decreased the sorafenib sensitivity of HCC cells (Lu *et al.*, 2018). MicroRNA (miR)-541 inhibited autophagy-dependent sorafenib resistance of HCC cells by directly targeting autophagy-related gene (ATG) 2A and Ras-related protein Rab-1B (Xu *et al.*, 2020).

In recent years, microRNAs (miRNAs) have shown great promise as therapeutic targets for cancer treatment. MiRNAs regulate gene function in diverse cellular activities including autophagy. MiR-375 is known to be an autophagy inhibitor in many disease conditions. As an example, miR-375 promoted inflammation and apoptosis

of acinar cells in severe acute pancreatitis by inhibiting ATG7-mediated autophagy (Zhao *et al.*, 2020). MiR-375 also inhibited autophagy to facilitate the sorafenib therapy for HCC (Zhao *et al.*, 2018). However, the mechanism of miR-375 in regulating autophagy-dependent sorafenib resistance in HCC remains largely unknown.

SIRT5 is a sirtuin family member that resides primarily in mitochondrial matrix and regulates cellular homeostasis (Kumar & Lombard, 2018). Overexpression of SIRT5 is associated with tumorigenesis in breast cancer, colorectal cancer, HCC and more (Greene *et al.*, 2019; Shi *et al.*, 2019; Zhang *et al.*, 2019). Garva and others found that SIRT5 positively regulated autophagy and proliferation of tumor cells under stress conditions (Garva *et al.*, 2019). However, there is no report on either SIRT5-mediated autophagy in HCC or the interaction between miR-375 and SIRT5. The authors used Jefferson database to predict whether SIRT5 mRNA and miR-375 have binding sites (https://cm.jefferson.edu/) and designed this study to verify the involvement of miR-375/SIRT5 axis in autophagy-mediated sorafenib resistance of HCC cells.

MATERIALS AND METHODS

Cell cultivation

A healthy liver cell line (L-02) and human HCC cell lines (HepG2 and Huh7) were purchased from the American Type Culture Collection. L-02, HepG2 and Huh7 cells were cultured in Dulbecco's modified Eagle medium (DMEM), MEM and high-glucose DMEM, respectively. All media were supplemented with 10% fetal bovine serum and 2 mM glutamine. These cells were incubated in a moist environment (37°C, 5% CO_2) and used for experiments until they reached the logarithmic growth phase.

Establishment of sorafenib-resistant HCC cell models

Sorafenib treatment was given to HepG2 and Huh7 cells at the logarithmic growth phase. The initial dose of sorafenib was set at 1 μmol/L. The culture medium was renewed every 24 hours, in which the sorafenib concentration increased by 0.25 μmol/L each time until reaching a total of 12 μmol/L. The half maximal inhibitory concentration (IC50) >10 μM indicated resistance to sorafenib. Sorafenib-resistant HepG2 and Huh7 cells (HepG2/so and Huh7/so) were continuously treated with sorafenib to enhance the drug resistance. The cells were observed and photographed under an inverted microscope.

Cell transfection and treatment

L-02 or HepG2/so cells were transfected with miR-375 mimic, miR-375 inhibitor, sh-SIRT5, pcDNA3.1-SIRT5 or their negative control (mimic NC, inhibitor NC, sh-NC or pcDNA3.1) in 3.5 cm culture dishes (2×10^6 cells per dish) using Lipofectamine 2000 (Invitrogen, California, USA). The plasmids and RNAs were provided by GenePharma (Shanghai, China). All other experiments were carried out 48 hours after the transfection.

For analyzing the effect of autophagy on sorafenib resistance of HCC cells, HepG2/so cells transfected with miR-375 mimic were treated with an autophagy activator rapamycin (RAP, 0.2 μg/10 μl; Cell Signaling Technology, MA, USA) for 24 hours and HepG2/so cells transfected with miR-375 inhibitor were treated with an autophagy inhibitor 3-Methyladenine (3-MA, 50 μM; Selleck, USA) for 24 hours. The use of RAP and 3-MA was based on existing literature (Ding *et al.*, 2021; Lendvai *et al.*, 2021).

CCK-8 assay

The assay was applied to measure the IC50 of parental and sorafenib-resistant HCC cells. The survival rates of HCC cells were measured 24 hours after treatment with sorafenib of different concentrations (1, 2, 4, 8, 16, 32 μmol/L). The influence of miR-375 or SIRT5 on the survival of sorafenib-resistant HCC cells was also assessed. Cells of each group were cultured in a 96-well plate where every well contained 1.5×10^4 cells. Cells in each well were incubated with 10 μl of CCK-8 reagent at 37°C for 3 hours. The absorbance value was measured at 450 nm.

Flow cytometry

Cells in each group were made into suspension for centrifugation at 2000 r/min. The cells were washed twice with phosphate-buffered saline (PBS) and then resuspended in binding buffer. Cell suspension (195 μL, about 1×10^5 cells) was mixed with 5 μL of Annexin-V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) solution. The cells were incubated in the dark for 10 minutes and their apoptosis rates were measured by a flow cytometer (BD Biosciences, Suzhou, China).

qRT-PCR

Total RNA was obtained from 4 ~ 5×10^4 cells per well using a TRIzol kit. cDNA reverse transcribed from the RNA was used as the template for qRT-PCR. The reaction was performed according to the instruction of SYBR Prime Script RT-PCR kit. The total reactants consisted of 0.8 μl of cDNA, 5.0 μl of SYBR Primix Ex Taq, 1.0 μl of primers and 3.2 μl of RNase H_2O. The thermal cycling was set as follows: 5 minutes at 95°C; 30 cycles of 15 seconds at 95°C, 30 seconds at 95°C and 40 seconds at 72°C. GAPDH served as a reference gene. Each sample had three duplicates. The results were analyzed using the $2^{-\Delta\Delta CT}$ method: $\Delta\Delta Ct = (Ct_{\text{target gene}}-Ct_{\text{reference gene}})_{\text{experimental group}}-(Ct_{\text{target gene}}-Ct_{\text{reference gene}})_{\text{control group}}$. Sequences of the primers used in the PCR are presented in Table 1.

Table 1. Primer sequences

miR-375-F	CACAAAATTTGTTCGTTCGGCT
miR-375-R	GTGCAGGGTCCGAGGT
SIRT5-F	ACAATGGCTCGTCCAAGTTC
SIRT5-R	CCAGTAACCTCCTGCTCCTCT
GAPDH-F	GACAGTCAGCCGCATCTTCT
GAPDH-R	GCGCCCAATACGACCAAATC

Note: F, forward; R, reverse.

Western blotting

Cells were cultured for 48 h before Western blotting analysis in which 1 ~ 2×10^6 cells per well were used. After two PBS washes, the cells were lysed in lysis buffer on ice for 45 minutes and shaken at 15-minute intervals. After quantification by a bicinchoninic acid (BCA) kit, proteins extracted from the cells were mixed with loading buffer and transferred onto a polyvinylidenefluoride

(PVDF) membrane after SDS-PAGE electrophoresis. Non-specific binding was blocked in 5% skim milk for 2 hours (room temperature). After that, the membrane was incubated with primary antibodies of SIRT5 (#8779, 1:1000), LC3II/I (#12741, 1:1000), p62 (#88588, 1:1 000), acetylated-lysine (#9814, 1:500) (Cell Signaling Technology, Beverly, MA, USA) or LDHB (ab53292, Abcam, Cambridge, MA, USA) at 4°C overnight and washed with PBS Tween 20 (PBST) for 3×15 minutes. The proteins were then incubated with the secondary antibody (ab6728, 1:2000, Abcam, Cambridge, MA, USA) for 1 hour. Protein expressions were reflected by chemiluminescence. GAPDH (Cell Signaling, #5174, 1:1000) acted as a reference protein.

Dual-luciferase reporter assay

MiR-375 was found to have a binding site on the 3'UTR of SIRT5 mRNA based on the analysis of jefferson (https://cm.jefferson.edu/). Wild and mutant SIRT5-3'UTR (WT-SIRT5 and MUT-SIRT5) were synthesized and cloned to luciferase reporter vectors and then co-transfected with miR-375 mimic or mimic NC into HEK-293T cells. Luciferase activities in the cells were assessed 48 hours after transfection using a fluorescent luminescence detector based on the instruction of the dual-luciferase reporter assay kit (Beyotime, Shanghai, China). Relative luciferase activity = Firefly luciferase activity/Renilla luciferase activity.

Statistical analysis

SPSS 18.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 7.0 (GraphPad Software Inc.) were applied for statistical analysis. Data were finally presented as mean ± standard deviation (S.D.). *T*-test and one-way analysis of variance were used to analyze the differences of two groups and multi-groups, respectively. Differences were deemed statistically significant when $P<0.05$.

RESULTS

MiR-375 is under-expressed in sorafenib-resistant HCC cells

The parental strains of HCC cells (HepG2 and Huh7) were continuously cultured in sorafenib of increasing concentrations to obtain sorafenib-resistant HCC cells (HepG2/so and Huh7/so). The morphology of HepG2 and Huh7 cells under the inverted microscope were changed from a plump, pebble-like shape (epithelial phenotype) into a spindle shape (mesenchymal phenotype) (Fig. 1A). CCK-8 assay detected that the IC50 of sorafenib in HepG2/so and

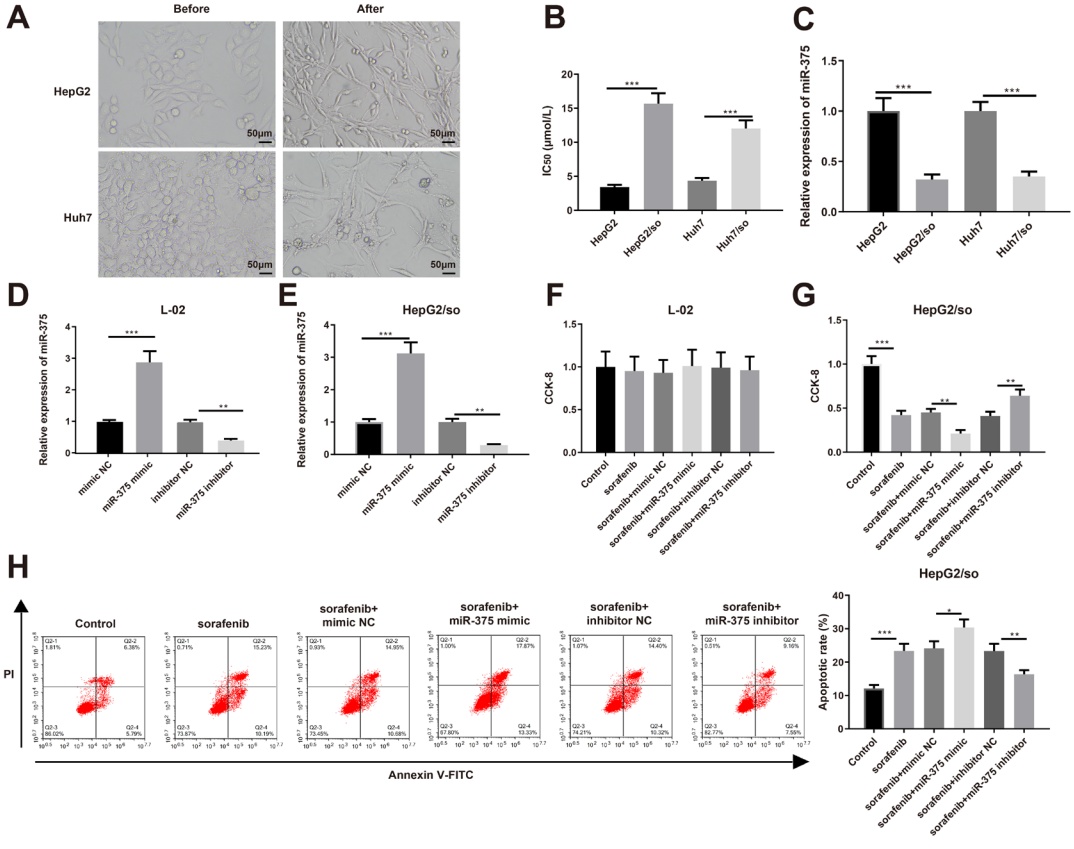

Figure 1. MiR-375 is under-expressed in sorafenib-resistant HCC cells
(**A**) The morphology of HepG2 and Huh7 cells under an inverted microscope. (**B**) CCK-8 assay measured the sorafenib IC50 of HepG2, Huh7, HepG2/so and Huh7/so cells. (**C**) the expression of miR-375 in HepG2, Huh7, HepG2/so and Huh7/so cells. (**D–E**) qRT-PCR detected the expression of miR-375 in HepG2/so (D) or L-02 (E) cells transfected with miR-375 mimic or miR-375 inhibitor. (**F**) CCK-8 assay assessed the survival of the transfected L-02 cells. CCK-8 assay (**G**) and flow cytometry (**H**) assessed the survival and apoptosis of the transfected HepG2/so cells after sorafenib treatment. N=3; **P*<0.05, ***P*<0.01, *** *P*<0.001; data were presented as mean ± S.D.; *T*-test was for comparison between two groups; one-way analysis of variance was for multi-group comparison; the Tukey test was for *post hoc* multiple comparisons after ANOVA; HCC, hepatocellular carcinoma; cell counting kit 8; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

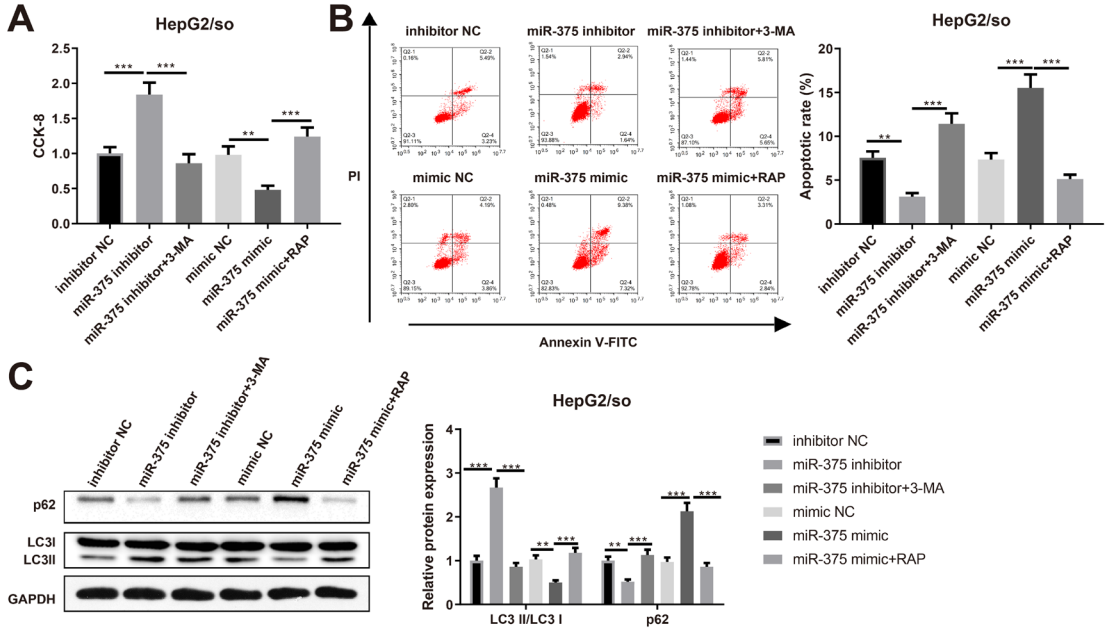

Figure 2. Autophagy acts on miR-375-mediated drug resistance of HCC cells
(**A**) CCK-8 assay assessed the survivability of HepG2/so cells; (**B**) Annexin-V-FITC/PI staining and flow cytometry assessed the apoptosis of HepG2/so cells; (**C**) Western blotting measured the expressions of p62, LC3I and LC3II in HepG2/so cells. N=3; **P*<0.05, ***P*<0.01, ****P*<0.001; data were presented as mean ± S.D; *T*-test was for comparison between two groups; one-way analysis of variance was for multi-group comparison; the Tukey test was for *post hoc* multiple comparisons after ANOVA; HCC, hepatocellular carcinoma; CCK-8, cell counting kit 8; FITC, fluorescein isothiocyanate; PI, propidium iodide; RAP, rapamycin; 3-MA, 3-Methyladenine..

Huh7/so cells was higher than in HepG2 and Huh7 cells (Fig. 1B, $P<0.05$), suggesting stronger tolerance of HepG2/so and Huh7/so cells to sorafenib.

MiR-375 was under-expressed in HepG2/so and Huh7/so cells compared to HepG2 and Huh7 cells (Fig. 1C, $P<0.05$). To investigate the potential effect of miR-375 expression on sorafenib resistance and on healthy liver cells, HepG2/so and L-02 cells were transfected with miR-375 mimic or miR-375 inhibitor. MiR-375 was up-regulated in the miR-375 mimic group and down-regulated in the miR-375 inhibitor group (Fig. 1D–E, $P<0.05$, *vs* the mimic NC or inhibitor NC group), suggesting successful cell transfection. The results of CCK-8 assay showed that transfection of miR-375 mimic/inhibitor had no significant impact on the survival of heathy liver cells (Fig. 1F). After the transfection, HepG2/so cells were exposed to sorafenib for 24 hours. Sorafenib treatment impeded the survival of HepG2/so cells (Fig. 1G, $P<0.05$). The survival of HepG2/so cells was further damaged by miR-375 mimic while improved by miR-375 inhibitor (Fig. 1G, $P<0.05$). The results of flow cytometry showed that miR-375 mimic promoted sorafenib-induced apoptosis of HepG2/so cells while miR-375 inhibitor attenuated the apoptosis (Fig. 1H, $P<0.05$).

Autophagy acts on miR-375-mediated drug resistance of HCC cells

The autophagy level in transfected HepG2/so cells was inhibited by 3-MA or activated by RAP. CCK-8 assay detected that 3-MA treatment impaired the viability of HepG2/so cells transfected with miR-375 inhibitor, while RAP treatment enhanced the viability of HepG2/so cells overexpressing miR-375 (Fig. 2A, $P<0.05$, *vs* the miR-375 inhibitor group or miR-375 mimic group). The apoptosis of HepG2/so cells was reduced in the miR-375 inhibitor group, while enhanced in the miR-375 inhibitor + 3-MA group (Fig. 2B, $P<0.05$, *vs* the inhibitor NC group and miR-375 inhibitor group, respectively). The apoptosis of HepG2/so cells was promoted in the miR-375 mimic group, while inhibited in the miR-375 mimic + RAP group (Fig. 2B, $P<0.05$).

Meanwhile, according to the Western blotting measurement of the expressions of p62, LC3I and LC3II, the autophagy of HepG2/so cells was inhibited in the miR-375 inhibitor + 3-MA group, while enhanced in the miR-375 mimic + RAP group (Fig. 2C, $P<0.05$, *vs* the miR-375 inhibitor group and miR-375 mimic group, respectively).

MiR-375 attenuates sorafenib resistance by mediating autophagy in HCC cells

According to the CCK-8 analysis of cell viability, HepG2/so cells were less susceptible to sorafenib than HepG2 cells ($P<0.05$). The drug resistance of HepG2/so cells was enhanced by miR-375 inhibitor and reduced by miR-375 mimic (Fig. 3A, $P<0.05$). The apoptosis rate of HepG2/so cells was significantly reduced compared to that of HepG2 cells ($P<0.05$). The number of apoptotic HepG2/so cells was decreased after transfection of miR-375 inhibitor, while increased after transfection of miR-375 mimic (Fig. 3B, $P<0.05$).

Furthermore, the LC3II/LC3I ratio was increased and p62 was decreased in HepG2/so cells compared to HepG2 cells ($P<0.05$). The above mentioned expression trends of p62, LC3I and LC3II in HepG2/so cells were promoted by miR-375 inhibitor, while perturbed by miR-375 mimic (Fig. 3C, $P<0.05$).

SIRT5 is targeted and down-regulated by miR-375

SIRT5 was detected to be a downstream target of miR-375 based on the bioinformatics analysis of jefferson (Fig. 4A). Dual-luciferase reporter assay was designed to confirm the potential regulation between miR-375 and SIRT5. miR-375 mimic attenuated the relative

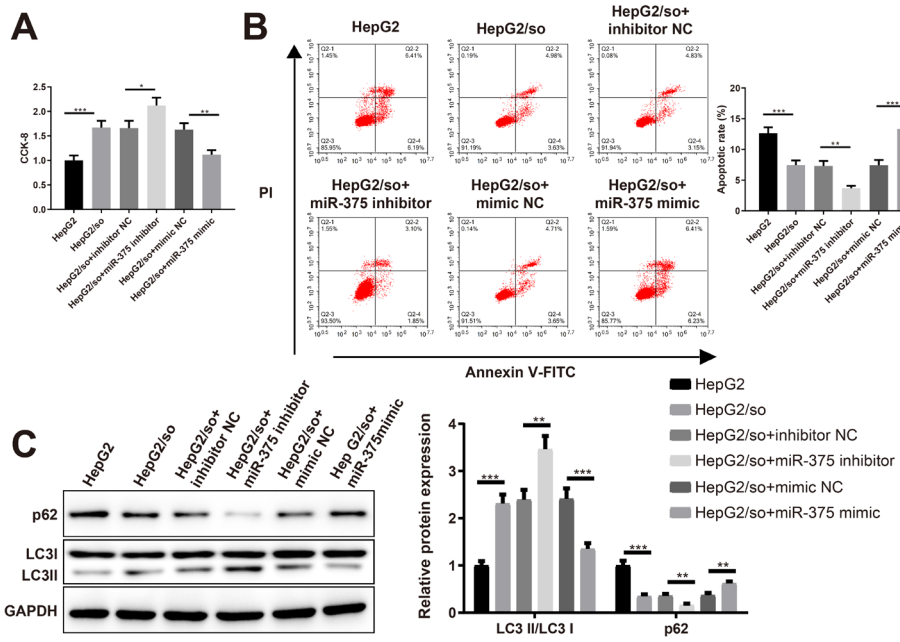

Figure 3. MiR-375 attenuates sorafenib resistance by mediating autophagy in HCC cells
(**A**) The survival rates of HepG2 and HepG2/so cells; (**B**) the apoptosis rates of HepG2 and HepG2/so cells; (**C**) the levels of p62, LC3I and LC3II in HepG2 and HepG2/so cells. N=3; **P*<0.05, ***P*<0.01, ****P*<0.001; data were presented as mean ± S.D.; *T*-test was for comparison between two groups; one-way analysis of variance was for multi-group comparison; the Tukey test was for *post hoc* multiple comparisons after ANOVA; HCC, hepatocellular carcinoma.

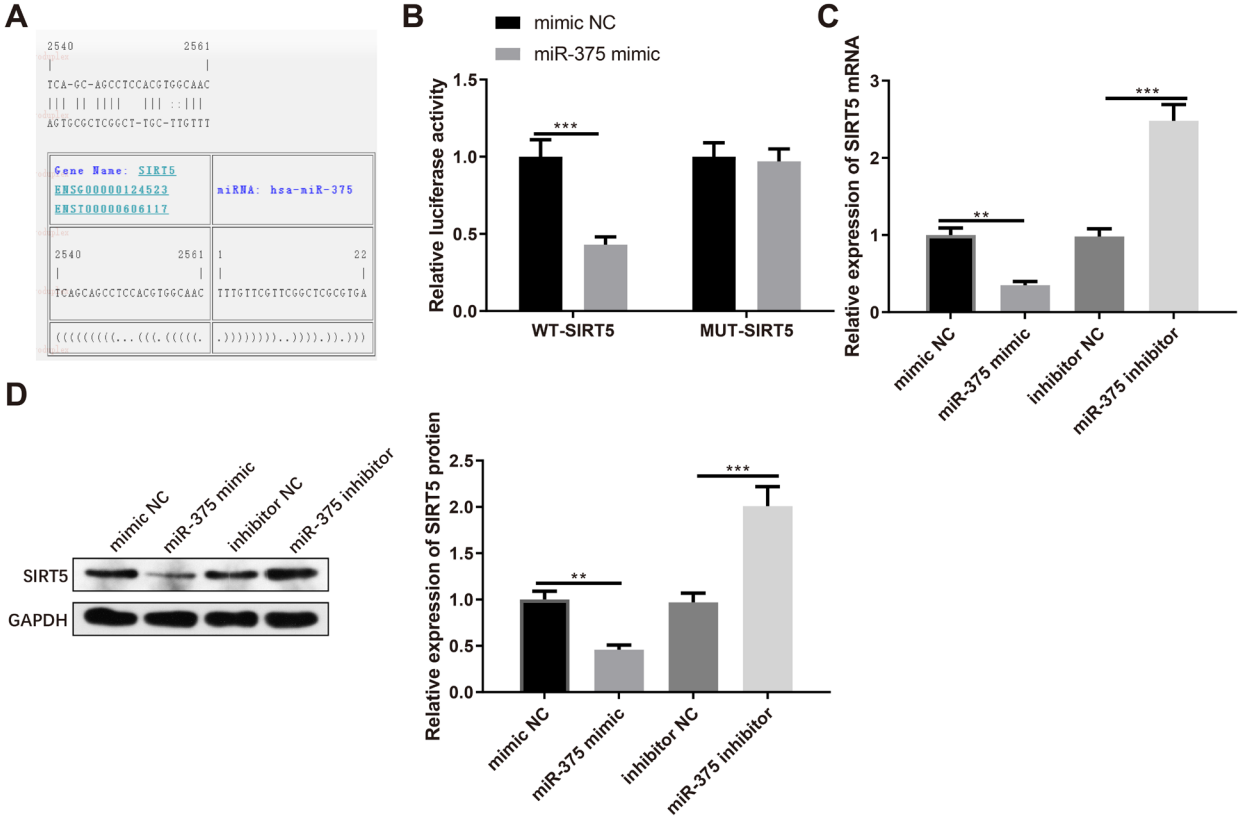

Figure 4. SIRT5 is targeted and down-regulated by miR-375
(**A**) The binding sites between miR-375 and SIRT5 were predicted by jefferson; (**B**) dual-luciferase reporter assay verified the binding between miR-375 and SIRT5; (**C–D**) the expression of SIRT5 in cells transfected with miR-375 mimic or miR-375 inhibitor. N=3; **P*<0.05, ***P*<0.01, ****P*<0.001; data were presented as mean ± S.D.; one-way analysis of variance was for multi-group comparison; the Tukey test was for *post hoc* multiple comparisons after ANOVA.

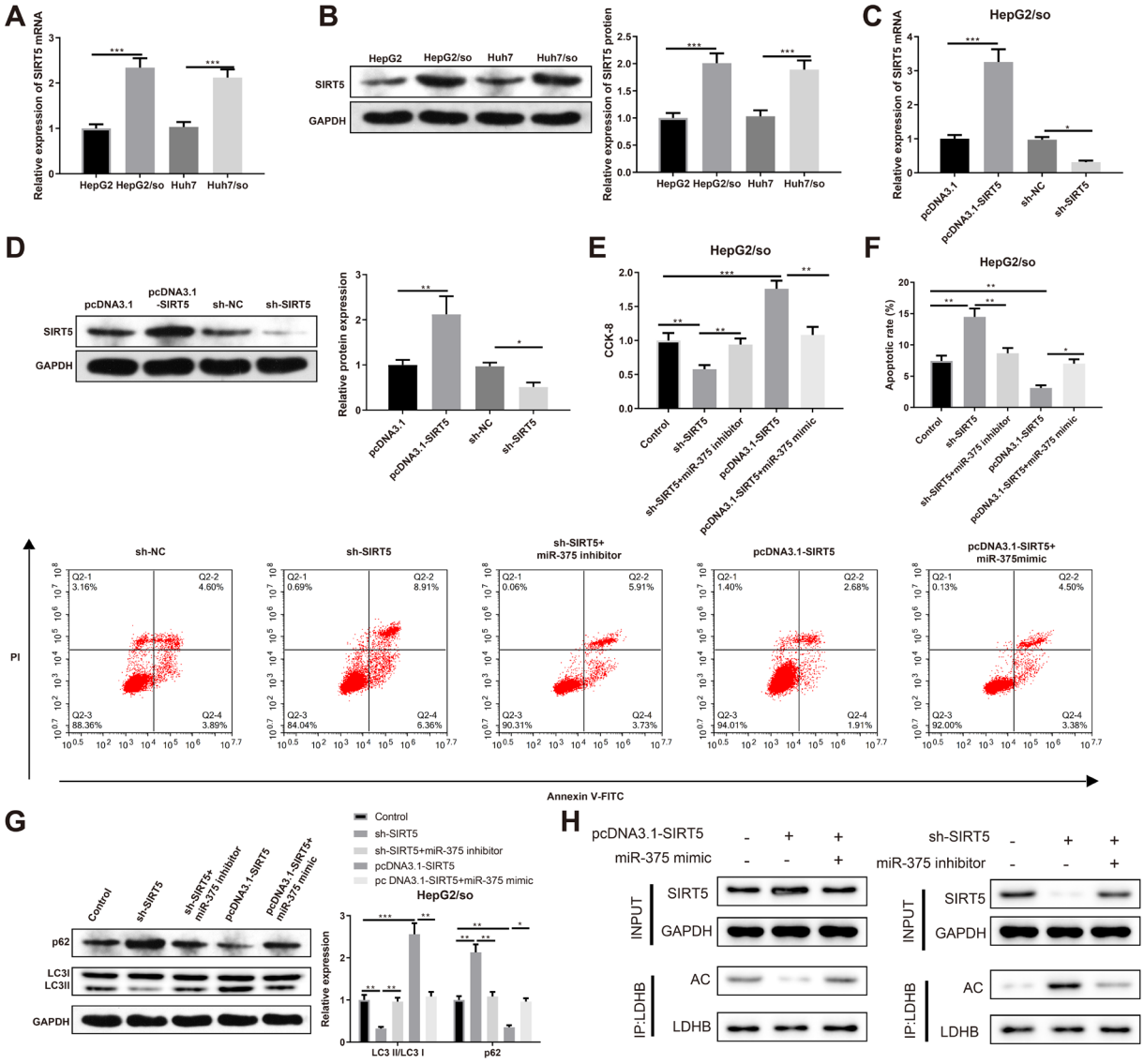

Figure 5. SIRT5 is over-expressed in sorafenib-resistant HCC cells and reverses the suppressive effect of miR-375 on sorafenib resistance

(**A–B**) The expression of SIRT5 in HepG2, Huh7, HepG2/so and Huh7/so cells. After HepG2/so cells were transfected with pcDNA3.1-SIRT5 or sh-SIRT5, qRT-PCR (**C**) and Western blotting (**D**) measured the expression of SIRT5 in HepG2/so cells; (**E**) CCK-8 assessed the survival rate of HepG2/so cells; (**F**) Annexin-V-FITC/PI staining and flow cytometry assessed the apoptosis of HepG2/so cells; Western blotting measured the expressions of p62, LC3I and LC3II (**G**) and the acetylation level of LDHB (**H**) in HepG2/so cells. N=3; **P*<0.05, ***P*<0.01, ****P*<0.001; data were presented as mean ± S.D.; *T*-test was for comparison between two groups; one-way analysis of variance was for multi-group comparison; the Tukey test was for *post hoc* multiple comparisons after ANOVA; HCC, hepatocellular carcinoma.

luciferase activity of WT-SIRT5 (Fig. 4B, *P*<0.01), but did not affect that of MUT-SIRT5. In cells, the expression of SIRT5 was negatively regulated by miR-375 (Fig. 4C–D, *P*<0.01).

SIRT5 is over-expressed in sorafenib-resistant HCC cells and reverses the suppressive effect of miR-375 on sorafenib resistance

According to the results of qRT-PCR and Western blotting, HepG2/so and Huh7/so cells had higher expressions of SIRT5 than HepG2 and Huh7 cells (Fig. 5A–B, *P*<0.05). To investigate the effect of SIRT5 on sorafenib resistance, SIRT5 was either knocked down or over-expressed in HepG2/so cells *via* transfection of sh-SIRT5 or pcDNA3.1-SIRT5 (Fig. 5C–D, *P*<0.05). SIRT5 inhibition aggravated the damage to the survival of HepG2/so cells whereas SIRT5 overexpression improved the survival (Fig. 5E, *P*<0.05). The suppressive or promotive effect of SIRT5 inhibition or overexpression on sorafenib resistance of HepG2/so cells was reversed by miR-375 inhibitor or miR-375 mimic (Fig. 5E, *P*<0.05). SIRT5 inhibition exacerbated sorafenib-induced apoptosis of HepG2/so cells, which was later attenuated by miR-375 inhibitor (Fig. 5F, *P*<0.05). SIRT5 overexpression ameliorated the deaths of sorafenib-treated HepG2/so cells, while the apoptosis rate was increased in the pcDNA3.1-SIRT5 + miR-375 mimic group (Fig. 5F, *P*<0.05).

SIRT5 promotes autophagy by catalyzing the deacetylation of LDHB (Shi *et al.*, 2019). The ratio of LC3II/LC3I was reduced, the expression of p62 was up-regulated and the acetylation level of LDHB was increased in the sh-SIRT5 group (*vs* the sh-NC group) and pcDNA3.1-SIRT5 + miR-375 mimic group (*vs* the

pcDNA3.1-SIRT5 + mimic NC group); different expression patterns of LC3II, LC3I and p62 and a decreased acetylation level of LDHB were found in the pcDNA3.1-SIRT5 group (*vs* the pcDNA3.1 group) and sh-SIRT5 + miR-375 inhibitor group (*vs* sh-SIRT5 + inhibitor NC group) (Fig. 5G–H, $P<0.05$).

The above experiment data exhibited that SIRT5 knockdown inhibited autophagy to augment the susceptibility of HCC cells to sorafenib, and miR-375 down-regulation abolished the assistance of SIRT5 knockdown to sorafenib treatment. In addition, SIRT5 overexpression enhanced the resistance to sorafenib via autophagy activation in HCC cells, while miR-375 up-regulation improved the sensitivity of SIRT5 overexpressing HCC cells to sorafenib. Taken together, miR-375 mediated the tolerance of HCC cells to sorafenib by regulating SIRT5.

DISCUSSION

HCC is the predominant primary liver cancer caused by both intrinsic and extrinsic risk factors (Ghouri *et al.*, 2017). As the first-line treatment option for HCC, sorafenib significantly improves the overall survival of HCC patients but the drug efficacy has been greatly limited by high its resistance rate (Niu *et al.*, 2017). Some biological processes in tumor microenvironment, including angiogenesis, inflammation, fibrosis, autophagy and viral reactivation, are associated with sorafenib resistance (Chen *et al.*, 2015). To further explore the molecular mechanisms underlying sorafenib resistance-related biological processes would help amplify the benefits of sorafenib. The present paper elucidates the regulatory mechanism of miR-375 in autophagy-mediated sorafenib resistance of HCC cells.

First of all, sorafenib-resistant HCC cells (HepG2/so and Huh7/so) were established. HepG2/so and Huh7/so cells obtained increased cell viability and decreased apoptosis compared to HepG2 and Huh7 cells. MiR-375 was found to be down-regulated in HepG2/so and Huh7/so cells compared with parental HepG2 and Huh7 cells. Sorafenib impaired the survival of HepG2/so cells and miR-375 overexpression further enhanced sorafenib-induced cell death. To confirm the potential regulation of endogenous miR-375 in HCC cells under sorafenib treatment, the expression of miR-375 was either up-regulated or down-regulated in HepG2/so cells. According to the measurement of cell viability and apoptosis, miR-375 inhibition made HepG2/so cells more resistant to sorafenib, while miR-375 overexpression increased the drug sensitivity. The assistance of miR-375 in anti-tumor therapy has already been uncovered. MiR-375 augmented the susceptibility of HCC cells to sorafenib by targeting the autophagy-related gene, ATG14 (Yang *et al.*, 2020). MiR-375 also inhibited the survival of fulvestrant-resistant breast cancer cells by restraining autophagy (Liu *et al.*, 2018).

Numerous existing scientific reports have authenticated that autophagy inhibition results in suppression on sorafenib resistance of HCC cells. For instance, SNHG16 promoted sorafenib resistance by enhancing autophagy via the miR-23b-3p/EGR1 axis in HCC (Jing *et al.*, 2020). LncRNA HANR enhanced autophagy-dependent sorafenib resistance of HCC cells by competing with ATG9A for miR-29b (Shi *et al.*, 2020). The LC3II/LC3I ratio was increased and p62 was decreased in HepG2/so cells in comparison with HepG2 cells, suggesting activated autophagy in sorafenib-resistant HCC cells. After transfection of miR-375 inhibitor or miR-375 mimic, the autophagy level was promoted or inhibited in HepG2/so cells. Autophagy activated or inhibited in HepG2/so cells by miR-375 inhibitor or miR-375 mimic was thereafter inhibited by 3-MA or promoted by RAP. The results of viability and apoptosis tests showed that autophagy manipulation counteracted with the effect of miR-375 on sorafenib resistance. Therefore, miR-375 inhibited sorafenib resistance by regulating autophagy in HCC cells.

Based on the bioinformatics analysis and dual-luciferase reporter assay, SIRT5 was found to be targeted and down-regulated by miR-375. SIRT5 was up-regulated in HepG2/so and Huh7/so cells compared with parental cells. SIRT5 is a vital metabolic regulator and promotes the progression of HCC in some cases (Chang *et al.*, 2018; Dang *et al.*, 2018; Tang & Yang, 2020). In the present study, SIRT5 inhibition suppressed autophagy and increased apoptosis of HepG2/so cells, while SIRT5 overexpression had the opposite effects. Down-regulation of miR-375 reversed the effects of SIRT5 inhibition, promoting sorafenib resistance of HepG2/so cells. On the other hand, up-regulation of miR-375 inhibited autophagy activated by SIRT5 overexpression and suppressed sorafenib resistance of HepG2/so cells.

Collectively, miR-375 mediates autophagy to enhance the sorafenib resistance of HCC cells partially by directly down-regulating SIRT5. MTDH, also known as AEG-1, is another target of miR-375 in mediating multidrug resistance of HCC cells (Xue *et al.*, 2017; Provvisiero *et al.*, 2019; Li *et al.*, 2021). Therefore, downregulation of MTDH may also contribute to miR-375-induced sorafenib sensitivity of HCC cells. Moreover, sorafenib can directly inhibit SLC7A11 (also called System Xc-) and consequently impede glutathione biosynthesis to induce ferroptosis in HCC cells (Li *et al.*, 2021). MiR-375 may also sensitize HCC cells to sorafenib-induced ferroptosis. Sorafenib resistance exists in most cases of HCC treatment. Despite this unsatisfactory outcome, sorafenib is still a potent drug for advanced HCC. The miR-375/SIRT5 axis discovered in the present study may serve as a new target for increasing sorafenib sensitivity and therefore improve the outcomes of HCC treatment.

Declarations

Acknowledgements. Not applicable.

Conflict of interests. The authors report no relationships that could be construed as a conflict of interest.

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