

Original Article

Long non-coding RNA CASC2 reduces sorafenib resistance in hepatocellular carcinoma cells via inhibition of miR-21-mediated PI3K/AKT pathways

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Received November 10, 2017; Accepted May 3, 2018; Epub March 15, 2019; Published March 30, 2019

Abstract: Hepatocellular carcinoma (HCC) is highly resistant to sorafenib treatment. This present study examined the relationship between lncRNA CASC2 expression and sensitivity of HCC cells to sorafenib. By selection and establishment of sorafenib-resistant cell lines, it was found that CASC2 was significantly downregulated in sorafenib-resistant HepG2 cells. In addition, overexpression of CASC2 could re-sensitize resistant HCC cells to sorafenib by reducing cell viability and promoting cell apoptosis. Overexpression of CASC2 also enhanced the anti-HCC efficacy of sorafenib *in vivo*. Dual luciferase reporter assay revealed that CASC2 might function as a competing endogenous RNA (ceRNA) for miR-21. miR-21 overexpression rescued functions induced by CASC2 overexpression in sorafenib-resistant HCC cells and might be associated with activation of PI3K/AKT pathways. In summary, this study's findings show that CASC2 might function as a potential therapeutic target to overcome sorafenib resistance in HCC.

Keywords: Hepatocellular carcinoma (HCC), sorafenib, CASC2, miR-21, PI3K/AKT pathway

Introduction

Hepatocellular carcinoma (HCC) is a major cause of cancer-associated mortality worldwide and its incidence is increasing [1]. HCC causes about 662,000 deaths annually, with about half of them happening in China [2]. Chemotherapy is a common component of postoperative or preoperative therapy for HCC. Presently, sorafenib, a multikinase inhibitor with antiangiogenic and antiproliferative effects, is the only chemotherapy drug extensively used in clinical practice as a first-line treatment for advanced HCC [3]. Its efficacy, however, is often impaired by the development of drug resistance [4]. Therefore, there is an urgent need to understand the mechanism of sorafenib resistance and explore new strategies to overcome sorafenib resistance in HCC.

Long non-coding RNAs (lncRNAs), a class of non-coding transcripts longer than 200 nucleotides with limited protein-coding potential, are gaining prominence due to their emerging role

in the regulation of multiple physiological and pathological processes [5]. A growing body of evidence has demonstrated that lncRNAs might be implicated in the development of chemoresistance in various cancers [6]. For example, lncRNA TUC338 might play an essential role in sorafenib-resistance of HCC cells [7]. lncRNA cancer susceptibility candidate 2 (CASC2), originally identified in endometrial cancer, serves as a tumor suppressor in many human cancers including non-small cell lung cancer, gastric cancer, and esophageal carcinoma [8-11]. In HCC, it has been previously reported that decreased expression of CASC2 contributes to development and progression of HCC [12, 13]. However, the association between CASC2 and sorafenib resistance in HCC requires further investigation.

Therefore, in the present research, a series of *in vitro* and *in vivo* experiments were carried out to explore the function of CASC2 in the progress of sorafenib resistance in HCC, along with its underlying mechanisms.

Table 1. The sequences of PCR primers

| Gene name | Primer sequences (5'-3') |
|-----------------------|---|
| CASC2 Forward primer | GCACATTGGACGGTGTTC |
| CASC2 Reverse primer | CCCAGTCCTTACAGGTCAC |
| GAPDH Forward primer | CGCTCTGCTCCTCCTGTTC |
| GAPDH Reverse primer | ATCCGTTGACTCCGACCTTAC |
| miR-21-RT | GTCGTATCCAGTGCAGGGTCCGAGGTATTTCGCACTGGATACGACTCAACA |
| U6-RT | ACCGTGGATGCAATGCCTAA |
| miR-21 Forward primer | TAGCTTATCAGACTGA |
| miR-21 Reverse primer | CTGGAGCAGCACAGCCAATA |
| U6 Forward primer | GTAGTCGGCGAAGGTCTCAC |
| U6 Reverse primer | ACCGTGGATGCAATGCCTAA |

Materials and methods

Cell culture and establishment of sorafenib-resistant cells

Human HCC HepG2 cells, obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China), were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin/streptomycin at 37°C in a humidified atmosphere with 5% CO₂. Sorafenib was purchased from Jinan Trio Pharmatech Co., Ltd. (Jinan, China). HepG2/SOR (sorafenib-resistant) cells were established from HepG2 cells after continuous exposure to 1-10 µM of sorafenib (Sigma, St. Louis, MO, USA) for more than 6 months.

Cell transfection

The full-length CASC2 sequence was synthesized and sub-cloned into pcDNA 3.1(+) vector (Invitrogen). The empty pcDNA3.1 vector was considered as negative control (NC). miR-21 mimics and corresponding negative control miR (miR-NC) were purchased from RiboBio (Guangzhou, China). Cell transfection was performed using Lipofectamine™ 2000 transfection reagent (Invitrogen), according to manufacturer instructions. After transfection for 48 hours, the cells were used for subsequent analysis.

RNA extraction and quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was extracted from cells using TRIzol Reagent (Gibco, Birmingham, MI, USA). For lncRNA, cDNA was synthesized using PrimeScript RT reagent Kit (TaKaRa, Dalian, China) and qRT-

PCR was performed using SYBR Premix Ex Taq (TaKaRa) on an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, USA). For miRNA, cDNA was synthesized using One Step Prime script miRNA cDNA Synthesis Kit (Qiagen, Valencia, CA, USA) and qRT-PCR was performed using miRNA-specific TaqMan® MiRNA Assay Kit (Applied Biosystems). GAPDH and U6 were used as an internal control for CASC2 and miR-21, respectively. Relative expression of individual genes was calculated using the 2^{-ΔΔCt} method [14]. Primers used in the reactions are listed in **Table 1**.

In vitro chemosensitivity assay

Chemosensitivity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, Sigma, USA) assay. Cells were cultured in 96-well plates treated with sorafenib (1, 5, 10, 20 µM). After 48 hours, the MTT solution (5 mg/mL, 20 µl) was added to each well. Following incubation for another 4 hours, the media was removed and 100 µl DMSO were added to each well. Absorbance was then measured at 450 nm using a MRX II microplate reader (Dynex, Chantilly, VA, USA).

Cell apoptosis analysis

Apoptosis rate of cells was detected using Annexin V-FITC apoptosis detection kit (BD Biosciences, San Jose, CA, USA). Following indicated treatment, the cells were trypsinized and washed with ice-cold PBS. Cell suspensions were then double stained with Annexin V-FITC and PI for 15 minutes at room temperature in the dark. The cells were then analyzed by flow cytometry (BD Bioscience) equipped with FlowJo9.1 software.

CASC2/miR-21 axis regulates HCC sorafenib resistance

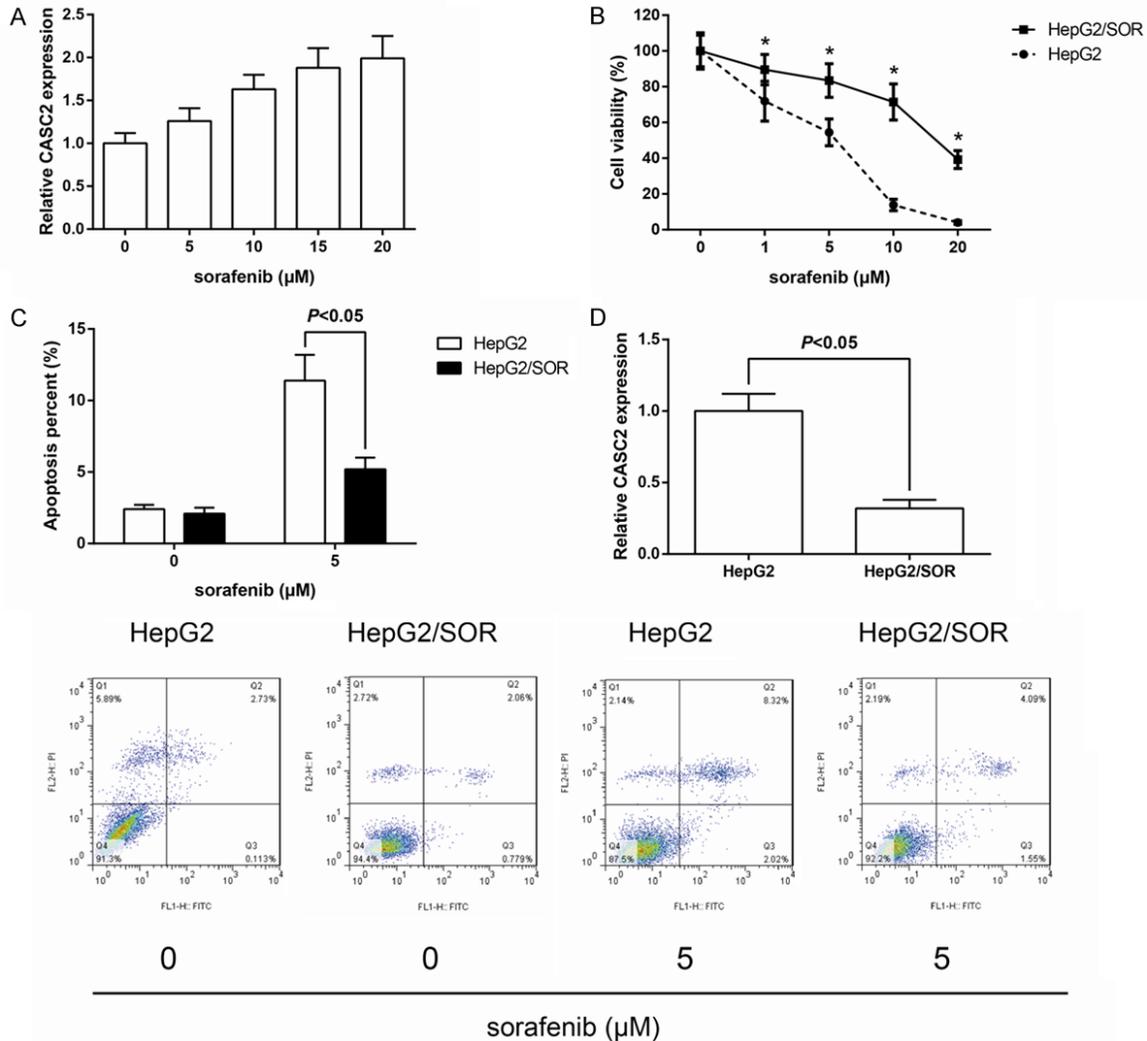


Figure 1. CASC2 is downregulated in sorafenib-resistant HCC cells. A: qRT-PCR analysis of CASC2 expression in HepG2 cells treated with different doses of sorafenib. B: MTT assay was performed to detect the proliferation of HepG2/SOR cells under sorafenib treatment. C: Flow cytometry was performed to measure the apoptosis rate of HepG2/SOR cells incubated with sorafenib (0, 5 μM). D: qRT-PCR analysis of CASC2 expression in HepG2/SOR cells. Data are presented as mean ± SD. * $P < 0.05$ versus HepG2 cells.

Western blot analysis

Cell samples were lysed using RIPA protein extraction reagent (Beyotime, Beijing, China) with proteinase inhibitor cocktail (Boster, Wuhan, China). Subsequently, equal amounts of protein were separated by 10% SDS-PAGE gel and then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Membranes were then blocked with 5% skim milk for 1 hour at room temperature and incubated with primary antibodies overnight at 4°C. This was followed by treatment with the secondary antibodies for 2 hours at room temperature. Immunoreactive bands were visualized

using a chemiluminescence reagent (ECL) kit (Pierce, Rockford, IL, USA) and band intensities were quantified using ImageJ software (NIH, Bethesda, USA). GAPDH was used as a control.

Luciferase reporter assay

Putative binding sites or mutation sites of the miR-21 seed sequence in CASC2 were cloned into psiCheck2 luciferase vector (Promega, Madison, WI, USA). HepG2 cells were placed on a 24-well plate and co-transfected with luciferase plasmids and miR-21 mimics or control. Firefly and Renilla luciferase activities were

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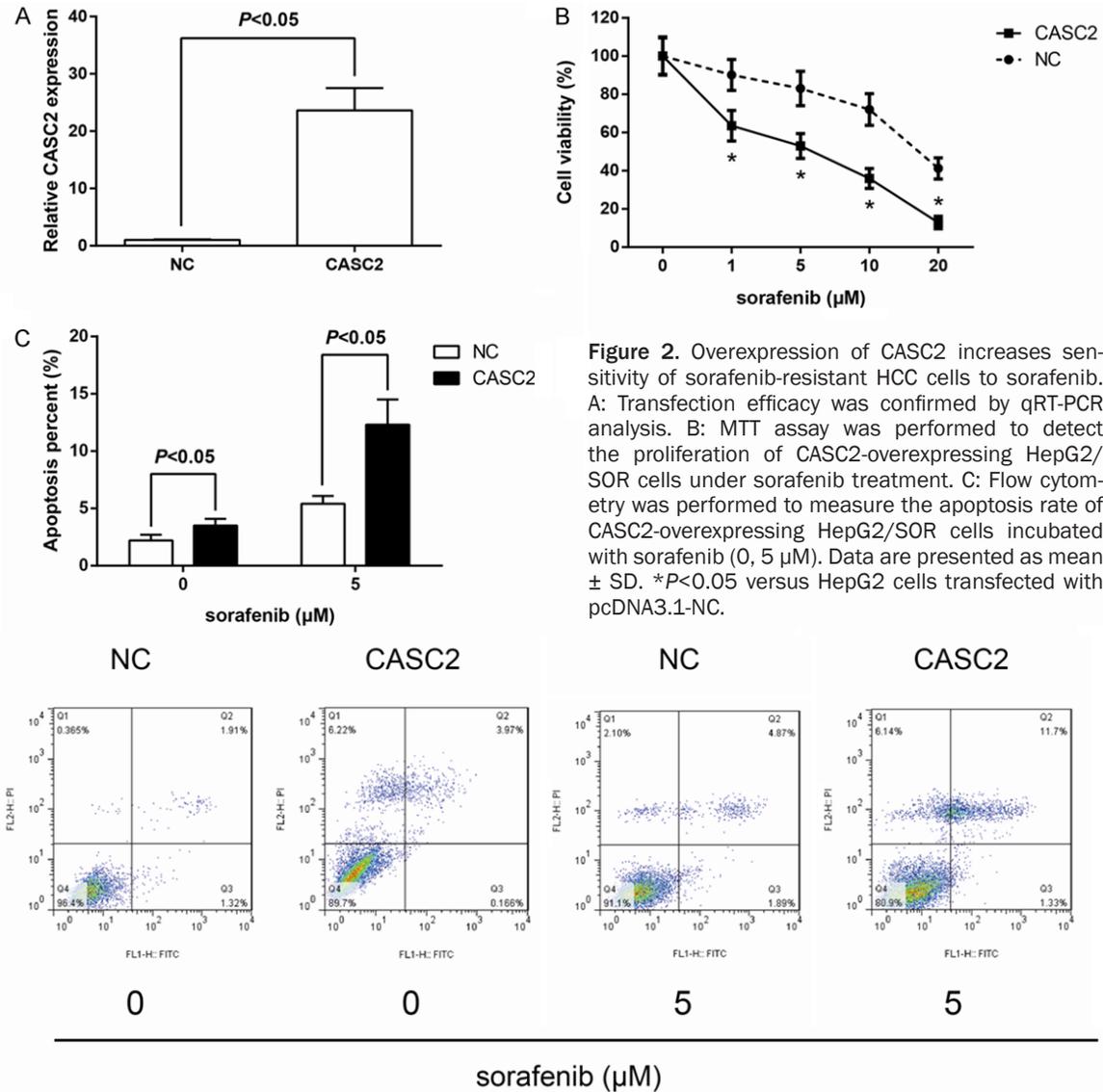


Figure 2. Overexpression of CASC2 increases sensitivity of sorafenib-resistant HCC cells to sorafenib. A: Transfection efficacy was confirmed by qRT-PCR analysis. B: MTT assay was performed to detect the proliferation of CASC2-overexpressing HepG2/SOR cells under sorafenib treatment. C: Flow cytometry was performed to measure the apoptosis rate of CASC2-overexpressing HepG2/SOR cells incubated with sorafenib (0, 5 μM). Data are presented as mean ± SD. * $P < 0.05$ versus HepG2 cells transfected with pcDNA3.1-NC.

measured using Dual-Luciferase Reporter Assay System (Promega) 48 hours after transfection. Firefly luciferase activity was normalized to Renilla luciferase activity.

In vivo tumor xenografts

A total of twenty male athymic BALB/c nude mice (4-6 weeks old), purchased from SLAC Laboratory Animal Ltd., Co. (Shanghai, China), were used in the present study. HepG2/SOR cells (1×10^6 cells in 0.1 ml PBS), stably transfected with pcDNA3.1-CASC2 and pcDNA3.1-NC, were subcutaneously injected in the hip back of nude mice. Beginning the eighth day, mice were orally administered 10 mg/kg sorafenib once daily and saline injection was

used as control. Tumors were measured every five days using a Vernier caliper and tumor volume was calculated using this equation: Volume = $0.5 \times L$ (length) $\times W^2$ (width). Twenty-eight days after cell injection, the tumor-bearing mice were killed. Tumors were excised and weighted. All experimental procedures involving animals were approved by the Animal Care and Experiment Committee of Sichuan Provincial People's Hospital (Chengdu, China).

Statistical analysis

All data are expressed as mean ± standard deviation (SD). Data were analyzed using GraphPad Prism version 6.0 (GraphPad Software, Inc., San Diego, CA, USA). Two-tailed Student's

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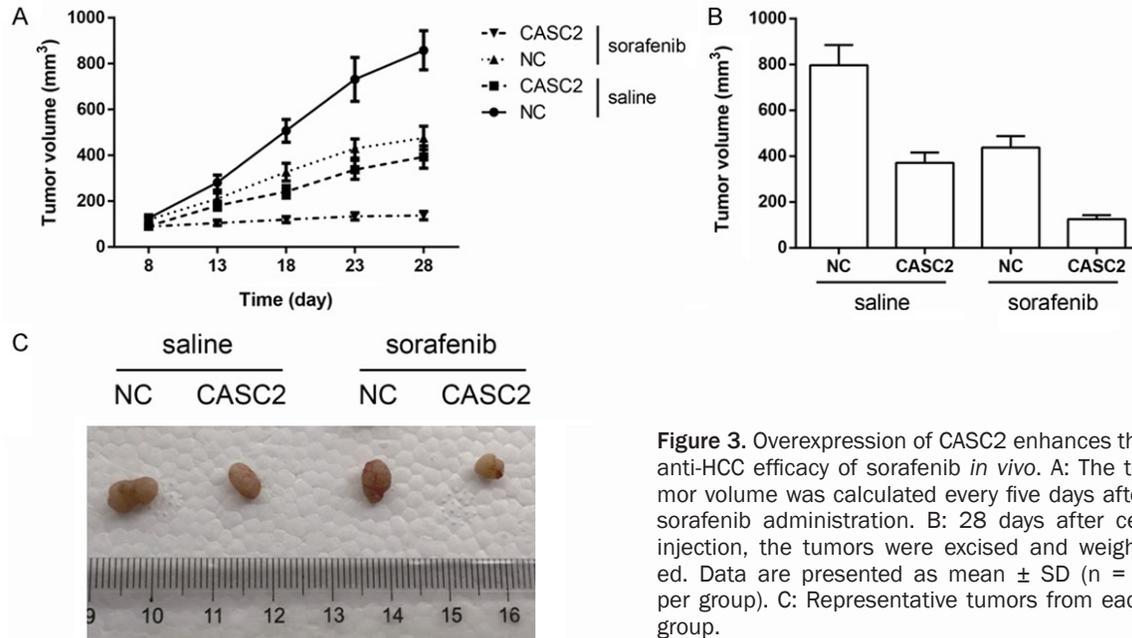


Figure 3. Overexpression of CASC2 enhances the anti-HCC efficacy of sorafenib *in vivo*. A: The tumor volume was calculated every five days after sorafenib administration. B: 28 days after cell injection, the tumors were excised and weighed. Data are presented as mean \pm SD (n = 5 per group). C: Representative tumors from each group.

t-test or one-way analysis of variance (ANOVA) were used for data analysis. Differences were considered statistically significant when *P*-values < 0.05.

Results

CASC2 was downregulated in sorafenib-resistant HCC cells

To examine whether sorafenib regulated CASC2 expression in HCC cells, cells were treated with increasing doses of sorafenib for 48 hours and then levels of CASC2 were determined. It was observed that CASC2 expression was remarkably increased by sorafenib in a dose-dependent manner (Figure 1A). Subsequently, this study established sorafenib-resistant HepG2 (HepG2/SOR) cell models by chronic exposure to sorafenib for at least 6 months. Results showed that HepG2/SOR cells were significantly more resistant to sorafenib treatment, demonstrated by increased cell viability (Figure 1B) and reduced cell apoptosis (Figure 1C). CASC2 expression was remarkably reduced in HepG2/SOR cells versus their parental counterparts (Figure 1D).

Overexpression of CASC2 increases sensitivity of sorafenib-resistant HCC cells to sorafenib

To test whether CASC2 overexpression may regulate sensitivity to sorafenib, pcDNA3.1-

CASC2 was transfected into HepG2/SOR cells. qRT-PCR analysis confirmed high expression levels of CASC2 after transfection (Figure 2A). Forced expression of CASC2 significantly enhanced sensitivity of HepG2/SOR cells to sorafenib-induced growth inhibition and apoptosis (Figure 2B, 2C).

Overexpression of CASC2 enhances anti-HCC efficacy of sorafenib in vivo

To further confirm the suppressive role of CASC2 to sorafenib resistance *in vivo*, effects of CASC2 on HCC tumor growth in nude mice receiving sorafenib were explored. It was found that the tumor volume of CASC2-overexpressing xenografts was significantly reduced compared to that of other xenografts under sorafenib treatment (Figure 3A). Average size and weight were also markedly reduced, 28 days after cell injection, in tumors derived from mice treated with CASC2-overexpressing HepG2/SOR cells (Figure 3B).

Overexpression of CASC2 suppresses PI3K/AKT signaling pathways in sorafenib-resistant HCC cells

To identify the molecular mechanism by which CASC2 elicits its effects, levels of key proteins in PI3K/AKT signaling pathways were investigated. Western blot analysis showed that phosphorylation levels of AKT and mTOR were sig-

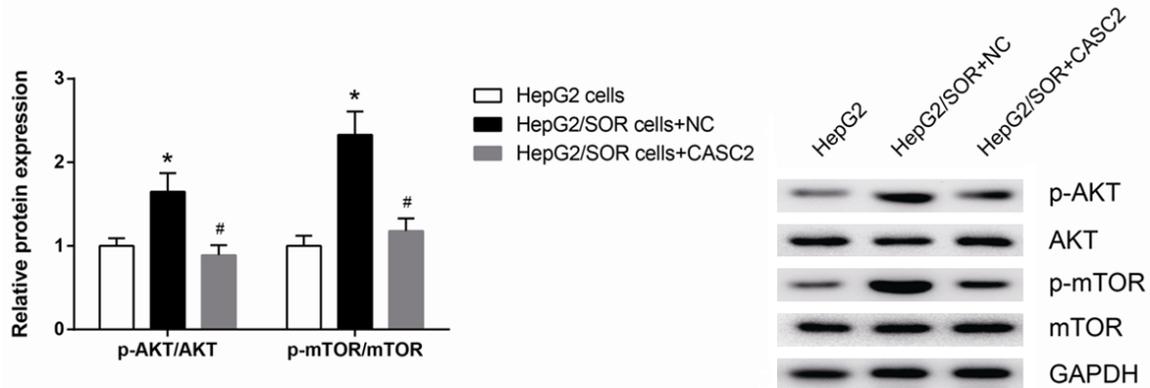


Figure 4. Overexpression of CASC2 suppresses PI3K/AKT signaling pathways in sorafenib-resistant HCC cells. Western blot analysis was performed to detect protein expression levels of p-AKT and p-mTOR. Data are presented as mean \pm SD. * $P < 0.05$ versus HepG2 cells, # $P < 0.05$ versus HepG2/SOR cells transfected with pcDNA3.1-NC.

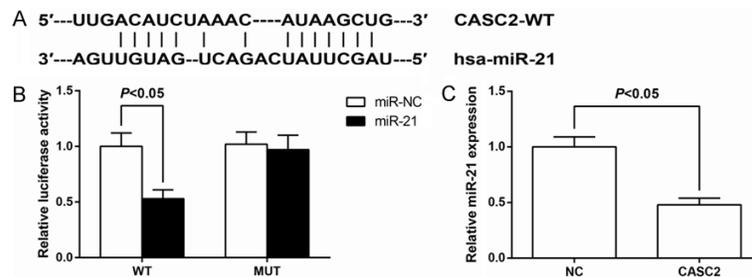


Figure 5. CASC2 binds to miR-21 and represses its expression in HCC cells. A: Schematic demonstration of the predicted binding sites between CASC2 and miR-21. B: Relative luciferase activities in HepG2 cells co-transfected with miR-21 mimics and luciferase reporters containing CASC2-WT or CASC2-MUT transcript. C: qRT-PCR analysis of miR-21 expression in CASC2-overexpressing HepG2 cells. Data are presented as mean \pm SD.

Additionally, qRT-PCR analysis revealed that overexpression of CASC2 in HepG2 cells led to decreased expression of miR-21 (Figure 5C).

Overexpression of miR-21 partially rescued the effects of CASC2 in sorafenib-resistant HCC cells

To further confirm that CASC2 exerts its biological function by targeting miR-21, HepG2/SOR cells were co-transfected with pcDNA3.1-CASC2 and miR-21 mimics. The resulting

data demonstrated that overexpression of miR-21 partially abrogated the effects of CASC2, resulting in increased cell viability (Figure 6A) and dramatically reduced apoptosis (Figure 6B), in the presence of sorafenib. Additionally, protein expression levels of p-AKT and p-mTOR were increased by miR-21 overexpression (Figure 6C).

CASC2 binds to miR-21 and represses its expression in HCC cells

To further clarify molecular mechanisms regarding the impact of CASC2 on sorafenib-resistant HCC, an online database (www.microRNA.org) was used to predict potential targets of CASC2. A potential miR-21 binding site was found in CASC2 (Figure 5A). It was hypothesized that CASC2 might serve as a competing endogenous RNA (ceRNA) by “sponging” miR-21 in sorafenib-resistant cells. Luciferase assay showed that miR-21 evidently reduced the luciferase activity of CASC2-WT but not that of CASC2-MUT in HepG2 cells (Figure 5B).

data demonstrated that overexpression of miR-21 partially abrogated the effects of CASC2, resulting in increased cell viability (Figure 6A) and dramatically reduced apoptosis (Figure 6B), in the presence of sorafenib. Additionally, protein expression levels of p-AKT and p-mTOR were increased by miR-21 overexpression (Figure 6C).

Discussion

Although a tremendous amount of effort has been invested in recent years, drug resistance remains a common problem associated with chronic treatment with anticancer drugs. Mechanisms of developing drug resistance in HCC are quite complicated. LncRNAs have increasingly been recognized as key regulators in cancer progression, especially regarding response to chemotherapy. This study aimed to

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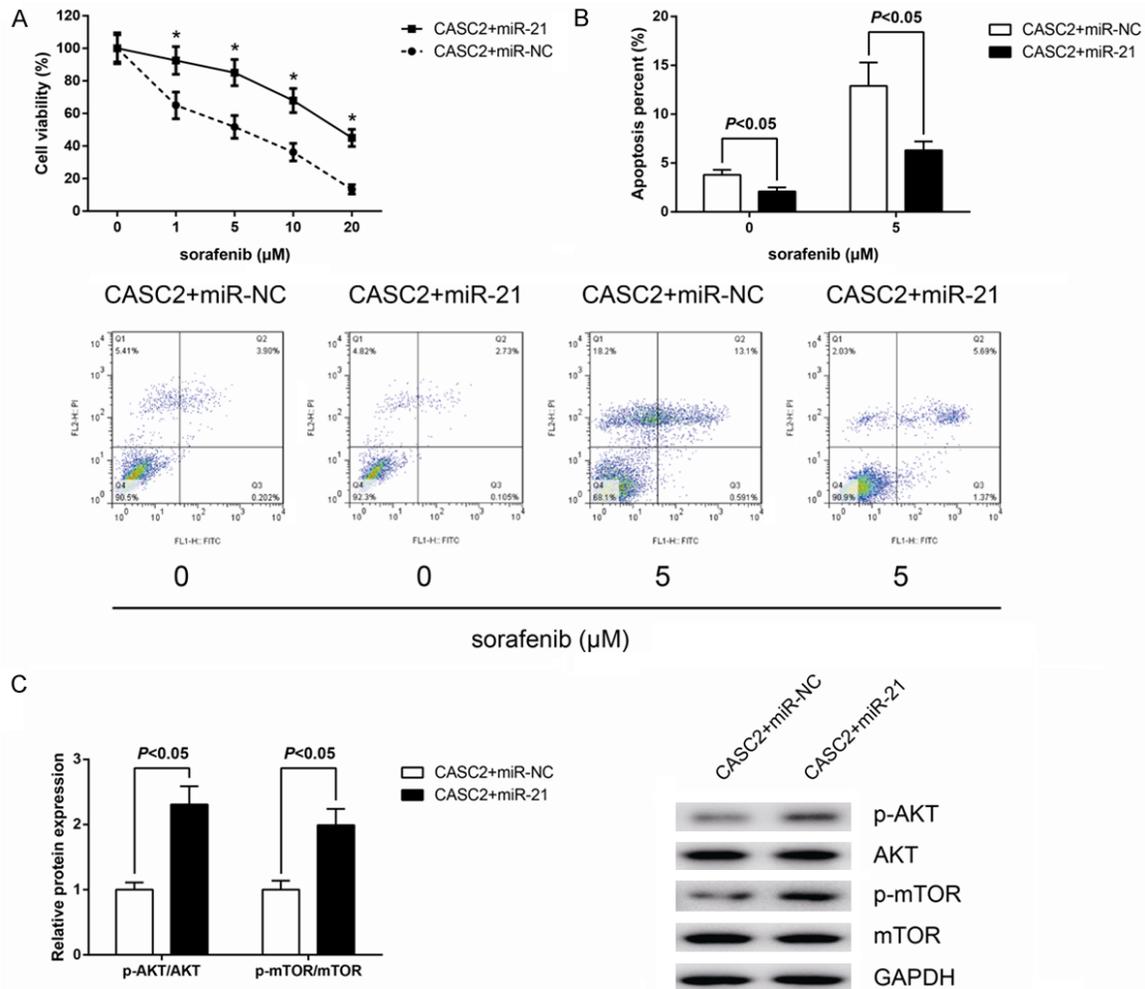


Figure 6. Overexpression of miR-21 partially rescued the effects of CASC2 in sorafenib-resistant HCC cells. **A:** MTT assay was performed to detect the effect of miR-21 on the proliferation of CASC2-overexpressing HepG2/SOR cells under sorafenib treatment. **B:** Flow cytometry was performed to detect the effect of miR-21 on the apoptosis of CASC2-overexpressing HepG2/SOR cells under sorafenib treatment. **C:** Western blot analysis of p-AKT and p-mTOR expression levels in HepG2/SOR cells co-transfected with pcDNA3.1-CASC2 and miR-21 mimics. Data are presented as mean \pm SD. * $P < 0.05$ versus HepG2/SOR cells co-transfected with pcDNA3.1-CASC2 and control miR.

investigate the underlying mechanisms of lncRNA CASC2 in the acquisition of sorafenib resistance in HCC.

This study found that, in HCC cells, downregulated CASC2 was responsible for an increase in resistance to sorafenib and CASC2 overexpression decreased cell viability and promoted cell apoptosis after sorafenib treatment, *in vitro*, thereby sensitizing HCC cells to sorafenib. *In vivo*, it was observed that sorafenib, in combination with CASC2 overexpression, suppressed tumor growth in a sorafenib-resistant HCC tumorigenic model. These results indicate that overexpression of CASC2 has the potential to reduce chemoresistance in response to sorafenib in HCC.

lncRNA-miRNA interactions are commonly implicated in various biological processes [15]. lncRNAs may function as competing endogenous RNAs (ceRNAs) or molecular sponges of miRNAs. Recent studies have shown that CASC2 functions as ceRNA to be involved in cancer progression [13, 16]. miR-21 acts as an 'onco-miR' upregulated in various human cancers and is involved in drug resistance [17, 18]. One previous study showed that CASC2 increased PTEN expression through directly inhibiting miR-21 in cisplatin-resistant cervical cancer cells [19]. This present study demonstrates that CASC2 acts as an endogenous sponge to downregulate miR-21 and overexpression of miR-21 partially rescues the effects of CASC2 in sorafenib-resistant HCC cells.

Cellular sensitivity to sorafenib is influenced by many different pathways [20]. The PI3K/AKT signaling pathway is a key pathway in multiple aspects of biological processes. It has been reported that human SMMC-7721 HCC cells exposed to sorafenib have reduced expression of PI3K/AKT [21] and aberrant activation of PI3K/AKT pathways plays a vital role in inducing drug resistance during the course of cancer treatment [22]. Therefore, the effects of CASC2 on PI3K/AKT signaling pathways in sorafenib-resistant HCC were also explored. This study's results showed that overexpression of CASC2 can inactivate PI3K/AKT pathways in sorafenib-resistant HCC cells, later rescued by miR-21 mimics.

In summary, this present study provides experimental evidence that overexpression of CASC2 improves responsiveness of sorafenib-resistant HCC cells to sorafenib treatment, at least in part by the inhibition of miR-21-mediated PI3K/AKT pathways. These results suggest that the CASC2/miR-21 signaling axis might be a novel therapeutic hint for sorafenib resistance in HCC patients.

Disclosure of conflict of interest

None.

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