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Original Paper

miR-191 Inhibition Induces Apoptosis Through Reactivating Secreted Frizzled-Related Protein-1 in Cholangiocarcinoma

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Key Words
Cholangiocarcinoma • miRNA • miR-191 • SFRP1 • Cell survival

Abstract
Background/Aims: Cholangiocarcinoma (CCA) is one of the most common malignant tumors of the biliary tract originating from biliary epithelial cells. Although many therapeutic strategies have been developed to treat CCA, the survival rate for CCA patients is still quite low. Thus it is urgent to elucidate the pathogenesis of CCA and to explore novel therapeutic targets. miR-191 has been shown to be associated with many human solid cancers, but the function of miR-191 in CCA is still poorly understood. ***Methods:*** We first investigated the expression level of miR-191 in human CCA tissues and cell lines with quantitative real-time PCR (qRT-PCR). The effects of miR-191 on CCA cells were determined by Cell Counting Kit-8 assay, colony formation assay and acridine orange/ethidium bromide staining. Finally, we utilized qRT-PCR, western blot and luciferase reporter assays to verify the miR-191 target gene. ***Results:*** We showed that miR-191 was up-regulated in CCA cell lines and patients. Knockdown of miR-191 by transfection of its inhibitor sequence blocked RBE cells viability and induced apoptosis of RBE cells. Both qRT-PCR and western blot analysis showed that the secreted frizzled-related protein-1 (sFRP1) level was negatively correlated with that of miR-191. Luciferase assay validated that sFRP1 was a direct target of miR-191. Moreover, knockdown of miR-191 led to suppression of Wnt/β-catenin signaling activation. Co-transfection of sFRP1 small interfering RNA (siRNA) and miR-191 inhibitor re-activated the Wnt/β-catenin signaling pathway as detected by an increased level of β-catenin and phosphorylation of GSK-3β, and restored the expression of survivin and c-myc in RBE cells. Co-transfection of sFRP1 siRNA with miR-191 inhibitor restored the colony formation ability and viability of RBE cells. ***Conclusion:*** Taken together, our results demonstrate a novel insight into miR-191 biological function in CCA . Our findings suggest that miR-191 is a potential therapeutic target of CCA treatment.

P.-C. Kang, K.-M. Leng and Y.-P. Liu contributed equally to this work.

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Introduction

Cholangiocarcinoma (CCA) is one of the most common malignancies originating from biliary epithelialcells [1]. CCA usually metastasize and cause relapse because it is commonly diagnosed in the advanced stage of the disease, and leads to poor prognosis [2-4]. Many studies have revealed that numerous genes are involved in CCA [5-9] and many therapeutic strategies have been developed to treat CCA [10-12]; however, the survival rate for CCA patients is still low [13]. In this case, the pathogenesis and progression of this malignancy remains to be investigated and new therapeutic targets need to be explored.

MicroRNAs (miRNAs) are 20 to 25 nucleotide-long non-coding RNAs transcripts that negatively regulate gene expression by directly binding to the 3′-UTR of target mRNA [14]. Some studies have concluded that over 60% of human genes are regulated by miRNAs [15]. Increasing evidences has demonstrated that miRNAs not only regulate various biological processes [16-18], but also are associated with the carcinogenesis and progression of diverse human cancers, including CCA [19-21]. Serum analysis revealed that miR-1281, miR-126 and miR-30b are significantly higher in CCA patients indicating a potential diagnostic value of these miRNAs in CCA [22]. The miR-17-92 cluster promotes CCA growth by decreasing expression of the tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) [23]. High expression of miR-21 contributes to CCA metastasis [24].

In this study, we focused on a novel cancer-related miRNA, miR-191. Studies have shown that miR-191 is involved in multiple human cancers' initiation and progression [25, 26]. Liu et al. reported that miR-191 plays key roles in pancreatic cancer and serves as an unfavorable prognostic marker for pancreatic cancer patients [27]. In addition, a recent study documented that miR-191 could facilitate breast cancer progression via affecting the P53-miR-191-SOX4 regulatory loop [28]. Commonly, miRNAs regulate downstream targets at the post-transcriptional level, and competing endogenous RNAs(ceRNAs) are the primary mechanism by which miRNAs are regulated *in vivo*. Different specific targets of miR-191 have been identified including TIMP3, MDM4-C and DAPK1 [29-31]. The findings above prompted us to further evaluate whether miR-191 exerts its effects via a similar mechanism in CCA. According to predictions made using "TargetScan and MicroRNA.org", secreted frizzled frizzled-related protein-1 (sFRP1) was selected as the target of miR-191.

In this study, we aimed to investigate the molecular mechanism underlying miR-191's role in CCA. First, we examined the expression of miR-191 in CCA cells and explored its effects on these cells' survival. The results indicated that miR-191was over-expressed in all CCA cell lines that we evaluated, and was associated with cell viability and apoptosis of CCA. Moreover, miR-191 regulated the Wnt/β-catenin signaling pathway by directly suppressing its target gene sFRP1. In addition, co-transfection of sFRP1 small interfering RNA (siRNA) with miR-191 inhibitor sequence reactivated the Wnt/β-catenin signaling pathway, and restored colony formation ability and regained cell viability. Thus, our work provides a vital role of miR-191 in CCA, and shows that miR-191 is a potential target of CCA therapy.

Materials and Methods

Human tissues and cell culture

A total of 21 pairs of CCA tissues and their adjacent normal tissues were obtained from patients who underwent surgical procedures at the Second Affiliated Hospital of Harbin Medical University. All of the patients provided written consent and approval was obtained from the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University. Human intrahepatic biliary epithelial cell (HiBECs), and all six CCA cell lines (QBC939, HUH28, HuCCT1, RBE, CCLP1 and TFK1) were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Dulbecco's modified Eagle medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (Invitrogen), 50 U/mL penicillin, and 50 μg/mL streptomycin (Invitrogen). Cells were maintained at 37°C in a humidified incubator at 5% CO2.

miRNA and siRNA transfection

miR-191 mimics (cat #. B06001), miR-191 inhibitor sequences (cat #. B05001), sFRP1 siRNA(cat #. A01001) and corresponding negative control (NC) sequence were all purchased from GenePharma, (Shanghai, China).

RBE cells were seeded 24 h before transfection. miR-191 mimics, inhibitor or sFRP1 siRNA was transfected using Lipofectamine 2000 (Invitrogen) with serum-free medium. Five hours after transfection, cells were changed to complete medium. Cell lysates were harvested at 48 h after transfection.

RNA extraction and quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. For regular gene expression analysis, RNAs were reverse transcribed into cDNA using Superscriptase II (Invitrogen). Quantitative real-time PCR (qRT-PCR) was performed using Power SYBR® Green PCR Master Mix (Life Technologies, Carlsbad, CA, USA). The following primer sets were used for qRT-PCR:

sFRP1-F: 5′- ACGTGGGCTACAAGAAGATGG -3′;

sFRP1-R: 5′- CAGCGACACGGGTAGATGG -3′;

GAPDH-F: 5′- AGCCTCCCGCTTCGCTCTCT -3′;

GAPDH-R: 5′- GCGCCCAATACGACCAAATCCGT -3′.

The Hairpin-itmiRNAs qPCR Quantitation Kit (Cat #. E01006, GenePhamar) was used to measure miR-191 expression. In brief, RNAs were reverse transcribed using miR-191 specific stem-loop reverse transcription primer to generate cDNA. The level of miR-191 was detected by qRT-PCR by specific primer set and molecular beacon probes. GAPDH was used for normalization.

Antibodies and western blotting

Whole-cell protein extracts collected from cells were prepared with RIPA lysis buffer containing a protease inhibitor cocktail (Roche, Basel, Switzerland). Nuclear proteins were isolated using a Minute Cytoplasmic and Nuclear Extraction Kit (Invent Biotechnologies, Inc., Plymouth, MN, USA). Equal amounts of proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane (Pall Corp., Port Washington, NY, USA). After blocking, the membranes were probed with primary antibodies against actin, survivin, c-myc (Santa Cruz Biotechnology, Dallas, TX, USA), β-catenin, glycogen synthase kinase 3 β (GSK3β), pSer9-GSK3β (Cell Signaling Technology, Danvers, MA, USA) and sFRP1 (Abcam, Cambridge, UK). After washing and incubating with rabbit or mouse secondary antibodies (Cell Signaling Technology), the blots were visualized by electrochemiluminescence reagent (GE Healthcare, Chicago, IL, USA).

Cell Counting Kit-8cell viability assay

RBE cells were tranfected with indicated reagents according to experimental aim. Twenty-four hours after transfection, cells were transferred into 96-well plates at a density of 2×10^3 cells per well. Cell viability was assessed using a Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto,Japan) at days 0, 2 and 4.

Colony formation assays

RBE cells were transfected with the indicated reagents according to experimental aim. Twenty-four hours after transfection, 800 cells were counted and seeded in 6-cm dishes. After 10 days, colonies were stained with 0.1% crystal violet in 20% methanol for 15 min. The samples were photographed and the numbers of visible colonies were counted.

Acridine orange/ethidium bromide (AO/EB) fluorescence staining

RBE cells were transfected with miR-191 inhibitor or control sequence. The cells were incubated with AO/EB solution for 5 min (Solarbio, Beijing, China). Cellular morphological changes were examined by fluorescence microscopy at 200×magnification. The percentage of apoptotic cells was calculated by the following formula: apoptotic rate (%) = (number of apoptotic cells)/ (number of all cells counted).

Luciferase reporter assay

The wild-type miR-191 binding site on sFRP1 (sFRP1-WT) and binding site mutant sequence (sFRP1-Mut) were subcloned into the pMIR-REPORT Luciferase vector (Ambion, Austin, TX,USA). RBE cells were seeded in 6-well plates, and transfected with the indicated components using Lipofectamine2000 for 48 h. Luciferase activity was assessed with the Dual Luciferase reporter 1000 Assay System (Promega, Madison, Wisconsin,USA). The activity of Renilla luciferase was used for normalization.

Data analysis

Data were obtained from at least three independent experiments and are presented as the mean ± standard deviation. Data were evaluated by unpaired Student's t test. The linear correlation coefficient (Pearson's *r*) was calculated to determine the correlation between miR-191 and sFRP1 expression in clinical samples. $P < 0.05$ was considered to represent a significant difference.

Results

miR-19 is up-regulated in CCA cells and specimens and maintains CCA cell survival

To test the expression of miR-191 in CCA cells, six CCA cell lines and HiBEC, which served as normal control, were collected. The level of miR-191 was measured by qRT-PCR. The data showed that although a diversity of expression levels was observed, all CCA cell lines harbored a much higher level of miR-191 compared with HiBECs (Fig. 1A). Moreover, 21 pairs of CCA tissues and their adjacent normal tissues were examined for the expression levels of miR-191. qRT-PCR results indicated that the expression level of miR-191 was significantly increased in CCA tissues compared with that in adjacent normal tissues (Fig. 1B). To uncover the effects of miR-191 on CCA, RBE cells were transfected with miR-191 inhibitor or its corresponding NC. CCK-8 assay and colony formation assay were performed to assess the cell viability. The data revealed that miR-191 inhibitor significantly reduced the level of miR-191 in RBE cells (Fig. 1C). CCK-8 assay showed that the cell viability was reduced by miR-191 knockdown (Fig. 1D). miR-191 inhibitor transfection also led to a decreased number of colonies formed (Fig. 1E). Consistent with these data, we found that knockdown of miR-191 led to an increased population of apoptotic cells as measured by AO/EB staining (Fig. 1F). Thus all these results indicate that miR-191 was over-expressed in CCA cells and maintains these cells' survival.

sFRP1 is a direct target of miR-191

The conventional mechanism by which miRNAs exert their effects is by binding to the 3′--UTR of target mRNA causing its degradation or translation inhibition. By interrogating a bioinformatics database using TargetScan and Microrna.org, we found that sFRP1 is a potential target of miR-191 (Fig. 2A). Both qRT-PCR and western blot analysis showed that sFRP1 level was negatively correlated with the level of miR-191 (Fig. 2B, C). Further, luciferase assay was performed to demonstrate whether sFRP1 was a direct target of miR-191. Intriguingly, reduced luciferase activity was observed in cells co-transfected with miR-191 mimic/sFRP1-WT. However, miR-191 mimic did not affect the luciferase activity in the sFRP1 MUT group (Fig. 2D). To determine the correlation between miR-191 expression and sFRP1 level, we examined the mRNA levels of sFRP1 in the same set of specimens, and qRT-PCR results indicated that the miR-191 expression levels were inversely correlated with sFRP1 expression (Fig. 2E, F). These data revealed that sFRP1 was a direct target of miR-191.

miR-191 downregulation interferes with Wnt/β-catenin signaling

It is well known that sFRP1 is a negative regulator of the Wnt/β-catenin signaling pathway. Since sFRP1 is a direct target of miR-191, we asked whether miR-191 could regulate Wnt/β-catenin signaling by targeting sFRP1. Western blot analysis revealed that miR-191 inhibitor transfection dramatically decreased β-catenin levels, especially in the nuclear fraction (Fig. 3A, B). The phosphorylation of GSK3β on Ser9 site was much higher

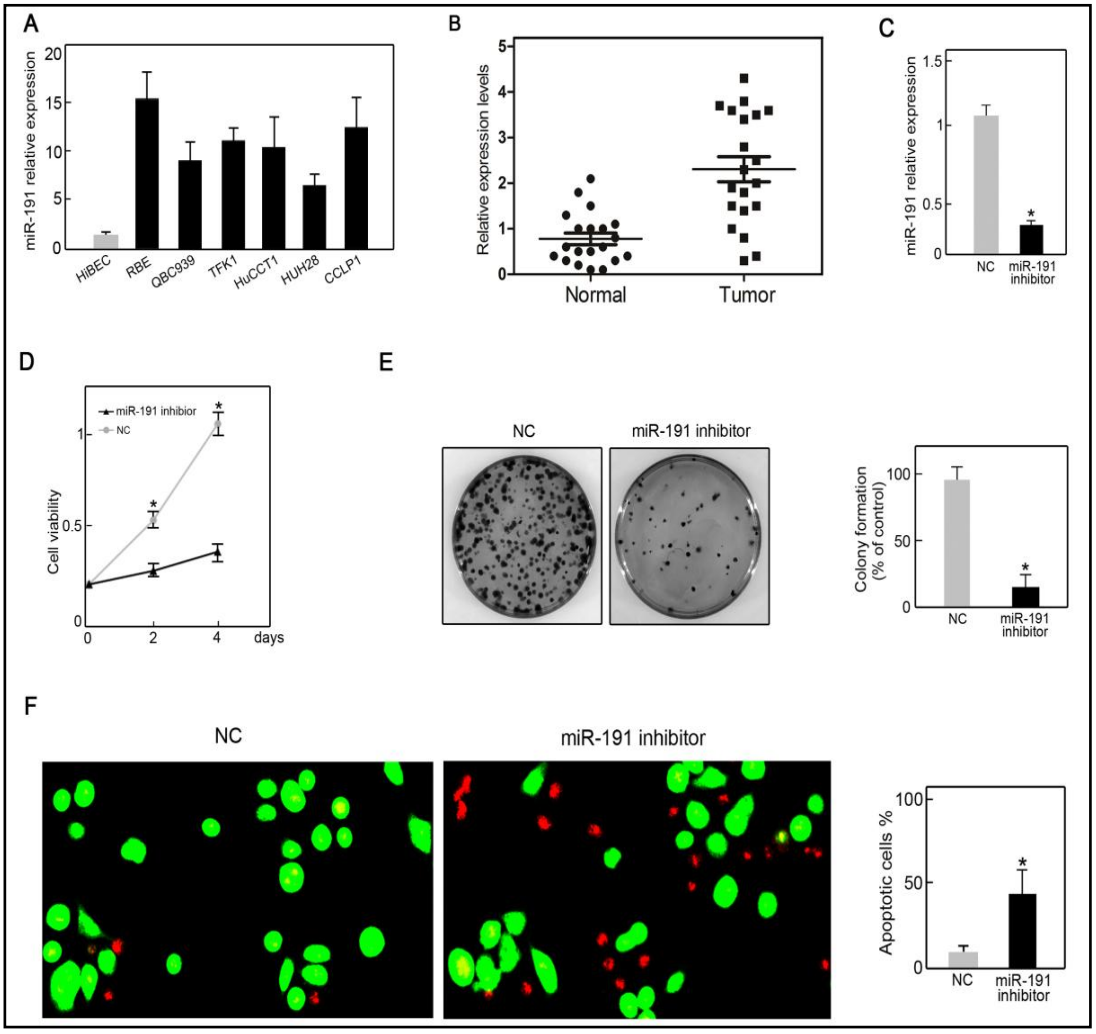

Fig. 1. miR-19 is up-regulated in CCA cells and maintainsCCA cell survival. (A). miR-191 was up-regulated in CCA cell lines. (B). Relative expression levels of miR-191 in adjacent normal tissues and CCA tissues. (C). miR-191 level was suppressed by miR-191 inhibitor transfection. (D). RBE cells transfected with miR-191 inhibitor or NC sequence were cultured for 4 days, and cell viability was analyzed by CCK-8 assay at days0, 2, and 4. (E). RBE cells transfected with miR-191 inhibitor or NC sequence were seeded into6-cm dishes at a density of 800 cells/dish. Colony formation was assessed by crystal violet staining. (F). RBE cells transfected with miR-191 inhibitor or NC sequence were subjected to AO/EB staining to detect changes in the nucleus. The orange-colored region indicates initiation of apoptosis. * $P<0.05$ vs. control (n = 3 independent experiments for each condition).

upon down-regulation of miR-191 compared with NC transfected cells, indicating blocked GSK3 β activity (Fig. 3C). The expression of two important target genes of Wnt/β-catenin signaling, survivin and c-myc, was reduced upon miR-191 inhibitor transfection (Fig. 3D). These data suggest that miR-191knockdown in RBE cells inhibited Wnt/β-catenin signaling.

miR-191 regulates Wnt/β-catenin signaling pathway via sFRP1

To determine whether miR-191 regulated Wnt/β-catenin signaling via its target gene sFRP1, sFRP1 siRNA was co-transfected with miR-191 inhibitor into RBE cells. Western blot analysis showed that sFRP1 expression was suppressed by sFRP1 siRNA (Fig. 4A). Western blot analysis also demonstrated that the increase in the level of GSK3β phosphorylation seen with miR-191 inhibition was reversed (Fig. 4B). Moreover, the expression of survivin

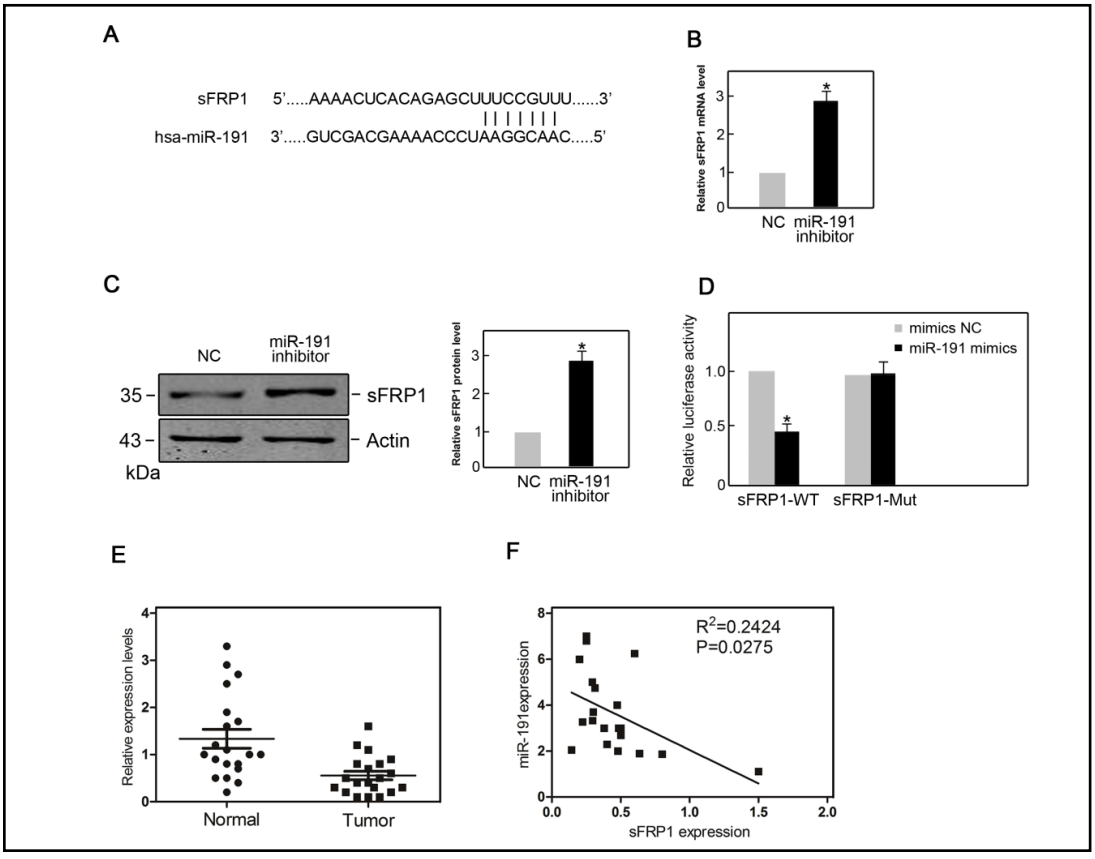

Fig. 2. sFRP1 is a direct target of miR-191. (A). Schematic diagram of the miR-191 binding site on sFRP1. (B). Knockdown of miR-181 increased the sFRP1 mRNA level. (C). Knockdown of miR-191 increased the sFRP1 protein level. (D). Luciferase reporter assay. RBE cells were co-transfected with a luciferase construct fused with the wild-type (sFRP1-WT) or site mutant (sFRP1-MUT) 3′-UTR of sFRP1 and miR-191 mimic or NC mimic. Luciferase activity is reported relative to that of Renilla. (E).Relative expression levels of sFRP1 in adjacent normal tissues and CCA tissues. (F). Correlation between miR-191 expression and sFRP1 expression in clinical samples.* P<0.05 vs. control (n = 3 independent experiments for each condition).

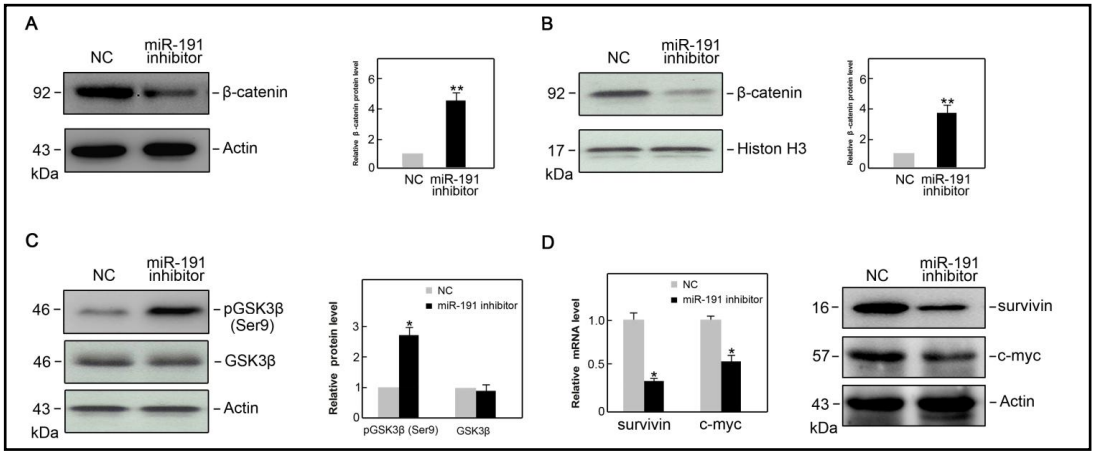

Fig. 3. miR-191 downregulation interferes with the Wnt/β-catenin signalingRBE cells were transfected with miR-191 inhibitor or NC sequence for 48 h. (A) . The level of β-catenin inwhole-cell lysates was measured by western blot. (B). Level of β-catenin in the nuclear fraction was measured by western blot. (C). miR-191 knockdown increased GSK3β phosphorylation at Ser9. (D). miR-191 knockdown reduced surviving and c-myc expression. * P<0.05, ** P<0.001 vs. control (n = 3 independent experiments for each condition).

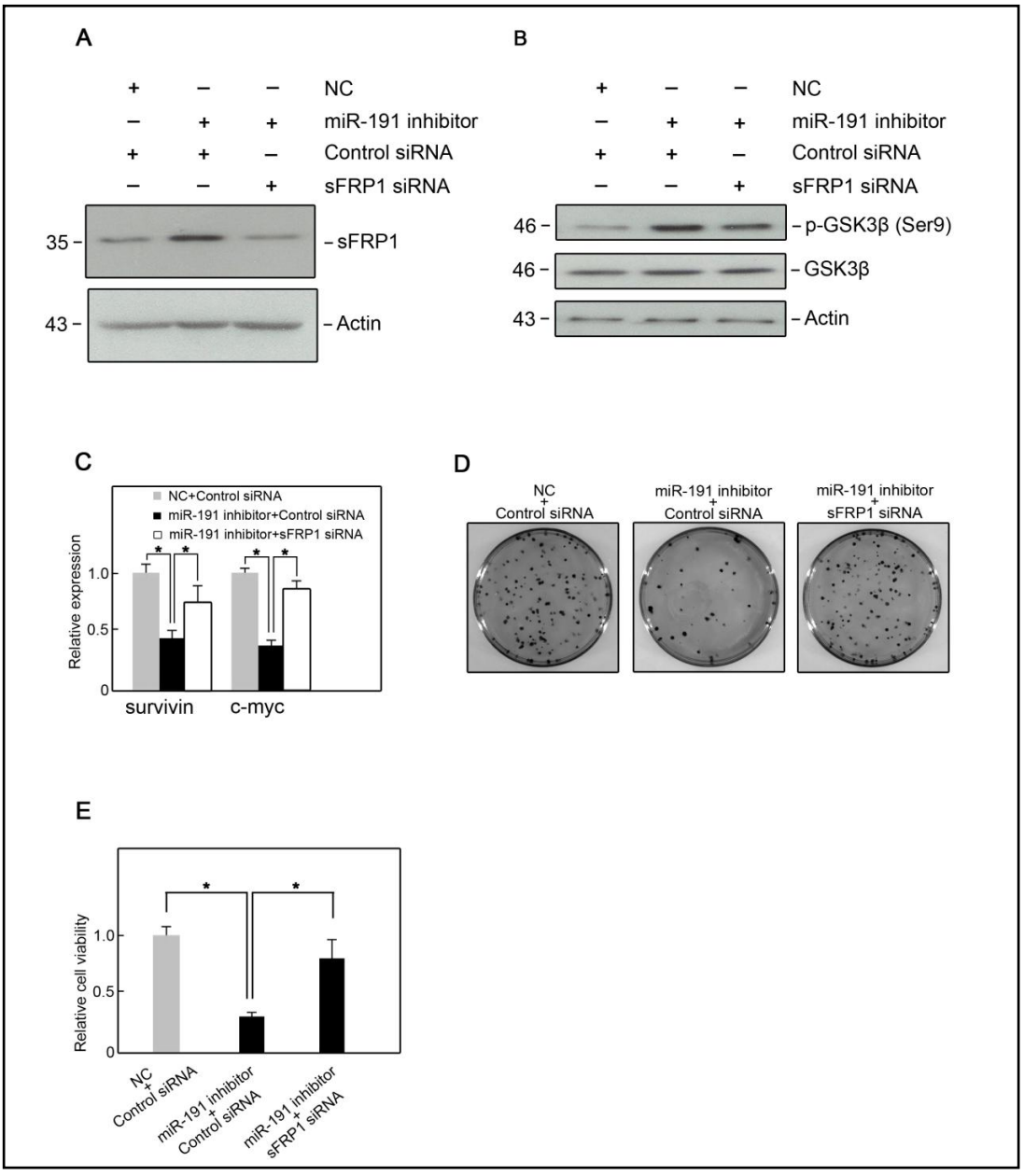

Fig. 4. miR-191 regulates the Wnt/β-catenin signaling pathway via sFRP1RBE cells were co-transfected with the indicated reagents for 48 h (A). The level of sFRP1 was measured by western blot. (B). sFRP1 siRNA co-transfection with miR-191 inhibitor reversed the increase in GSK3β phosphorylation observed when RBE cells were treated with miR-191 inhibitor alone. (C). sFRP1 siRNA co-transfection with miR-191 inhibitor restored surviving and c-myc expression. (D). sFRP1 siRNA co-transfection with miR-191 inhibitor restored the colony formation ability of RBE cells. (E). sFRP1 siRNA co-transfection with miR-191 inhibitor restored cell viability in RBE cells. *P<0.05 vs. control (n = 3 independent experiments for each condition).

and c-myc was restored by sFRP1 siRNA co-transfection (Fig. 4C). Colony formation assay validated that co-transfection of sFRP1 siRNA with miR-191 inhibitor restored the formation ability of A59 cells (Fig. 4D). CCK-8 assay showed that RBE cell viability was regained by sFRP1 siRNA co-transfection (Fig. 4E). These results indicated that miR-191 regulates Wnt/β-catenin signaling via sFRP1 and thus participates in maintaining RBE cell viability.

Discussion

It is crucial to understand the molecular mechanisms of CCA carcinogenesis and its progression for developing new effective therapeutic strategies. Aberrant expression of miRNAs often occurs in CCA and plays critical roles in its progression [32]. Thus, it is critical to uncover the function of miRNAs in CCA and their mechanisms. In this research, we focus on miR-191 to investigate its molecular mechanism in CCA. We found that miR-191 was over-expressioned in all six CCA cell lines compared with HiBECs. Knock-down of miR-191 inhibited RBE cell growth and the impaired the colony formation ability.

As is well known, miRNAs suppress target gene expression usually by binding the 3′-UTR of target mRNAs. Many genes, such as Egr-1, C/EBPβ, Tmod2 and ChK2, have been identified as miR-191 targets [33-36]. Here we screened potential target genes of miR-191 using the TargetScan and MicroRNA.org and found sFRP1 to be a viable candidate. Using miR-191 mimics or inhibitor to alter the expression level of miR-191 in RBE cells, we found that the sFRP1 level was negatively correlated with miR-191 expression. This observation is consistent with the conventional manner by which miRNAs regulate target expression. Luciferase reporter assay validated that sFRP1 is a direct target of miR-191.

It is well documented that sFRP1 is a negative regulator of the Wnt/β-catenin signaling pathway [37, 38]. sFRP1 can bind to the Wnt protein though its CRD domain [39]. This interaction between sFRPs and Wnt proteins prevents the Wnt protein binding to frizzled receptors and in turn shuts down Wnt/β-catenin signaling [40]. Masses of studies have shown that Wnt/β-catenin signaling pathway is quite critical for CCA carcinogenesis and its progression [41, 42]. We identified sFRP1as a direct target of miR-191, and further found that miR-191 regulates the Wnt/β-catenin signaling pathway via targeting sFRP1. Knockdown of miR-191 inactivated Wnt/β-catenin signaling characterized by decreased β-catenin level, increased GSK3β phosphorylation and reduced expression of two important target genes of Wnt/β-catenin signaling, survivin and c-mycBy co-transfection of sFRP1 siRNA with miR-191 inhibitor sequence, we found that the Wnt/β-catenin signaling pathway was reactivated and the colony formation ability of RBE cells was restored, indicating that miR-191 indeed restored Wnt/β-catenin signaling via sFRP1.

Based on these findings, we demonstrate a novel insight into miR-191 biological function in CCA. Our findings suggest that miR-191 is a potential therapeutic target of CCA treatment.

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Disclosure Statement

The authors declare that there is no conflict of interests regarding the publication of this paper.

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