

ORIGINAL ARTICLE

miR-449a regulates biological functions of hepatocellular carcinoma cells by targeting SATB1

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Summary

Purpose: To investigate whether miR-449a can regulate the biological functions of hepatocellular carcinoma (HCC) cells by targeting special AT-rich sequence binding protein 1 (SATB1).

Methods: qRT-PCR and western blot were carried out to detect the expression of miR-449a and SATB1 in normal human hepatocyte cell line HL-7702 and in HCC cells SMMC-7721, Hep3B, HepG2, and Bel-7402. miR-449a-mimics, miR-negative control (miR-NC), specifically inhibited SATB1 RNA (si-SATB1), specifically overexpressed SATB1 RNA (sh-SATB1), and negative control RNA (Si-NC) were transfected into the Hep3B and Bel-7402 cells. MTT assay, Transwell assay and flow cytometry were conducted to detect cell proliferation, invasion, and apoptosis. Dual luciferase reporter assay was performed to determine the relationship between miR-449a and SATB1.

Results: miR-449a was highly but SATB1 was poorly expressed in HCC cells. According to the cell experiments, the up-regulation of miR-449a expression could inhibit the proliferation and invasion of HCC cells, promote their apoptosis, and significantly reduce SATB1 expression. The inhibition of SATB1 expression could inhibit the proliferation and invasion and promote apoptosis. The dual luciferase reporter assay confirmed that there was a targeted regulatory relationship between miR-449a and SATB1.

Conclusion: miR-449a can inhibit the proliferation and invasion of HCC cells and promote their apoptosis through the targeted regulation of SATB1, so it is expected to become a potential therapeutic target for this disease in clinical practice.

Key words: miR-449a; SATB1; biological function; hepatocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors in the digestive system, with its incidence ranking 6th and its mortality ranking 2nd around the world [1]. Because of the unapparent symptoms of early HCC, 70-80% of patients are diagnosed in advanced stage missing their best treatment time. Their average survival time is shorter than 1 year, and the 5-year survival

rate of the untreated patients with advanced HCC is less than 5% [2]. At present, the pathogenesis, diagnosis, and treatment of HCC have been widely studied. However, the exact pathogenesis of HCC remains unclear, and the 5-year survival rate of the disease has not significantly increased [3], which emphasizes the importance of the early diagnosis and disease treatment.

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Received: 17/03/2020; Accepted: 20/04/2020

microRNA (miRNA, miR) is a single-stranded non-coding RNA with a length of about 23 nucleotides [4]. Recent and in-depth studies have shown that miR is abnormally expressed in tumors and functions as an oncogene and as a tumor suppressor gene, so it is expected to become a therapeutic target for cancers [5,6]. As an important member of the miR family, miR-449a is involved in the pathogenesis of various tumors. According to Wang et al, miR-449a inhibits the proliferation, migration, and invasion of esophageal squamous carcinoma cells, so it can be used as a new target for the treatment of this disease [7]. According to Luo et al, miR-449a expression is down-regulated in tissues and cell lines of patients with non-small cell lung cancer (NSCLC), and miR-449a can regulate the migration and invasion of NSCLC cells by targeting c-Met [8]. These studies suggest that miR-449a plays an important role in the development and progression of tumors. However, there are currently few reports of miR-449a in HCC, and the relationship between miR-449a and HCC is still unclear. The prediction by TargetScan, an online prediction software, shows that special AT-rich sequence binding protein 1 (SATB1) is a potential target site for miR-449a. SATB1 is a specifically expressed matrix attachment region binding protein, and its expression is closely related to the proliferation, differentiation, and other biological functions of cells [9]. Some studies have shown that SATB1 is highly expressed in lung adenocarcinoma, and silencing its expression can significantly reduce the proliferation, invasion, and migration of lung adenocarcinoma cells, as well as significantly increase the apoptotic rate of the cells [10]. SATB1 also regulates the process of epithelial-mesenchymal transition (EMT), thus affecting the development and progression of bladder cancer [11]. The above studies demonstrate that SATB1 is involved in the pathogenesis of malignant diseases. Therefore, we suspect that miR-449a may regulate the biological functions of HCC cells by targeting SATB1.

Accordingly, miR-449a may regulate the biological functions of HCC cells by targeting SATB1. This study was conducted to demonstrate this hypothesis and explore the relationship between miR-449a and HCC.

Methods

Major experimental supplies

Reagents and equipment: CCK-8 assay kit (Beyotime Biotechnology, Shanghai, China, C0037); Transwell kit, DMEM, phosphate buffered saline (PBS), fetal bovine serum (FBS), and penicillin-streptomycin (Thermo Fisher Scientific, China, 15140122); RIPA reagent, BCA protein kit, ECL luminescence kit, trypsin, and Lipofectamine™ 2000 transfection reagent (Thermo Scientific, USA, 89900, 23250, 32209, 90059, 11668019); β -actin primary antibody and horseradish peroxidase (HRP)-labeled goat anti-mouse IgG secondary antibody (R&D, USA, MAB8929, HAF007); TRIzol extraction kit (CHUNDUBIO, Wuhan, China, CDLG-4396); reverse transcription kit (TIANGEN, Beijing, China, FP209); Annexin V-FITC apoptosis kit (Shanghai Yuduo Biotechnology Co., Ltd., China, YDQ955); dual luciferase reporter gene detection kit (Solarbio, Beijing, China, D0010); PCR instrument (ABI, USA, 7500); flow cytometer (BD, USA, FACS Canto II); multifunctional microplate reader (BioTek, USA, DLK0001622). All primers were designed and synthesized by TaKaRa BIO, China. Normal human hepatocyte cell line HL-7702 and HCC cell lines SMMC-7721, Hep3B, HepG2, and Bel-7402 were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences.

Cell culture

After the cell concentration was adjusted to 40-60%, the cells were cultured in DMEM (containing 10% FBS) at 37°C and with 5% CO₂. After adherently growing to about 90%, they were conventionally digested with trypsin and then passaged once every 24 h. Cells in the logarithmic growth phase were selected for subsequent experiments. qRT-PCR was conducted to detect differences in the expression level of miR-449a between each group.

Cell line transfection

Cells with the greatest difference in the miR-449a expression level between two groups were selected and transfected with the Lipofectamine™ 2000 kit, with the steps strictly carried out in accordance with the kit instruction. miR-449a-mimics, miR-negative control (miR-NC), specifically inhibited SATB1 RNA (si-SATB1), specifically overexpressed SATB1 RNA (sh-SATB1), and negative control RNA (Si-NC) were used for transfection.

qRT-PCR detection of miR-449a expression

The total RNA was extracted from the cells in each group in strict accordance with the instruction of the total RNA kit. After the concentration and purity of the extract-

Table 1. Primer sequences

Groups	Upstream (5'→3')	Downstream (5'→3')
miR-449a	GCTGGCAGTGTATTGTTA	GTGCAGGGTCCGAGGT
β -actin	CTCGCTTCGGCAGCACATATACT	ACGCTTCACGAATTTGCGTGTC

ed total RNA were detected by a DR6000 ultraviolet-visible spectrophotometer, the quality of it was verified by 1% denatured agarose gel electrophoresis. The extracted total RNA was taken out for PCR amplification with U6 considered as an internal reference. The reaction system was as follows: 10 μ L of 2 \times Talent qPCR PreMix, each 1.25 μ L of upstream and downstream primers, 100ng of cDNA, and water supplemented to 20 μ L. The reaction conditions were as follows: pre-denaturation at 95°C for 3min, denaturation at 95°C for 5s, and annealing and extension at 60°C for 15s, for a total of 40 cycles. The data were analyzed using $2^{-\Delta\Delta C_t}$. Primer sequences involved are shown in Table 1.

Western blot detection of SATB1 expression

The cultured cells were collected for extracting total protein using RIPA lysis method, and the protein concentration was adjusted to 4 μ g/ μ L using bicinchoninic acid (BCA). After 6% SDS-PAGE was used for electrophoretic separation, the protein was transferred to PVDF membrane, stained in Ponceau S staining solution, soaked in PBST for 5min, and then washed for 3 times. Next, the membrane was sealed with 5% skimmed milk powder for 2 h, added with SOX1 and β -actin primary antibody (1:1000), and then sealed overnight in a refrigerator at 4°C. After washed to remove the primary antibody, the membrane was added with HRP-labeled goat anti-rabbit secondary antibody (1:5000), incubated at 37°C for 1 h, and then rinsed with PBS over 5min for 3 times. After the membrane was developed in the dark, the excess liquid on the membrane was absorbed dry with filter papers, and the ECL kit was used for luminescing and developing. The protein bands were scanned to analyze their gray values using Quantity One. The relative expression of proteins = the gray values of target protein bands / the gray values of β -Actin protein bands.

CCK-8 assay of cell proliferation

After transfection for 24 h, the cells were collected, adjusted to 4×10^6 cells/well, inoculated into a 96-well plates, and then cultured for 24 h, 48 h, 72 h, and 96 h. Each well was added with CCK solution (10 μ L) and basic medium (DMEM) (90 μ L), and then cultured at 37°C for 2 h. Next, the optical density (OD) values of each group were measured by microplate reader at 570nm.

Transwell detection of cell invasion

After transfection for 24 h, the cells were collected, adjusted to 5×10^4 cells/well, inoculated into a 6-well plate, washed with PBS for 2 times, and then inoculated in the upper chamber. DMEM culture solution (200 μ L) was added to the upper chamber, while DMEM (containing 20% FBS) (500mL) was added to the lower chamber. After the upper chamber was cultured at 37°C for 48 h, the matrix and cells in the chamber that did not pass through the membrane surface were wiped off. After the Transwell was washed with PBS for 3 times and the cells were fixed with paraformaldehyde for 10 min, the upper chamber was washed with double distilled water for 3 times, and then stained with 0.5% crystal violet after drying. The cell invasion was observed with a microscope.

Flow cytometry detection of apoptosis

The transfected cells were digested with 0.25% trypsin and washed twice with PBS. Then, they were added with binding buffer (100 μ L), prepared into a 1×10^6 cells/mL suspension, successively added with the AnnexinV-FITC and propidium iodide (PI) reagents, and then incubated in the dark for 5 min. Flow cytometer was used for detection, and the experiment was repeatedly carried out for 3 times to obtain the average value.

Statistics

In this study, SPSS 22.0 (SPSS, Inc., Chicago, IL, USA) was used for data processing. The comparison of means between two groups was analyzed by t-test, while the comparison between more than two groups was analyzed by one-way ANOVA. The later pairwise comparison was analyzed by Dunnett-t test. When $p < 0.05$, the difference was statistically significant.

Results

Expression of miR-449a and SATB1

According to the RT-PCR, the expression of miR-449a in HCC cells (SMMC-7721, Hep3B, HepG2, and Bel-7402) was significantly lower than that in normal human hepatocyte cell line

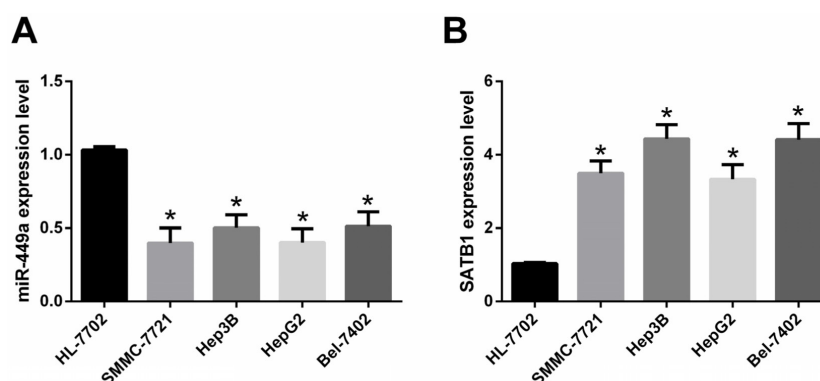


Figure 1. Expression of miR-449a and SATB1. **A:** The expression of miR-449a in HCC cells was significantly lower than that in the HL-7702 hepatocytes. **B:** The expression of SATB1 in HCC cells was significantly higher than that in the HL-7702 hepatocytes. * $p < 0.05$.

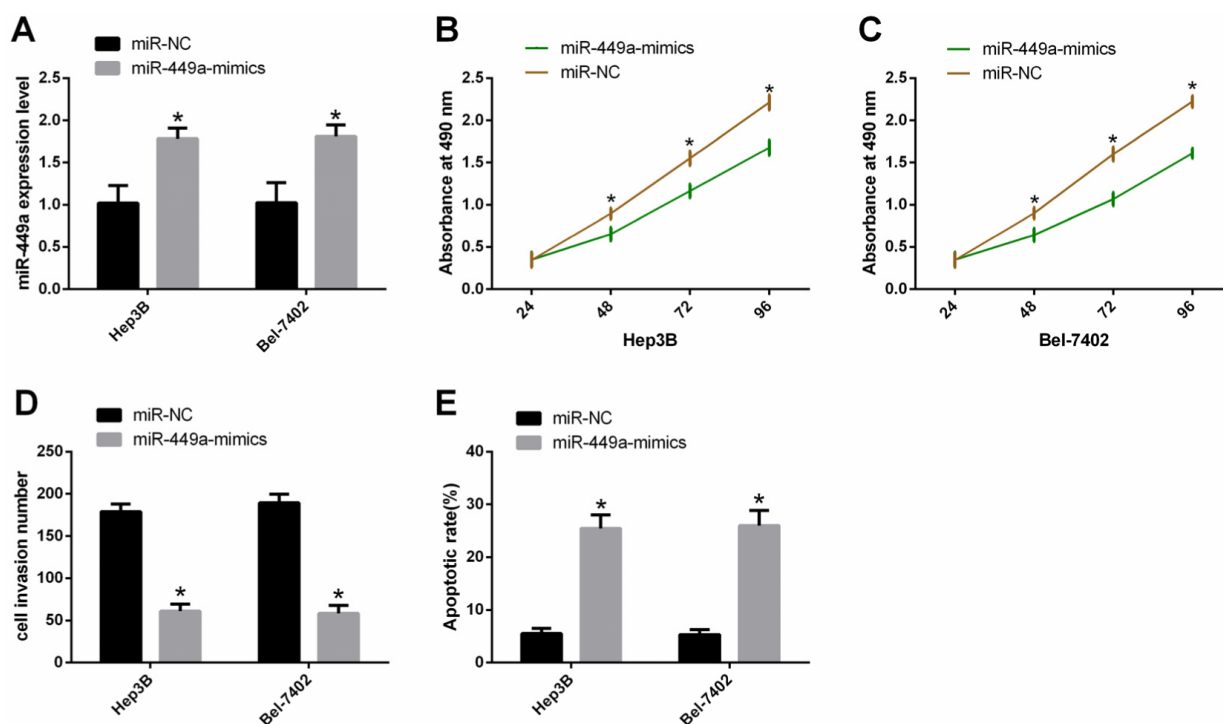


Figure 2. Effects of miR-449a on the biological functions of HCC cells. **A:** The expression of miR-449a in the cells transfected with miR-449a-mimics was significantly higher than that in those transfected with miR-NC. **B/C:** The proliferation of the cells transfected with miR-449a-mimics was significantly lower than that in those transfected with miR-NC. **D:** The invasion of the cells transfected with miR-449a-mimics was significantly lower than that in those transfected with miR-NC. **E:** The apoptotic rate of the cells transfected with miR-449a-mimics was significantly higher than that in those transfected with miR-NC. * $p < 0.05$.

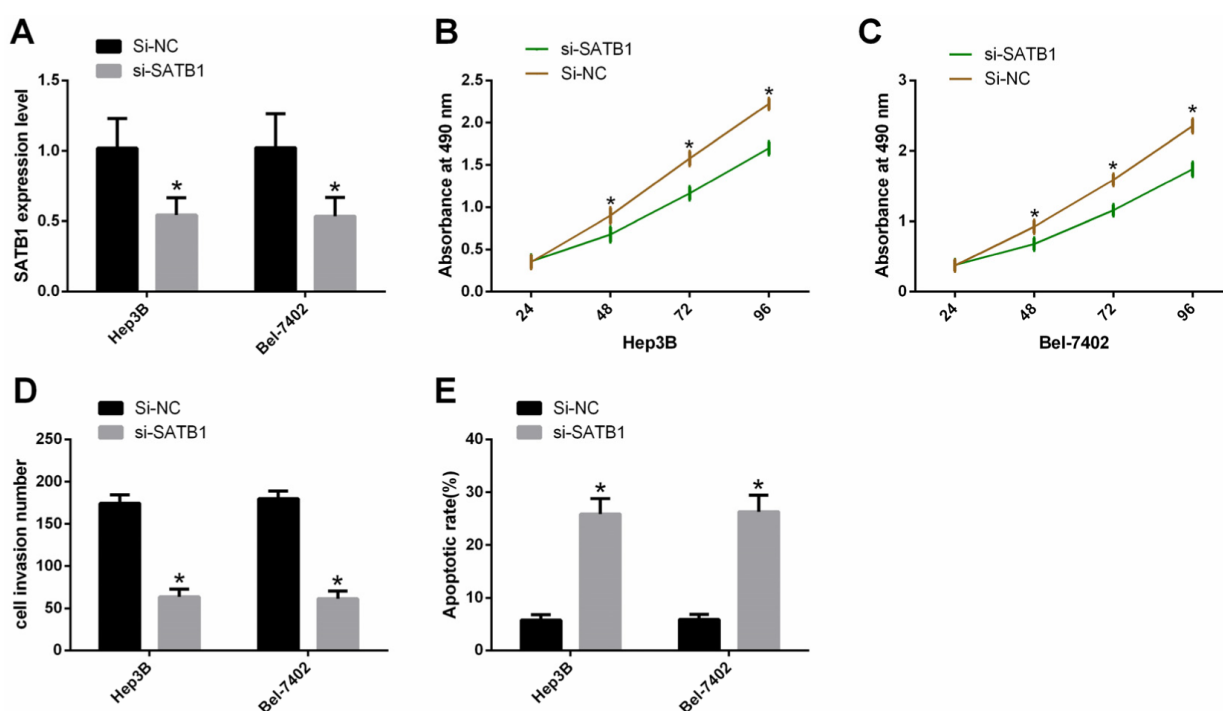


Figure 3. Effects of SATB1 on the biological functions of HCC cells. **A:** The expression of SATB1 in the cells transfected with si-SATB1 was significantly lower than that in those transfected with Si-NC. **B/C:** The proliferation of the cells transfected with si-SATB1 was significantly lower than that in those transfected with Si-NC. **D:** The invasion of the cells transfected with si-SATB1 was significantly lower than that in those transfected with Si-NC. **E:** The apoptotic rate of the cells transfected with si-SATB1 was significantly higher than that in those transfected with Si-NC cells. * $p < 0.05$.

(HL-7702) ($p < 0.05$), while the expression of SATB1 was significantly higher than that in the HL-7702 hepatocytes ($p < 0.05$). The expression of miR-449a in the Hep3B and Bel-7402 cell lines was the lowest, so these two cell lines were selected for transfection and subsequent experiments (Figure 1).

Effects of miR-449a on the biological functions of HCC cells

The Hep3B and Bel-7402 cells were transfected with miR-449a-mimics and miR-NC. The results showed that the expression of miR-449a in the cells transfected with miR-449a-mimics was significantly higher than that in those transfected with miR-NC ($p < 0.05$). According to the detection of the biological functions, compared with those transfected with miR-NC, the cells transfected with miR-449a-mimics had significantly lower proliferation and invasion, but significantly higher apoptotic rate (Figure 2).

Effects of SATB1 on the biological functions of HCC Cells

The Hep3B and Bel-7402 cells were transfected with si-SATB1 and Si-NC. The results showed that the expression of SATB1 in the cells transfected with si-SATB1 was significantly lower than that in those transfected with Si-NC ($p < 0.05$). According to the detection of the biological functions, compared with those transfected with Si-NC, the cells

transfected with si-SATB1 had significantly lower proliferation and invasion, but significantly higher apoptotic rate (Figure 3).

Dual luciferase reporter assay

Targetscan7.2 was used to predict the target gene downstream of miR-449a, so as to further verify the relationship between miR-449a and SATB1. The prediction showed that there was a targeted binding site between them. According to the dual luciferase reporter assay, the luciferase activity of SATB1-3'UT Wt significantly decreased after miR-449a was overexpressed ($p < 0.05$), while the luciferase activity significantly increased after miR-449a expression was inhibited ($p < 0.05$). The expression had no effect on the luciferase activity of SATB1-3'UTR Mut ($p > 0.05$). According to the Western Blot, the protein expression of SATB1 in the cells transfected with miR-449a-mimics was significantly reduced ($p < 0.05$), while the expression in those transfected with miR-449a-inhibitor was significantly increased ($p < 0.05$) (Figure 4).

Rescue experiment

In the rescue experiment, the Hep3B and Bel-7402 cells were co-transfected with miR-449a-mimics+sh-SATB1. According to the detection of the biological functions, there were no significant differences between the cells transfected with miR-449a-mimics+sh-SATB1 and with miR-NC in terms

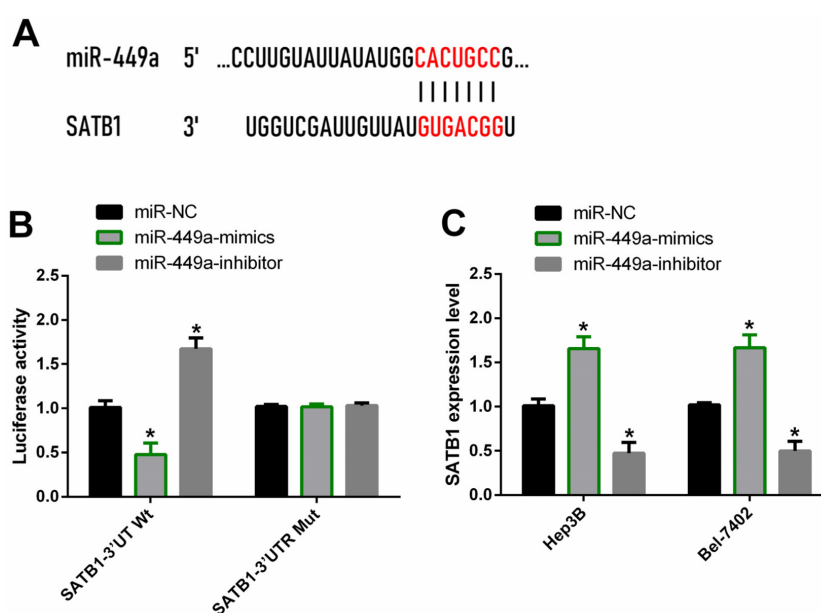


Figure 4. Dual luciferase reporter assay. **A:** There was a targeted binding site between miR-449a and SATB1. **B:** The luciferase activity of SATB1-3'UT Wt significantly decreased after miR-449a was overexpressed, while the luciferase activity significantly increased after miR-449a expression was inhibited. The expression had no effect on the luciferase activity of SATB1-3'UTR Mut. **C:** The protein expression of SATB1 in the cells transfected with miR-449a-mimics was significantly reduced, while the expression in those transfected with miR-449a-inhibitor was significantly increased. * $p < 0.05$.

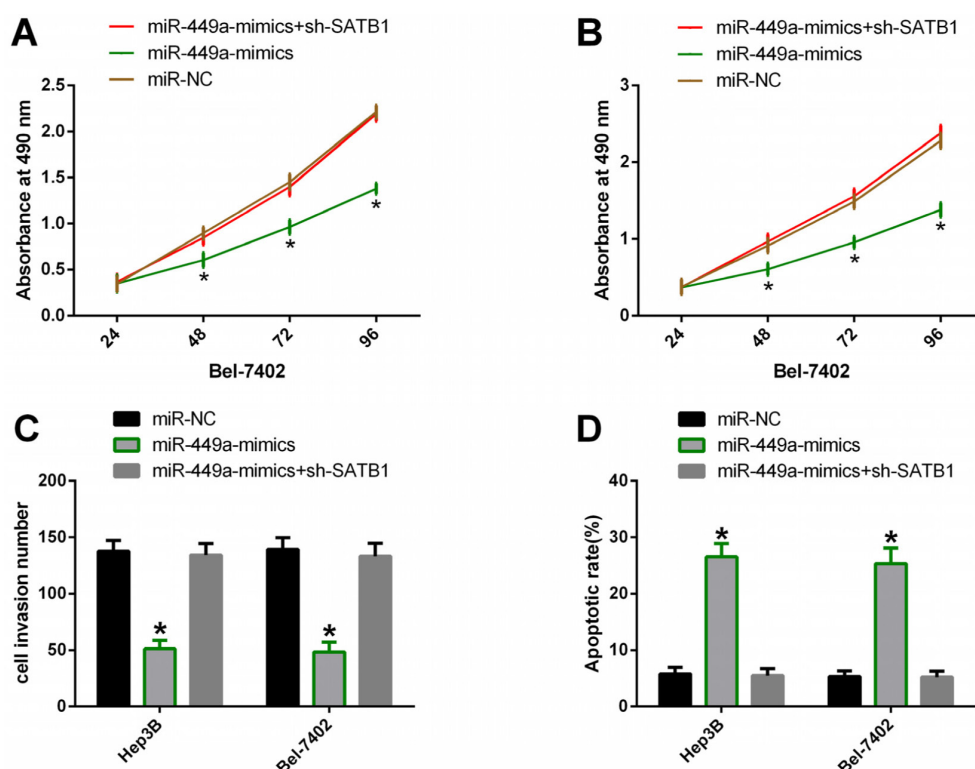


Figure 5. Effects of co-transfection with miR-449a-mimics+sh-SATB1 on biological functions of HCC cells. **A/B:** The proliferation of the cells transfected with miR-449a-mimics+sh-SATB1 was not different from those transfected with miR-NC, but significantly higher than those transfected with miR-449a-mimics. **C:** The invasion of the cells transfected with miR-449a-mimics+sh-SATB1 was not different from those transfected with miR-NC, but significantly higher than those transfected with miR-449a-mimics. **D:** The apoptotic rate of the cells transfected with miR-449a-mimics+sh-SATB1 was not different from those transfected with miR-NC, but significantly lower than those transfected with miR-449a-mimics. * $p < 0.05$.

of proliferation, invasion, and apoptosis. Compared with those transfected with miR-449a-mimics, the cells transfected with miR-449a-mimics+sh-SATB1 had significantly higher proliferation and invasion, but significantly lower apoptotic rate (Figure 5).

Discussion

In recent years, the incidence and mortality of HCC have increased year by year, but the disease still cannot be systematically treated. Although the research on the treatment has risen to the gene level, there is still no specific molecular target for the treatment [12]. Early HCC is very difficult to be diagnosed and advanced HCC is characterized by high recurrence and easy metastasis, which leads to the poor prognosis of this disease [13]. Therefore, to fully understand the mechanism of the development and disease progression plays an important role in improving the therapeutic and diagnostic effects in HCC.

As a short-chain non-coding RNA, miR shows outstanding performance in various fields because it is involved in the growth, proliferation, apopto-

sis, and other biological functions of cells [14,15]. As a highly conserved miR that belongs to miR-449 cluster [16], miR-449a has been proved to be closely related to the development and progression of tumors. For example, the up-regulation of miR-449a expression inhibits the proliferation of human neuroblastoma cells, which is completed by miR-449a regulating Bax and Bcl-2 proteins [17]. miR-449a is poorly expressed in gastric cancer cells, and the down-regulation of its expression promotes the growth and inhibits apoptosis of the cells, which confirms that miR-449a functions as a tumor suppressor gene in gastric cancer [18]. A previous study has shown that miR-449a is poorly expressed in HCC cells [19], which is consistent with our results. This indicates that miR-449a may be involved in the pathogenesis of the disease. However, the relationship between miR-449a and HCC has not been clarified yet. Therefore, in order to understand the relationship, subsequent cell experiments were carried out. The results showed that, compared with those transfected with miR-NC, the cells transfected with miR-449a-mimics had significantly lower proliferation and invasion

rates, but significantly higher apoptotic rate. This suggests that miR-449a plays the role of a tumor suppressor in HCC cells and is expected to become a therapeutic target for this disease. However, the way in which miR-449a affects the biological functions remains unclear.

As an endogenous small fragment RNA interference factor, miR directly regulates its target proteins to affect the biological functions of cells [20]. In this study, the prediction by the TargetScan showed that SATB1 was a potential target site for miR-449a. SATB1, which is the organizer and transcription factor of chromatin, affects gene expressions and then regulates cell differentiation, proliferation, apoptosis, etc [21]. Recent studies have found that SATB1 is abnormally expressed in malignant tumors and functions as an oncogene. It is highly expressed in esophageal cancer cells, and down-regulation of its expression reduces the proliferation, survival, and invasion of the cells [22]. Additionally, it is highly expressed in prostate cancer (PCa) tissues and cells. The up-regulation of its expression promotes the proliferation and migration of PCa cells, while the down-regulation of its expression inhibits the proliferation and migration. Moreover, the inhibition of SATB1 reverses EMT by up-regulating E-cadherin and down-regulating vimentin [23].

There have also been related studies on SATB1 in liver cancer. According to Tu et al, the up-regulation of SATB1 promotes the cycle progress and proliferation of liver cancer cells, and reduces the apoptotic rate of the cells; SATB1 also regulates EMT-related genes [24]. In this study, the expression of SATB1 was up-regulated in HCC cells; compared with those transfected with Si-NC, the cells transfected with si-SATB1 had significantly lower proliferation and invasion rates, but significantly higher apoptotic rate. This reveals that SATB1 also plays the role of an oncogene in HCC cells and may be a therapeutic target for this disease.

Previous studies have found that SATB1 can be targeted by a variety of miRs to affect the development and progression of malignant diseases. miR-23a inhibits the proliferation of osteosarcoma

cells by targeting SATB1 [25] and miR-495 inhibits renal cell carcinoma by inhibiting the expression of SATB1 [26]. These findings show that miR/SATB1 axis plays a regulatory role in the development and progression of tumors. In this study, the proliferation, invasion, and apoptosis of the cells transfected with miR-449a-mimics+sh-SATB1 were not different from those in the cells transfected with miR-NC, but different from those in the cells transfected with miR-449a-mimics. This indicates that miR-449a is closely related to SATB1. Therefore, the rescue experiment was carried out and the results showed that the luciferase activity of SATB1-3'UT Wt significantly decreased after miR-449a was overexpressed, while the luciferase activity significantly increased after miR-449a expression was inhibited. The expression had no effect on the luciferase activity of SATB1-3'UTR Mut. The protein expression of SATB1 in the cells transfected with miR-449a-mimics significantly decreased, while the expression in those transfected with miR-449a-inhibitor significantly increased. These findings indicate that there is a targeted regulatory relationship between miR-449a and SATB1, i.e. the up-regulation of miR-449a can inhibit SATB1 expression and then affects the biological functions of HCC cells.

This study still has some limitations. First, tumor formation in nude rats was not conducted, so we were unable to know whether the injection of miR-449a-mimics could relieve the tumor formation. Second, the clinical values of miR-449a and SATB1 for patients with HCC were not explored, which led to incomplete results. Therefore, we hope that more experiments will be added to supplement these deficiencies in future research.

In summary, miR-449a can reduce the proliferation and invasion of HCC cells and increase the apoptotic rate of the cells through targeting SATB1, so it is expected to become a therapeutic target for the disease in clinical practice.

Conflict of interests

The authors declare no conflict of interests.

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