

Long noncoding RNA ZFAS1 promotes hepatocellular carcinoma proliferation by epigenetically repressing miR-193a-3p

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Abstract. – **OBJECTIVE:** Hepatocellular carcinoma (HCC) is one of the most common malignant tumors. Long noncoding RNAs (lncRNAs) play important roles in many diseases. Therefore, the aim of this study was to investigate the role of lncRNA ZFAS1 in the development of HCC and to explore its underlying mechanism.

PATIENTS AND METHODS: Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was utilized to detect ZFAS1 expression in tissue samples of HCC patients. Subsequently, Cell Counting Kit-8 (CCK-8) assay, colony formation assay, and EdU incorporation assay were performed to detect the function of ZFAS1 in vitro. Furthermore, mechanism assays were performed to explore the interaction between ZFAS1 and miR-193a-3p.

RESULTS: ZFAS1 was significantly highly expressed in HCC tissues than that of adjacent normal tissues. The growth ability of HCC cells was markedly inhibited after ZFAS1 was silenced. However, the growth ability of HCC cells was remarkably promoted after ZFAS1 overexpression. Moreover, RT-qPCR results revealed that miR-193a-3p was significantly down-regulated via the overexpression of ZFAS1. However, miR-193a-3p was significantly up-regulated via the knockdown of ZFAS1. Further experiments showed that miR-193a-3p was a direct target of ZFAS1 in HCC.

CONCLUSIONS: ZFAS1 could enhance the proliferation of HCC cells by suppressing miR-193a-3p, which might be a potential therapeutic target in HCC.

Key Words:

Long noncoding RNA, ZFAS1, Hepatocellular carcinoma (HCC), MiR-193a-3p.

Introduction

Hepatocellular carcinoma (HCC) is one of the common primary liver cancers, which accounts for the second leading cause of cancer-related deaths¹. HCC is defined as a primary tumorigenesis in the liver. Meanwhile, it mainly occurs in patients suffering from chronic liver cirrhosis or hepatitis B or C. Currently, HCC-related mortality is significantly higher in developing countries, especially in China, where hepatitis B virus (HBV) is particularly prevalent among people^{2,3}. During the past several years, therapeutic advances have been achieved in HCC prevention and management. However, the prognosis of patients with HCC remains poor. Approximately 600,000 patients die of HCC annually⁴, with 5-year overall survival rate lower than 20%^{5,6}. Therefore, it is urgent to understand the underlying molecular mechanism of HCC and improve the prognosis of HCC patients.

Long noncoding RNAs (lncRNAs) are known as transcripts longer than 200 nt, with no protein-coding function. Some studies⁷ have uncovered the vital role of lncRNAs in the regulation of cellular biological processes. For instance, lncRNA LINC00261 promotes the migration of gastric cancer cells by regulation of epithelial-mesenchymal transition. The knockdown of lncRNA MALAT1 remarkably suppresses cell viability and mobility in renal cell cancer *via* activating the PI3K/Akt signaling pathway⁸. In addition, significantly inhibited cell migration can be observed by the knockdown of lncRNA MALAT1 in esophageal squamous cell carcinoma⁹.

MicroRNAs (miRNAs) are another type of non-coding RNAs with 19–22 nucleotide in length. Previous studies¹⁰ have reported that miRNAs participate in numerous biological progressions in many diseases, including cancers. For example, miR-219-5p suppresses cell growth and migration in malignant melanoma through targeting BCL-2. Overexpression of miRNA-370 promotes the proliferation of human prostate cancer cells through the down-regulation of FOXO1¹¹. Furthermore, miRNA-613 functions as a tumor suppressor in the progression of colorectal cancer by targeting FMNL2¹².

LncRNA ZFAS1 plays an important role in tumor biology and development. However, the exact function of lncRNA ZFAS1 in HCC and the potential molecular mechanism have not been fully elucidated.

Patients and Methods

Tissue Samples

A total of 60 cancer tissues and paired adjacent normal tissues were obtained from HCC patients who underwent surgery at Jining No.1 People's Hospital. All fresh tissues collected from surgery were stored immediately at -80°C for use. This investigation was approved by the Ethics Committee of Jining No.1 People's Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Human HCC cell lines (Bel-7402 and HepG2) and normal liver epithelial cell line (L02) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) consisting of 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and penicillin/streptomycin. Besides, the cells were maintained in an incubator containing 5% CO_2 at 37°C .

Cell Transfection

The cDNA oligonucleotides specifically targeting ZFAS1 (sh-ZFAS1) and lentivirus against ZFAS1 were synthesized by GenePharma (Shanghai, China). Subsequently, they were cloned into a pLenti-EF1a-EGFP-F2A-Puro vector (Biosettia Inc., San Diego, CA, USA). After that, sh-ZFAS1

and ZFAS1 lentivirus were transfected into Bel-7402 and HepG2 HCC cells according to the instructions of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), respectively. 48 h later, Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was used to verify the transfection efficiency.

RNA Extraction and RT-qPCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was utilized to separate total RNA in tissues and cells. Subsequently, the extracted RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA) through the reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The primer sequences used for RT-qPCR were as follows: ZFAS1 primers forward: 5'-CTATTGTCCTGCCGTTAGAG-3', reverse: 5'-GTCAGGAGATCGAAGGTTGTAG-3'; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers forward: 5'-CCAAAATCAGATGGGGCAATGCTGG-3' and reverse 5'-TGATGGCATGGACTGTGGTCATTCA-3'. The thermal cycle was as follows: pre-denaturation at 95°C for 1 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s.

Cell Counting Kit-8 (CCK-8) Assay

The proliferation of HCC cells in 96-well plates was monitored every 24 h by the CCK-8 assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). The absorbance at 450 nm was detected by a spectrophotometer (Thermo Fisher Scientific, Rockford, IL, USA).

Colony Formation Assay

HCC cells were first seeded into 6-well plates for 10 days. After that, formed colonies were fixed with 10% formaldehyde for 30 min and stained with 0.5% crystal violet for 5 min. Image-Pro Plus 6.0 (Silver Spring, MD, USA) was used for data analysis.

Ethynyl Deoxyuridine (EdU) Incorporation Assay

According to the manufacturer's protocol, the EdU Kit (Roche, Mannheim, Germany) was utilized to monitor the proliferation of transfected cells. Zeiss Axiophot Photomicroscope (Carl Zeiss, Oberkochen, Germany) was performed to capture representative images.

Luciferase Reporter Gene Assay

The Luciferase reporter gene assay kit (Promega, Madison, WI, USA) was used to detect the luciferase activity of HCC cells. Briefly, the 3'-UTR of ZFAS1 cloned into the pGL3 vector (Promega, Madison, WI, USA) was identified as wild-type (WT) 3'-UTR. QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) was used for site-directed mutagenesis of the miR-193a-3p binding site in ZFAS1 3'-UTR, which was named as mutant (MUT) 3'-UTR. Then, the cells were transfected with WT-3'-UTR or MUT-3'-UTR and miR-ctrl or miR-193a-3p for 48 h, respectively. Finally, the luciferase assay was conducted on the dual Luciferase reporter assay system (Promega, Madison, WI, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (SPSS, Chicago, IL, USA) was used

for all statistical analysis. Independent-sample *t*-test was selected when appropriate. $p < 0.05$ was considered statistically significant.

Results

ZFAS1 Expression Level in HCC Tissues and Cells

First, the ZFAS1 expression in 60 HCC patients' tissues and 2 HCC cell lines was detected via RT-qPCR. Results showed that ZFAS1 was significantly up-regulated in HCC tissue samples compared to that of adjacent normal tissues (Figure 1A). Meanwhile, ZFAS1 expression was significantly higher in HCC cells than in L02 cells (Figure 1B). In our study, the HepG2 and Bel-7402 cell lines were chosen for overexpression and knockdown of ZFAS1, respectively. The transfection efficiency was confirmed by RT-qPCR (Figure 1C and 1D).

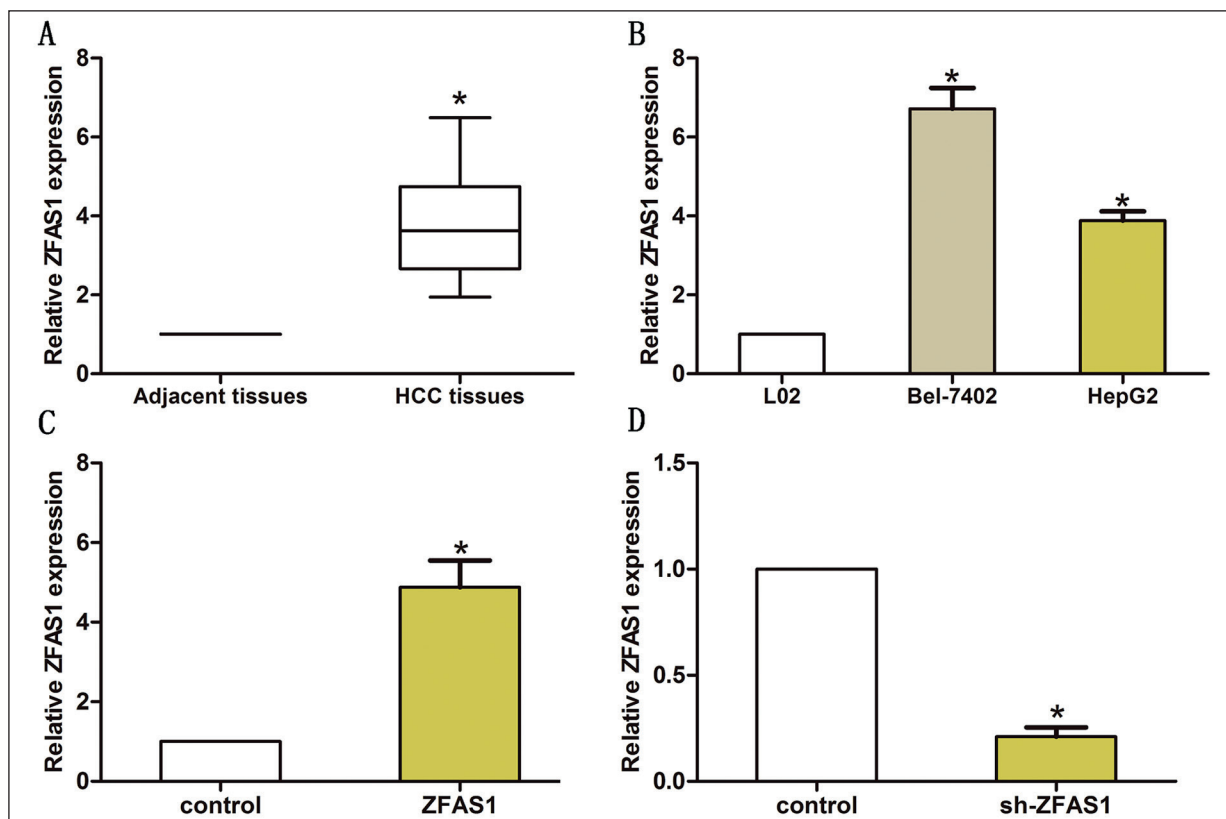


Figure 1. Expression level of ZFAS1 was significantly up-regulated in HCC tissues and cell lines. **A**, ZFAS1 expression increased significantly in HCC tissues compared with adjacent tissues. **B**, Expression level of ZFAS1 relative to GAPDH in human HCC cell lines and L02 (normal liver epithelial cell) was determined by RT-qPCR. **C**, ZFAS1 expression in HepG2 HCC cells transfected with ZFAS1 lentivirus and control vector was detected by RT-qPCR. **D**, ZFAS1 expression in Bel-7402 HCC cells transfected with sh-ZFAS1 and control vector was detected by RT-qPCR. GAPDH was used as an internal control. Data were presented as mean $\pm$ standard error of the mean. * $p < 0.05$.

Overexpression of ZFAS1 Promoted Proliferation of HepG2 HCC Cells

The results of the CCK8 assay showed that overexpression of ZFAS1 significantly enhanced the growth ability of HepG2 cells (Figure 2A). The colony formation assay also revealed that the number of formed colonies increased remarkably after ZFAS1 was overexpressed in HepG2 cells (Figure 2B). Moreover, the results

of EdU incorporation assay demonstrated that the percentage of EdU positive cells increased significantly after overexpression of ZFAS1 in HepG2 cells (Figure 2C).

Silence of ZFAS1 Suppressed Proliferation of Bel-7402 HCC Cells

The results of the CCK-8 assay showed that silence of ZFAS1 significantly inhibited the

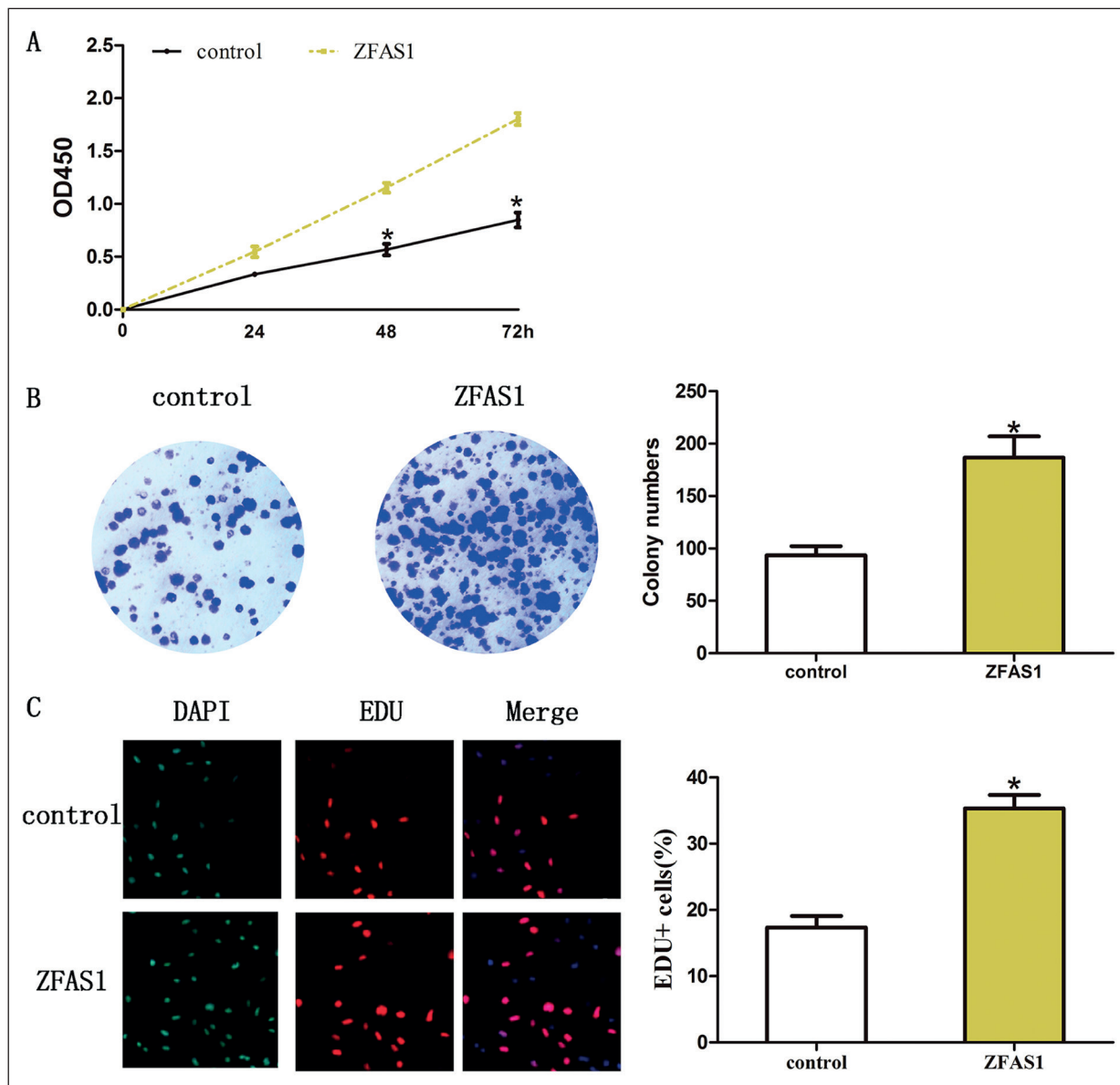


Figure 2. Overexpression of ZFAS1 promoted HepG2 HCC cell proliferation. **A**, CCK8 assay showed that overexpression of ZFAS1 significantly enhanced the proliferation in HepG2 HCC cells. **B**, Colony formation assay showed that the number of colonies increased significantly *via* overexpression of ZFAS1 in HepG2 HCC cells (magnification $\times 40$). **C**, EdU incorporation assay showed that the number of EdU positive cells increased significantly *via* overexpression of ZFAS1 in HepG2 HCC cells (magnification $\times 20$). The results represented the average of three independent experiments (mean $\pm$ standard error of the mean). * $p < 0.05$, as compared with control cells.

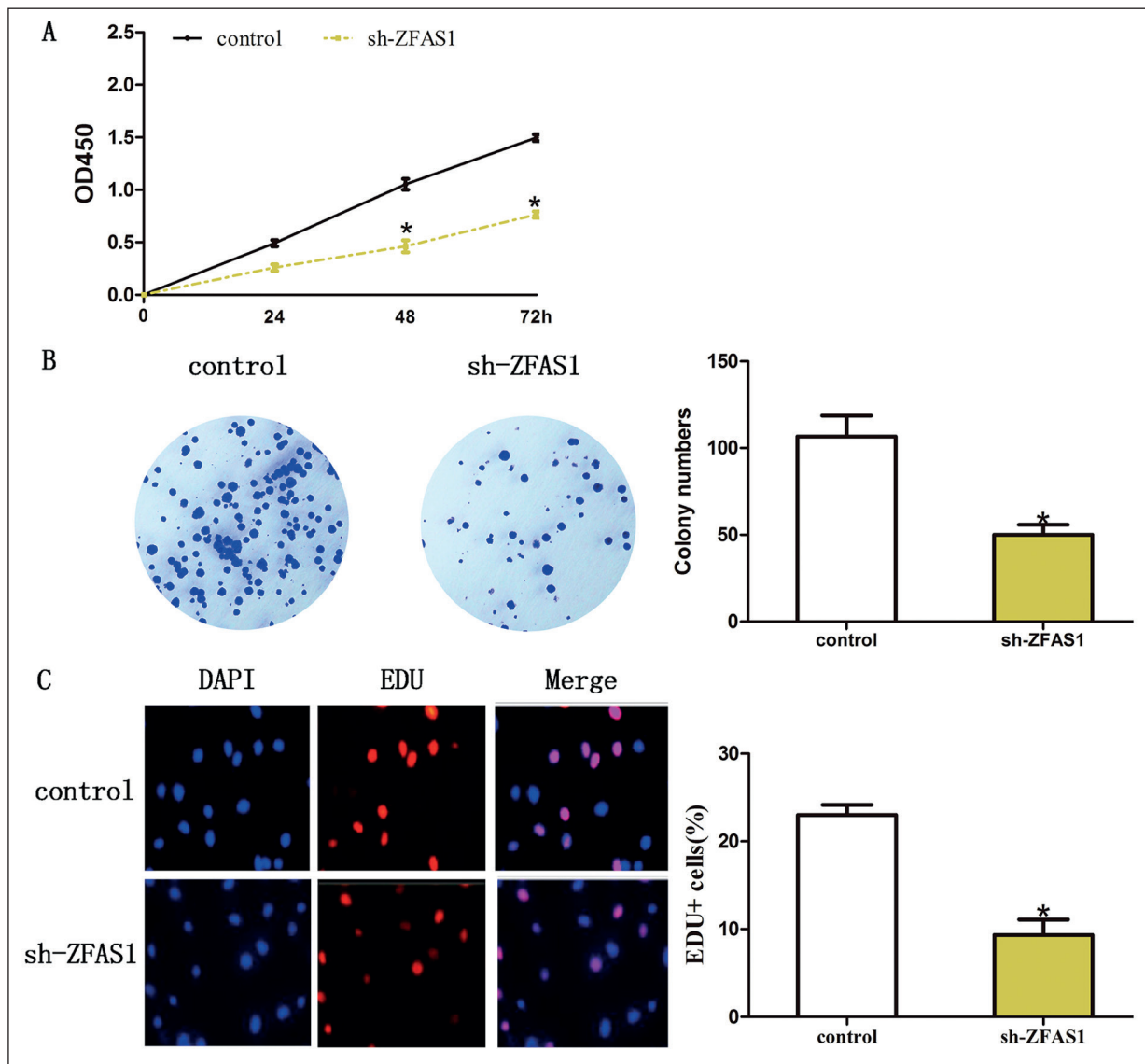


Figure 3. Knockdown of ZFAS1 inhibited Bel-7402 HCC cell proliferation. **A**, CCK8 assay showed that the silence of ZFAS1 significantly repressed the proliferation of Bel-7402 HCC cells. **B**, Colony formation assay showed that the number of colonies decreased remarkably *via* silence of ZFAS1 in Bel-7402 HCC cells (magnification $\times 40$). **C**, EdU incorporation assay showed that the number of EdU positive cells decreased significantly *via* silence of ZFAS1 in Bel-7402 HCC cells (magnification $\times 40$). The results represented the average of three independent experiments (mean $\pm$ standard error of the mean). * $p < 0.05$, as compared with control cells.

growth ability of Bel-7402 cells (Figure 3A). The colony formation assay indicated that the number of formed colonies was remarkably reduced after ZFAS1 was silenced in Bel-7402 cells (Figure 3B). Furthermore, the results of EdU incorporation assay illustrated that the percentage of EdU positive cells was remarkably reduced after the silence of ZFAS1 in Bel-7402 cells (Figure 3C).

Interaction Between MiR-193a-3p and ZFAS1 in HCC

DIANA LncBASE Predicted v.2 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex-predicted) was used to search for miRNAs that contained complementary base with ZFAS1. MiR-193a-3p is a tumor suppressor gene that has been found to suppress cancer cell proliferation. Therefore,

among these miRNAs, we focused on miR-193a-3p which was interacted with ZFAS1 (Figure 4A). Indeed, the RT-qPCR assay showed that the expression of miR-193a-3p was significantly lower in ZFAS1 lentivirus cells than in control

cells (Figure 4B). However, the expression of miR-193a-3p was markedly higher in sh-ZFAS1 cells than in control cells (Figure 4C). Luciferase reporter gene assay revealed that co-transfection of ZFAS1-WT and miR-193a-3p largely

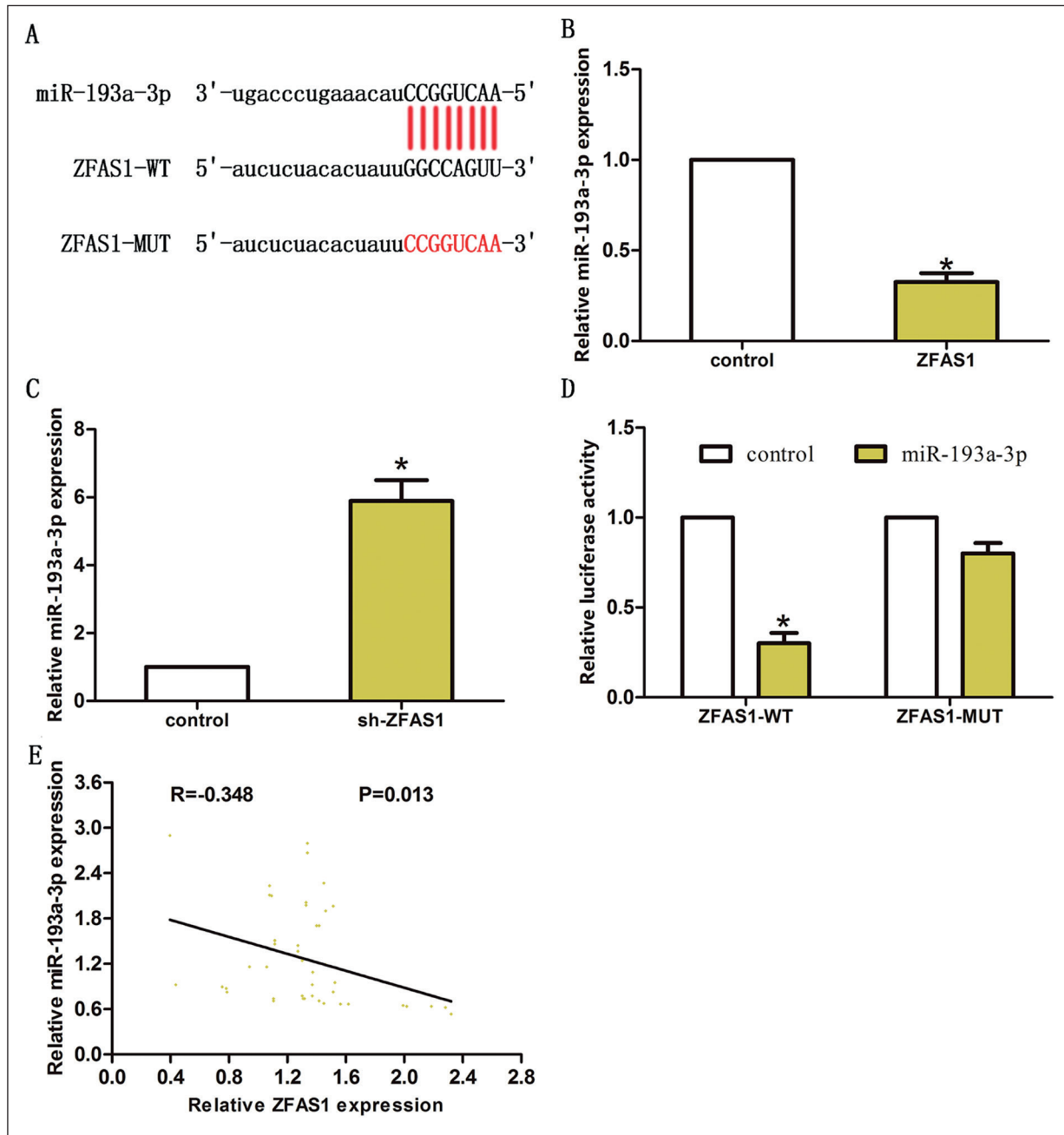


Figure 4. Reciprocal repression between ZFAS1 and miR-193a-3p. **A**, Binding sites of miR-193a-3p on ZFAS1. **B**, MiR-193a-3p expression decreased in ZFAS1 lentivirus group compared with control group. **C**, MiR-193a-3p expression increased in sh-ZFAS1 group compared with control group. **D**, Co-transfection of miR-193a-3p and ZFAS1-WT strongly decreased luciferase activity, while co-transfection of miR-193a-3p and ZFAS1-MUT did not change luciferase activity. **E**, Linear correlation between the expression level of miR-193a-3p and ZFAS1 in HCC tissues. The results represented the average of three independent experiments Data were presented as the mean $\pm$ standard error of the mean. * $p < 0.05$.

decreased the luciferase activity (Figure 4D). Furthermore, correlation analysis demonstrated that miR-193a-3p expression was negatively correlated with ZFAS1 expression in HCC tissues (Figure 4E).

Discussion

In the past decades, the morbidity and mortality of HCC have been increasing worldwide, especially in developing countries. Hepatocarcinogenesis is a complex process, which is associated with the accumulation of genetic and epigenetic changes during the HCC progression. Currently, lncRNAs have been shown as important regulators of HCC. For example, through the regulation of miR-202-5p, lncRNA NORAD enhances the progression of HCC *via* the modulation of TGF- β pathway¹³. The down-regulation of lncRNA P7 facilitates the proliferation of HCC cells through the modulation of the STAT1-MAPK signal pathway. Meanwhile, it is associated with unfavorable prognosis of HCC patients¹⁴. As a sponge of miR-149, lncRNA SNHG8 enhances tumorigenesis and metastasis in HCC¹⁵.

lncRNA ZFAS1 (lncRNA ZNFX1 antisense RNA 1), located on the chromosome 20q13.13, has been found involved in the progression of many diseases. Gao et al¹⁶ have indicated that ZFAS1 participates in the development of carcinogenesis. For instance, ZFAS1 promotes the proliferation of glioma cells through the activation of the Notch signaling pathway. ZFAS1 is up-regulated in gastric cancer and can induce epithelial-mesenchymal transition *in vitro*¹⁷. ZFAS1 enhances the malignancy of ovarian cancer by regulating Sp1¹⁸. In this study, we found that ZFAS1 was significantly up-regulated in HCC tissues and cell lines. Besides, the silence of ZFAS1 significantly repressed the proliferation of HCC cells, while the overexpression of ZFAS1 promoted the proliferation of HCC cells. The above results indicated that ZFAS1 promoted the proliferation of HCC and might act as an oncogene.

Recently, Ren et al¹⁹ have revealed that lncRNAs interact with microRNAs in many diseases. Meanwhile, microRNAs expression can be activated by lncRNAs. For example, the silence of SNHG7 inhibits cell growth and migration in glioblastoma through the inhibition of miR-5095. Knockdown of lncRNA TUG1 depresses the proliferation and invasion of osteosarcoma cells *via* sponging miR-153²⁰. Furthermore, lncRNA

SPRY4-IT1 functions as an oncogene in bladder cancer through the up-regulation of EZH2 by sponging miR-101-3p²¹.

Numerous microRNAs have been reported as dysregulated in various types of cancer. Recent studies have suggested that miR-193a-3p functions as a tumor suppressor in multiple malignant tumors. For example, miR-193a-3p serves as a tumor suppressor in lung cancer and inhibits the development of lung cancer *via* targeting KRAS. The proliferation and metastasis of renal cell carcinoma are regulated by miR-193a-3p²³. MiR-193a-3p suppresses the process of pathogenesis in colorectal cancer by targeting KRAS²⁴. In the present study, the potential interaction between miR-193a-3p and ZFAS1 was predicted through the bioinformatics software. Subsequent experiments showed that miR-193a-3p could directly bind to ZFAS1 through the luciferase reporter gene assay. In addition, miR-193a-3p expression was significantly suppressed by the up-regulation of ZFAS1. However, the downregulated ZFAS1 expression could induce a reverse outcome. Furthermore, the expression of miR-193a-3p was negatively correlated with ZFAS1 in HCC tissues. All these results indicated that ZFAS1 might promote tumorigenesis of HCC through directly targeting miR-193a-3p.

Conclusions

In sum, ZFAS1 was remarkably up-regulated in HCC tissues. Furthermore, it could facilitate cell proliferation in HCC by targeting miR-193a-3p. These findings suggested that ZFAS1 might contribute to therapy for HCC as a prospective target.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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