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Circ_0000064 adsorption of microRNA-143 promotes malignant progression of hepatocellular carcinoma

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Abstract. – OBJECTIVE: The aim of this study was to investigate the expression characteristics of circ_0000064 in hepatocellular carcinoma, and to further explore the underlying mechanism.

PATIENTS AND METHODS: Real time quantitative-Polymerase Chain Reaction (qPCR) was used to detect the expression of circ_0000064 in 42 hepatocellular carcinoma tissues and adjacent normal tissues. Meanwhile, the relationship between circ_0000064 expression and clinical indicators, as well as the prognosis of patients with hepatocellular carcinoma, were detected. QPCR was applied to measure circ_0000064 level in hepatocellular carcinoma cell lines as well. Subsequently, the circ_0000064 knockdown model was successfully constructed using lentivirus in hepatocellular carcinoma cell lines. Cell counting kit-8 (CCK-8), colony formation assay, and flow apoptosis were performed to analyze the influence of circ_0000064 on the biological functions of hepatocellular carcinoma cells. The potential mechanism was explored using cell recovery experiments. In addition, the relationship between circ_0000064 and microRNA-143 was finally explored.

RESULTS: QPCR results showed that the expression level of circ_0000064 in hepatocellular carcinoma tissues was remarkably higher than that of adjacent normal tissues, and the difference was statistically significant. Compared with patients with lower expression of circ_0000064, patients with higher expression of circ_0000064 exhibited remarkably higher pathological stage and lower overall survival rate. *In vitro* experiments demonstrated that the proliferation ability of the cells was remarkably reduced after the transfection of si-circ_0000064, while cell apoptosis ability significantly increased when compared with the NC group. Meanwhile, qPCR results indicated that microRNA-143 expression was negatively correlated with circ_0000064 expression in hepatocellular carcinoma. Luciferase reporter gene assay indicated that circ_0000064 could be targeted by microRNA-143 through their binding site. In addition, the cell recovery experiment confirmed that circ_0000064 and microRNA-143 could be mutually regulated, which affected the malignant progression of hepatocellular carcinoma together.

CONCLUSIONS: Circ_0000064 level was remarkably upregulated in hepatocellular carcinoma and was associated with high pathological stage and poor prognosis of patients. In addition, circ_0000064 significantly promoted proliferation and inhibited apoptosis of hepatocellular carcinoma cells via modulating microRNA-143.

Key Words:
Circ_0000064, MicroRNA-143, Hepatocellular carcinoma, Proliferation.

Introduction

The latest epidemiological survey of malignant tumors[1,2] shows that the incidence and mortality rate of liver cancer ranks 5th and 2nd worldwide. Currently, there are about 782,500 new cases of liver cancer around the world every year, with 745,500 deaths[3,4]. In China, although the morbidity and mortality of liver cancer have greatly decreased in recent years, the morbidity and mortality of liver cancer still rank 3rd among male cancer patients. Meanwhile, the incidence and mortality of liver cancer rank 6th and 4th among all female malignancies, respectively[5-7]. Therefore, it is of great significance to search for novel molecular markers to predict the recurrence of liver cancer after surgery. Furthermore, it is also important to study the molecular mechanism of the occurrence and metastasis of liver cancer, and to find new therapeutic targets[8-10].

CircularRNAs (circRNAs) are a kind of closed circular single-stranded RNAs. They were previ-

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ously considered as rare RNAs and only a "waste product" produced during RNA splicing without any function[11,12]. In recent years, high-throughput sequencing has shown that a large number of circRNAs exist in eukaryotic cells, including human cells[12,13]. For example, more than 10% of genes in tested cells or tissues are capable of producing circRNAs[14]. Multiple circRNAs are highly abundant, which are also cell or tissue specific[15]. All the above findings suggest that these highly expressed circRNAs are functional rather than waste products from the splicing process[15,16]. Several studies[17,18] have demonstrated that circRNAs can adsorb microRNAs or bind proteins. Meanwhile, the changes in the expression of circRNAs can cause changes in the expression levels of tumor-related genes, thus affecting the occurrence and development of malignancies. Currently, there are very few reports[19] on the role of circ_0000064 in the development of tumors. Therefore, the aim of this study was to comprehensively analyze the expression and biological effects of circ_0000064 in hepatocellular carcinoma, and to preliminarily explore the underlying molecular mechanism.

MiRNA expression and gene regulation have been confirmed closely related to the occurrence and development of hepatocellular carcinoma[20]. Currently, the research ideas of most scholars are screening differentially expressed miRNAs in disease progression, discussing their correlation with the occurrence, development, and prognosis of diseases, and searching for targets for further elucidation of the mechanism of disease occurrence and targeted disease treatment[20,21]. In recent years, it has been found that microRNA-143 is widely studied to various degrees in a variety of tumors. However, the exact role of microRNA-143 in hepatocellular carcinoma has not been fully elucidated[22,23]. Therefore, in this study, we investigated whether circ_0000064 mediated the proliferation and apoptosis of hepatocellular carcinoma by regulating microRNA-143. Our findings might help to provide an experimental basis for the clinical application of microRNA-143.

Patients and Methods

Patients and Hepatocellular Carcinoma Samples

Totally 42 pairs of invasive hepatocellular carcinoma tissues and corresponding adjacent normal tissues were collected from patients who received surgery. All collected tissues were preserved at -80°C for use. The collection of clinical specimens was approved by the Ethics Oversight Committee. Informed consent was obtained from patients and their families before the study.

Cell Lines and Reagents

Six human HCC cell lines (Bel-7402, HepG2, MHCC88H, SMMC-7221, Huh7, Hep3B) and one human normal liver cell line (LO2) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM) medium and fetal bovine serum (FBS) were purchased from American Life Technologies (Gaithersburg, MD, USA). All cells were cultured in DMEM high glucose medium containing 10% FBS, penicillin (100 U/mL), streptomycin (100 μg/mL), and maintained in an incubator with 5% CO_2 at 37°C. The cell passage was performed when cells reached the 80-90% of confluence.

Cell Transfection

The negative control group (si-NC) and lentivirus containing circ_0000064 knockdown sequence (si-circ_0000064) were purchased from Shanghai Jima Company (Shanghai, China). The cells were first plated in 6-well plates and grown to a cell density of 40%. Subsequently, cell transfection was performed according to the manufacturer's instructions. 48 h later, the cells were harvested for real-time quantitative polymerase chain reaction (qPCR) analysis and cellular functional experiments.

Cell Proliferation Assay

48 h after transfection, the cells were collected and plated into 96-well plates at a density of 2000 cells per well. After culture for 24 h, 48 h, 72 h, and 96 h, respectively, the Cell Counting Kit-8 (CCK-8) reagent (Dojindo Molecular Technologies, Kumamoto, Japan) was added in the cells, followed by incubation for 2 h in the dark. Optical density (OD) value of each well at the wavelength of 490 nm was measured by a microplate reader.

Colony Formation Assay

After 48 h of transfection, the cells were collected and seeded into a 6-well plate with 200 cells per well. Then, the cells were cultured in complete medium for 2 weeks. After cell clones were formed, the medium was removed, and the cells were washed twice with Phosphate-Buffered Saline (PBS). Subsequently, the cells were fixed

with 2 mL of methanol for 20 min, and stained with 0.1% crystal violet staining solution for 20 min. Finally, the cell colonies were photographed under a light-selective environment, and the number of colonies was counted.

Flow Cytometry

Bel-7402 and Hep3B cells in logarithmic growth phase were seeded into 6-well plates. After 24 hours of drug treatment, the cells were collected and washed twice with PBS. After re-suspension in the binding solution, the cells were incubated at room temperature for 15 min in the dark. Next, 5 μL of Annexin V-FITC (fluorescein isothiocyanate) and propidium iodide (PI) was added in the cells and gently mixed. The cell apoptosis rate was measured *via* flow cytometry analysis (FACSCalibur; BD Biosciences, Detroit, MI, USA).

QPCR

The total RNA in the cells was first extracted according to the instructions of TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Initially, the extracted RNA was treated with DNase I to remove genomic DNA and re-purify the RNA. Subsequently, RNA was reverse transcribed into cNDA according to the Prime Script Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) instructions. Real-time PCR was performed according to the instructions of SYBR® Premix Ex Taq™ kit (TaKaRa, Otsu, Shiga, Japan). PCR reaction was performed using the StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The Bio-Rad PCR instrument was used to analyze and process the data with software iQ5 2.0 (Bio-Rad, Hercules, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were used as internal parameters for mRNA and miRNA, respectively. The gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study were as follows: circ_0000064, F: 5'-GCGAGGCATCCGCTGCTGACTC-3', R: 5'-GCTAGCCATGTGTAAGACGGA-3'; microRNA-143, F: 5'-GCCGTATAGACAGCCTGACTG-3', R: 5'-AGGTGCGTCCGTAGCGTAG-3'; U6: F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', R: 5'-CGCTTCAGAATTTGCGTGTCAT-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Dual Luciferase Reporter Assay

A reporter plasmid was first constructed, in which a specific fragment of target promoter was inserted in front of the Luciferase expression sequence. The transcription factor expression plasmid to be detected was co-transfected with the reporter plasmid into Bel-7402 and Hep3B cells or other cell lines. If the transcription factor could activate the target promoter, the Luciferase gene would be expressed. Meanwhile, the amount of Luciferase expression was directly proportional to the intensity of the transcription factor. A specific Luciferase substrate was added, and the Luciferase reacted with the substrate to generate fluorescence. By measuring the intensity of the fluorescence, the activity of the Luciferase was detected to determine whether the transcription factor could interact with the target promoter fragment.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 5 V5.01 software (La Jolla, CA, USA). The Student's *t*-test was used to compare the difference between the two groups. One-way ANOVA was applied to compare the differences among different groups, followed by the post-hoc test (Least Significant Difference). The experiments were repeated for at least three times, and the experimental data were expressed as mean ± standard deviation. $p<0.05$ was considered statistically significant.

Results

Circ_0000064 was Highly Expressed in Hepatocellular Carcinoma Tissues and Cell Lines

To determine the expression level of circ_0000064 in hepatocellular carcinoma, 42 pairs of tumor tissues and adjacent normal tissues were first collected. The expression of circ_0000064 in the hepatocellular carcinoma tissues and adjacent normal tissues was detected by qPCR. The results showed that circ_0000064 was significantly elevated in the hepatocellular carcinoma tissues when compared with adjacent normal tissues (Figure 1A). This suggested that circ_0000064 might play an oncogenic role in hepatocellular carcinoma. In addition, circ_0000064 expression was remarkably higher in hepatocellular carcinoma cell lines than human

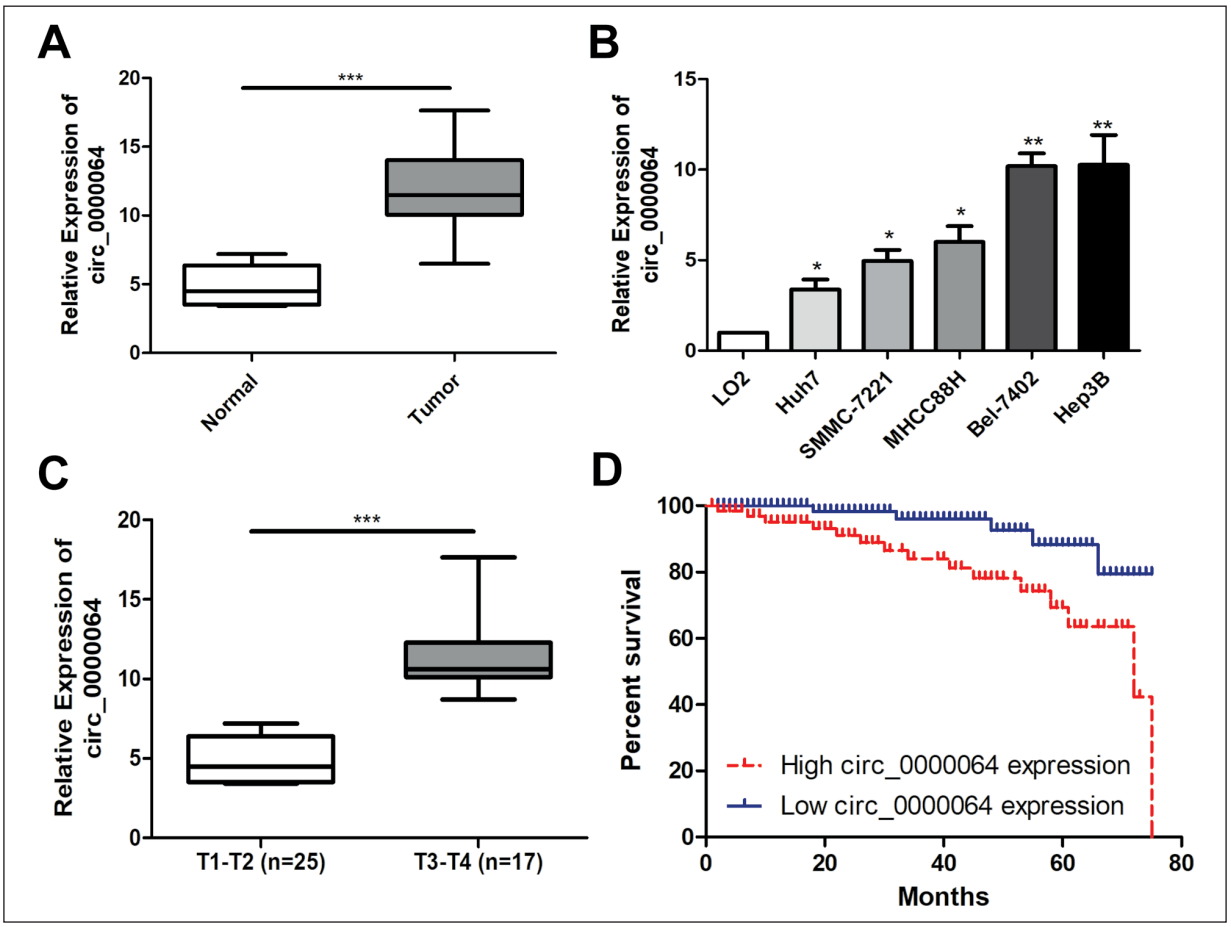

Figure 1. Circ_0000064 was highly expressed in hepatocellular carcinoma tissues and cell lines. **A,** QPCR was used to detect the expression of circ_0000064 in hepatocellular carcinoma tumor tissues and adjacent tissues. **B,** QPCR was used to detect the expression level of circ_0000064 in hepatocellular carcinoma cell lines. **C,** QPCR was used to detect the expression level of circ_0000064 in patients with different pathological stages of hepatocellular carcinoma. **D,** Kaplan Meier survival curve of hepatocellular carcinoma patients based on circ_0000064 expression. The data were expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

normal liver cell line (LO2), and the difference was statistically significant (Figure 1B). These results suggested that circ_0000064 was highly expressed in hepatocellular carcinoma tissues and cell lines.

Circ_0000064 Expression was Correlated with Pathological Staging and Overall Survival of Patients with Hepatocellular Carcinoma

Based on the mRNA expression of circ_0000064, the hepatocellular carcinoma patients were divided into high circ_0000064 expression group and low circ_0000064 expression group. Subsequently, the relationship between the expression of circ_0000064 and age, sex, pathological stage, lymph node metastasis, and distantness of hepatocellular carcinoma patients was evaluated. As shown in Table I, high expression of circ_0000064 was positively correlated with the pathological stage of hepatocellular carcinoma, whereas was not associated with age, gender, distant metastasis, and lymph node metastasis (Figure 1C).

Meanwhile, the relationship between the expression of circ_0000064 and the prognosis of patients with hepatocellular carcinoma was explored as well. The Kaplan-Meier survival curves showed that high expression of circ_0000064 was remarkably associated with poor prognosis of HCC (p<0.05; Figure 1D). Therefore, we concluded that circ_0000064 was correlated with pathological staging and overall survival of hepatocellular carcinoma patients.

Table I. Association of circ_0000064 and miR-143 expression with clinicopathologic characteristics of hepatocellular carcinoma.

Parameters	Number of cases	circ_0000064 expression			miR-143 expression		
		Low (%)	High (%)	*p*-value	Low (%)	High (%)	*p*-value
Age (years)				0.346			0.414
< 60	17	10	7		6	11	
≥ 60	25	11	14		12	13	
Gender				0.355			0.533
Male	21	12	9		8	13	
Female	21	9	12		10	11	
T stage				0.028			0.018
T1-T2	25	16	9		7	18	
T3-T4	17	5	12		11	6	
Lymph node metastasis				0.107			0.094
No	27	16	11		9	18	
Yes	15	5	10		9	6	
Distance metastasis				0.116			0.276
No	25	15	10		9	16	
Yes	17	6	11		9	8	

Knockdown of Circ_0000064 Inhibited Cell Proliferation and Promoted Cell Apoptosis

To investigate the function of circ_0000064 *in vitro*, the knockdown circ_0000064 lentiviral vector was constructed. After transfection of the circ_0000064 lentiviral vector in Bel-7402 and Hep3B cell lines, the qPCR assay was performed to verify the interference efficiency. The results showed that the transfection of lentiviral vector and the expression of circ_0000064 were significantly downregulated (Figure 2A). After knockdown of circ_0000064 in Bel-7402 and Hep3B cell lines, the CCK-8, colony formation assay, and flow cytometry were used to detect cell proliferation and apoptosis, respectively. The results showed that the proliferation ability of cells was remarkably reduced in circ_0000064 silenced group when compared with the NC group (Figures 2B, 2C). In addition, circ_0000064 silenced group exhibited significantly decreased cell apoptosis when compared with the si-NC group (Figure 2D). These results demonstrated that circ_0000064 silencing could inhibit cell proliferation and promote cell apoptosis in hepatocellular carcinoma.

Circ_0000064 could Bind to MicroRNA-143

As shown in Figure 3A, to further verify the targeting effect of microRNA-143 on circ_0000064, we performed the Dual-Luciferase Reporter Gene Assay. The results showed that the overexpression of microRNA-143 remarkably attenuated the Luciferase activity of wild-type circ_0000064 vector ($p<0.05$). This further indicated that circ_0000064 could target microRNA-143 through this binding site. Subsequently, the overexpressing/knockdown microRNA-143 vector was transfected into HCC cells as well, the qPCR results showed that the expression level of circ_0000064 was remarkably downregulated after microRNA-143 overexpression, while the level of circ_0000064 remarkably increased in microRNA-143 inhibitor group (Figure 3B). After circ_0000064 was knocked down in Bel-7402 and Hep3B cell lines, the expression level of microRNA-143 was remarkably up-regulated, and the difference was statistically significant (Figure 3C). These results revealed that circ_0000064 could target microRNA-143, and they could affect mutual expression.

MicroRNA-143 was Lowly Expressed in Hepatocellular Carcinoma Tissues and Cell Lines

Next, we detected the expression of microRNA-143 in 42 pairs of hepatocellular carcinoma tissues and corresponding normal tissues, as well as hepatocellular carcinoma cell lines. The results showed that the expression level of microRNA-143 was remarkably downregulated in hepatocellular carcinoma tissues compared with corresponding normal tissues (Figure 3D). Subsequently, we found that microRNA-143 was

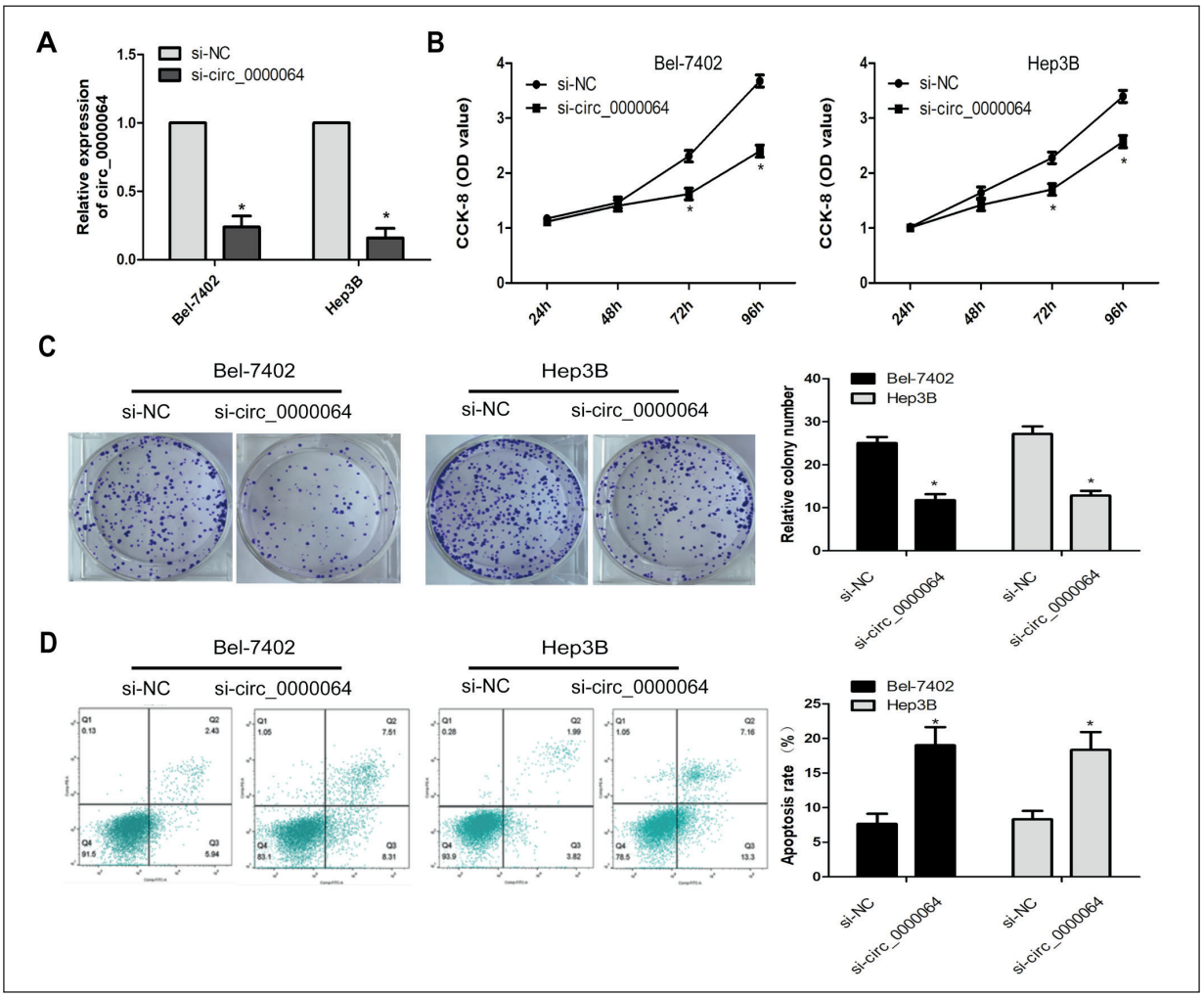

Figure 2. Silencing circ_0000064 inhibited the proliferation and promoted the apoptosis of hepatocellular carcinoma cells. **A,** QPCR verified the interference efficiency after transfection of circ_0000064 knockout vector in Bel-7402 and Hep3B cell lines. **B,** CCK-8 assay detected the effect of silencing circ_0000064 on the proliferation of Bel-7402 and Hep3B cells. **C,** Colony formation assay was performed to detect the proliferation of Bel-7402 and Hep3B cells with circ_0000064 knockdown (magnification × 10). **D,** Flow cytometry assay was performed to detect the effect of silencing circ_0000064 on the apoptosis of Bel-7402 and Hep3B cell lines. The data were expressed as mean ± SD, *$p<0.05$.

lowly expressed in hepatocellular carcinoma cell lines as well (Figure 3E). Meanwhile, qPCR results showed that circ_0000064 expression was negatively correlated with microRNA-143 expression in the hepatocellular carcinoma tissues (Figure 3F). At the same time, we analyzed the relationship between microRNA-143 expression with pathology and prognosis of patients with hepatocellular carcinoma. As shown in Table I, the low expression of microRNA-143 was positively correlated with the pathological stage of hepatocellular carcinoma, whereas it was not associated with age, gender, lymph node metastasis, and distant metastasis.

Circ_0000064 Modulated MicroRNA-143 Expression in Hepatocellular Carcinoma Tissues and Cell Lines

To further explore the mechanism in which circ_0000064 promoted the malignant progression of hepatocellular carcinoma, we hypothesized that there might be a relationship between circ_0000064 and microRNA-143 through bioinformatics analysis. After that, we silenced microRNA-143 in cells that had already silenced by circ_0000064 to further explore the interaction between circ_0000064 and microRNA-143 in hepatocellular carcinoma. QPCR assay was used to detect the transfection efficiency of circ_0000064

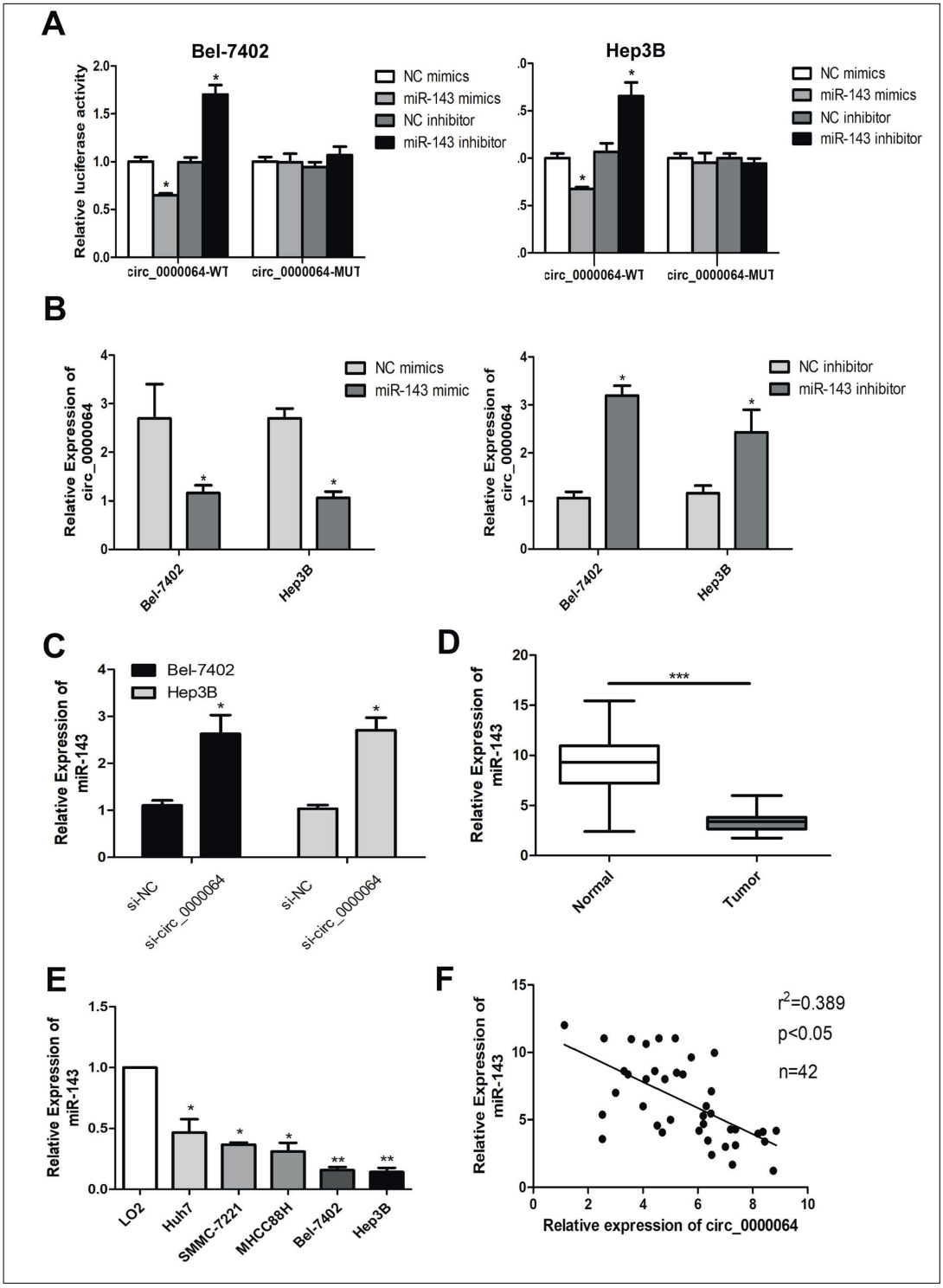

Figure 3. Circ_0000064 could direct target to miR-143. **A,** Dual-Luciferase reporter gene assay verified the direct targeting of circ_0000064 and miR-143. The results in Bel-7402 and Hep3B cells indicated that the overexpression of miR-143 significantly attenuated the Luciferase activity of the wild-type circ-0000064 vector. **B,** Overexpression/silencing of miR-143 significantly reduced/increased the expression level of circ_0000064. **C,** Silencing circ_0000064 expression significantly increased miR-143 expression level. **D,** QPCR was used to detect the expression of miR-143 in hepatocellular carcinoma tissues and adjacent normal tissues. **E,** QPCR was applied to verify the mRNA expression level of miR-143 after the transfection of circ_0000064 in hepatocellular carcinoma cell lines. **F,** There was a significant negative correlation between the expression levels of circ_0000064 and miR-143 in hepatocellular carcinoma tissues. The data were expressed as mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

after co-rotation (Figure 4A). Subsequent CCK-8, colony formation assay, and flow apoptosis found that microRNA-143 could counteract the effect of circ_0000064 on the proliferation and apoptosis of hepatocellular carcinoma cells (Figures 4B-4D).

Discussion

Hepatocellular carcinoma is a highly malignant tumor, which is described as the second killer of cancer in China. Meanwhile, it seriously threatens the health of Chinese people, eventually leading to huge economic and social burden[1-4]. Currently, the main clinical treatment methods for hepatocellular carcinoma include surgical resection, radiotherapy, chemotherapy, embolization, interventional therapy, and molecular targeted drug therapy, etc.[5,6]. Although great progress has been made in the treatment of hepatocellular carcinoma, its long-term survival rate has not been remarkably improved. This is mainly due to the reason that the highly malignant and aggressive growth of HCC is

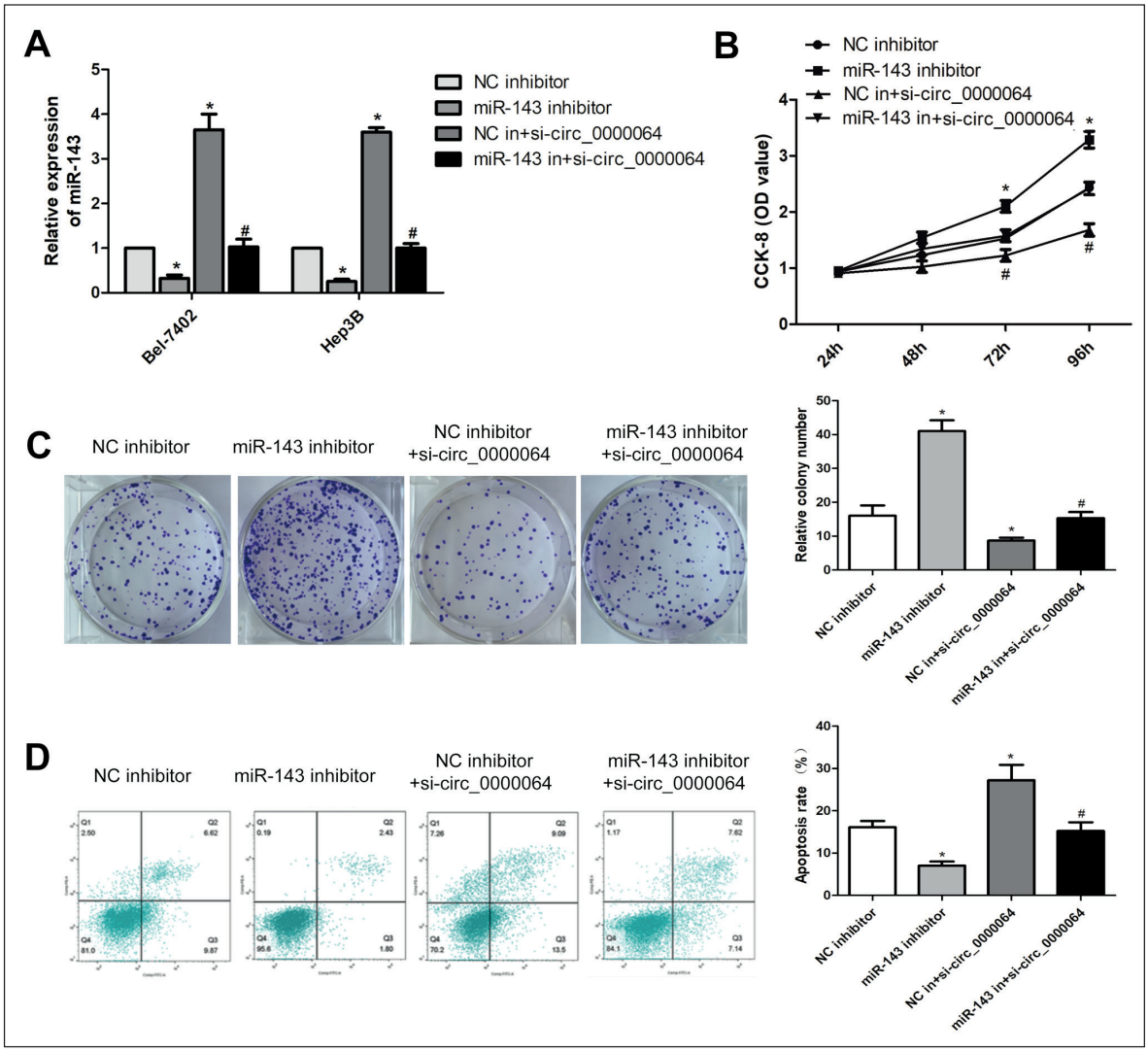

Figure 4. Circ_0000064 regulated the expression of miR-143 in hepatocellular carcinoma tissues and cell lines. **A,** Circ_0000064 expression in cell lines co-transfected with circ_0000064 and miR-143 was detected by qPCR. **B,** CCK-8 assay detected the effect of circ_0000064 and miR-143 on the proliferation of the hepatocellular carcinoma cells after co-transfection. **C,** Colony formation assay was used to detect the number of hepatocellular carcinoma positive clone-forming cells after co-transfection of circ_0000064 and miR-143 (magnification × 10). **D,** Flow cytometry assay was used to detect the apoptosis of hepatocellular carcinoma cells after co-transfection of circ_0000064 and miR-143. The data were expressed as mean ± SD, *$p<0.05$.

likely to cause recurrence and metastasis after treatment[7-10]. Therefore, revealing the pathogenesis of hepatocellular carcinoma is extremely important to guide its treatment.

Currently, several literatures[11-14] have reported that circRNAs can be used as diagnostic markers for diseases. In this study, we determined that circ_0000064 could promote the malignant progress of hepatocellular carcinoma. Circ_0000064 expression was found remarkably higher in tumor tissues than that of the corresponding normal tissues. Meanwhile, its expression was positively correlated with pathological stage and poor prognosis of hepatocellular carcinoma. The results convinced us that circ_0000064 might play an important role in promoting the development of hepatocellular carcinoma. To further study the molecular mechanism of circ_0000064 in the development of hepatocellular carcinoma, cellular experiments were conducted *in vitro*. CCK-8 assay, colony formation assay, and flow apoptosis demonstrated that silencing circ_0000064 in bel-7402 and Hep3B cell lines could significantly inhibit the proliferation and promote the apoptosis of the hepatocellular carcinoma cells.

CircRNAs can affect tumor development by adsorbing microRNAs, binding proteins, and translating proteins[20,21]. In this study, the bioinformatics methods were used to predict the possible interaction between microRNA-143 and circ_0000064. We found that circ_0000064 sequence contained a miR-143 binding site. The Dual-Luciferase Reporter Gene Assay and other molecular biology experiments showed that circ_0000064 could direct combine with its downstream miR-143. Meanwhile, miR-143 was not enriched by a circ_0000064 vector that mutated miR-143 binding site, further verifying the binding relationship between circ_0000064 with miR-143. To further explore the roles of circ_0000064 and microRNA-143 in the regulation of hepatocellular carcinoma cell lines, their expression levels were detected by qPCR. The results demonstrated that circ_0000064 expression was negatively correlated with microRNA-143 expression. In addition, we found that microRNA-143 could counteract the effect of circ_0000064 on hepatocellular carcinoma cells through recovery assay. Besides, microRNA-143 expression significantly decreased in hepatocellular carcinoma tissues than of adjacent normal tissues. Furthermore, microRNA-143 remarkably inhibited the proliferation and promoted the apoptosis of hepatocellular carcinoma cells. In summary, the findings suggested that circ_0000064 inhibited the expression of microRNA-143, thereby promoting the proliferation and inhibiting the apoptosis of hepatocellular carcinoma cells.

Conclusions

Circ_0000064 expression was remarkably upregulated in hepatocellular carcinoma and was closely correlated with pathological stage and poor prognosis of patients. Furthermore, circ_0000064 might be able to promote hepatocellular carcinoma proliferation and inhibit cell apoptosis by regulating microRNA-143.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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