CircRNA_0000502 promotes hepatocellular carcinoma metastasis and inhibits apoptosis through targeting microRNA-124

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Summary

Purpose: To investigate the expression level of circ_0000502 in hepatocellular carcinoma (HCC), and to further explore whether it can promote the malignant progression of HCC by targeting and binding to microRNA (miR)-124.

Methods: Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the expression level of circ_0000502 in 40 pairs of HCC tissue specimens and adjacent ones, and to analyze the relationship between circ_0000502 expression and prognosis of patients with HCC. QRT-PCR was used to verify the expression of circ_0000502 in HCC cells. The circ_0000502 knockdown model was constructed using lentivirus in HCC cell lines, and cell counting KIT-8 (CCK-8), Transwell and flow cytometry assays were used to figure out the effect of circ_0000502 on the function of HCC cells. Lastly, luciferase reporter gene assay was applied to verify the relationship between circ_0000502 and miR-124.

Results: QRT-PCR results indicated that the level of circ_0000502 in HCC tissues was significantly higher than that in adjacent ones. Compared with patients with low expression of circ_0000502, patients with high expression of circ_0000502 had a lower overall survival rate compared with the negative control (NC) group. The proliferation, invasion and migration ability of circ_0000502 knockdown group significantly decreased, while on the contrary cell apoptosis increased. QRT-PCR results revealed that the expression of miR-124 and circ_0000502 mRNA in HCC tissues was negatively correlated. Also, the result of luciferase reporter gene assay demonstrated that circ_0000502 could be targeted by miR-124 via this binding site.

Conclusion: High expression of circ_0000502 was significantly positively correlated with poor prognosis of HCC. Besides, circ_0000502 promoted the malignant progression of HCC by regulating miR-124 expression.

Key words: Circ_0000502, miRNA-124, hepatocellular carcinoma, malignant progression

Introduction

Primary liver cancer is one of the most common malignant tumors in the world. Hepatocellular carcinoma (HCC) is one of the most common types of primary hepatic malignancies [1-3]. China is one of the high-incidence areas of hepatitis B, and liver cancer patients account for more than half in the world [4,5]. However, due to lack of the early and specific clinical manifestations of HCC, patients are often in advanced stage when they first consult physicians [6,7]. The most effective treatment for liver cancer is radical resection. The current rate of radical resection is low and is one of the main reasons for poor prognosis of liver cancer [8,9]. Therefore, early warning and early interven-

tion of liver cancer are of great significance. It is particularly important to further study the molecular mechanism of liver cancer and the improvement of early diagnostic rates [10,11].

In recent years, a large number of non-coding RNAs, especially miRs and long non-coding RNAs, have attracted more and more attention in the studies on the biological function regulation and clinical diagnosis of liver cancer [12,13]. The discovery and further recognition of circular RNA have enriched the family of post-transcriptional regulators [14,15]. An increasing number of cyclic RNA molecules that interact with each other and control molecular modification are involved in the regulation at post-transcriptional level [16]. Research has found and confirmed that circular RNA is involved in the regulation of various tumor cell biological functions, including colorectal cancer, esophageal squamous cell carcinoma, gastric cancer, breast cancer and basal cell carcinoma [17,18]. In different tumor types, circular RNA may play a role as a tumor suppressor gene or an oncogene [18]. However, there are few reports on the involvement of circular RNA in the regulation of hepatocarcinogenesis and its molecular mechanisms. The expression profile of hepatoma-related circular RNA still needs in-depth research [19,20]. There are very few reports in the current literature on the role of circ_0000502 in the development of tumors [21]. Our data indicated that the expression of circular RNA is abundant in liver cancer tissues, and circ_0000502 with increased expression in liver cancer was further selected as the research object. Therefore, this experiment comprehensively analyzed the expression and biological roles of circ_0000502 in HCC. The study also explored the molecular mechanism of the regulation of HCC.

miRs are non-coding small fragments of RNA that are involved in various physiological and pathological processes by regulating the expression of their target genes [22,23]. The genes encoding miRs are mostly located in intergenic or intron regions of the coding gene and exist as single copies, multiple copies or gene clusters [23]. Mature miRs recognize each other through incomplete base pairing of the 5-8 nucleotides (nt) seed sequence at the 5' end with the miR regulatory element (MRE) of the target gene mRNA 3'-UTR. The two mechanisms of inhibition of protein translation and initiation of mRNA degradation enable post-transcriptional regulation of target gene expression [23]. In recent years, studies have found that miR-124 has different degrees of involvement in HCC [24,25]. The primary purpose of this study was to investigate whether circ_0000502 promotes the malignant progression of HCC by targeting miR-124, pro-

viding thus experimental evidence for its clinical application.

Methods

Patients and hepatocellular carcinoma samples

In this study, 40 pairs of HCC tissue and their corresponding adjacent tissues were collected and stored at -80°C. Signed informed consents were obtained from all participants before the study entry. This study was approved by the Ethics Committee of our hospital.

Cell lines and reagents

Six human HCC cells lines (Bel-7402, HepG2, MH-CC88H, 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 Medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Rockville, MD, USA). The HCC cell lines were cultured in a high glucose DMEM containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL). All cells were cultured in an incubator at 37°C with 5% CO_2 and passaged with 1% trypsin + ethylene diamine tetraacetic acid (EDTA) for digestion when grown to 80-90% confluence.

Transfection experiment

The negative control sequence (NC) and the lentivirus containing the circ_0000502 knockdown sequence (sh-circ_0000502) were purchased from Shanghai GenePharma Company (Shanghai, China). Cells were plated in 6-well plates and grown to a cell density of 40%, followed by the transfection experiment. After 48 h, cells were collected for quantitative real-time polymerase chain reaction (qRT-PCR) analysis and cell function experiments.

Cell proliferation assays

After 48 h of transfection, cells were harvested and plated into 96-well plates. The cells were cultured for 24 h, 48 h, 72 h and 96 h. CCK-8 (Dojindo Laboratories, Kumamoto, Japan) reagent was added. After incubation for 2 h, the optical density (OD) value of each well was measured with a microplate reader at 490 nm of absorption wavelength.

Flow cytometry

Flow cytometry was performed using the method of the combination of Annexin V-FITC (fluorescein isothiocyanate) (Merck, Billerica, MA, USA) and Propidium Iodide (PI). First, the cell density was adjusted to about 1×10^6 cells/ml, the medium was removed, and the cells were washed twice with phosphate buffered saline (PBS). Sencond, the cells were gently resuspended with 0.5 mL of pre-cooled 1×binding buffer, and 1.25 μL of Annexin V-FITC was added for incubating the cells at room temperature in the dark for 15 min. Centrifugation was performed at 1000×g for 5 min at room temperature, and the supernatant was discarded. Then, the cells were gently

resuspended with 0.5 mL of pre-cooled l×binding buffer and incubated with 10 μL of PI. The sample was placed on ice and stored in the dark, and immediately analyzed by flow cytometry (BD, Franklin Lakes, NJ, USA).

Transwell cell migration and invasion assay

After transfection for 48 h, cells were digested, centrifuged and resuspended in medium without FBS to adjust the density to $5×10^5$ cells/mL. 200 μL of cell suspension ($1×10^5$ cells) were added to the upper chamber, and 700 μL of medium containing 20% FBS were added to the lower chamber. According to the different migration abilities of each cell line, cells were put back into the incubator and continued to be cultured. After that, the transwell chamber was clipped, washed 3 times with 1×PBS, and placed in methanol for 15 min for cell fixation. After the chamber was stained in 0.2% crystal violet for 20 min, the cells on the upper surface of the chamber were carefully wiped off with water and a cotton swab. The perforated cells stained in the outer layer of the basement membrane of the chamber were observed under microscope, and 5 fields of view were randomly selected.

QRT-PCR

After the cells were treated accordingly, 1 mL of TRIzol (Invitrogen, Carlsbad, CA, USA) was used to lyse the cells, and total RNA was extracted. The initially extracted RNA was treated with DNase I to remove genomic DNA and repurify the RNA. RNA reverse transcription was performed according to the Prime Script Reverse Transcription Kit (TaKaRa, Tokyo, Japan) instructions, and RT-PCR was performed according to the SYBR® Premix Ex TaqTM (TaKaRa, Tokyo, Japan) kit instructions. The PCR reaction was performed using the StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The Bio-Rad (Hercules, CA, USA) PCR instrument was used to analyze and process the data with the software iQ5 2.0. The glyceraldeyde 3-phosphate dehydrogenase (GAPDH) and U6 genes were used as internal parameters, and the gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method. The following primers were used for the qRT-PCR reaction: Circ_0000502 F: 5'-ACAAUAU-CAAAAGAGGAAAG-3'; R: 5'-AAAGGAAGUGGGUAGGAG-GAAAG-3'; GAPDH F: 5'-CGCTCTCTGCTCCTCCTGTTC-3'; R: 5'-ATCCGTTGACTCCGACCTTCAC-3'; miRNA-124 F: 5'-TGCGGTAAGGCACGCGGGAAT-3'; R: 5'-GGTACAAA-CAGGGAGGGA-3'; U6 F: 5'-ATGGCTATAAATAGATA-CACGC-3'; R: 5'-GGTACAAACAGGGAGGGA-3'

Dual luciferase reporter assay

A reporter plasmid was constructed in which a specific fragment of the 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 HepG2 cells or other related cell lines. By measuring the intensity of the fluorescence, the activity of the luciferase could be determined to figure out whether the transcription factor could interact with the target promoter fragment.

Statistics

Statistical analyses were performed using GraphPad Prism 5 V5.01 software (La Jolla, CA, USA). Statistical differences between the two groups were analyzed using Student's *t*-test. Comparison between groups was made using one-way ANOVA test followed by *Post Hoc* test (Least Significant Difference). Independent experiments were repeated at least three times for each analysis and data were expressed as mean±standard deviation (x±s). $P<0.05$ was considered statistically significant.
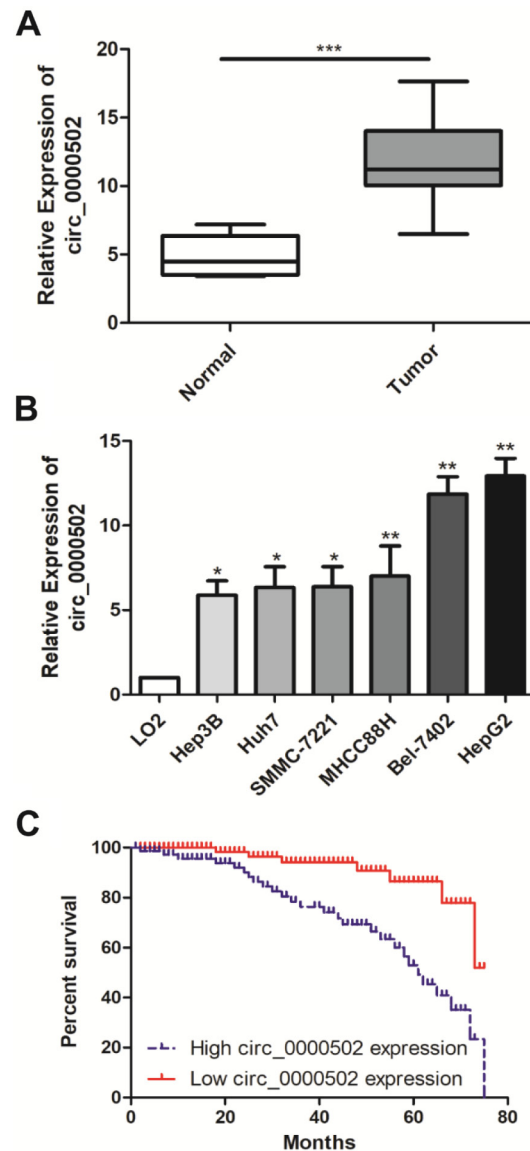

Figure 1. Circ_0000502 was highly expressed in hepatocellular carcinoma tissues and cell lines. **(A):** qRT-PCR was used to detect the difference in expression of circ_0000502 in hepatocellular carcinoma tumor tissues and adjacent tissues; **(B):** qRT-PCR was used to detect the expression level of circ_0000502 in hepatocellular carcinoma cell line. **(C):** Kaplan-Meier survival curve of hepatocellular carcinoma patients based on circ_0000502 expression revealed that the prognosis of patients with high expression was significantly worse than that of the low expression group. Data are shown as mean ± SD (*p<0.05, **p<0.01, ***p<0.001).

Results

Circ_0000502 was highly expressed in hepatocellular carcinoma

In order to determine the expression characteristics of circ_0000502 in HCC, qRT-PCR was used to detect the difference in expression of circ_0000502 in HCC tissues and adjacent tissues. The results showed that circ_0000502 was elevated in HCC tissues compared with adjacent tissues (Figure 1A). Circ_0000502 was significantly expressed in HCC cells compared to normal liver cell lines (LO2) (Figure 1B). To explore the relationship between the expression of circ_0000502 and the prognosis of patients with HCC, we collected relevant follow-up data. Kaplan-Meier survival curves revealed that high expression of circ_0000502 was significantly correlated with poor prognosis of HCC, and the higher the expression level of circ_0000502, the worse the prognosis (p<0.05; Figure 1C).

Knockdown of circ_0000502 inhibited cell proliferation, invasion, migration, and promoted cell apoptosis

To investigate the function of circ_0000502 in HCC, we constructed a circ_0000502 knockdown lentiviral vector. After transfecting the vector in Bel-7402 and HepG2 cell lines, the interference efficiency was verified by qRT-PCR (Figure 2A). After circ_0000502 was knocked down, CCK-8, transwell, and flow cytometry assays were used to analyze cell proliferative ability, invasiveness, migratory capacity and cell apoptosis. The results showed that
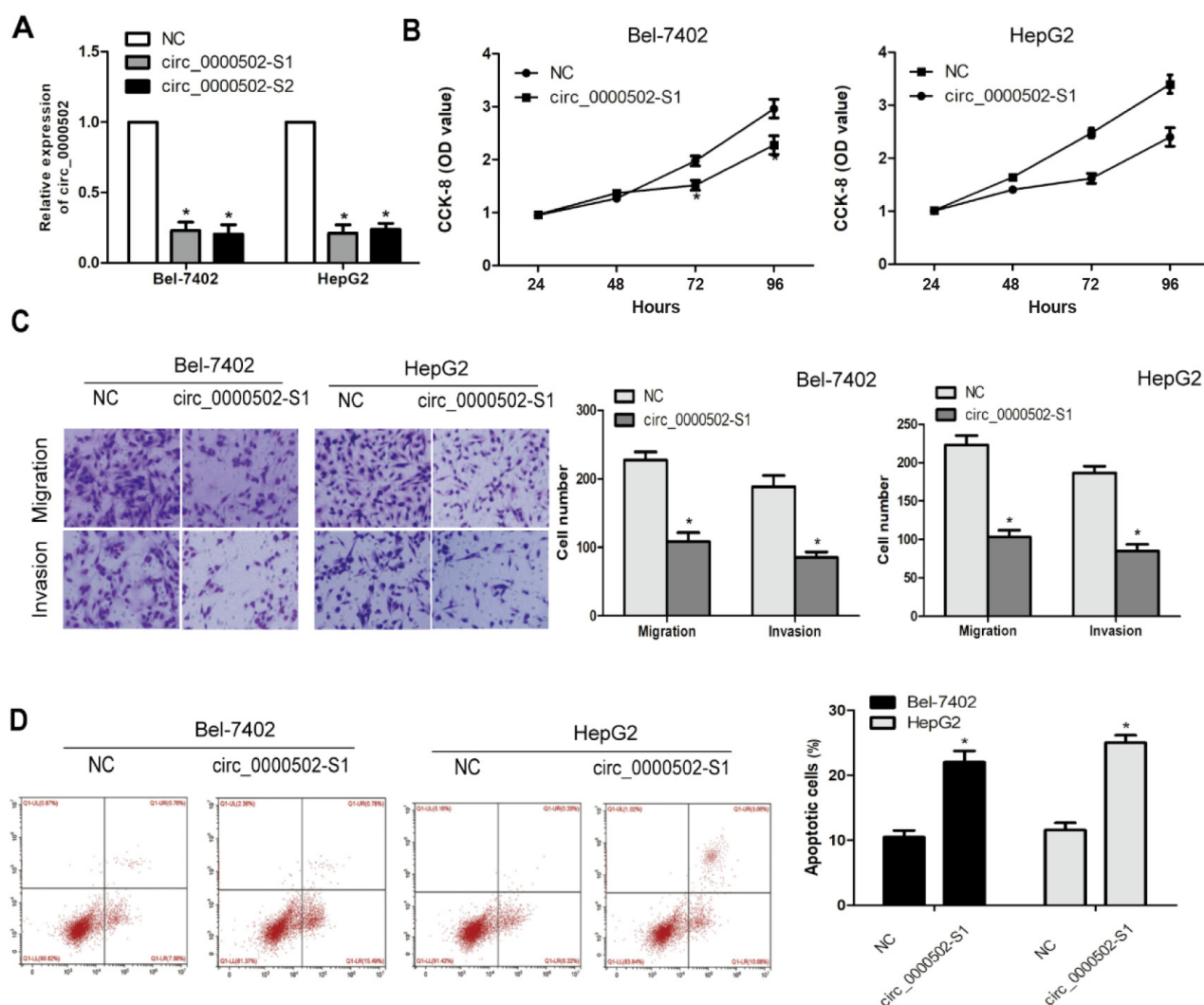

Figure 2. Silencing circ_0000502 inhibited the progression of hepatocellular carcinoma cells. **(A):** qRT-PCR verified the interference efficiency of si-circ_0000502 in Bel-7402 and HepG2 cell lines; **(B):** CCK-8 assay detected the effect of silencing circ_0000502 on cell proliferation of Bel-7402 and HepG2 cell lines; **(C):** Transwell assay detected the effect of silencing circ_0000502 on invasion and migration of hepatocellular carcinoma cells in Bel-7402 and HepG2 cell lines; **(D):** Flow cytometry assay detected the effect of silencing circ_0000502 on cell apoptosis of Bel-7402 and HepG2 cell lines. The data are shown as mean ± SD (*p<0.05).

compared with NC, the cell proliferative ability and invasiveness in the circ_0000502 silenced group was significantly reduced (Figure 2B, 2C), while cell apoptosis was notably increased (Figure 2D).

Circ_0000502 bound to miRNA-124

As shown in Figure 3A, luciferase reporter gene assay results demonstrated that circ_0000502 can be targeted by miR-124 *via* this binding site. In addition, qRT-PCR detected the level of miR-124 in 40 pairs of HCC tissues and its corresponding paracancer tissues, as well as in HCC lines, and the results showed that miR-124 level in both HCC tissues and cell lines was significantly decreased compared with paracancer tissues or normal he-

patic cell line (Figure 3B, 3C). Subsequently, it was found that circ_0000502 and miR-124 showed a negative correlation in the expression levels in HCC tissues (Figure 3D). In addition, it was found that after circ_0000502 was knocked down in HCC cell lines, the expression of miR-124 was significantly increased (Figure 3E).

Overexpression of miRNA-124 inhibited cell proliferation, invasion and migration, and promoted cell apoptosis

To investigate the function of miR-124 in HCC, we constructed an overexpressing miR-124 lentiviral vector. After transfection of the vector in HCC cell lines, the overexpression efficiency
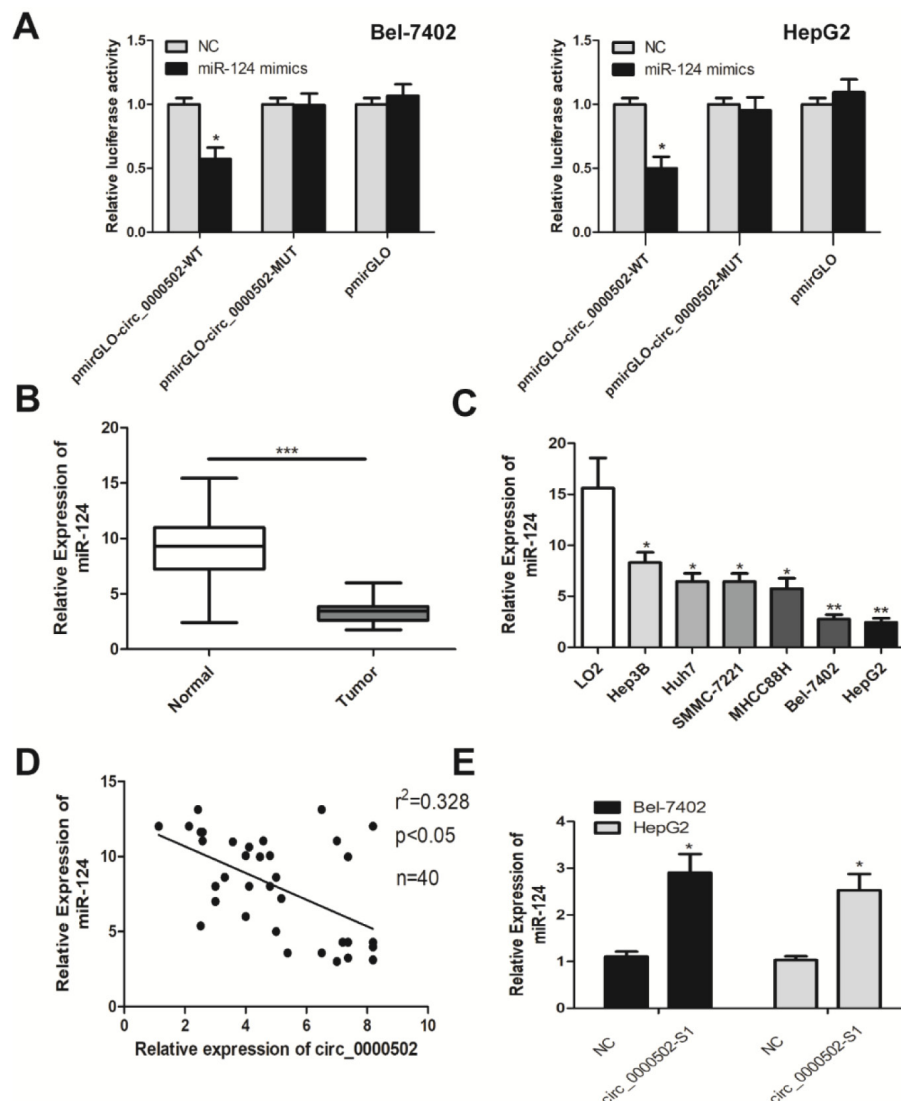

Figure 3. Circ_0000502 can directly target miR-124. **(A):** Dual luciferase reporter gene assay confirmed the direct targeting of circ_0000502 and miR-124; **(B):** QRT-PCR detected the differential expression of miR-124 in hepatocellular carcinoma and adjacent tissues; **(C):** QRT-PCR verified the mRNA expression level of miR-124 after transfection of circ_0000502 in hepatocellular carcinoma cell lines; **(D):** the expression level of circ_0000502 and miR-124 in hepatocellular carcinoma tissues was significantly negatively correlated; **(E):** Downregulation of circ_0000502 significantly enhanced the expression level of miR-124. Data are shown as mean ± SD (*p<0.05, **p<0.01, ***p<0.001).

was confirmed with qRT-PCR (Figure 4A). After overexpressing miR-124 in the above cells, CCK-8, transwell assay and flow cytometry were used to detect cell proliferative capacity, invasiveness, migration and cell apoptosis. The results indicated that compared with miR-NC, the former three cell functions in miR-124 overexpression group were significantly reduced (Figure 4B, 4C), while cell apoptosis was notably increased (Figure 4D).

Circ_0000502 modulated microRNA-124 expression in hepatocellular carcinoma

To further explore the ways in which circ_0000502 promotes the progression of HCC, we found a possible relationship between circ_0000502 and miR-124 through relevant bio-informatics analysis. In addition, to further explore the interaction between circ_0000502 and miR-124 in HCC cells, we silenced miR-124 in a HCC cell line with knockdown of circ_0000502, and the qRT-PCR assay was used to verify the the co-transfection efficiency (Figure 5A). Subsequently, through the transwell and flow cytometry experiments, we found that miR-124 could counteract the effect of circ_0000502 on invasiveness and migratory ability of HCC cells (Figure 5B and 5C).

Discussion

Due to the high frequency of metastasis and recurrence rate of HCC, the 5-year overall survival rate of patients with liver cancer is still poor.
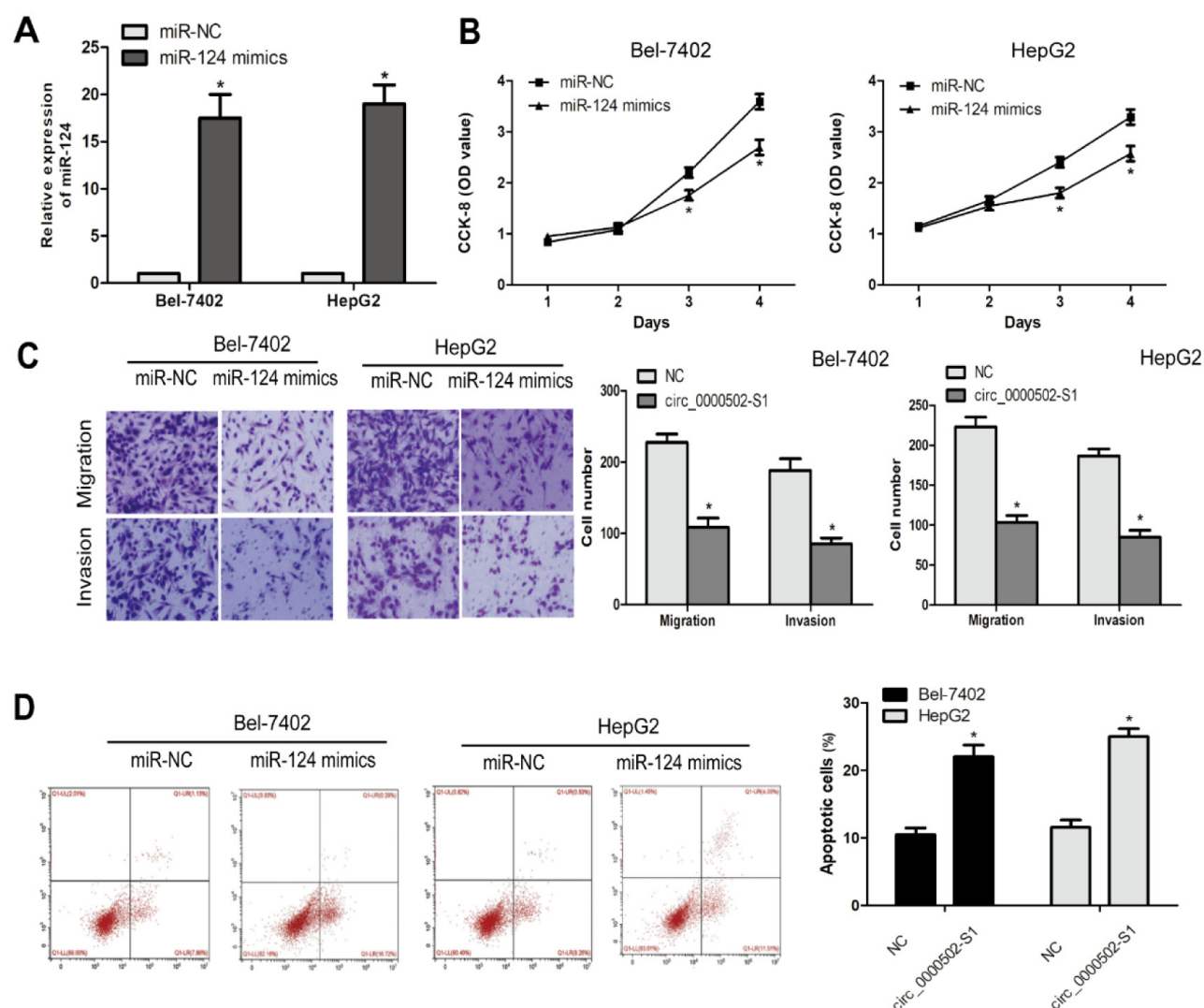

Figure 4. Overexpression of miR-124 inhibited malignant progression of hepatocellular carcinoma cells. **(A):** QRT-PCR verified the efficiency of miR-124 overexpression vector in Bel-7402 and HepG2 cell lines; **(B):** CCK-8 assay detected the effect of overexpressed miR-124 on cell proliferation of Bel-7402 and HepG2 cell lines; **(C):** Transwell assay detected the effect of overexpressed miR-124 on invasion and migration of hepatocellular carcinoma cells in Bel-7402 and HepG2 cell lines; **(D):** Flow cytometry detected the effect of overexpressed miR-124 on cell apoptosis of Bel-7402 and HepG2 cell lines. Data are shown as mean ± SD (*$p < 0.05$).

Therefore, early detection, diagnosis and treatment are particularly important [1-4]. Circular (circ) RNA has recently been shown to be involved in the regulation of mammalian gene expression. Its special cyclic structure allows it to exist very stably in organisms, it enters exosomes and has become a hot research molecule [14-16]. The circ_RNA has no 5' to 3' polarity and does not have a poly A tail structure, which is resistant to exonuclease digestion, so it is more stable than normal RNA, suggesting that circ_0000502 can be used as a marker for tumor diagnosis [17,21]. Functional studies have also shown that circ_RNA 0000502 plays a vital role in regulating hepatoma cell growth, migra-
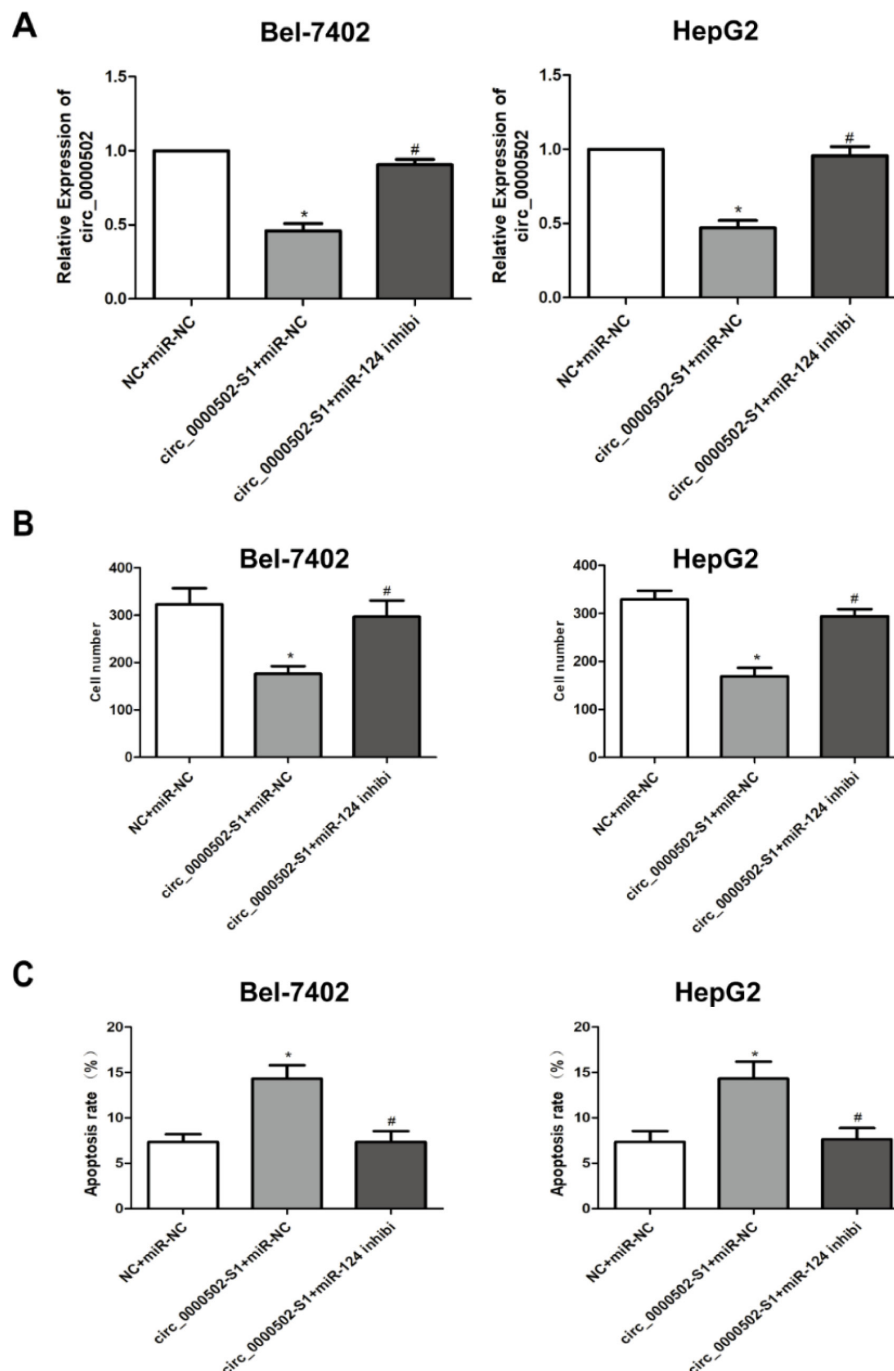

Figure 5. Circ_0000502 can regulate the expression of miR-124 in hepatocellular carcinoma tissues and cell lines. **(A):** Circ_0000502 expression level was examined by qRT-PCR in the HCC cell lines that were co-transfected with circ_0000502 and miR-124; **(B):** Transwell assay was used to detect the invasion and migration of hepatocellular carcinoma cells after co-transfection of circ_0000502 and miR-124; **(C):** Flow cytometry was performed to examine the cell apoptosis after co-transfection of circ_0000502 and miR-124 in liver cancer cell lines. Data are shown as mean ± SD (*p<0.05), compared with NC⁺ miR-NC; #p<0.05 compared with circ 0000502-S1+miR-NC.

tion and invasion. The circ_RNA is derived from the exon 2 of its parent gene and does not contain an intron structure. Therefore, based on previous experimental studies, we further explored whether it has the role of "miR sponge". Circ_0000502 is a newly discovered long-chain non-coding RNA [21]. Studies have shown that up-regulation of circ_0000502 can predict poor prognosis of osteosarcoma and promote malignant progression of osteosarcoma through spongy miR-1238 [21]. In the present study, the HCC knockdown cell line established by lentivirus was selected, and circ_0000502 was selected as a candidate for related CDs of malignant progression of HCC, and the relationship between circ_0000502 and the development of HCC was finally determined. Through tissue validation, we found that circ_0000502 level in HCC tissues was notably higher than that in adjacent tissues and was related to poor prognosis of this cancer and therefore, we believe that circ_0000502 might play a part in promoting HCC progression. In order to further study the molecular mechanism of circ_0000502 in the development and progression of liver cancer, experiments were performed in *in vitro* cells. Subsequently, CCK-8, transwell and flow cytometry experiments were carried out. It was found that knockdown of circ_0000502 could inhibit HCC cell proliferation, invasion and migration while promoting cell apoptosis.

The occurrence of tumors is likely to be regulated by competing endogenous RNAs (ce) [26]. After the "ceRNA hypothesis" was presented in 2011, researchers validated the hypothesis from different levels of bioinformatics, cell biology, and animal models [26,27]. ceRNA molecules, such as miRs, circular RNAs, pseudogenes, etc., are thought to be able to play a role in regulating their own expression levels by competing for miR receptor elements (MRE) to bind to the same miR [27]. Post-transcriptional regulation of genes has been complemented by the ceRNA hypothesis, providing new ideas for transcriptome research in the development of diseases such as tumors [28]. Cyclic RNA is a new member of the ceRNA family. Unlike linear RNA, circular RNA has no 5' end cap structure and 3' terminal polyadenylation (polyA) tail structure, but a covalent bond to form a closed loop structure that is connected end to end., naturally and widely present in eukaryotic cell lines of different races [29]. Recently, some circRNAs have been shown to contain multiple conserved miR binding sites that regulate gene expression, and the development and progression of tumors are regulated by these epigenetic changes [29]. Therefore, in-depth study of the important signaling pathways and the expression profiles of node molecules in the development of HCC will greatly promote the development of HCC diagnosis. The results of this experiment showed that miR-124 was less expressed in HCC tissues and could inhibit cell proliferation, invasion and migration while promoting apoptosis of HCC cells. We used bioinformatics to analyze the circ-0000502 sequence containing a binding site for miR-124, and verified the direct binding of circ_0000502 and downstream miR-124 through dual luciferase reporting assay, In addition, the deletion of the miR-124 binding site in circ_0000502 vector led to its failure to enrich miR-124, further validating the binding relationship between circ_0000502 and miR-124. Meanwhile, the expression levels of circ_0000502 and miR-124 were confirmed as negatively correlated in HCC cell lines. Additionally, we performed cell recovery experiment and found that miR-124 could counteract the role of circ_0000502 in HCC cell lines. In summary, the findings suggest that circ_0000502 can inhibit the expression of miR-124 and promote the proliferation of HCC cells.

Conclusions

Circ_0000502 was significantly associated with the prognosis of HCC. Also, circ_0000502 may promote the proliferation, invasion and migration of this cancer through regulating miR-124.

Acknowledgements

Conflict of interests

The authors declare no conflict of interests.

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