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Circ_0000515 drives the progression of hepatocellular carcinoma by regulating MAPK10

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Abstract. – OBJECTIVE: To explore the expression pattern and clinical significance of circ_0000515 in hepatocellular carcinoma (HCC), as well as the molecular mechanism.

PATIENTS AND METHODS: Fifty HCC patients were recruited, and their cancer tissues and adjacent normal ones were collected for detecting the differential expression of circ_0000515. The relationship between circ_0000515 and clinical parameters in HCC patients was analyzed. Circ_0000515 knockdown model was generated by lentivirus transfection in Hep3B and MHCC88H cells that were highly expressed with circ_0000515. Regulatory effects of circ_0000515 on phenotypes of Hep3B and MHCC88H cells were examined by Cell Counting Kit-8 (CCK-8) and transwell assay. Target gene of circ_0000515 was verified by Dual-Luciferase reporter assay, and its involvement in HCC progression was detected by rescue experiments. In vivo xenograft model was generated in nude mice aiming to elucidate the role of circ_0000515 in regulating HCC growth.

RESULTS: Circ_0000515 was highly expressed in HCC tissues and cell lines. High level of circ_0000515 predicted advanced stage, high incidence of lymphatic metastasis, and low disease-free survival and overall survival in HCC. Knockdown of circ_0000515 attenuated proliferative and migratory abilities in Hep3B and MHCC88H cells. MAPK10, as the target gene binding circ_0000515, was negatively regulated by circ_0000515. Rescue experiments and in vivo xenograft model both indicated that circ_0000515 aggravated the malignant progression of HCC by targeting MAPK10.

CONCLUSIONS: Circ_0000515 is upregulated in HCC tissues and cell lines. It can be used for predicting tumor staging, lymphatic metastasis, and prognosis in HCC. Circ_0000515 aggravates the malignant progression of HCC by downregulating MAPK10.

Key Words:
Circ_0000515, MAPK10, HCC, Malignant progression.

Introduction

Primary liver cancer is one of the most prevalent malignant tumors in the world. Its diagnostic rate and cancer mortality rank fifth and third, respectively[1,2]. In China, there are about 400,000 new cases of hepatocellular carcinoma (HCC), accounting for 50% of the global number[3-5]. According to the histological subtypes, primary liver cancer is divided into HCC, cholangiocarcinoma (ICC) and combined hepatocellular cholangiocarcinoma (cHCC-CC). HCC makes up the majority subtype of liver cancer[6]. The aggravation, metastasis and recurrence of HCC are complicated, involving multiple factors and genes[7,8]. It is generally believed that genetic and epigenetic alterations are responsible for the malignant transformation of cancer cells[8,9]. Very recently, noncoding RNAs have been well concerned because of their biological functions in regulating HCC development and clinical diagnosis[10,11].

CircRNAs differ from linear noncoding RNAs that lack 3' and 5' end[12,13]. They have a closed covalent structure[13,14]. CircRNAs are extensively expressed in eukaryotic cells, and highly conserved among species. Typically, circRNAs are specifically expressed and resistant to RNA degradation, which exert vital significances in disease diagnosis[14-16]. Through literature review, circ_0000515 is a cancer-associated circRNA[17,18]. This study aims to elucidate the clinical significance of circ_0000515 in the progression of HCC, and the molecular mechanism. The constructed circRNA/target gene/cell phenotype network provides a novel idea in the clinical diagnosis and treatment of HCC.

Patients and Methods

Patients and HCC Samples

A total of 50 primary liver cancer patients undergoing surgery in the Department of Hepato-

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biliary Surgery, the Second People's Hospital of Yunnan Province and were pathologically confirmed as HCC were recruited. Inclusion criteria: patients with no severe diseases in other organs, those undergoing no post-operative radiotherapy and those with normal liver function before operation. Exclusion criteria: patients with distant metastasis of tumors, those complicated with other malignancies, those with mental disease, those complicated with myocardial infarction, heart failure or other chronic diseases, those with abnormal liver function prior to operation, or those previously exposed to radioactive rays. Resected tumor tissues and paracancerous ones were labeled and preserved at -80°C for use. Their clinical parameters were collected for Chi-square test, and follow-up data were used for survival analysis by Kaplan-Meier method. This investigation was approved by the research Ethics Committee of the Second People's Hospital of Yunnan Province and complied with the Helsinki Declaration. Informed consent from patients or their families was obtained prior to sample collection.

Cell Lines and Reagents

Human HCC cell lines (Bel-7402, HepG2, MHCC88H, Huh7, Hep3B) and normal hepatocytes (HL-7702) were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). They were cultivated in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) at 37°C with 5% CO_2. In culture medium, 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin were added. Cell passage was conducted using 1×trypsin + EDTA (ethylenediaminetetraacetic acid) when cells were grown to 80-90% confluence.

Transfection

Cells were cultivated to adherence and 40-60% density in the 6-well plate. In each well, 1 ml of enhancer + 1 μL of polybrene + transfection lentivirus (MOI 10-30) was added. After 8-12 h cell culture, complete medium was replaced. At 24-48 h of transfection, fluorescence intensity of cells was observed using a fluorescence microscope. The ratio of GFP-positive cells reached more than 90% indicated the successful transfection.

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 2.5×10^3 cells/well. At day 1, 2, 3 and 4, absorbance value at 450 nm of each sample was recorded using the Cell Counting Kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Transwell Migration Assay

200 μL of serum-free suspension (1×10^5 cells) was applied on the top of transwell chamber. In the bottom, 700 μL of complete medium was applied. After 24 h of incubation, cells in the bottom were fixed in methanol for 15 min, dyed with crystal violet for 30 min, and counted using a microscope (Olympus, Tokyo, Japan).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cellular RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Extracted RNAs were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) using PrimeScript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The obtained cDNA underwent qRT-PCR using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was the internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. Circ_0000515: Forward: 5'-GGTCAGACTGGGCAGGAGAT-3', Reverse: 5'-GAGTGACAGGACGCACTCAG-3'. MAPK10: Forward: 5'-GAGAAACACCAACAGCAGGC-3', Reverse: 5'-TCGAATCCCTGACAAAAGGCA-3'. GAPDH: Forward: 5'-CCTGGCACCCAGCACAAT-3', Reverse: 5'-GCTGATCCACATCTGCTGGAA-3'.

Western Blot

Cells were lysed in radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) on ice for 15 min, followed by centrifugation at 14000×g, 4°C for 15 min. The concentration of cellular protein was determined by bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). Protein samples with the adjusted same concentration was separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and loaded on polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was cut into small pieces according to the molecular size and blocked in 5% skim milk for 2 h. They were incubated with primary and secondary antibodies, followed by band exposure and grey value analyses.

Dual-Luciferase Reporter Assay

HEK293T cells were inoculated in a 24-well plate. Binding sites in the 3'UTR of circ_0000515

and MAPK10, and the mutant sequences were inserted into pMIR vectors. They were co-transfected into HEK293T cells with pcDNA3.1-NC or pcDNA3.1-MAPK10, respectively. Luciferase activity was measured after 48 h of co-transfection.

Tumorigenicity Assay

This investigation was approved by the Animal Ethics Committee of the Second People's Hospital of Yunnan Province Animal Center. A total of 15 male nude mice with 8 weeks old were classified in three groups. They were subcutaneously administrated with Hep3B cells co-transfected with sh-NC+si-NC, sh-NC+si-MAPK10 or sh-circ_0000515+si-MAPK10, respectively. Tumor size was weekly recorded. Six weeks later, mice were sacrificed for harvesting HCC tissues, followed by measurement of tumor weight, and detection of circ_0000515 and MAPK10 levels in tumors. Tumor volume = (Tumor width^2 × tumor length)/2.

Statistical Analysis

Data were expressed as mean ± standard deviation, and processed by GraphPad Prism 5 V5.01 (La Jolla, CA, USA). Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ was considered as statistically significant.

Results

Expression Pattern and Clinical Significance of Circ_0000515 In HCC

Compared with paracancerous tissues, circ_0000515 was upregulated in HCC tissues (Figure 1A). Similarly, circ_0000515 was highly expressed in HCC cell lines (Figure 1B). Hep3B and MHCC88H cell lines expressed the highest level of circ_0000515 among the five detected HCC cell lines, which were selected for the following experiments.

Based on the mRNA level of circ_0000515 in HCC tissues we collected, recruited HCC patients were divided into high circ_0000515 expression group and low circ_0000515 expression group, re-

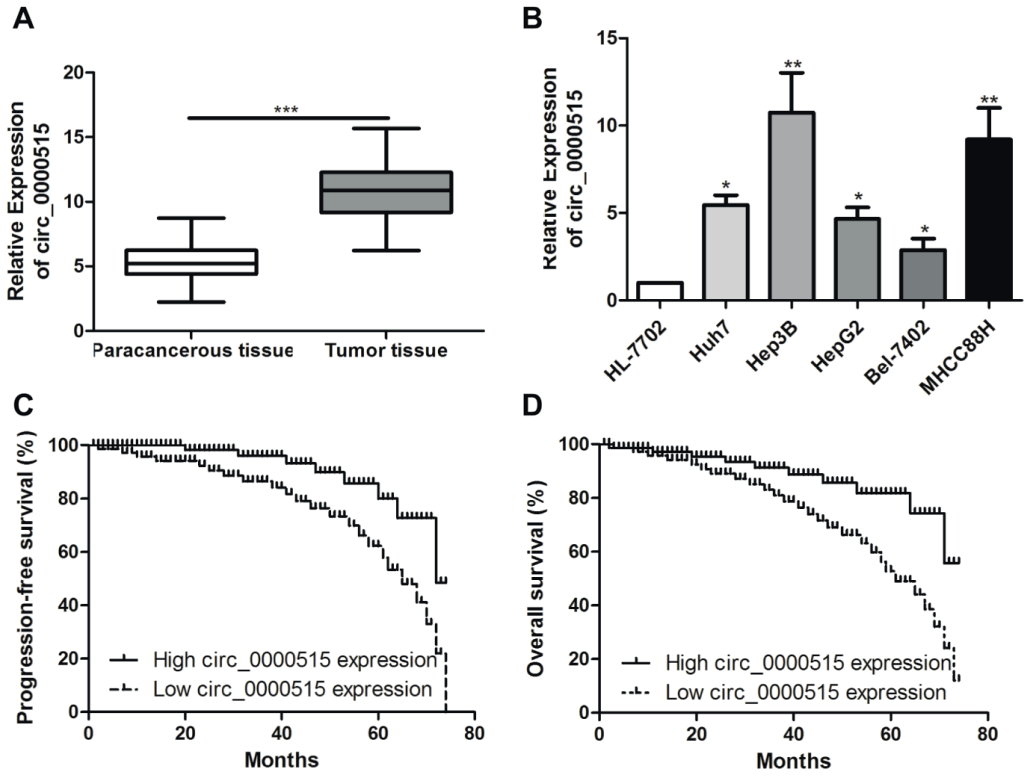

Figure 1. Expression pattern and clinical significance of circ_0000515 in HCC. **A,** Differential expressions of circ_0000515 in HCC tissues and paracancerous ones. **B,** circ_0000515 levels in HCC cell lines. **C,** Kaplan-Meier curves of progression-free survival in HCC patients based on circ_0000515 levels. **D,** Kaplan-Meier curves of overall survival in HCC patients based on circ_0000515 levels. Data were expressed as mean±SD; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

spectively. The correlation between circ_0000515 level and clinical parameters in HCC patients was analyzed by Chi-square test. It is found that circ_0000515 level was positively correlated to tumor staging and incidence of lymphatic metastasis in HCC patients (Table I). Kaplan-Meier method indicated that high level of circ_0000515 predicted poor progression-free survival and low overall survival in HCC (Figure 1C, 1D). The AUC value of 0.827 (95% CI=0.776-0.917) by the ROC curve was obtained, suggesting the diagnostic and prognostic value of circ_0000515 in patients with hepatocellular carcinoma.

Circ_0000515 Contributed to Cell Proliferation In HCC In Vitro

Transfection of sh-circ_0000515 markedly downregulated circ_0000515 in Hep3B and MHCC88H cells, indicating the effective transfection (Figure 2A). CCK-8 assay showed that knockdown of circ_0000515 reduced viability in HCC cells (Figure 2B). In addition, migratory ability was attenuated by knockdown of circ_0000515 as well (Figure 2C).

Circ_0000515 Was Bound to MAPK10 In Vitro

Bioinformatics website (miRBase, StarBase and TargetScan) was used to illustrate the potential cirRNAs binding circ_0000515, and the results found that MAPK10 was predicted as a potential target binding circ_0000515. Western blot analyses uncovered that protein level of MAPK10 was remarkably upregulated by the knockdown of circ_0000515 in Hep3B and MHCC88H cells (Figure 3A). Based on the predicted binding sites in the 3'UTR of circ_0000515 and MAPK10, Dual-Luciferase reporter assay was conducted. It is confirmed that circ_0000515 was bound to MAPK10 (Figure 3B). In HCC tissues, circ_0000515 was negatively correlated to MAPK10 (Figure 3C).

MAPK10 Reversed Proliferative and Migratory Changes Induced by Circ_0000515 In HCC Cells

We thereafter elucidated the co-regulation of circ_0000515 and MAPK10 on HCC cell phenotypes. Firstly, MAPK10 level was detected to be higher in HCC cells with co-silence of circ_0000515 and MAPK10 than those with MAPK10 knockdown (Figure 4A). The knockdown of MAPK10 largely enhanced proliferative and migratory abilities in Hep3B and MHCC88H cells. Notably, the enhanced abilities were partially abolished by co-silence of circ_0000515 (Figure 4B, 4C).

Circ_0000515 Stimulated In Vivo Growth of HCC by Negatively Regulating MAPK10

The above data have demonstrated the *in vitro* functions of circ_0000515 in aggravating the malignant phenotypes of HCC cells. Subsequently, an *in vivo* xenograft model was generated in nude mice by administrating transfected Hep3B cells. Tumor volume and tumor weight in xenografted HCC tissues were lower in nude mice with *in vivo*

Table I. Association of circ_0000515 expression with clinicopathologic characteristics of hepatocellular carcinoma.

Parameters	No. of cases	circ_0000515 expression		*p*-value
		Low (%)	High (%)	
Age (years)				0.741
<60	23	13	10	
≥60	27	14	13	
Gender				0.522
Male	22	13	9	
Female	28	14	14	
T stage				0.047
T1-T2	25	17	8	
T3-T4	25	10	15	
Lymph node metastasis				0.025
No	29	12	7	
Yes	21	5	16	
Distance metastasis				0.441
No	29	17	12	
Yes	21	10	11	

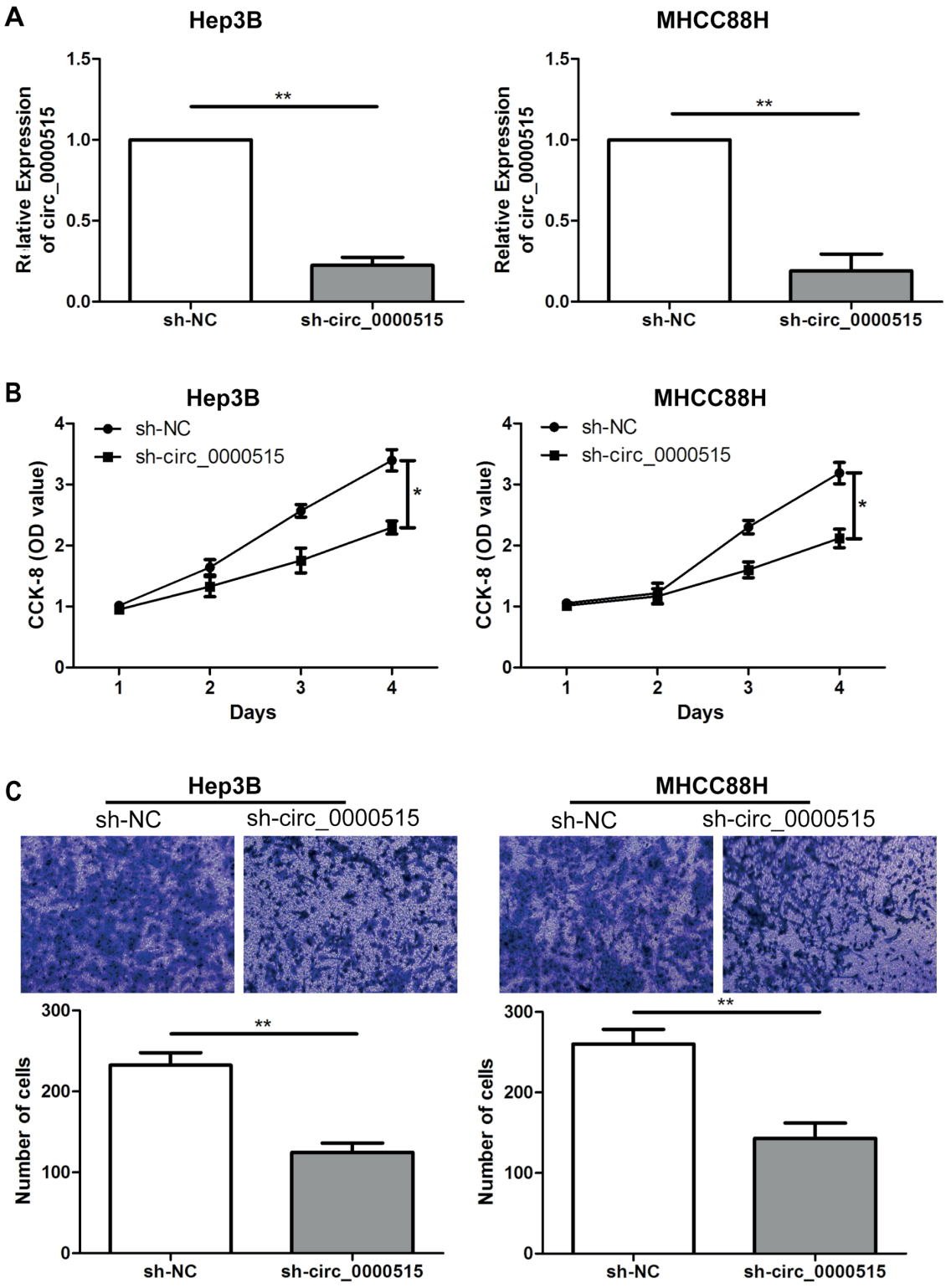

Figure 2. circ_0000515 contributed to cell proliferation in HCC *in vitro.* **A,** Transfection efficacy of sh-circ_0000515 in Hep3B and MHCC88H cells. **B,** Viability in Hep3B and MHCC88H cells with circ_0000515 knockdown. **C,** Migration in Hep3B and MHCC88H cells with circ_0000515 knockdown (magnification: 40×). Data were expressed as mean±SD; $*p < 0.05$, $**p < 0.01$.

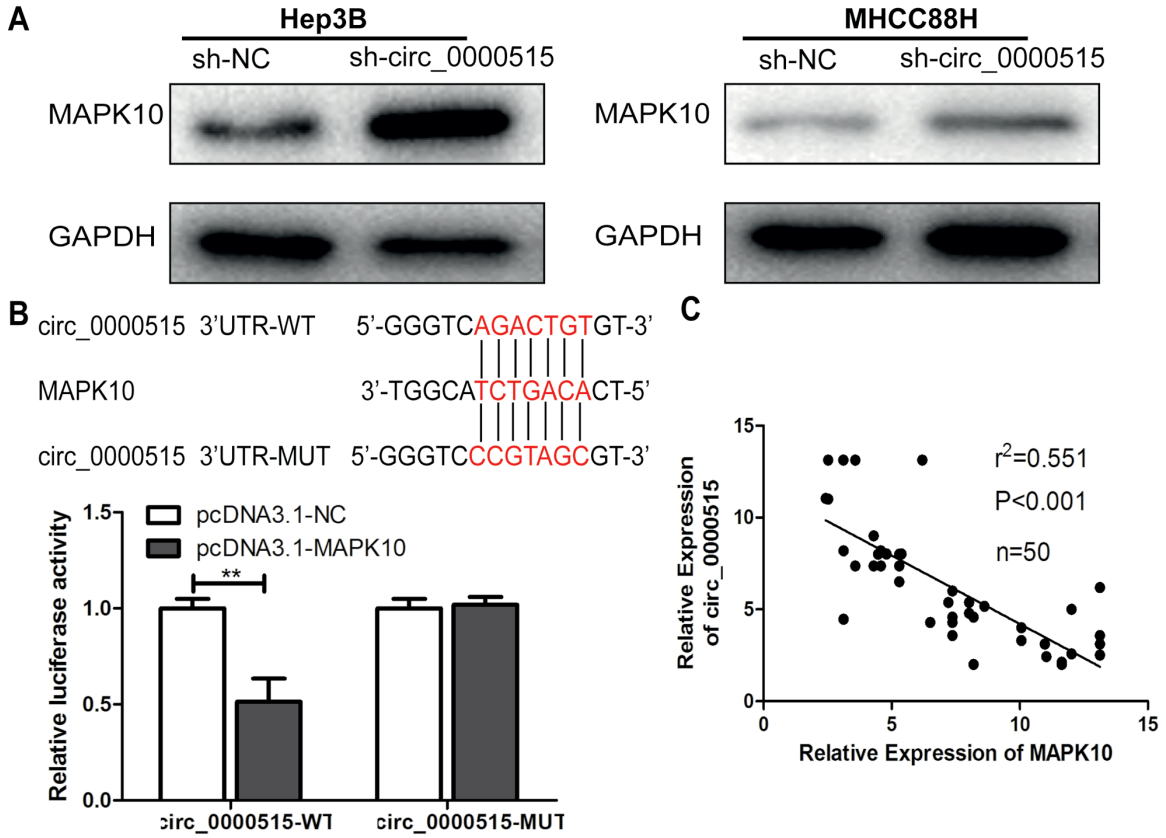

Figure 3. circ_0000515 was bound to MAPK10 *in vitro*. **A,** Protein level of MAPK10 in Hep3B and MHCC88H cells with circ_0000515 knockdown. **B,** Binding relationship between circ_0000515 and MAPK10. **C,** A negative correlation between circ_0000515 and MAPK10 in HCC tissues. Data were expressed as mean±SD; $^{**}p < 0.01$.

knockdown of circ_0000515 than those of controls, indicating that circ_0000515 drove the growth of HCC. However, mice with *in vivo* knockdown of both circ_0000515 and MAPK10 displayed a faster growth rate of HCC than those with *in vivo* knockdown of circ_0000515 (Figure 5A, 5B). HCC tissues were harvested from mice after sacrifice. As expected, circ_0000515 was lowly expressed in HCC tissues collected from mice with *in vivo* knockdown of circ_0000515, which was reversed by co-silence of MAPK10 (Figure 5C). Positive expression of MAPK10 was negatively regulated by circ_0000515 in xenografted HCC tissues as well (Figure 5D). It is concluded that circ_0000515 stimulated tumorigenicity of HCC in nude mice by negatively regulating MAPK10.

Discussion

Rapid progression, high incidences of recurrence and metastasis of HCC attribute to the unsatisfactory efficacy of clinical treatment[4-6]. Surgery is the preferred method for HCC. However, the resection rate of HCC is lower than 30% and the postoperative 5-year survival remains 30-40%[6-8]. For HCC patients who can be operated, postoperative recurrence and metastasis are the major factors influencing the prognosis. For HCC patients in the progressive stage who cannot be operated, effective therapies aiming to improving their survival are lacked[6,7]. Traditional chemotherapy and radiotherapy are less effective. At present, molecular targeted drugs for tumor treatment have been highlighted[7,8].

The vital functions of ncRNAs in regulating gene expressions and cellular behaviors have been gradually identified[10,11]. CircRNAs are a novel type of ncRNAs[12,13]. The gradual understanding of circRNAs not only enriches the regulatory network of competitive endogenous RNA (ceRNA), but also provides a new direction in clarifying the mechanisms of disease development and treatment[14-16]. Abnormally ex-

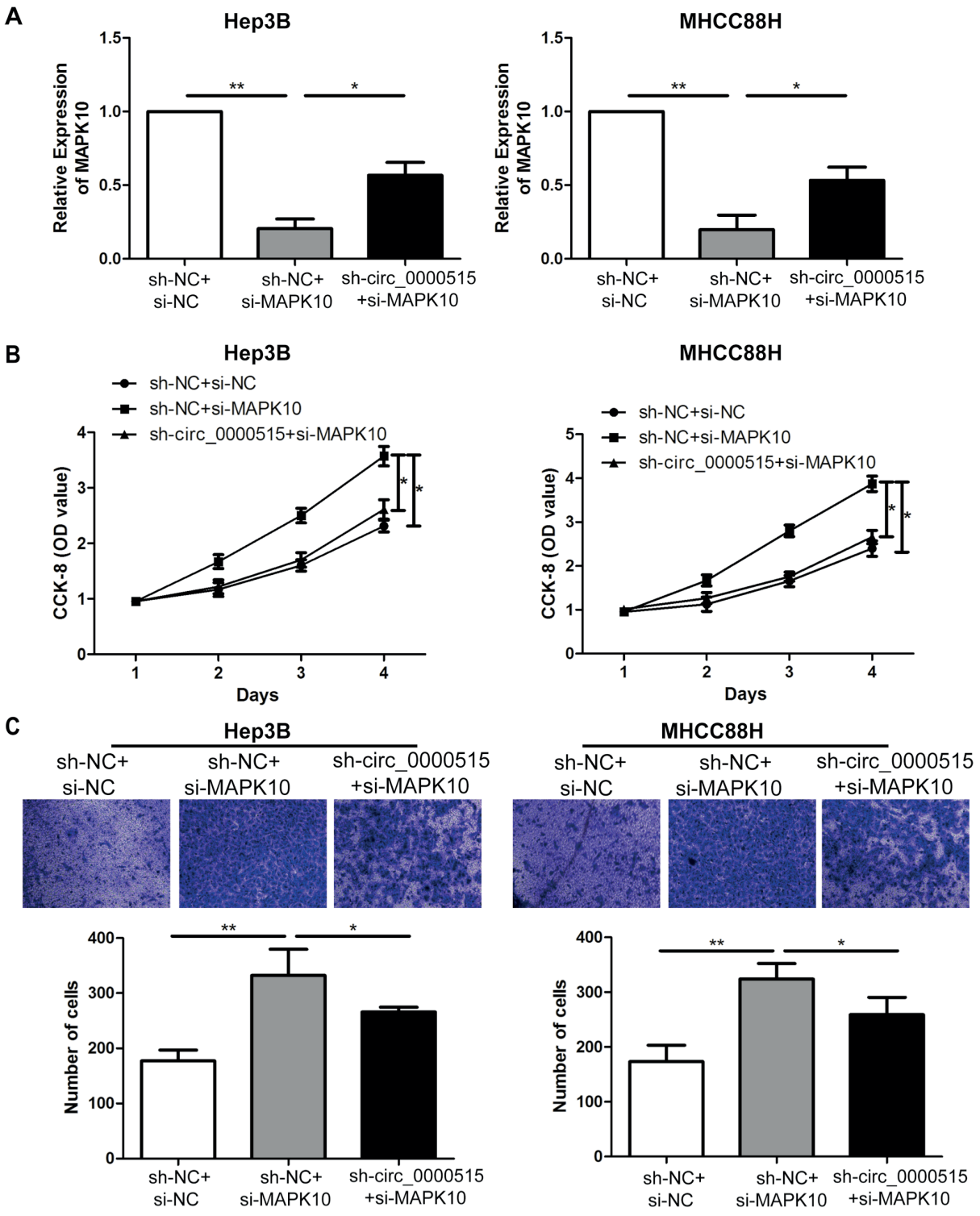

Figure 4. MAPK10 reversed proliferative and migratory changes induced by circ_0000515 in HCC cells. **A,** MAPK10 level in Hep3B and MHCC88H cells co-regulated by circ_0000515 and MAPK10. **B,** Viability in Hep3B and MHCC88H cells co-regulated by circ_0000515 and MAPK10. **C,** Migration in Hep3B and MHCC88H cells co-regulated by circ_0000515 and MAPK10 (magnification: 40×). Data were expressed as mean±SD; *$p < 0.05$, **$p < 0.01$.

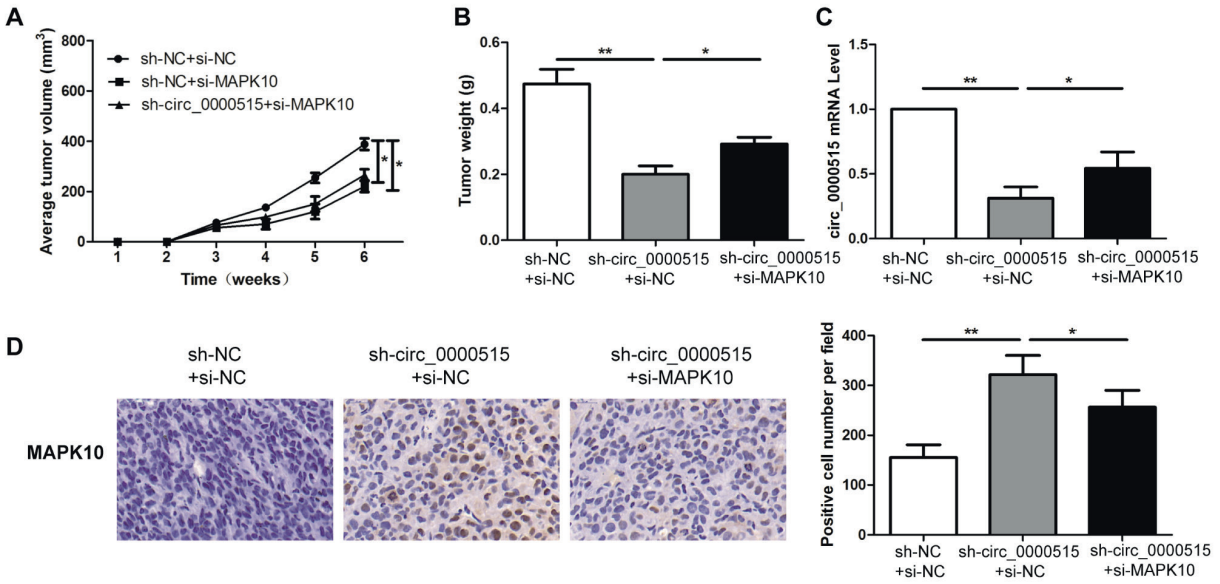

Figure 5. circ_0000515 stimulated *in vivo* growth of HCC by negatively regulating MAPK10. **A,** Average tumor volume in nude mice with xenografted HCC. **B,** Tumor weight in nude mice with xenografted HCC. **C,** circ_0000515 level in HCC tissues harvested from nude mice. **D,** Positive expression of MAPK10 in HCC tissues harvested from nude mice (magnification: 200×). Data were expressed as mean±SD; **p* < 0.05, ***p* < 0.01.

pressed circ_0000515 has been detected in many types of tumors, which has a relation to tumor progression[17,18]. Our findings uncovered that circ_0000515 was upregulated in HCC tissues and cell lines. By analyzing follow-up data of recruited HCC patients, it is shown that high level of circ_0000515 was correlated to advanced tumor staging, high incidence of lymphatic metastasis, and poor survival in HCC. Subsequently, Hep3B and MHCC88H cell lines expressing relatively high level of circ_0000515 were used for generating circ_0000515 knockdown models. CCK-8 and transwell assay indicated the promotive effect of circ_0000515 on proliferative and migratory abilities in HCC cells.

Using the online bioinformatic website, MAPK10 was predicted to be the target gene of circ_0000515, and their binding relationship was verified by Dual-Luciferase reporter assay. We thereafter identified a negative interaction between the expression levels of circ_0000515 and MAPK10 in HCC cells. Downregulated MAPK10 was responsible for the promotive effects of circ_0000515 on proliferative and migratory abilities in HCC. Thus, we have already detected the *in vitro* functions of circ_0000515 on HCC cell phenotypes. Its *in vivo* functions were then explored by generating xenograft model in nude mice. Through a series of animal procedures, it is suggested that circ_0000515 stimulated tumorigenicity of HCC in nude mice *via* downregulating MAPK10. To sum up, our study observed the expression pattern of circ_0000515 in HCC profiling, and its carcinogenic role during the progression of HCC. Through competitively binding MAPK10, circ_0000515 blocks the binding between MAPK10 and its target genes, thus driving proliferative and migratory abilities in HCC. Our findings demonstrate the molecular mechanism of circ_0000515 on regulating HCC cell phenotypes for the first time. In addition, we provide a potential therapeutic target for clinical treatment of HCC.

Conclusions

The above results demonstrated that circ_0000515 is upregulated in HCC tissues and cell lines. It can be used for predicting tumor staging, lymphatic metastasis and prognosis in HCC. Circ_0000515 aggravates the malignant progression of HCC by downregulating MAPK10.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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