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Circ_0091579 promotes proliferative ability and metastasis of liver cancer cells by regulating microRNA-490-3p

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Abstract. – **OBJECTIVE:** To explore the role of Circular RNA 0091579 in the progression of liver cancer (LCa) and its molecular mechanism.

PATIENTS AND METHODS: Quantitative polymerase chain reaction (qPCR) was used to detect circ_0091579 expression levels in LCa tissues and adjacent tissues, which was further verified in LCa cells and normal liver epithelial cells. After circ_0091579 was knocked down in [illegible] and HepG2 cells, cell counting kit-8 (CCK-8 [illegible] cloning and transwell assays were performed to verify the effect of circ_0091579 on cell proliferative ability and metastasis of LCa cells. The starBase database was used to search microRNAs that could interact with circ_0091579 and the Dual-Luciferase reporter [illegible] was used to verify their binding relationship.

RESULTS: circ_0091579 [illegible] highly expressed in HCC and HCC cells [illegible] *vitro* [illegible] showed that down-regulation of circ_0091579 expression could remarkably inhibit the proliferative ability and metastasis [illegible] HCC cells. Bioinformatics software predicted the binding sites between circ_0091579 and microRNA-490-3p, and dual-luciferase reporter gene assay confirmed the binding relationship between circ_0091579 and microRNA-490-3p. qPCR results showed that microRNA-490-3p was remarkably down-regulated in LCa tissues. *In vitro* experiments confirmed that overexpression of microRNA-490-3p inhibited the proliferative ability and metastasis of HCC cells.

CONCLUSIONS: circ_0091579 is abnormally highly expressed in LCa tissues and cells. Down-regulation of circ_0091579 can inhibit the proliferative ability and metastasis of HCC cells by regulating microRNA-490-3p, thus accelerating [illegible] of the tumor.

Key Words:

[illegible] Circular RNA 0091579, MicroRNA-490-3p, Cell proliferative ability and metastasis.

Introduction

Liver cancer (LCa) is one of the most malignant tumors worldwide[1]. Hepatitis virus infection is considered as the main reason for the high incidence [illegible] LCa, and advanced fibrosis in the [illegible] of hepatitis is a high-risk factor for the occurrence of LCa[2]. Studies have suggested that there are numerous mechanisms [illegible] occurrence of hepatocellular carcinoma. Among them, the dysfunction of TGF-β, VEGF, cox-2, PPAR, and other signaling pathways is considered as an important molecular mechanism for the occurrence of hepatocellular carcinoma induced by hepatitis[3]. Despite the continuous progress in the treatment of hepatocellular carcinoma in recent years, the overall treatment expectation of hepatocellular carcinoma is still at a poor level due to the high postoperative recurrence rate and metastasis rate[4]. In addition, many patients with hepatocellular carcinoma have lost the opportunity of surgery and other treatment when diagnosed, which has a huge impact on the quality of life and long-term prognosis[5]. Therefore, it is urgent to explore new therapeutic targets for diagnosis and treatment of LCa.

In recent years, non-coding RNA has been found to play an important role in a variety of tumors[6]. Circular RNA (circRNA) was a newly discovered non-coding RNA and was considered to play an important role in tumor progression[7]. Since its tail can form a closed loop structure by covalent bond, rather than the cap-like or poly(A) tail-like structure of common linear RNA, it is called the circular RNA[8]. circRNAs can be divided into four main categories, includ-

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ing exonic circRNAs, intronic circRNAs from introns, exon-intron circRNAs, and intergenic circRNAs[9]. In addition to tumors, circRNAs have been found to play an important role in a variety of diseases, including nervous system diseases and cardiovascular diseases[9]. Due to the special circular structure of circular RNA, it has high stability, high expression abundance and high conservatism compared with linear RNA. Therefore, circular RNA has certain advantages in serving as a new target for tumor prediction and tumor therapy. Similar to other non-coding RNAs, circular RNA can also play an important regulatory role in gene cleavage and protein transcription[10]. The involvement of circRNAs in tumor progression has been studied. For example, circVAPA in colorectal cancer can promote tumor progression by adsorbing microRNA-101[11]. Circ_0067934 was found to be abnormally highly expressed in pituitary tumor and can lead to poor prognosis of the tumor[12]. Circular RNA cTFRC can promote the proliferative ability and metastasis of bladder cancer cells by regulating the expression of downstream microRNA107[13].

As a newly discovered circRNA, [illegible] of circ_0091579 in LCa has not been [illegible]. Circ_0091579 is located at chrX:13279[illegible] 132888203, and the shear sequence length [illegible] 1145bp. The annotated gene of [illegible] 0091579 [illegible] the circBase database[14] ([illegible] circbase.org/cgi-bin/listsearch.cgi) [illegible] sapiens glypican 3, NCBI [illegible] NM_001164617). Fu et [illegible] have [illegible] high [illegible] of [illegible] LCa, and Zhang et al[16] have [illegible] found [illegible] has abnormal [illegible] in [illegible] is associated with [illegible] of LCa. [illegible], circ_0091579 [illegible] as a newly discovered [illegible], but the [illegible] mechanism of [illegible] in the [illegible] LCa has not been [illegible] in detail.

[illegible] the expression level of [illegible] LCa [illegible] and cell lines was [illegible] PCR. Circ_0091579 [illegible] circ_0091579 interfering [illegible] and [illegible] ability and metastasis [illegible] cells [illegible] determined by cell counting [illegible] (CCK-8), plate cloning and transwell [illegible] also revealed that microR[illegible]490-3p could act as a downstream [illegible] mechanism of circ_0091579 and was also [illegible] in the role of circ_0091579 in the [illegible] of LCa.

Patients and Methods

Clinical Sample Collection

This study was approved by [illegible] Ethics Committee of Nantong First People's [illegible]. All patients signed the experimental [illegible] consent form. All clinical samples [illegible] in the refrigerator at -80°C [illegible] ex vivo [illegible] ported to the laboratory [illegible] liquid [illegible]. [illegible] of 40 pairs of [illegible] tissues were included in the [illegible] samples were confirmed by [illegible] tissue [illegible] liver tissue.

Cell [illegible]

[illegible] cell lines (HepG2, Huh-7, Hep-3B and [illegible] and immortal normal [illegible] line (LO2) [illegible] from the [illegible] Academy of Sciences Cell Bank [illegible] (Shanghai, [illegible]). All LCa cells were [illegible] in [illegible] Memorial Institute-1640 ([illegible]) [illegible] (Invitrogen, Carlsbad, [illegible]), [illegible] the LO2 cell line was [illegible] Modified Eagle's Medium (DMEM) [illegible] Carlsbad, CA, USA). [illegible] media were supplemented with 10% fetal [illegible] (FBS) (Cat. 10082-147; Gibco, [illegible], NY, USA) and 1% penicillin/streptomycin (Cat. No. 15140-12; Invitrogen, Carlsbad, CA, USA). All cells were cultured in a 5% CO_2, 37°C cell culture incubator.

Cell Transfection

Small interfering RNAs (siRNAs) targeted for knockdown of circ_0091579, mimics for overexpression of the microRNA-490-3p gene, and their corresponding negative references were synthesized at Shanghai Genoma (Shanghai, China). Oligonucleotide fragments were transfected into LCa cells at a final concentration of 50 nM. The transfection procedure was performed according to the Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) product protocol and the transfection efficiency was determined by quantitative Real-time polymerase chain reaction (qRT-PCR).

Nuclear Separation

Cytoplasmic separation was performed using the PARISTM kit (Invitrogen, Carlsbad, CA, USA) and operated according to the product instructions. Briefly, cells were collected, added with the lysate, and then centrifuged to separate the nucleus and cytoplasmic components. The upper component was absorbed into the RNA-free tube as the cytoplasmic fraction. Next, the cell pellet was added with the nuclear lysate. The cytoplasmic fraction and nuclear lysate were then mixed with 2X Lysis/Binding solution and absolute ethanol. Samples were collected after filtration and washing. The RNA component in the sample was eluted with an eluent and then used for further measurement. U6 and 18S were used as positive references in the nucleus and cytoplasm, respectively, to calculate the relative amount of RNA in the nucleus and cytoplasm.

RNA Extraction and Quantitative PCR

Total RNA extraction was extracted from cell lines and clinical tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) kit according to the manufacturer's instructions. RNA reverse transcription was performed using the Prime Script RT Reagent Kit (TaKaRa, Dalian, China) kit. Quantitative PCR was performed using the SYBR Premix Ex Taq (TaKaRa, Dalian, China) kit and following the AB 7500 Real-time PCR system (Applied Biosystems, Fost[illegible] CA, USA). U6 was used as the microRNA-[illegible]3p internal reference, while glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the cyclic rna0091579 internal reference. The primer sequences are as follows: circ[illegible] forward: 5'-TGAGCCAGTGGTCAGT[illegible]AA-3[illegible] reverse: 5'-GTGGAGTCAGGCTT[illegible]GTAG-3[illegible] GAPDH forward: 5'-GTCA[illegible]GATT[illegible]TATT-3', Reverse: 5'-[illegible]GC[illegible]AAUUUGCGUGUCAU-3'; [illegible]RNA-[illegible] forward: 5'-CGTGGATC[illegible]CTTCAAC[illegible]GGTGGTG-3'; revers[illegible]AGAATTCA[illegible]GCAGGAAGAGTA[illegible]ACT[illegible]3'; U6 forward: 5'-GGAGC[illegible]AGATCCC[illegible]AAAT-3', reverse: 5'- GGCT[illegible]TGTCATACTT[illegible]ATGG-3'

CCK-8 Experiment

[illegible] cell proliferation ability was measured using [illegible] kit (Do[illegible] Laboratories, Kumamoto, Japan) and the procedure was carried out according to the manufacturer's instructions. Approximately 1 × [illegible] LCa cells were seeded in 96-well plates and 10 μL of CCK-8 reagent was added at [illegible]2 h, respectively. After 2 h of incubation, optical density (OD) value of each well was measured using the Varioskan Flash Spectrum R[illegible] (Thermo Fisher Scientific, Waltham, MA, USA) [illegible] 450 nm with 5 replicate wells.

Plate Cloning Experiment

24 h after transfection, LCa cells w[illegible] in 6-well plates and cultured for 2 weeks under standard conditions. The resulting colonies were fixed using 4% paraformaldehyde for 15 min, washed and stained with 0.1% crystal violet solution for 15 min at room temperature[illegible] cell colonies were photographed and analyzed.

Transwell Cell Migration Experiment

Cell migration ability [illegible] measured using transwell cell permeability [illegible] a polycarbonate (PC) membrane [illegible]Corning, Co[illegible]Y, USA) as the experimental chamber. 24 h after transfection, LCa cells were uniformly mixed in 200 μL of serum-free medium and added in the upper layer of the chamber, and 700 [illegible] of complete medium containing 20% serum was added to the lower compartment of the chamber to induce migration of the LCa [illegible] to the lower layer. After 48 [illegible] culture, the upper cells migrated through the polycarbonate membrane to the lower layer, and the [illegible]xed with 4% paraformaldehyde for 15 min, washed and stained with 0.1% crystal violet stain for 15 min at room temperature using [illegible] DM4000B microscope (Leica, Wetzlar, Germany). Five different fields of view were randomly selected and the migrated cells were counted and statistically analyzed.

Dual Luciferase Reporter Gene Experiment

Wild type and mutant circ_0091579 were cloned into the pGL3-control vector. 5 x 10^4 LCa cells were seeded in 24-well plates. The corresponding plasmids were co-transfected with the microRNA-490-3p mimics and the negative control for 48 h, respectively. Then, the luciferase activity was measured using a dual luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 7.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Experimental data were expressed as mean ± standard error of the data (mean ± SEM). Two-tailed *t*-test was used to analyze the difference between two groups, and circ_0091579 expression level and microRNA-490-3p correlation were analyzed using Pearson correlation. $p<0.05$ was considered statistically significant.

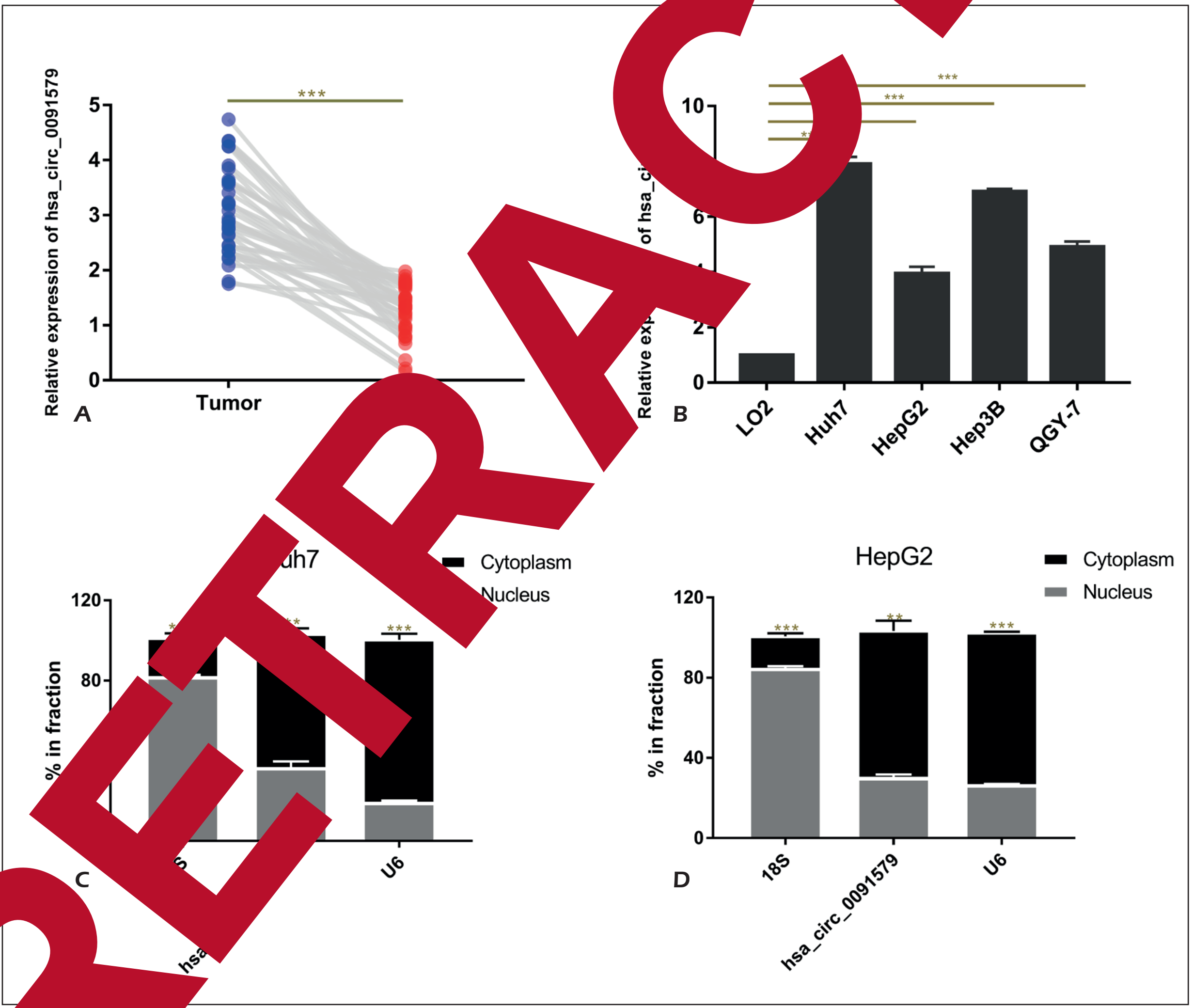
Results

Circ_0091579 Was Highly Expressed in LCa Tissues and Cells

A total of 40 pairs of LCa tissues and corresponding adjacent tissues were included in the study. We determined the expression of circ_0091579 by quantitative PCR and found that the expression level of circ_0091579 in LCa tissues was remarkably higher than that in normal liver tissues, and the difference was statistically significant (Figure 1A); Meanwhile, Circ_0091579 expression was measured in human hepatoma cell lines (HepG2, Huh-7, Hep-3B and QGY-77) and immortal normal liver cell line (LO2), and it was found that circ_0091579 also showed high expression in LCa cell lines (Figure 1B). We selected HepG2 and Huh-7 cells for subsequ[illegible] ments and used nuclear separation [illegible]niques [illegible] determine the expression of circ_0[illegible]579 in cells. Results showed that the main ex[illegible]on region of circ_0091579 was located in the cy[illegible] (Figure 1C, 1D). These results reve[illegible]d that c[illegible]1579 was highly expressed in [illegible] tissues and [illegible] was mainly located in th[illegible]ytoplas[illegible]

Knock-Down of [illegible]09[illegible]9 Inhibited Pro[illegible]ati[illegible]ty and Metasta[illegible] of LCa

To furthe[illegible]estigate the r[illegible]c_0091579 in the pr[illegible] of LCa, we [illegible]ecifically silenced [illegible]009[illegible]igure 2A) in HepG2 and



[illegible]ure 1. Circular RNA 0091579 is highly expressed in liver cancer tissues and liver cancer cells. **A**, Circular RNA 0091579 [illegible]ly expressed in liver cancer tissues. **B**, Circular RNA 0091579 is highly expressed in liver cancer cell lines. **C**, The nu[illegible]m separation experiment in Huh7 cells, Circular RNA 0091579, is mainly distributed in the cytoplasm. **D**, Nuclear RNA [illegible]ation assay in HepG2 cells, Circular RNA 0091579, is mainly distributed in the cytoplasm. **$p<0.01$; ***$p<0.001$

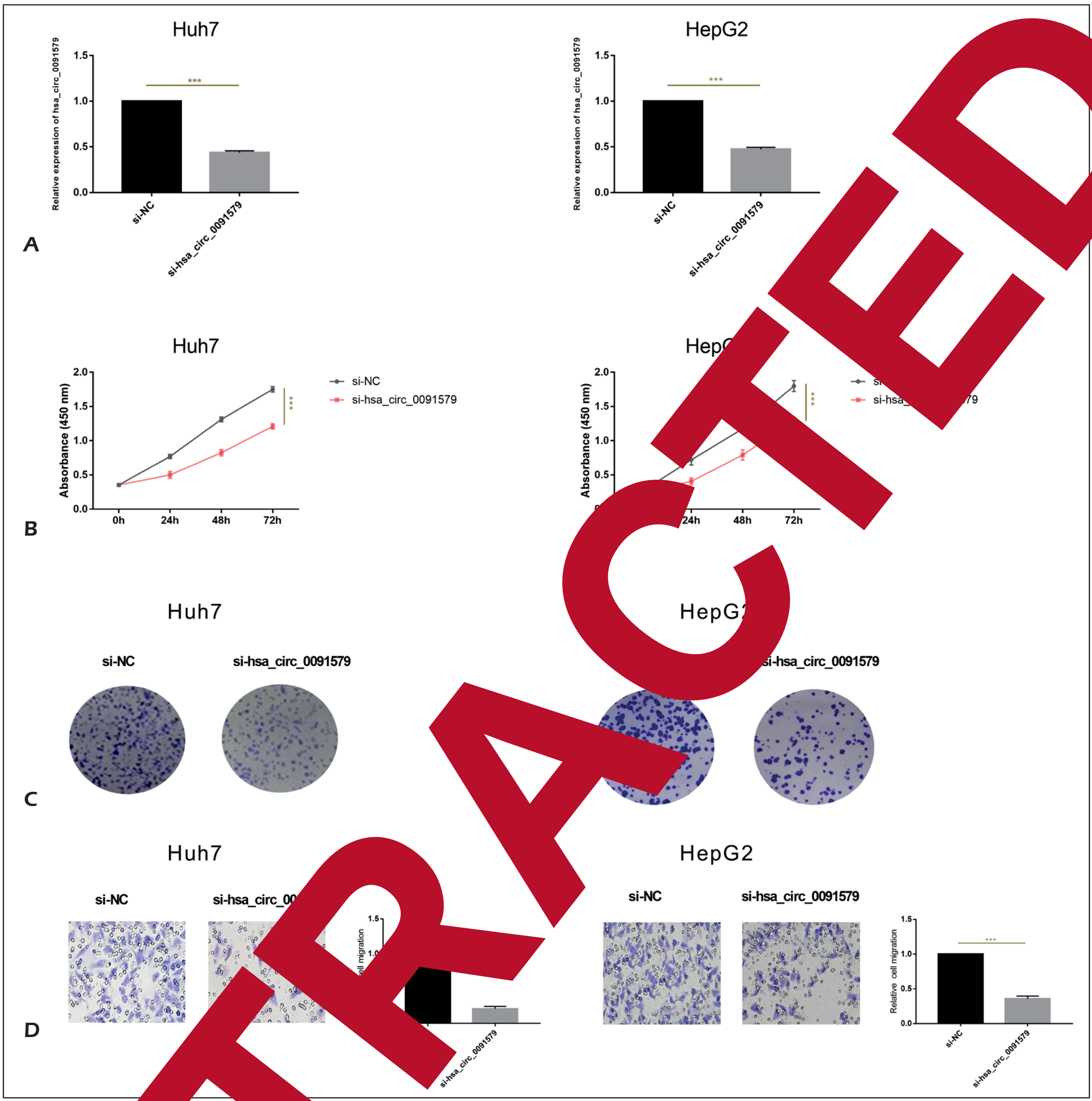


Figure 2. K[illegible]ckdown of Circ[illegible]NA 0091579 inhibits proliferative ability and metastasis of liver cancer cells. **A**, After transfectio[illegible]-Circular RNA 00[illegible] the Huh7 and HepG2 cell lines, Circular RNA 0091579 expression was down-regulated. **B**[illegible]he CCK-8 assay and c[illegible]colony formation assays examined changes in cell proliferation capacity following knockd[illegible] of Circ[illegible]RNA 0091579 in Huh7 and HepG2 cell lines. **D**, The transwell migration assay detects changes in cell [illegible]tion ca[illegible] after knockdown of Circular RNA 0091579 in Huh7 and HepG2 cell lines. (Magnification: 20X). **[illegible]

Hu[illegible]ells usi[illegible]-circ_0091579 transfection and[illegible]cted the cell viability in HepG2 and H[illegible]CK-8 and plate cloning experi[illegible]ts, respectively. After circ_0091579 knock[illegible]n, the proliferation ability of HepG2 and H[illegible]cells was remarkably decreased (Figure 2[illegible] 2C). We also used transwell assay to measure cell migration ability and found that after knocking down circ_0091579, the ability of LCa cells migration was also inhibited, and the number of migrated cells was reduced (Figure 2D), which suggested that knock-down of circ_0091579 could inhibit proliferative ability and metastasis of LCa cells.

Circ_0091579 Could Regulate microRNA-490-3p Expression

We predicted mi-RNAs that may bind to circ_0091579 through the starBase database (http://starbase.sysu.edu.cn) and found 19 possible mi-RNAs with binding relationships, including microRNA-490-3p (Figure 3A). Previous studies have found that microRNA-490-3p is remarkably down-regulated in HCC. Through bioinformatics prediction, we found that circ_0091579 can bind to the 3'UTR region of microRNA-490-3p (Figure 3B), which were further confirmed by dual-luciferase reporter gene assay (Figure 3C). Subsequently, we verified by quantitative PCR in LCa tissues and corresponding adjacent tissues that microRNA-490-3p had significant low expression in tumor tissues, and the difference was statistically significant (Figure 3D). Pearson test revealed a negative correlation between microRNA-490-3p and circ_0091579 in LCa tissues, R=-0.4525, $p<0.01$ (Figure 3E). These results indicated that microRNA-490-3p was regulated by circ_0091579 in hepatocarcinoma tissues and cells, which might be a vital target for circ_0091579 in LCa.

Overexpression of microRNA-490-3p Inhibited Proliferative Ability and Metastasis of Hepatoma Cells

In order to further confirm [illegible] of microRNA-490-3p in liver cancer cells, we used microRNA-490-3p mimics to specifically overexpress microRNA-490-3p in liver cancer cells HepG2 and Huh-7 (Figure 4A). Subsequently, cell proliferation was verified by CCK-8 and [illegible] cloning experiments, and it was found that after transfection with microRNA-490-3p mimics, the proliferation of HepG2 and Huh-7 cells was significantly reduced (Figure 4B, 4C). Subsequently, transwell was used to determine the migration ability of cells, and it was also found that after transfection with microRNA-490-3p mimics the migration ability of HepG2 and Huh-7 cells was significantly reduced (Figure 4D). These results suggested that overexpression of microRNA-490-3p inhibited proliferative ability and metastasis of hepatoma cells

Discussion

Currently, the diagnosis and treatment of LCa are faced with many challenges and the clinical prognosis is often poor since most LCa patients lost the opportunity of treatment at the time of diagnosis[17]. With the development of large-scale high-throughput sequencing technology, circRNAs may become an important target for most tumor researchers. Numerous studies have shown that circRNAs play a key role in the progression of LCa. For example, down-regulation of circ_DYNC1H1 can [illegible] the proliferative ability and metastasis of LCa cells, suggesting that circ_DYNC1H1 plays a pro-cancer role in LCa[18]. Circ_SETD2 can inhibit the growth of LCa by absorbing microRNA-421[19]. Similarly, circRNAs can also be used to diagnose and predict tumor progression. For example, circRNA SMARCA5 was found to be applicable [illegible] diagnosis and detection of LCa progression. Previous studies have shown that circ_0091579 is abnormally highly expressed in LCa and may be associated with poor prognosis of LCa. We found through a series of experiments that circ_0091579 expression was remarkably upregulated in LCa tissues and LCa cells, and could promote the proliferative ability and metastasis of LCa cells. The gene of circ_0091579 is located in the coding gene GPC3, which is considered [illegible] important oncogene in the malignant progression of liver cancer. GPC3 belongs to the proteoglycan family and is attached to the cell surface *via* glycosylphosphatidylinositol anchors, which can regulate the signaling activity of several growth factors, including Wnt. GPC3 is expressed in most hepatocellular carcinomas (HCC), but not in normal and cirrhotic or benign liver injury. Filmus et al[21] indicated that GPC3 promotes the progression of liver cancer through activating Wnt signal pathway. Wu et al[22] suggest that GPC3 and AFP can also be used as important indicators for the diagnosis and detection of LCa progression. These studies suggest that circ_0091579 plays an important role in the progression of LCa. Our results showed that circ_0091579 was up-regulated in LCa tissues and cells and can promote the proliferative ability and metastasis of LCa cells.

Although microRNAs themselves do not encode proteins, many authors have shown that microRNAs play a particularly pivotal role in the occurrence and progression of LCa[23]. CircRNAs regulating microRNAs expression are considered to be one of the main mechanisms by which circRNAs play a role in tumor progression[24]. MicroRNA-490-3p has been found to play a key role in prostate cancer, esophageal cancer, and ovarian cancer[25-28].

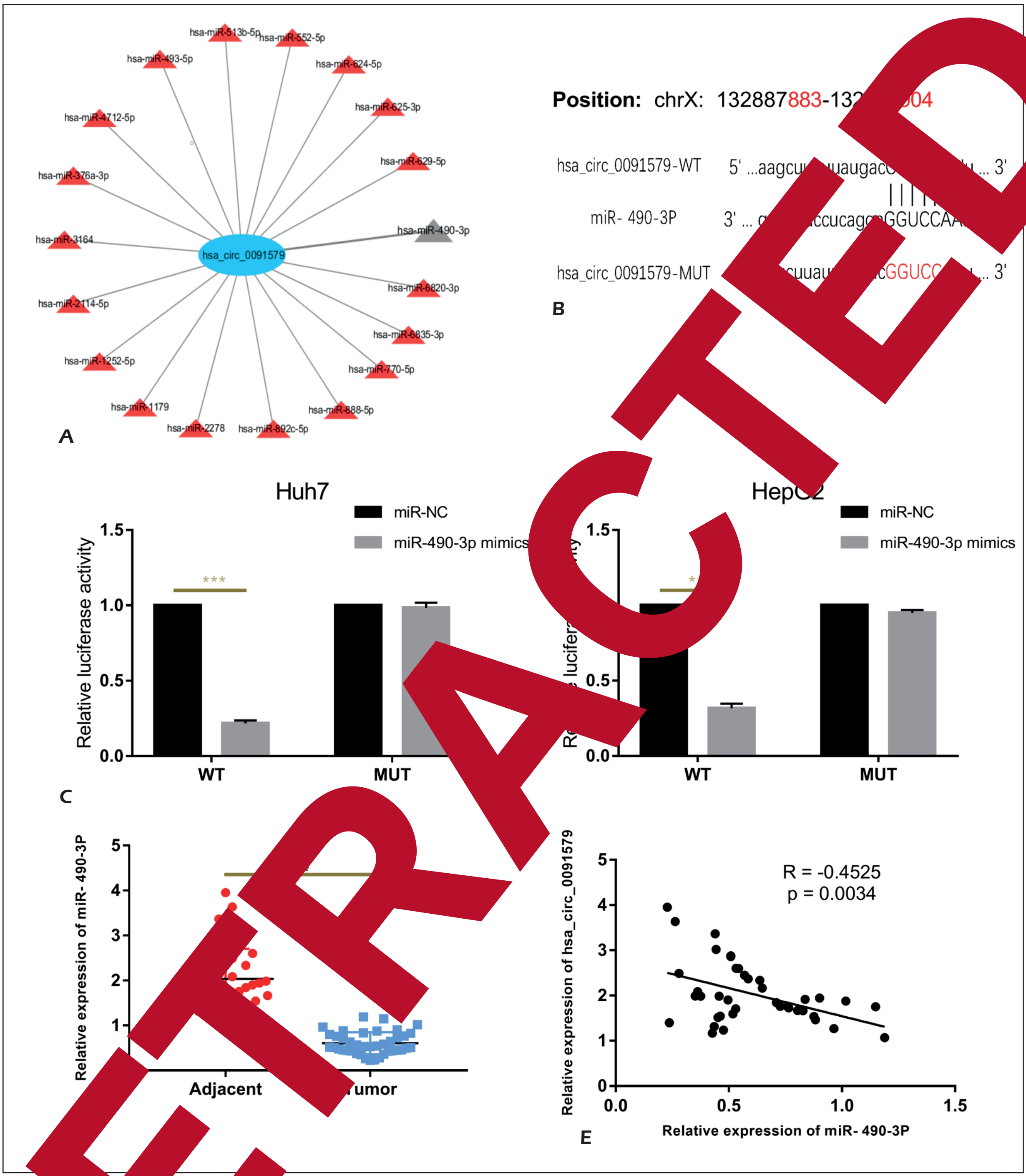


Figure [illegible] r RNA [illegible] 79 is capable of binding to miR-490-3p. **A**, The starbase database predicts the possible binding [illegible] -0003 [illegible] ni-R [illegible] **B**, Prediction of binding sites of miR-490-3p and circ-0003998 by sequence alignment. **C**, The [illegible] ferase [illegible] he assay confirmed that miR-490-3p binds to circ-0003998 in the hepatoma cell lines Huh7 and Hep [illegible], Quantit [illegible] PCR for the expression of miR-490-3p in hepatocellular carcinoma and paracancerous tissues. E, The [illegible] ssion of Circular RNA 0091579 in liver cancer tissues was significantly negatively correlated with the expression of mi [illegible] .001.

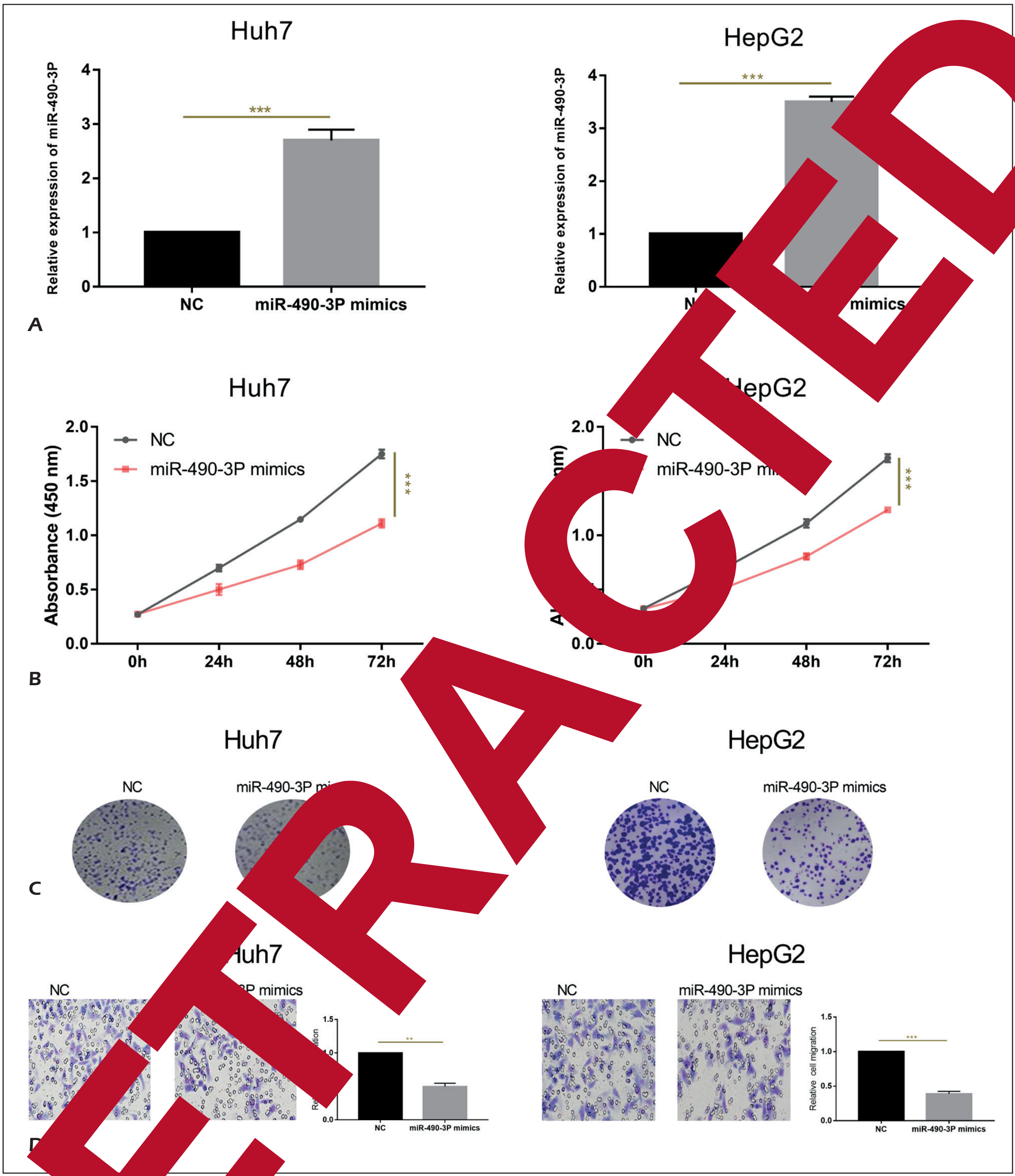


Figure 4. [illegible] xpressi[illegible] miR-490-3p inhibits proliferative ability and metastasis of liver cancer cells. **A**, After trans- [illegible] of mi[illegible] s in the Huh7 and HepG2 cell lines, miR-490-3p expression was up-regulated. **B-C**, The CCK-8 [illegible] cell clo[illegible] ion experiments showed that interference with miR-490-3p in Huh7 and HepG2 cells inhibited cell proli[illegible] on CCK-8 [illegible] y (**B**), cell clone formation assay (**C**). **D**, After miR-490-3p was disrupted in the Huh7 and HepG2 cell lines [illegible] migration ability was reduced. (Magnification: 20X). *$p<0.05$; **$p<0.01$

Li et al[29] showed the low expression of microRNA-490-3p in 749 LCa samples by bioinformatics method, which may serve as the basis for the diagnosis of LCa, but the corresponding functional test was not completed. We confirmed by database prediction and quantitative PCR that miR-490-3p was an important molecular target for circ_0091579 to play a carcinogenic role in liver cancer. At the same time, after correlation analysis of the two gene expression in LCa tissue, it was detected that there was a negative correlation between the expressions of circ_0091579 and microRNA - 490-3 p, which suggested that microRNA-490-3p may play a key role in the circ_0091579 induced LCa progress. Subsequently, after overexpression of microRNA-490-3p in LCa cells, we found that the proliferative ability and metastasis ability of LCa cells was remarkably inhibited, suggesting that microRNA-490-3p played a pivotal protective role in the process of LCa.

Conclusions

The above findings indicated by quantitativ[illegible] that circ_0091579 was abnormally highly e[illegible] in LCa tissues and cells, and verified by [illegible]8, plate cloning and transwell assay that circ_00[illegible] can promote the proliferative ability and metast[illegible] of LCa cells. Then, we found that th[illegible] expression [illegible] microRNA-490-3p was remarka[illegible] regulated in LCa, and circ_0091579 cou[illegible] gulat[illegible] expression of microRNA-490-3p. [illegible] er cell [illegible] riments confirmed that microRN[illegible] 3p co[illegible] proliferative ability a[illegible] me[illegible] LCa cells. These results sugges[illegible] hat circ[illegible] 79 can promote the progressi[illegible] f LCa by reg[illegible] the expression of micr[illegible] 0-3p.

Confli[illegible] Interests

The a[illegible]s declar[illegible] conflict of interest.

[illegible]nding [illegible]owle[illegible]ments

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