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# The role of hsa_circ_0000285 in metastasis of hepatocellular carcinoma

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**Abstract. – OBJECTIVE: The importance of circular RNAs in malignant tumors causes more attention in researchers. Hepatocellular carcinoma (HCC) is one of the most ordinary malignant tumors. Hsa_circ_0000285 was explored to identify how it functions in the metastasis of HCC.**

**PATIENTS AND METHODS: Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was utilized to detect hsa_circ_0000285 expression in HCC patients' tissues. Hsa_circ_0000285 lentivirus and shRNA was constructed for the transfection of HCC cells. Wound healing assay, transwell assay, and Matrigel assay were conducted to identify the function of hsa_circ_0000285 in HCC cells. Furthermore, mechanism assays were performed to uncover the interaction between hsa_circ_0000285 and miR-599.**

**RESULTS: Hsa_circ_0000285 was signifi ly higher-expressed in HCC samples com to that in adjacent samples. The migrated le th of HCC cells was reduced after hsa_circ_000 was silenced, while the migrated l h of H cells was increased after hsa_ci 285 w overexpressed. Moreover, the nber migrat ed and invaded HCC cells was circ_0000285 was silence whil numb of migrated and invaded l cells w creased after hsa_circ_000 s over ressed. Moreover, RT-qPCR result vealed that miR-599 was downr ated via expression of hsa_circ_000 5, while miR-5 was upregulated via kn ow hsa_circ_0000285. Further experime owed hat miR-599 was a direct t of circ_0000285 in HCC.**

**CO ONS: circ_0000285 could enha cell stasis of HCC by targeting miR-599 potential therapeutic target in HC**

*Key Words:*
Circular RNA, Hsa_circ_0000285, Hepatocellular carcinoma, MiR-193a-3p.

## Introduction

Liver cancer is the 2nd cau er-related deaths worldwide. The her cellula cinoma (HCC) is one of the pri liver ca ers, accounting for almost 9 % of imar ver cancers[1]. HCC cells ha strong in nd metastasis ability, whi as s usly a cted people's health and qu lity . The fore, it is of great significanc r the al agnosis and treatment of o study pathogenesis of HCC and fi effec therapeutic targets.

th the rap velopment of bioinformatics transcriptome equencing technology, scientists have realized that this highly stable and conserved cyclic NA in mammalian cells may have v ant functions. As a kind of non-coding RNA widely existing in mammalian cells, ular RNAs (circRNAs) are considered to have important regulatory functions in gene expression and other life activities because of its high expression and stability in the cytoplasm and evolutionary conservatism among species[3]. Currently, the research of circRNAs in tumors mainly focuses on digestive system tumors. Compared with normal tissues, the expression of circRNA-CDR1as in HCC increased which was negatively correlated with the expression of anti-cancer microRNA-7. Further experiments *in vitro* showed that circRNA-CDR1as could promote the proliferation and invasion of HCC by binding with microRNA-7[4]. The expression of CircRNA-SETD3 in HCC and cell lines decreased significantly. Functional studies showed that circRNA-SETD3 could play a regulatory role by targeting microRNA-421. Survival analysis showed that the low expression level of circRNA-SETD3 was associated with poor prognosis and larger tumor volume, suggesting that circRNA-SETD3 might be a predictor of

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HCC[5]. Circ_0000885 is a novel circRNA which has been recently reported to be significantly overexpressed in patients with osteosarcoma[6]. To research the role of hsa_circ_0000285 in HCC, the expression was detected, and related protein and mechanism were studied.

## Patients and Methods

### Tissue Samples

A total of 54 cancer tissues and paired adjacent tissues were obtained from HCC patients who underwent surgery at the Xuzhou Central Hospital. No radiotherapy or chemotherapy was performed before the surgery. All fresh tissues were stored immediately at –80°C. This research was approved by the Ethics Committee of Xuzhou Central Hospital. Signed written informed consents were obtained from all participants before the study.

### Cell Culture

Human HCC cell lines (Bel-7402 and HepG2) and a normal liver epithelial cell line (L02) purchased from American Type Culture tion (ATCC; Manassas, VA, USA). Cells cultured with Dulbecco's Modified Eagle's dium (DMEM; Gibco, Rockville, MD, USA) a 10% fetal bovine serum (FBS; Life T nologies, Gaithersburg, MD, USA) in an in ntaining 5% $CO_2$ at 37°C.

### Cell Transfection

After HCC cells were ultu 4 h on 6-well plates, cells wer nsfected a_ circ_0000285 lentivirus mpty vector u Lipofectamine 3000. A lls were cultured for 24 h on 6-well s, e transfected with cDNA oligonucleotides s y targeting hsa_circ_0 285 (shRNA; ePharma; Shanghai, Chi r negative control using Lipofectamine 30 Invitr n, Carlsbad, CA, USA). Those GFP ve were sen for following experime

### RNA ion an Time-qua Polymerase Chain Re on (

l R acted from cultured CC cel es *via* TRIzol reagent (Invitrogen, Carlsba SA) and then reverse-transcribed to complem eoxyribose nucleic acids (cDNAs) through th reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd China). Following are the primers for CR: circRNA_0000285 prim forward T-GTTGGTGGATCCTGT GCA-3′, se 5′-TGGTGGGTAGACC GTTGT -3′; Glyceraldehyde 3-phosphat dehydrogenase (GAPDH) primer forward AAC-CAGATGGGGCA GCT -3′ and reverse 5′-TGATGGCA GACT GGGCATCCA-3′. The thermal cy lows: sec at 95°C, 5 sec for 40 cycles 35 s 60°C.

### Wound ling Assay

After tion, HCC cells were seeded in 6-well es bated in a DMEM medium overnight. Then, e scratched with a plastic and cultured i um-free DMEM. Each a was repeated in triplicate independently. relate distance was viewed under a light microscope (Olymp Tokyo, Japan) at 48 h.

### y and Matrigel Ass

After transfection, $1\times10^5$ cells in 200 µL serum-free DMEM were replanted in the top chamber (Corning, NY, USA) with or without Matrigel (BD Biosciences, Bedford, MA, USA). DMEM and FBS were added to the lower chamber. Then, they were cultured overnight in an incubator supplemented with 5% $CO_2$ at °C. The top surface of chambers was treated with methanol for 30 min after wiped by a cotton swab. Next, they were stained in crystal violet for 20 min. Five fields were randomly chosen under a Leica DMI4000B microscope (Leica Microsystems, Heidelberg, Germany).

### Luciferase Assays

Luciferase reporter gene assay kits (Promega, Madison, WI, USA) were used to detect the luciferase activity of HCC cells. Briefly, the 3′-untranslated region (3′-UTR) of hsa_circ_0000285 cloned into the pGL3 vector (Promega, Madison, WI, USA) was identified as wild-type (WT) 3′-UTR. Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used for site-directed mutagenesis of the miR-599 binding site in hsa_circ_0000285 3′-UTR, which was named as mutant (MUT) 3′-UTR. Cells were transfected with WT-3′-UTR or MUT-3′-UTR and miR-ctrl or miR-599 for 48 h. Then, the luciferase assay was conducted on the dual luciferase reporter assay system (Promega, Madison, WI, USA).

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### Statistical Analysis

All statistical analyses were performed by Statistical Product and Service Solutions (SPSS) 20.0 (SPSS, Chicago, IL, USA). Independent-sample *t*-test was selected when appropriate. Moreover, $p<0.05$ was considered a statistically significant difference.

## Results

### Hsa_circ_0000285 Expression Level in HCC Tissues and Cells

RT-qPCR analysis performed in 54 paired HCC tissues and adjacent tissues showed that hsa_circ_0000285 expression was upregulated in HCC tissues, which was shown in Figure 1A. Moreover, its expression in Bel-7402 and HepG2 cells and a normal liver epithelial cell (L02) were also detected. As was shown in Figure 1B, the hsa_circ_0000285 expression level of HCC cells was higher compared to that of L02.

### Knockdown of Hsa_circ_0000285 Suppressed Cell Migration and Invasion of HCC Cells

To explore the effects of hsa_circ_0 in cell migration and invasion of HCC cel HepG2 cell line was used for the knockdow hsa_circ_0000285. Then, RT-qPCR was utiliz for detecting the transfection effici (Figure 2A). As shown in Figure 2B, hs 00285 knockdown reduced cell mi th of HepG2 cells. As shown in Fig 2C, t of migrated cells was remark d afte

hsa_circ_0000285 was knock HepG2 cells. As was shown in Figu , the of invaded cells was remarka ecreased af a_ circ_0000285 was knock n in HepG2 s.

### Overexpression of Hsa_ci Promoted Cell M ation and on of HCC Cells

To explore t ffect hsa irc_0000285 in cell migrati on of cells, Bel-7402 cell lin was the expression of hsa_circ_0 85. Th R was utilized for detect the transfecti ficiency (Figure 3A). As Figure 3B, hsa_circ_0000285 overe ssi d the cell migrated length of Bel-7402 cell wn in Figure 3C, the nu of migrated s was remarkably increased after hsa_circ_0000285 was overexpressed in Bel-7402 cells. As was shown in Figure the number nvaded cells was remarkably ased afte a_circ_0000285 was overexp 02 cells.

### The Interaction Between MiR-599 Hsa_circ_0000285 in HCC

r RNA Interactome (https://circinter- ia.nih.gov/) was used to find the miRAs that contained complementary base with sa_circ_0000285. As miR-599 was a tumor uppressor and was able to suppress cancer cell oliferation, we focused on miR-599 among hese miRNAs which was interacted with hsa_circ_0000285 (Figure 4A). Indeed, the RT-qPCR assay showed that the expression of miR-599 was

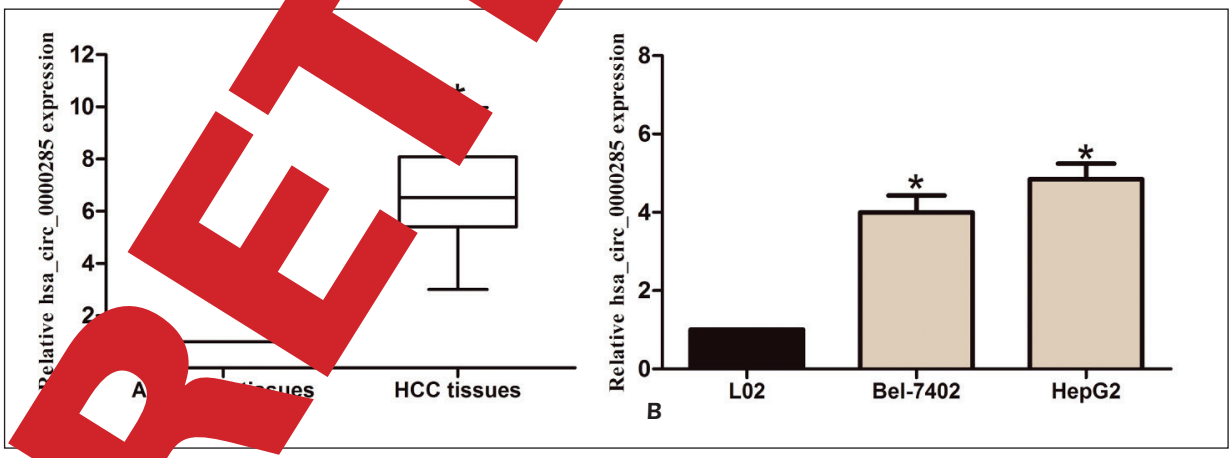


**Figure** ion levels of hsa_circ_0000285 were increased in HCC tissues and cell lines. **A**, Hsa_circ_0000285 expression was sig increased in the HCC tissues compared with adjacent tissues. **B**, Expression levels of hsa_circ_0000285 relative to GAP e determined in the human HCC cell lines and L02 (normal liver epithelial cell) by RT-qPCR. GAPDH was used as an inte al control. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

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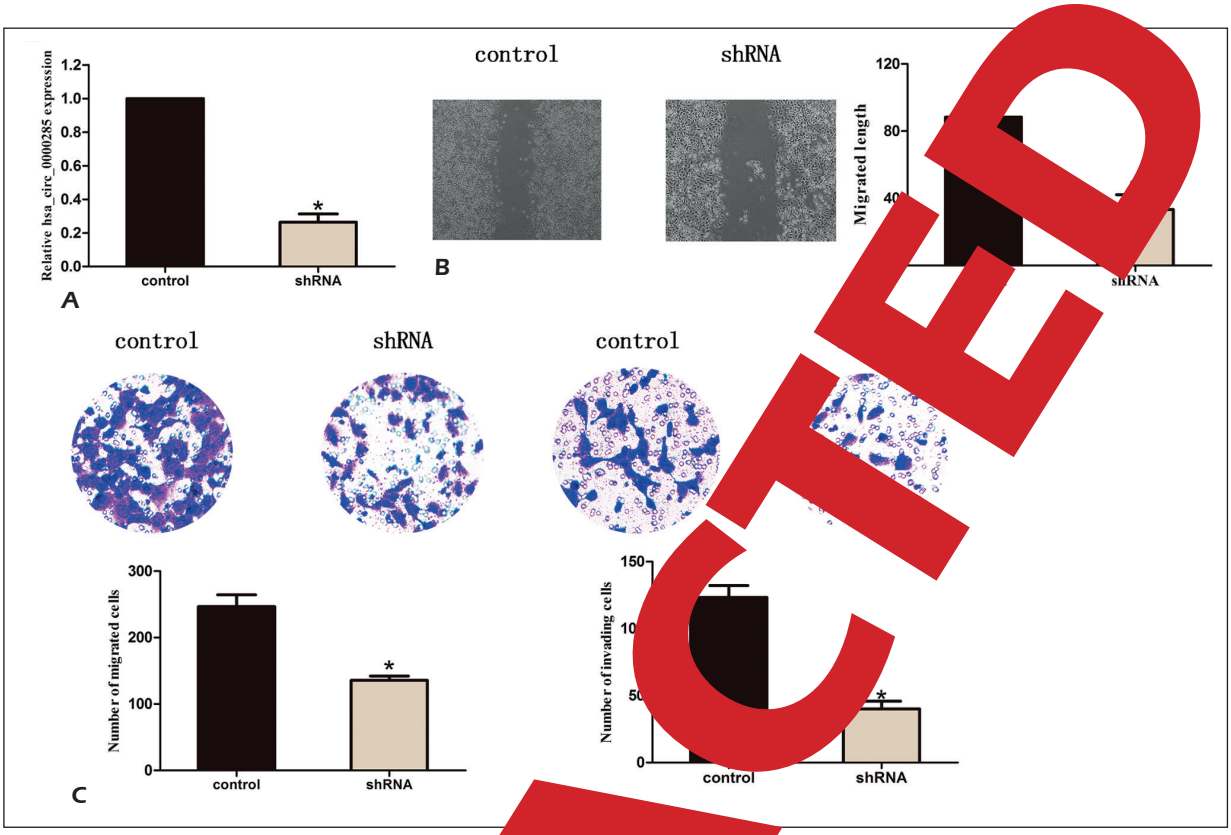


**Figure 2.** Knockdown of hsa_circ_0000285 inhib… epG2 c… on. **A**, Hsa_circ_0000285 expression in HepG2 cells transfected with shRNA and control was detecte… T-qP… **B**, Wound healing assay showed that the migrated length of HepG2 cells in shRNA group was significantly decre… ed with control group in HCC cells (magnification: 40×). **C**, Transwell assay showed that knockdown of hsa_circ_0… ignificantly repressed cell migration in HCC cells (magnification: 40×). **D**, Matrigel assay showed that k…kdown of hs… 0000285 significantly repressed cell invasion in HCC cells (magnification: 40×). The results represe… ge of three… endent experiments (mean ± standard error of the mean). *$p<0.05$, as compared with the control.

higher in shRNA group than… group (Figure 4B), while the expr…ssion… 599 was lower in hsa_circ_0000… ntivirus… han that empty vector grou… ure 4C). Mea… le, luciferase assay reve… o-transfection of hsa_circ_0000285-W… and… 9 largely decreased the luciferase activity, … co-transfection of hsa_c… 0000285-MUT… miR-599 had no effect… the luciferase activity either (Figure 4D). F… herm… the correlation analysis demonstrat… mi… 99 exp… ion level negatively correla… circ… 0285 expression in HCC tissues (r…

## …ussion

…g RNA (ncRNA) is a hotspot in the researc… lecular mechanism, new tumor markers an… eutic targets of cancers. From microRNA to lo… non-coding RNA, a large number of basic studies have confirmed that ncRNA has important biological functions in the occurrence and development of a variety of cancers. As a result, ncRNA may be an effective marker for early diagnosis of tumors or a potential target for drug therapy. In recent years, circRNA has become another star molecule after lncRNA and has attracted wide attention. In view of its specific expression, complex regulatory mechanism and close relationship with the occurrence and development of cancer, it has become the latest hotspot in the field of research, including colorectal cancer, breast cancer, bladder cancer and so on[7-9].

HCC is the fifth and seventh most common malignant tumors in men and women, and has the third highest mortality rate among all cancers[10]. The disease is characterized by a high recurrence rate after radical resection and resistance to medication. In addition, due to the lack of accurate early diagnosis and effective treatment, most patients with HCC are in advanced stage when di-

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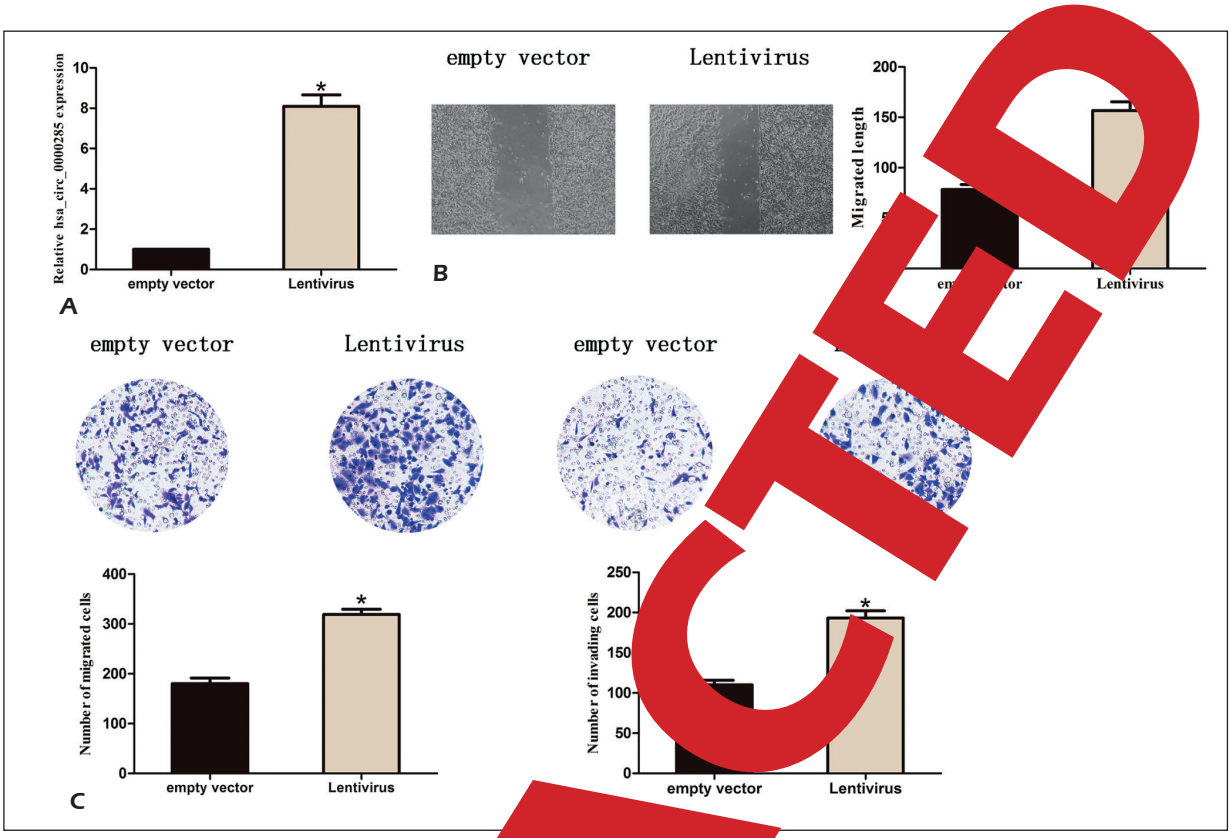


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**Figure 3.** Overexpression of hsa_circ_0000285 p d Bel-7 oliferation. **A**, Hsa_circ_0000285 expression in Bel-7402 cells transfected with hsa_circ_0000285 len and ty vector was detected by RT-qPCR. **B**, Wound healing assay showed that the migrated length of Bel-7402 cel us group was markedly increased compared with empty vector group in Bel-7402 cells (magnification: 40×). **C**, ll assay showed that overexpression of hsa_circ_0000285 significantly enhanced cell migration in Bel cells (mag on: 40×). **D**, Matrigel assay showed that overexpression of hsa_circ_0000285 significantly promoted on in Bel- lls (magnification: 40×). The results represent the average of three independent experiments (mea error of the an). *$p$<0.05, as compared with the control cells.

agnosed, which leads to p f most patients. Therefore, new idate bio for diagnosis, prognosis a eatment are tly need.

Hsa_circ_0000 s a RNA which has been initially explored in na geal carcinoma and blad cancer[11, 12]. The ression of hsa_circ_0000 was significantly associated with tumor s diffe iation, lymph node metastasis, dis is and mor-Node-Metastasis stage uld b erved as a prognostic biomarker cancers. \ In this resear found t _circ_0000285 was upre HCC samp s. Besides, the silence of circ ssed cell migration and i while overexpression of hsa 0285 promoted cell migration and invasion cells. The above results indicated that hsa_c 00285 promoted metastasis of HCC and might act as an oncogene.

At present, the functional recognition and mechanism of circRNA are still very limited. Competitive adsorption of microRNA is one of the mechanisms that have been studied extensively at present. Since circRNAs are highly expressed in the cytoplasm, studies have found that circRNAs can interfere with the biological regulation mediated by microRNAs by adsorbing specific microRNAs in the cytoplasm through their microRNAs adsorption sites[13]. The circRNA-CDR1as/miRNA-7 axis has been reported to be involved in brain tumors, lung cancer, osteosarcoma, urogenital tumors and other diseases[14]. Low expression of circRNA-SETD3 in HCC can bind to microRNA-421 which is closely related to tumor size growth and poor prognosis[5]. In addition, many circRNAs have been found to bind to multiple microRNAs, suggesting that these circRNAs may regulate the level of target genes by regulating the microRNAs.

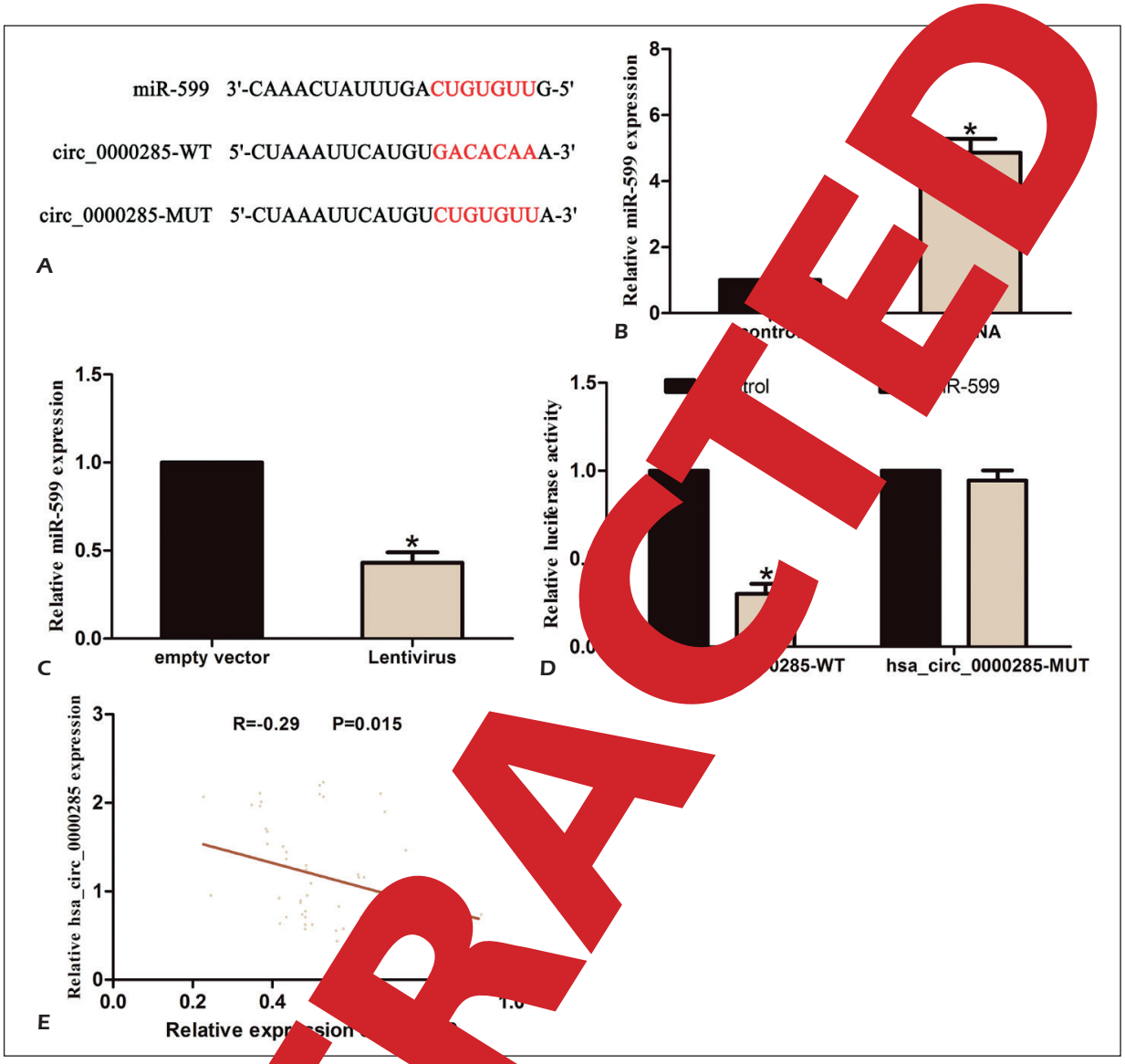


**Figure 4.** Reciprocal repres[illegible]between hsa_circ[illegible]285 and miR-599. **A**, Binding sites of miR-599 on hsa_circ_0000285. **B**, MiR-599 expression wa[illegible]t in shRNA group compared with control group. **C**, MiR-599 expression was decreased in hsa_circ_0000285 lentivi[illegible]red with empty vector group. **D**, Co-transfection of miR-599 and hsa_circ_0000285-WT strongly decreased [illegible]lucifera[illegible]while co-transfection of miR-599 and hsa_circ_0000285-MUT did not change the luciferase activity either. **E**, Linear[illegible]n between the expression level of miR-599 and hsa_circ_0000285 in HCC tissues. The results [illegible]sent the average o[illegible]e independent experiments Data are presented as the mean ± standard error of the mean. *$p<0$[illegible]

Circular RN[illegible]e was used to predic[illegible]otentia[illegible]miRNAs of hsa_circ[illegible]and miR-[illegible]9 was selected for ou[illegible]ther[illegible]599 is a novel miRNA, w[illegible]has[illegible]d to a novel tumor suppr[illegible]C[15].

In th[illegible]t report, miR-599 could directly bind to h[illegible]_0000285 through a luciferase assay. In addition, the miR-599 expression could be suppressed by the upregulation of hsa_circ_0000285, while miR-599 expression could be promoted by the downregulation of hsa_circ_0000285. Furthermore, the expression of miR-599 was negatively correlated with hsa_circ_0000285 in HCC tissues. All these results indicated that hsa_circ_0000285 might promote tumorigenesis of HCC by directly targeting miR-599.

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## Conclusions

We showed that hsa_circ_0000285 was remarkably upregulated in HCC tissues and could facilitate cell metastasis in HCC by targeting miR-599. These findings suggest that hsa_circ_0000285 may contribute to therapy for HCC as a prospective target.

### Conflict of Interests

The Authors declared that they have no conflict of interests.

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