**Original Article**

**Suppressive role of microRNA-29 in hepatocellular carcinoma via targeting IGF2BP1**

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**Abstract:** Hepatocellular carcinoma (HCC) is the most common primary liver cancer, ranking as the second leading cause of male cancer death worldwide. MicroRNA-29 (miR-29) has been demonstrated to act as a tumor suppressor in HCC. However, the regulatory mechanism of miR-29 underlying HCC growth and metastasis still remains obscure. In the present study, we showed that the expression of miR-29 was significantly reduced in HCC tissues and cell lines, and low miR-29 expression was associated with disease progression and shorter survival time of HCC patients. In vitro experiments showed that restoration of miR-29 expression caused a significant reduction in HCC cell proliferation, migration and invasion. Insulin like growth factor 2 mRNA binding protein 1 (IGF2BP1) was identified as a novel target gene of miR-29. The expression of IGF2BP1 was significantly increased in HCC tissues and cell lines. Moreover, IGF2BP1 was negatively regulated by miR-29 at the post-transcriptional levels in HCC cells. Furthermore, overexpression of IGF2BP1 attenuated the suppressive effects of miR-29 on the proliferation, migration, and invasion of HCC cells. According to these above findings, our study suggests that miR-29 may play a suppressive role in HCC growth and metastasis through directly targeting IGF2BP1. Therefore, miR-29 may be used as a potential candidate for the treatment of HCC.

**Keywords:** Hepatocellular carcinoma, microRNA, insulin like growth factor 2 mRNA binding protein 1, IGF2BP1, tumor suppressor

**Introduction**

As one of the most common malignant cancers worldwide, hepatocellular carcinoma (HCC), with a rapidly increased incidence, brings about a large amount of deaths every year [1, 2]. In recent years, the treatment outcomes for advanced HCC have been not improved, despite great efforts have been paid on surgical resection combined with radiotherapy and/or chemotherapy [2]. Understanding the molecular mechanism underlying HCC growth and metastasis may be benefit for the development of novel therapeutic strategies for this disease.

MicroRNAs (miRs) are a class of 18-25 nucleotides in length non-coding RNAs. It has been well established that miRNAs can directly bind to 3’ untranslated regions (3’UTRs) of their target mRNAs, and lead to mRNA degradation or translation inhibition, and thus act as important regulators for gene expression [3, 4]. In recent years, miRs have been demonstrated to be involved in many physiological and pathological processes, such as development, differentiation, angiogenesis, as well as tumorigenesis [5-12]. Recently, many miRs have been reported to play promoting or suppressive roles in liver cancers including HCC [13, 14]. For instance, MiR-935 promotes liver cancer cell proliferation and migration by targeting SOX7 [15]. MiR-133b inhibits hepatocellular carcinoma cell progression by targeting Sirt1 [16].

Among these miRs, miR-29 has been found to generally act as a tumor suppressor [17, 18]. For instance, Muniyappa et al. found that miR-29 could regulate the expression of numerous proteins and reduce the invasiveness and proliferation of lung and pancreatic cancer cell lines [18]. Cui et al. showed that miR-29 inhibited gastric cancer cell proliferation and induce cell cycle arrest through inhibiting the expression of p42.3 [19]. MiR-29 has recently been reported to play a suppressive role in HCC [20, 21]. Xiong et al. showed that miR-29 could inhi-
miR-29 inhibits HCC

**Table 1. Association between miR-29 expression and clinicopathological characteristics in hepatocellular carcinoma**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Cases (n=64)</th>
<th>Low miR-29 level (n=30)</th>
<th>High miR-29 level (n=34)</th>
<th>P value</th>
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<tr>
<td>Age (years)</td>
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<tr>
<td>&gt;55</td>
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<tr>
<td>Tumor size (cm)</td>
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<tr>
<td>&gt;5 cm</td>
<td>22</td>
<td>13</td>
<td>9</td>
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<tr>
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<td>16</td>
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<td>10</td>
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<tr>
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<td>11</td>
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<td>I-II</td>
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<tr>
<td>Absent</td>
<td>15</td>
<td>6</td>
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</table>

*Means the difference has statistical significance.

**bit HCC growth in vitro and in vivo, and induce cell apoptosis, and downregulation of miR-29 was significantly associated with worse disease-free survival of HCC patients [20]. Lin et al. reported that miR-29 could inhibit HCC metastasis through targeting TET1 [21]. However, the molecular mechanism of miR-29 underlying HCC growth has not been fully uncovered.

Therefore, our study aimed to investigate the regulatory mechanism of miR-29 underlying HCC growth and metastasis.

**Materials and methods**

**Tissue collection**

All experiments in our study were approved by the Ethics Committee of Xiangya Hospital, Central South University. A total of 64 HCC tissues as well as their paired adjacent tissues were collected from our department at Xiangya Hospital from October 2010 to June 2012. The written informed consents have been obtained from these HCC patients. Tissues were immediately snap-frozen in liquid nitrogen after surgical removal, and stored at -80°C. The clinical characteristics were summarized in Table 1.

**Cell culture and transfection**

Normal liver THLE-3 cells and four common human HCC cell lines including LM3, HepG2, Hep3B, and SMCC7721 were purchased from the Cell Bank of Xiangya Medical School, Central South University. These cells were cultured in DMEM (Thermo Fisher, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Thermo Fisher) in a humidified incubator (Thermo Fisher) containing 5% CO₂ at 37°C. HepG2 cells were transfected with scramble miR (miR-NC, Yearthbio, Changsha, China), miR-29 mimics (Yearthbio), negative control (NC) inhibitor (Yearthbio), miR-29 inhibitor (Yearthbio), or co-transfected with miR-29 mimics and pc-DNA3.1-IGF2BP1 plasmid (Yearthbio), or miR-29 mimics and blank pcDNA3.1 vector (Yearthbio), respectively, using Lipofectamine 2000 (Thermo Fisher), according to the manufacturer’s instruction. After 48 h for transfection, the expression analysis was performed using real-time PCR or western blot.

**Real-time PCR assay**

Total RNA of tissues and cells was extracted using Trizol Reagent (Thermo Fisher), according to the manufacturer’s instruction. After that, RNA was converted into cDNA using the Reverse Transcription Kit (Thermo Fisher). For mRNA expression detection, Standard SYBR-Green RT-PCR Kit (Takara) was used to conduct real-time PCR using on ABI 7500 thermocycler (Thermo Fisher), according to the manufacturer’s instruction. For miR expression detection, PrimeScript® miRNA RT-PCR Kit (Takara, Tokyo, Japan) was used to conduct real-time PCR using on ABI 7500 thermocycler (Thermo Fisher), according to the manufacturer’s instruction. U6 or GAPDH were used as an internal reference for miR or mRNA expression, respectively. The reaction condition was
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95°C for 3 min, followed by 40 cycles of 95°C for 30 sec and 60°C for 30 sec. The expression analysis was conducted using the 2^ΔΔCt method.

**Western blot assay**

Cells were lysed in RIPA buffer, and the lysates were then centrifuged at 12,000×g for 30 min at 4°C. BCA protein assay kit (Beyotime Biotechnology, Shanghai, China) was used to determine the protein concentration, according to the manufacturer’s instruction. The protein (60 μg) was then separated in 10% SDS-PAGE gel, which was then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was then blocked in 5% non-fat milk (Mengniu, Beijing, China) overnight at 4°C. After that, the membrane was incubated with rabbit anti-human IGF2BP1 and GAPDH antibodies (Abcam, Cambridge, MA, USA) at room temperature for 3 h. Then, the membrane was incubated with the mouse anti-rabbit secondary antibody (Abcam) at room temperature for 1 h. The immunoblots on the membrane were visualized using an enhanced chemiluminescence (ECL) kit (Thermo Fisher).

**MTT assay**

MTT assay was used to examine cell proliferation. HepG2 cells (50000 cells per well) in 100 μL of serum-free DMEM containing 0.5 g/L MTT (Sigma, USA) were seeded in a 96-well plate, and then incubated at 37°C for 0 h, 24 h, 48 h and 72 h. After that, the medium was removed, and 50 μL of DMSO (Sigma) was added. After incubated at 37°C for 10 min, the A570 of cells was measured using a spectrophotometer (UV-3600, Shimadzu, Kyoto, Japan).

**Transwell assay**

Transwell assay was used to examine cell invasion using transwell chambers (BD, USA). HepG2 cell suspension (5×10^5 cells/ml) was prepared in DMEM, 300 μl of which was added into the upper chamber. After that, 500 μL of DMEM added with 10% FBS was added into the lower chamber. After incubated in 37°C for 24 h, a cotton-tipped swab was used to wipe out those HepG2 cells not through the pores. After that, the filter was fixed in 90% alcohol, and stained by crystal violet (Sigma). Cells through the pores were counted and photographed under an inverted microscope (IX71, Olympus, Tokyo, Japan).

**Wound healing assay**

HepG2 cells were cultured to full confluence in 24-well plates. A plastic scriber was used to create wounds (about 1 mm width). After that, HepG2 cells were washed with DPBS (Thermo Fisher), and then cultured in DMEM added with 10% FBS 37°C for 48 h. After that, HepG2 cells were photographed under an inverted microscope (IX71, Olympus, Tokyo, Japan).

**Luciferase reporter assay**

The putative target genes of miR-29 were predicted using Targetscan (www.targetscan.org/) and miRanda (www.microrna.org), according to the manufacture’s instruction. The wild type (WT) of IGF2BP1 3’-UTR were constructed using PCR. The mutant type (MT) of IGF2BP1 3’-UTR was generated using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), according to the manufacturer’s instruction. The WT or MT IGF2BP1 3’-UTR was inserted into the psiCHECK vector (Promega, Madison, WI, USA), generating WT-IGF2BP1 3’-UTR or MT-IGF2BP1 3’-UTR plasmid, respectively. HepG2 cells a 24-well plate were co-transfected with 100 ng of WT-IGF2BP1 3’-UTR or MT-IGF2BP1 3’-UTR plasmid, and 50 nM of miR-29 mimics or miR-NC, respectively, using Lipofectamine 2000. After transfection for 48 h, the renilla and firefly luciferase activities were detected using the Dual-luciferase Reporter Assay System (Promega), according to the manufacturer’s instruction. After that, Renilla luciferase activity was normalized to firefly luciferase activity.

**Statistical analysis**

The data were expressed as the mean ± standard deviation (SD). SPSS 19.0 (SPSS, Armonk, NY, USA) was used to perform statistical analysis. Student t test was conducted to examine the statistical correlation of data between two groups, while one-way ANOVA was used to examine the statistical correlation of data among more than two groups. Besides, chi-square test was performed to study the association between miR-29 expression and clinical characteris-
miR-29 inhibits HCC

Figure 1. MiR-29 is downregulated in HCC. A: Real-time PCR was used to examine the miR-29 levels in hepatocellular carcinoma (HCC) tissues compared to adjacent non-tumor tissues. **P<0.01 vs. Adjacent. B: The HCC patients with low expression of miR-29 showed shorter survival time compared with those with high miR-29 levels.

Results

MiR-29 is downregulated in HCC, associated with HCC progression and poor prognosis

The regulatory mechanism of miR-29 underlying HCC progression still remains largely unknown. Here we firstly conducted real-time PCR to examine the miR-29 expression in HCC tissues and adjacent non-tumor tissues. The miR-29 levels were significantly decreased in HCC tissues compared to adjacent non-tumor tissues (Figure 1A). The clinical significance of miR-29 expression in HCC was then investigated. Our data indicated that low miR-29 expression was significantly associated with poor differentiation, node metastasis, and clinical stage in HCC (Table 1). In addition, those HCC patients with low miR-29 levels showed shorter survival time, when compared with those with high miR-29 expression (Figure 1B). Accordingly, our data demonstrate that the reduced expression of miR-29 associated with HCC progression and poor prognosis.

Restoration of miR-29 decreases the proliferation, migration and invasion of HepG2 cells

To further confirm these above findings, we detected the expression of miR-29 in several common HCC cell lines including LM3, HepG2, Hep3B, and SMCC7721. The normal liver THLE-3 cells were used as the control group. As indicated in Figure 2A, miR-29 was also significantly downregulated in HCC cell lines compared with THLE-3 cells.

As HepG2 cells showed the most significant decrease in miR-29 expression, we used this cell line in the following experiments in vitro. To restore the expression levels of miR-29 in HepG2 cells, miR-29 mimic were used to perform cell transfection. Our data indicated that transfection with miR-29 mimic caused a significant increase in the expression of miR-29 (Figure 2B). MTT assay, wound healing assay and transwell assay were then conducted to determine the cell proliferation, migration and invasion, respectively. As indicated in Figure 2C-E, restoration of miR-29 expression significantly reduced the proliferation, migration and invasion of HepG2 cells. Therefore, miR-29 may play a suppressive role in HCC growth and metastasis.

IGF2BP1, upregulated in HCC, is identified as a novel target gene of miR-29

As miRs function through regulating the expression of their target genes, the putative target genes of miR-29 were predicted. As shown in Figure 3A, IGF2BP1 was a putative target gene of miR-29. To confirm this targeting relationship, the luciferase vectors containing WT or MT of IGF2BP1 3′-UTR were constructed, respectively (Figure 3B). Luciferase reporter as-
miR-29 inhibits HCC

Figure 2. Restoration of miR-29 decreases the proliferation, migration and invasion of HepG2 cells. (A) Real-time PCR was used to determine the miR-29 levels in HCC cell lines including LM3, SMCC7721, HepG2, and Hep3B, compared to normal human liver THLE-3 cells. **P<0.01 vs. THLE-3. HepG2 cells were transfected with miR-29 mimic or scramble miR (miR-NC), respectively. (B) Real-time PCR was used to determine the miR-29 expression. (C) MTT assay. (D) wound healing assay, and (E) transwell assay were used to determine cell proliferation, migration and invasion, respectively. **P<0.01 vs. miR-NC.

say data further showed that the luciferase activity was significantly decreased in HepG2 cells co-transfected with miR-29 mimic and WT-IGF2BP1-3’UTR plasmid, which was elimi-
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IGF2BP1 is negatively regulated by miR-29 at the post-transcriptional levels in HepG2 cells

After that, we examined the effects of miR-29 expression on the protein expression of IGF2BP1 in HepG2 cells. As shown in Figure 4A, overexpression of miR-29 led to a significant increase in the expression of IGF2BP1 protein levels compared to the control. These findings suggest that the reduced expression of miR-29 may contribute to the upregulation of IGF2BP1 in HCC tissues and cell lines.

As miRs negatively regulate the expression of their targets at the post-transcriptional levels, we then examined the expression of IGF2BP1 in HCC tissues and cell lines. The protein levels of IGF2BP1 were significantly higher in HCC tissues compared to adjacent non-tumor tissues (Figure 3D). As indicated in Figure 3E, the IGF2BP1 protein levels were also increased in HCC cells compared to THLE-3 cells. These findings suggest that the reduced expression of miR-29 may contribute to the upregulation of IGF2BP1 in HCC tissues and cell lines.

As no previous study has revealed their targeting relationship, the present study for the first time reports that IGF2BP1 is a novel target gene of miR-29.

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miR-29 inhibits HCC

reduction of IGF2BP1 protein expression in HepG2 cells. To further confirm these findings, HepG2 cells were transfected with miR-29 inhibitor or NC inhibitor, respectively. After transfection, the miR-29 levels were significantly decreased in miR-29 inhibitor group compared with NC inhibitor group (Figure 4B). We then found that knockdown of miR-29 significantly enhanced the protein expression of IGF2BP1 (Figure 4C). Therefore, miR-29 negatively regulates the protein expression of IGF2BP1 in HepG2 cells.

Overexpression of IGF2BP1 attenuates the suppressive effects of miR-29 of HCC cells

Based on the above findings, we speculated that IGF2BP1 might be involved in miR-29-me-
miR-29 inhibits HCC

Figure 5. Overexpression of IGF2BP1 attenuates the suppressive effects of miR-29 of HCC cells. MiR-29-overexpressing HepG2 cells were transfected with pcDNA3.1-IGF2BP1 plasmid or blank pcDNA3.1 vector, respectively. (A, B) Real-time PCR and western blot was used to determine the mRNA and protein expression of IGF2BP1. (C) MTT assay; (D) wound healing assay; and (E) transwell assay were used to determine cell proliferation, migration and invasion, respectively. **P<0.01 vs. MiR-29+ blank.
miR-29 inhibits HCC

diated malignant phenotypes of HepG2 cells. To clarify this speculation, miR-29-overexpressing HepG2 cells were transfected with pcDNA3.1-IGF2BP1 plasmid or blank pcDNA3.1 vector, respectively. As indicated in Figure 5A, 5B, the IGF2BP1 levels were significantly increased in the miR-29+IGF2BP1 group compared with miR-29+ blank group. MTT assay, wound healing assay and transwell assay were then conducted to determine the cell proliferation, migration and invasion, respectively. Our data showed that the proliferation, migration and invasion of HepG2 cells were significantly increased in miR-29+IGF2BP1 group compared with miR-29+ blank group, indicating that overexpression of IGF2BP1 attenuates the suppressive effects of miR-29 of the malignant phenotypes of HCC cells. These findings suggest that miR-29 may inhibit HCC growth and metastasis via targeting IGF2BP1.

Discussion

The present study investigated the underlying regulatory mechanism of miR-29 in HCC, and showed that miR-29 was significantly downregulated in HCC tissues and cell lines, which was significantly associated with disease progression as well as shorter survival time of HCC patients. In vitro experiments data indicated that restoration of miR-29 expression caused a significant reduction in HCC HepG2 cell proliferation, migration and invasion. IGF2BP1, significantly upregulated in HCC, was identified as a novel target of miR-29, and its protein expression was negatively regulated by miR-29 in HepG2 cells. Moreover, overexpression of IGF2BP1 attenuated the suppressive effects of miR-29 on the malignant phenotypes of HepG2 cells.

MiR-29 has been demonstrated to play a suppressive role in different cancers types, such as breast cancer [22], acute megakaryoblastic leukemia [23], ovarian cancer [24], and gastric cancer [25]. Recently, miR-29 was found to play a suppressive role in HCC. For instance, Xiong et al. found that miR-29 was downregulated in HCC, which was significantly associated with poor prognosis of HCC patients. Moreover, they showed that miR-29 could sensitize HCC cells to apoptosis triggered by either serum starvation and hypoxia or chemotherapeutic drugs, and inhibit HCC cell growth in vitro and in vivo [20]. In the present study, we also found that miR-29 was downregulated in HCC tissues and cell lines, and low expression of miR-29 was associated with the poor differentiation, metastasis, and advanced clinical stage in HCC. We further showed that restoration of miR-29 significantly decreased HepG2 cell proliferation, migration and invasion.

Moreover, several targets of miR-29 have been identified in HCC, including TET1, Bcl-2 and Mcl-1 [20, 21]. We speculated that other targets of miR-29 may also play important roles in HCC. In this study, bioinformatics analysis indicated that IGF2BP1 was a putative target gene of miR-29. We then conducted luciferase reporter gene assay, and confirmed their target relationship. IGF2BP1 is a member of the IGF2BP family, and contains four K homology domains and two RNA recognition motifs [26]. IGF2BP1 can bind to and stabilize the mRNAs of some genes, such as c-MYC, MKI67, IGFl2, and beta-actin, through which it participates in regulating cell survival and proliferation [26-28]. Recently, IGF2BP1 was reported to promote tumor cell proliferation, migration and invasion in several cancers including HCC [27-29]. Knockdown of IGF2BP1 could significantly decrease HCC growth in vivo, suggesting that IGF2BP1 may become a promising molecular target for HCC treatment [28]. Moreover, several miRs have been reported to directly target HCC, and thus function as tumor suppressors. For instance, miR-1275 has suppressive effects on HCC tumor growth through targeting IGF2BPs and IGF1R [30]. MiR-625 could inhibit HCC cell migration and invasion by targeting IGF2BP1 [31]. In this study, we found that IGF2BP1 was significantly upregulated in HCC tissues and cell lines, and IGF2BP1 was negatively regulated by miR-29 at the post-transcriptional level in HepG2 cells. Moreover, we showed that overexpression of IGF2BP1 attenuated the suppressive effects of miR-29 on the proliferation, migration and invasion of HepG2 cells, which further suggests that the inhibitory effects of miR-29 on HCC is probably through inhibition of IGF2BP1.

To our knowledge, our study for the first time demonstrates that miR-29 plays a suppressive role in the proliferation, migration and invasion of HCC cells through inhibition of IGF2BP1. These findings suggest that miR-29 may be used as a promising therapeutic candidate for HCC treatment.
Disclosure of conflict of interest

None.

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