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Original Article

miR-497 inhibits the carcinogenesis of hepatocellular carcinoma by targeting the Rictor/Akt signal pathway

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Abstract: MicroRNAs (miRNAs) are involved in regulating various physiologic and pathologic processes of different human diseases including hepatocellular carcinoma (HCC). Our research aimed to investigate the role of miR-497 in migration, invasive ability of HepG2-GS cells and the regulating mechanism. In this study, Rictor was identified as a target gene of miR-497 by informatic software, including Microcosm Targets, miRanda, and TargetScan. MiR-497 or Rictor were silenced or overexpressed in HepG2-GS cells through transfection. The functional assay results showed that Rictor knockdown inhibited cancer cell proliferation, migration and invasion. Overexpression of Rictor inversed the effects of miR-497 on cancer cells growth inhibition. miR-497 regulated protein kinase B, PKB (Akt) signaling pathway by targeting Rictor. MiR-497 increased chemo-sensitivity of HepG2-GS through regulation of Rictor. In conclusion, our research demonstrated that miR-497 inhibits the proliferation, invasion, metastasis, and chemotherapy resistance of hepatoma cells by targeting of Rictor/Akt signal pathway, and miR-497. Thus, Rictor has the potential to be a explored as a biomarker or therapeutic target for diagnosis and treatment of HCC.

Keywords: Hepatocellular carcinoma, miR-497, Rictor/Akt signal pathway

Introduction

Hepatocellular carcinoma (HCC), a primary malignancy of the liver, is the third leading cause of cancer death worldwide [1]. The morbidity and mortality of HCC have increased in many countries over the last few decades [2]. Due to the difficulty in early diagnosis, aggressive nature, and resistance to chemotherapy, the 5-year survival rate of HCC is poor [3]. Researchers predict that the mortalities of HCC in Northern and Central Europe, North and Latin America were much higher than that in most European countries and the Americas up to 2020 [4]. Hence, it is important to investigate the molecular mechanism of HCC carcinogenesis, to search for molecular targets and possible drugs and therapeutic stategies for HCC treatment.

MicroRNAs (miRNAs), which are small and highly conserved noncoding RNAs, are involved in regulating physiologic and pathologic processes including cell proliferation and death, hematopoietic differentiation, and immunity and are usually complementary to the 3'untranslated region (3'-UTR) of target genes [5-7]. Accumulating studies indicate that miRNAs contribute to the development and progression of most human cancers through various mechanisms including the PI3K/Akt signaling pathway [8, 9]. The downregulated expression of miR-497 was found in several types of human cancer [10, 11] and overexpression of miR-497 was able to inhibit cancer cell proliferation, migration, and invasion [12]. Some studies showed that downregulation of miR-497 was related to angiogenesis and metastasis in HCC [13]. However, the role of miR-497 in HCC carcinogenesis and the associated mechanism remain unclear. This study aimed to investigate the role of miR-497 inmigration, invasive ability of HCC cells and the regulating mechanism.

Materials and methods

Cell culture

SK-HEP-1 and Huh-7 cell lines were purchased from American Type Culture Collection

(Manassas, VA, USA). SK-HEP-1 and Huh-7 cells were maintained in Eagle's Minimum Essential Medium (Gibco, Grand Island, NY, USA) (SK-HEP-1) or Dulbecco's Modified Eagle Medium (DMEM) (Huh-7) containing 10% (v/v) fetal bovine serum (FBS) with penicillin and streptomycin, at 37°C with 5% CO_2.

Cell transfection

The miR-497 mimics (miR-497), miR-497 inhibitor (miR-497-inh), miRNA negative control and miRNA inhibitor negative control were purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China) and transfected into SK-HEP-1 and Huh-7 cells by Lipofectamine2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The sequences were referred from other research [14].

miR-497 target gene Rictor predictions, and 3'UTR luciferase reporter assay

Rictor was predicted as a targetfor miR-493 and the binding sites were analyzed by Microcosm Targets, miRanda, and TargetScan. The 3'-UTR of Rictor containing miR-497 binding sites was cloned downstream of luciferase gene in the pGL-3 vector (Promega, Madison, WI). SK-HEP-1 and Huh-7 cells were transfected with different miRNA and luciferase activity was measured by the dual-luciferase reporter assay system (Promega, Madison, WI).

Proliferation, migration, and invasion ability

To investigate the effects of miR-497 on the ability of proliferation, migration and invasion, transwell migration, invasion assays, and wound healing assay were performed. SK-HEP-1 and Huh-7 cells were transfected with Rictor deficient plasmid (siRictor) or negative control. The proliferation ability was evaluated by MTT assay as described previously [15]. For transwell assay, transfected cells were plated onto the non-coated membrane of each well in the upper chamber (24 well, diameter 6.5 mm, pore size 8 μm) coated with matrigel extracellular matrix gel (ECM) (BD Bioscience, San Jose, CA, USA). Medium in thebottom chamber contained added hepatocyte growth factor (HGF) (Invitrogen, Carlsbad, CA, USA). The incubation times for migration and invasion was 24 h and 48 h, respectively. Cells on the upper membrane surface were removed and cells on the undersurface were fixed with ethanol (v/v 95%) and stained with crystal violet for 30 min. The number of migrated or invaded cells was counted under an inverted microscope (400×, six random fields). For the wound healing assay, cells were seeded in 96-well flat-bottom microplate. Wounds were assembled with pipette tips and cells were observed at 0 h and 48 h by an inverted microscope.

RNA extraction and quantitative real-time (RT-PCR)

Total RNAs were extracted using from cells using Trizol reagent (Invitrogen Life Technologies, Carlsbad, California). To quantitate Rictor expression, Equal amounts of RNA were used to synthesize cDNA by cDNA Synthesis kit (Invitrogen Life Technologies, CA, California). Quantitative real-time PCR was performed by using SYBR Green PCR Master Mix reagent kits (Promega Corporation, Madison, WI, USA). All samples were normalized to GADPH (internal control).

Protein extraction and western blot analysis

SK-HEP-1 and Huh-7 cells were harvested and lysed in cold RIPA buffer with protease and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, Mo. USA) and centrifuged (12,000 g for 10 min) to collect cell lysates. After concentrations quantification and degeneration, equal amount of proteins were separated on Invitrogen™ NuPAGE™ Bis-Tris gels (10%) and transferred onto PVDF membranes (Millipore, MA). After blocking, the membranes were incubated with primary antibodies, including anti-Rictor antibody (#9476, 1:1000, Cell Signaling Technology Inc., Beverly, MA, USA), anti-β-actin antibody (#8457, 1:1000, Cell Signaling Technology, Inc., Beverly, MA, USA) respectively. After washing with TBST for 3 times, the membranes were subsequently incubated with secondary antibodies conjugated with horseradish peroxidase (1:10000) for 2 hours at room temperature. After incubation with secondary antibodies (1:10000), the proteins were measured by ECL system according to the manufacturer's instructions.

Statistical analysis

All data are presented as the means ± standard deviation (SD). Data were analyzed using one-way ANOVA with Tukey's tests using Prism 6 (San Diego, CA, USA). $P < 0.05$ was considered significant.

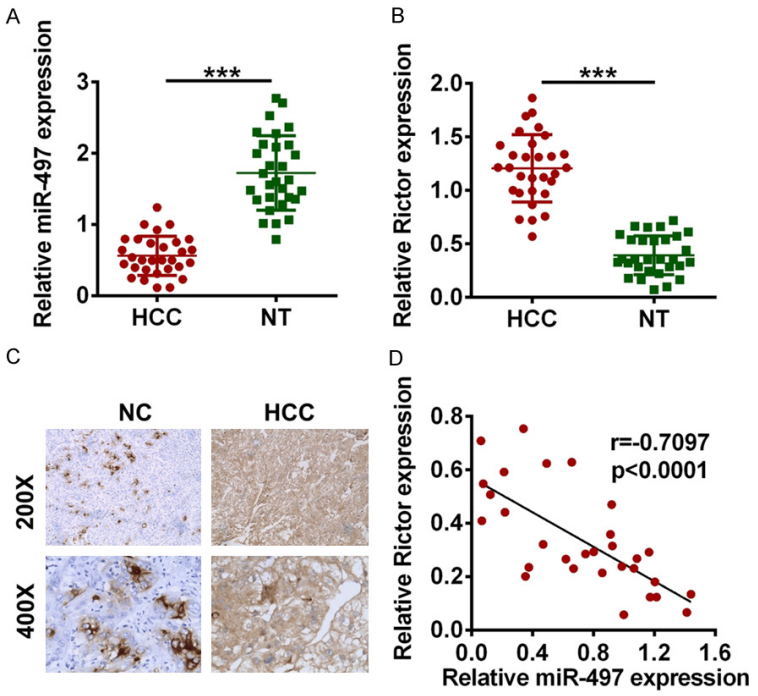

Figure 1. Level of miR-497 and Rictor in carcinoma tissue and para-carcinoma tissue of HCC patients. A. Level of miR-497 mRNA in HCC patients analyzed by RT-PCR. B. Level of Rictor mRNA in HCC patients analyzed by RT-PCR. C. Expression of Rictor in carcinoma tissue and para-carcinoma tissue of HCC patients analyzed by immunohistochemical staining. D. The linear regression of miR-497 and Rictor expression. ***P < 0.001 compared to HCC.

Results

The levels of miR-497 and Rictor in HCC tissue and para-carcinoma tissues

The expression levels of miR-497 and Rictor in HCC tissues and corresponding para-carcinoma tissues of HCC patients were detected by RT-PCR. As shown in **Figure 1A** and **1B**, the level of miR-497 in HCC tissue was lower than in NT group, while Rictor was higher than in the NT group ($P < 0.05$). Immunohistochemical staining of Rictor in HCC patients showed that the Rictor expression in HCC tissue was higher than NT group ($P < 0.05$) (**Figure 1C**). Linear regression was used to analyze the relationship of Rictor and miR-497, and results showed that the level of Rictor was negatively correlated with miR-497 (**Figure 1D**) (r = -0.7097).

miR-497 overexpression inhibited cancer cell proliferation, migration and invasion

To explore the effects of miR-497 on migratory and invasive abilities of cancer cells, we performed proliferation, wound healing, transwell migration, and invasion assays upon miR-497 overexpression by transfection of miR-497 mimics (SK-HEP-1, Huh-7) or miR-497 knockdown with miR-497-inhibitor in human hepatoma cells. The transfection efficiency of the knockdown was confirmed by RT-PCR analysis. The expression of miR-497 (**Figure 2A**) in the miR-497 mimic transfected group was remarkably increased compared with the miR-NC group ($P < 0.001$). The miR-497 level in the miR-497-inh group was remarkably decreased compared with the NC-inh miR group ($P < 0.001$). MTT results showed that miR-497 overexpression inhibited the cell viability in both SK-HEP-1 and Huh-7 cells (**Figure 2B**) ($P < 0.01$ at 3th day). However, knockdown of miR-497 increased the cell viability c compared with NC-inh group ($P < 0.001$ at 3th day). Then the effect of miR-497 on cell invasion was investigated. The transwell assay results showed that the invasion ability of the miR-497 overexpression group was decreased compared with the miR-NC group (**Figure 2C**). Conversely, the cell invasion ability in miR-497 group was increased compared with that of NC-inh group. Furthermore, the wound healing assay showed that miR-497 overexpression cells migrated more slowly compared with the miR-NC group (**Figure 2D**). The migration of miR-497 knockdown cells was faster than the NC-inh group. The effects of miR-497 on cell mobility were similar in SK-HEP-1 and Huh-7 cells. Taken together, these results suggest that overexpression of miR-497 inhibits HepG2-GS cells' proliferative and invasive ability.

Rictor is a target gene of miR-497

To predict a miR-497 target, three bioinformatic databases (TargetScan, miRanda, and PicTar) were used. Rictor was selected as a putative miR-497 target due to its inhibitory effect in tumor progression and metastasis. The complementary sequence of miR-497 was found on the site of the 3'-UTR of Rictor mRNA (**Figure**

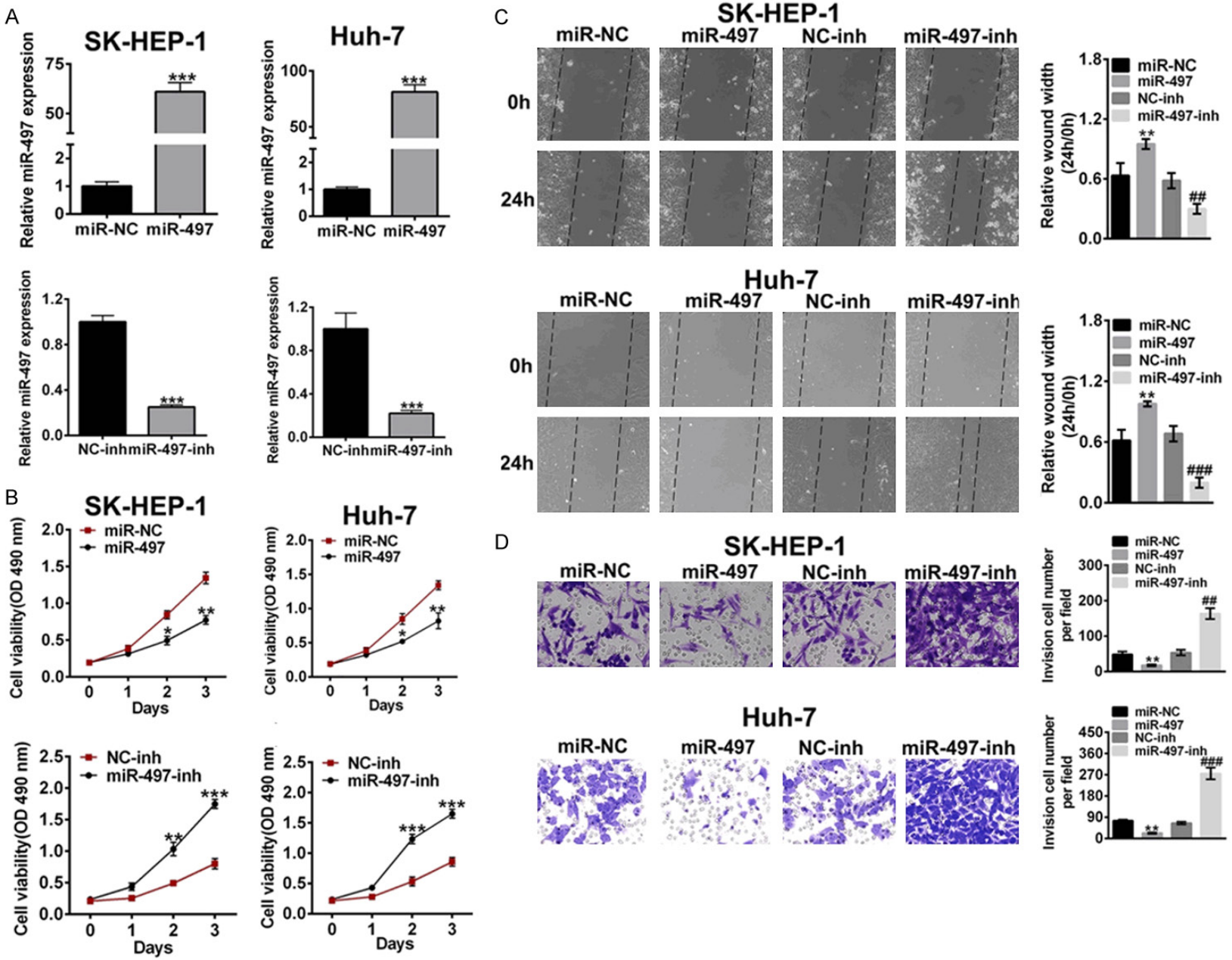
A
SK-HEP-1
Huh-7
Relative miR-497 expression
miR-NC
miR-497
NC-inh
miR-497-inh
B
Cell viability(OD 490 nm)
Days
C
0h
24h
Relative wound width (24h/0h)
D
Invision cell number per field

Figure 2. Effect of miR-497 overexpression or knockdown on the proliferation and motility of SK-HEP-1 and Huh-7 cells. A. miR-497 expression of miR-497 mimics or inhibitor transfected cells by RT-PCR. B. Proliferation of SK-HEP-1 and Huh-7 cells detected by MTT assay. C. Invasion of SK-HEP-1 and Huh-7 cells detected by transwell assay. D. Migration of SK-HEP-1 and Huh-7 cells in the wound healing assay. $^{**}P < 0.01$ compared to miR-NC, $^{***}P < 0.001$ compared to miR-NC, $^{\#\#}P < 0.01$ compared to NC-inh, $^{\#\#\#}P < 0.001$ compared to NC-inh.

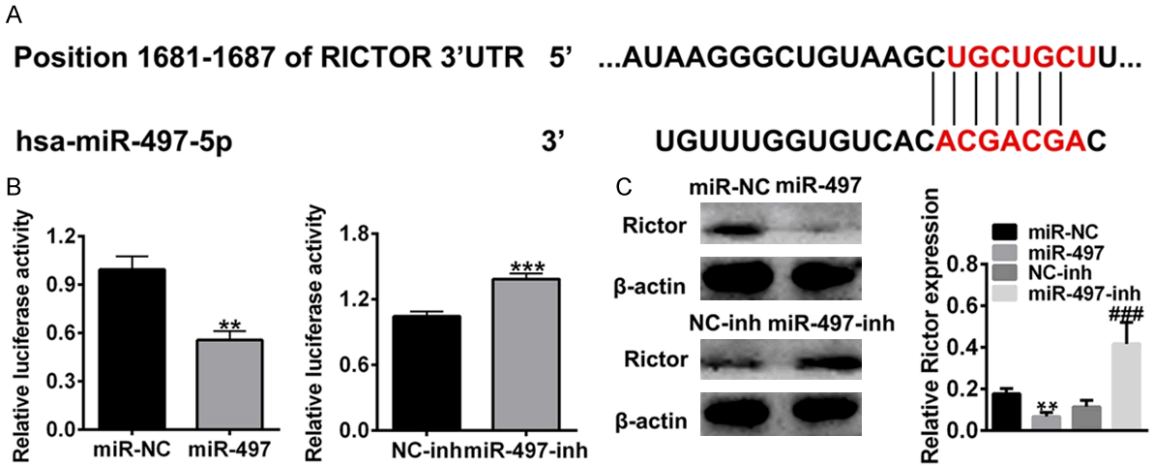

Figure 3. Rictor is a direct target of miR-497. A. Complementary sequence of miR-497 binding sites in Rictor 3'-UTR regions. B. The luciferase activities of miR-497 mimics or miR-497-inh transfected cells at 48 h after the transfection. C. Rictor expression of miR-497 or miR-497-inh transfected cells analyzed by western blotting. $^{**}P < 0.01$ compared to miR-NC, $^{***}P < 0.001$ compared to miR-NC, $^{\#\#\#}P < 0.001$ compared to NC-inh.

3A). To investigate the regulation of miR-497 on *Rictor*, 293T cells were transfected with Rictor luciferase reporter plasmid, with their 3'-UTR regions containing of miR-497 binding sites or corresponding mutant sites, and the 3'-UTR luciferase activity was measured. As shown in **Figure 3B**, miR-497 overexpression (miR-497) reduced the transcriptional activity of *Rictor* compared with control (miR-NC), and miR-497 inhibitor (miR-497-inh) increased the transcriptional activity of *Rictor* compared to negative control (NC-inh). Furthermore, the protein expression of Rictor was in accordance with mRNA levels (**Figure 3C**). These results suggested that miR-497 can negatively regulate *Rictor* by directly targeting its 3'-UTR.

Rictor knockdown inhibited cancer cell proliferation, migration, and invasion

To explore the effects of Rictor on migratory and invasive abilities of HCC cancer cells, we performed proliferation, wound healing, transwell migration, and invasion assays in Rictor overexpression (Rictor group) human hepatoma cells (SK-HEP-1, Huh-7) or Rictor knockdown cells (SiRictor#1 and SiRictor#2). The transfection efficiency was confirmed by RT-PCR. The Rictor mRNA (**Figure 4A**) and protein level (**Figure 4B**) in the Rictor overexpression group (Rictor) were remarkably increased compared with the control ($P < 0.001$). The level of Rictor mRNA and protein were decreased in siRNA#1 and #2 group than siNC. The level of siRNA#1 was lower than #2, which indicated that the sequence of #1 was more efficiency than #2. MTT assay results showed that the proliferation of HCC cells was increased in Rictor overexpression cells compared with control cells (**Figure 4C**) ($P < 0.01$ at 3rd day). Conversely, the proliferation of HCC cells were decreased in the Rictor knockdown group (siRictor) compared with the siNC group ($P < 0.01$ at 3rd day). We then investigated the role of Rictor on cell invasion. The invasion assay results showed that Rictor overexpression increased the invasive ability of the cells compared with controls (**Figure 4D**). The invasion ability of cell was decreased in Rictor knockdown cells compared with siNC group. Furthermore, the wound healing assay showed that Rictor overexpression cells migrated faster compared with control (**Figure 4E**). The migration of Rictor knockdown cells was lower than the siNC group. The results were similar in SK-HEP-1 and Huh-7. Taken together, these results suggest that Rictor knockdown inhibits cell proliferative and invasive ability.

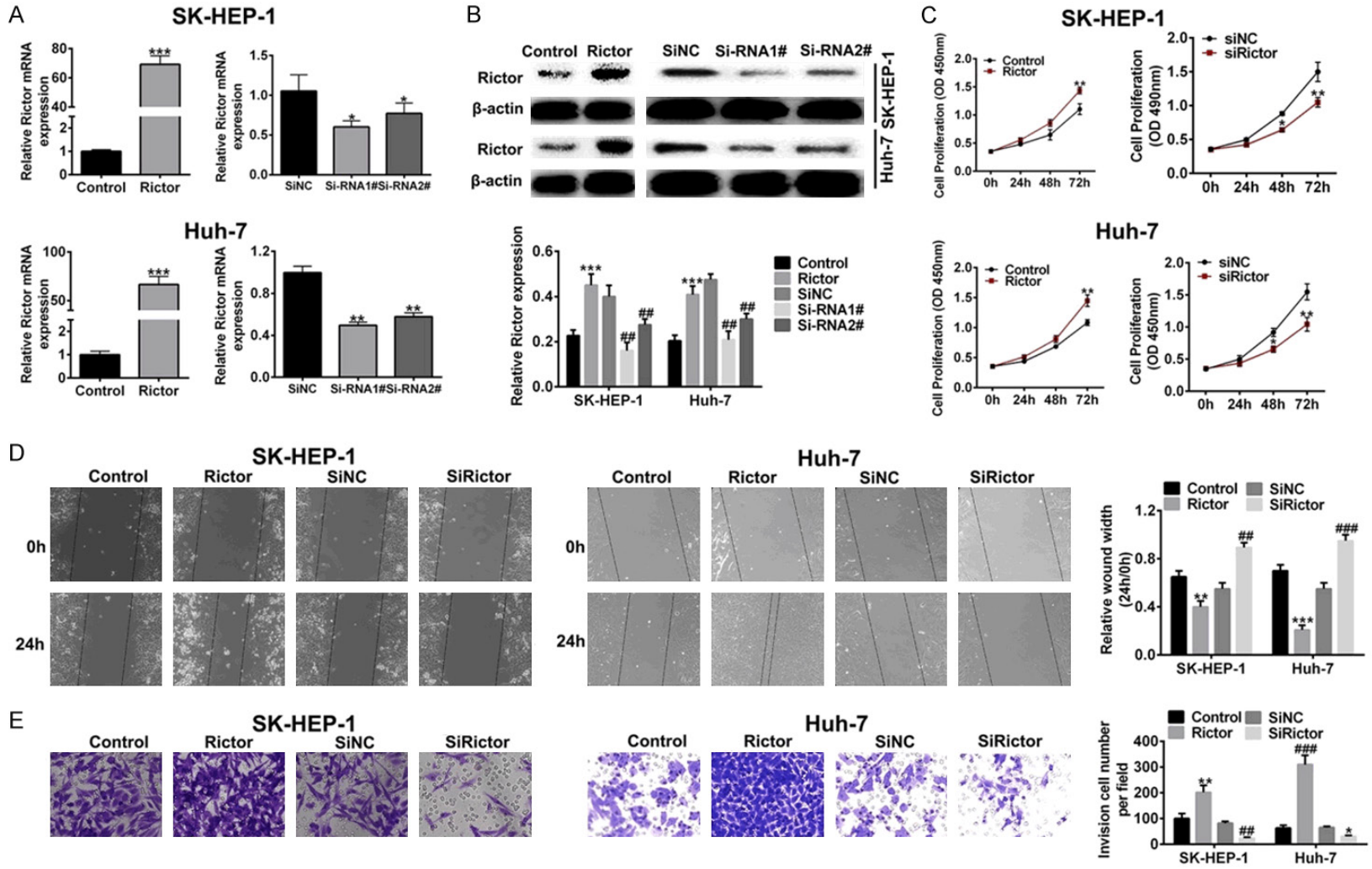

Figure 4. Proliferation, migration and invasion of Rictor overexpression or knockdown cells (SK-HEP-1 and Huh-7). A. The expression of Rictor overexpression or knockdown cells by RT-PCR. B. The expression of Rictor overexpression or knockdown cells by western blotting. C. Proliferation of SK-HEP-1 and Huh-7 cells detected by MTT assay. D. Invasion of SK-HEP-1 and Huh-7 cells detected by transwell assay. E. Migration of SK-HEP-1 and Huh-7 cells in the wound healing assay. $^{**}P < 0.01$ compared to control, $^{***}P < 0.001$ compared to control, $^{\#\#}P < 0.01$ compared to siNC, $^{\#\#\#}P < 0.001$ compared to siNC.

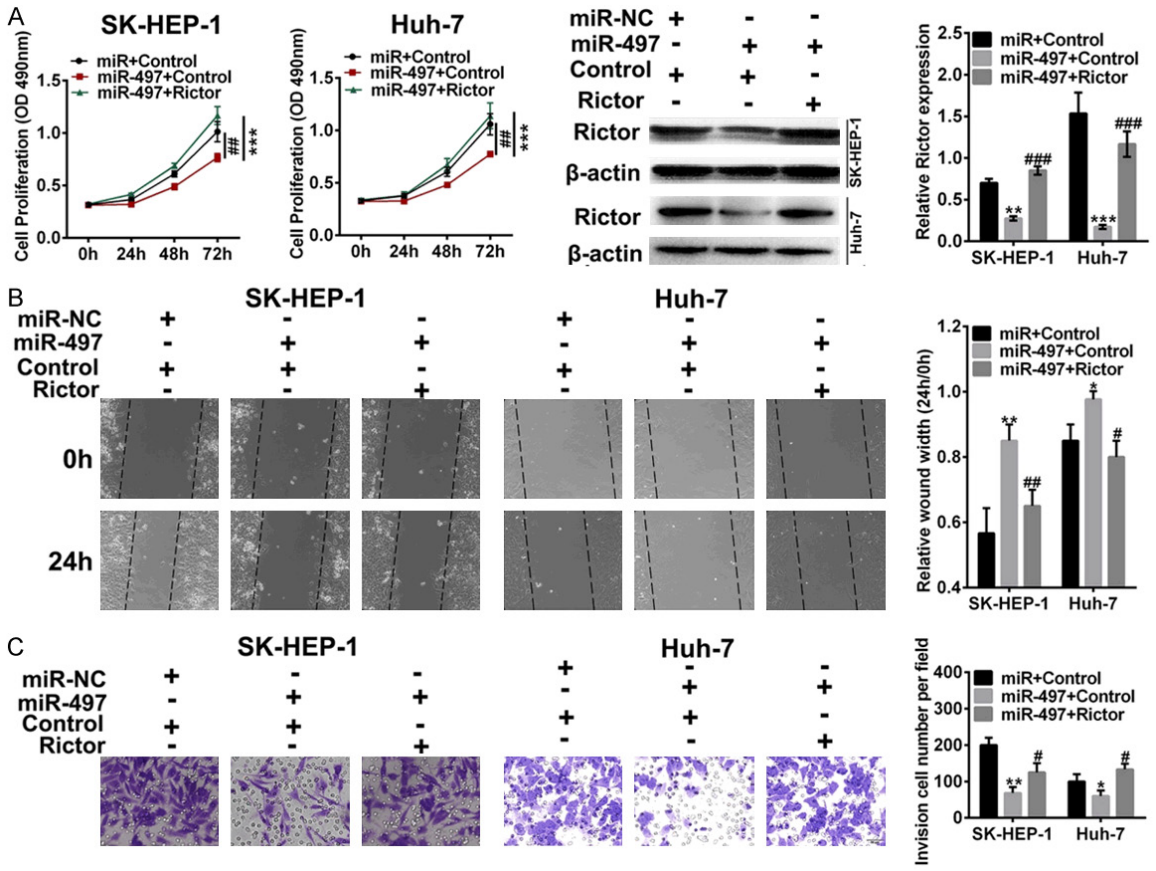

Figure 5. Proliferation, migration and invasion of Rictor and miR-497 overexpression cells (SK-HEP-1 and Huh-7). A. Proliferation of SK-HEP-1 and Huh-7 cells detected by MTT assay. Rictor expression was analyzed by RT-PCR and Western blotting ofSK-HEP-1 and Huh-7 cells. B. Migration of SK-HEP-1 and Huh-7 cells in the wound healing assay. C. Invasion of SK-HEP-1 and Huh-7 cells detected by transwell assay. $^{**}P < 0.01$ compared to miR + control, $^{***}P < 0.001$ compared to miR + control, $^{\#\#}P < 0.01$ compared to miR-497 + control, $^{\#\#\#}P < 0.001$ compared to miR-497 + control.

Rictor overexpression reversed the effects of miR-497 overexpression

To explore the effects of miR-497 on migratory and invasive abilities of cancer cells and to determine its regulatory effects on Rictor, SK-HEP-1 and Huh-7 cells were transfected with miR-497 agomir and/or Rictor plasmid. Proliferation, wound healing, transwell migration, and invasion assays were performed in different groups (miR + Control, miR-497 + control, miR-497 + Rictor). As shown in **Figure 5A**, MTT results showed that the proliferation of cancer cells was inhibited by miR-497 overexpression, and the effects were reversed by Rictor overexpression. miR-497 overexpression remarkably decreased the expression of Rictor, whereas the Rictor overexpression inversed the effects of miR-497. Then the role of miR-497 on cell invasion was evaluated. The invasion assay results showed that the miR-497 overexpression inhibited the invasion ability, and the effects were inversed by Rictor overexpression (**Figure 5B**). Furthermore, the wound healing assay showed that miR-497 overexpression inhibited the migration ability, and the inhibition effects were inversed by Rictor overexpression (**Figure 5C**). Taken together, these results suggest that miR-497 overexpression inhibits SK-HEP-1 and Huh-7 cell proliferative and invasive ability, while Rictor reverses the effects.

miR-497 regulated Akt signaling pathway by targeting Rictor

As our previous results demonstrated that miR-497 inhibited SK-HEP-1 and Huh-7 cell proliferation, migration and invasion by targeting Rictor, and other research showed that Rictor directly regulates the phosphorylation of Akt at

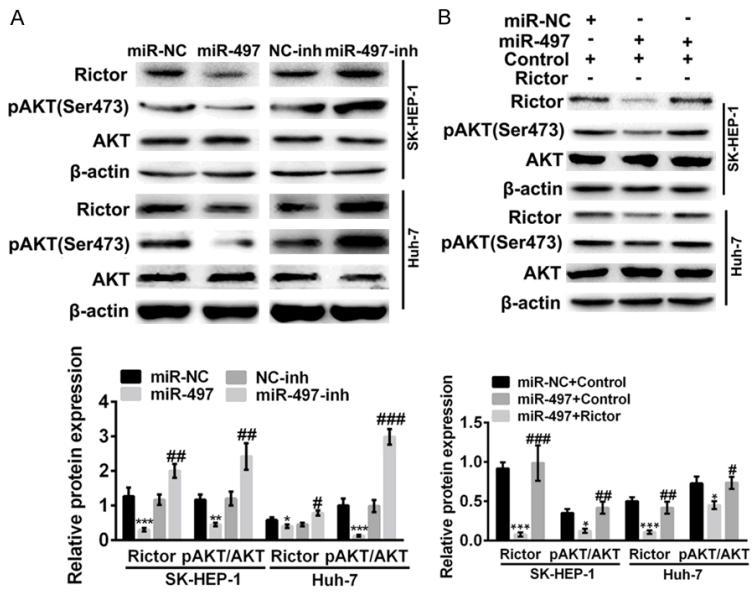

Figure 6. Levels of pAKT (Ser473), total AKT, and Rictor in SK-HEP-1 and Huh-7 cells. A. Cells transfected with miR-497 and/or Rictor by western blotting. B. Cells transfected with miR-497, miR-497-inh or negative control by western blotting. $^{**}P < 0.01$ compared to miR-NC (or miR-NC + control for B), $^{***}P < 0.001$ compared to miR-NC (or miR-NC + controlfor B), $^{\#}P < 0.05$ compared to NC-inh (or miR-497 + control for B), $^{\#\#}P < 0.01$ compared to NC-inh (or miR-497 + control for B), $^{\#\#\#}P < 0.001$ compared to NC-inh (or miR-497 + control for B).

Ser-473, we further explored whether the Akt signaling pathway was involved in miR-497-induced cell growth inhibitory effects. We analyzed the phosphorylation of Aktor total Akt in miR-497 overexpression (miR-497) or miR-497-Inh transfected cells. As shown in **Figure 6A**, miR-497 overexpression decreased the expression of Rictor and pAKT (Ser-473) but had no effects on the expression of total AKT, miR-497 inhibitor (miR-497-inh) led to the opposite effects of miR-497 expression. When cells were co-transfected with miR-497 and Rictor, the level of Rictor, pAKT/AKT was significantly increased compared with miR-497 overexpression group ($P < 0.01$ vs miR-497 + control) and close to controls (miR-NC + Control).

Discussion

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and displays vascular abnormalities and active metastasis [1]. Accumulating studies indicate that MicroRNAs (miRNAs) play important roles in tumorigenesis, metastasis and prognosis in multiple cancers including HCC [9]. Researchers found a general downregulation of miRNAs in human tumors compared with normal tissues [11] and miR-497 downregulation was found in various tumors including HCC [16]. In our studies, we found that overexpression of miR-497 inhibited the proliferation, invasion, and metastasis of human hepatoma cells (SK-HEP-1, Huh-7), which is consistent with previous report [14]. Additionally, we demonstrated that Rictor was a direct target of miR-497.

Rictor, a central component of the rapamycinin sensitive complex of mTOR (mTORC2) which is involved in various cellular processes, is up-regulated in several human cancers and related to a poor prognosis [6]. Researchers found that inhibition of Rictor (mTORC2 component) inhibits tumor growth in pancreatic cancer models [5], and gastric cancer [7]. However, the role of Rictor in HCC progression is still largely unknown. Our results indicated that the growth inhibitory effects of miR-497 were reversed by Rictor overexpression, which indirectly confirmed that Rictor was the target of miR-497 and related with HCC severity. Previous studies demonstrated that Rictor or mTOR directly regulated the phosphorylation of Akt at Ser-473 [17]. The Akt is activated in many human cancers and inhibition of Rictor inhibits Akt activity [18]. Another study showed that Rictor overexpression in melanocytes disrupted the negative feedback of activated Akt and stimulate melanoma proliferation [19]. The level of total and phosphorylated Akt was related to the severity of gliomas, and Rictor plays an important role in proliferation of glioblastoma [20]. Furthermore, altering Rictor inhibits tumor progression in prostate cancer model [21]. In this study, miR-497 mimics and inhibitor were used to demonstrated that miR-497 could inhibit cell proliferation, migration and invasion by targeting Rictor, and Rictor further regulating the phosphorylation of Akt. These results were in accordance with other studies.

MiR-497 is an important regulator in tumor progression including HCC, but the detail mechanism has not been sufficiently reported so far.

Our research demonstrated that miR-497 inhibits the proliferation, invasion, metastasis of hepatoma cells *in vitro* by target Rictor/Akt signal pathway. Therefore, miR-497 or Rictor can be potential targets for treatment of HCC. However, the effects of miR-497 on the HCC *in vivo* will be further investigated to confirm its regulation in HCC progression. If the results of *in vivo* research are in accordance with *in vitro* results, miR-497 may be an important biomarker for HCC diagnosis and a potential treatment target.

Disclosure of conflict of interest

None.

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