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MicroRNA-181a inhibits autophagy by targeting Atg5 in hepatocellular carcinoma

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1. ABSTRACT

Available evidence suggests that autophagy may serve as a tumor suppressor in cases of chronic liver disease and liver cirrhosis and that autophagic deficiency may lead to hepatocellular carcinoma (HCC). Recent studies suggested that the development of several tumor types could be regulated by microRNA-181a. However, the role of miR-181a in the autophagy of HCC remains unclear. In this study, we aimed to investigate the role of miR-181a in the autophagy of HCC. We found that the mRNA expression of miR-181a is higher but the level of autophagy is lower in human HCC compared to normal liver tissue. A luciferase assay confirmed that Atg5 is the target gene of miR-181a. Moreover, the results showed that an miR-181a sponge increased apoptosis in HepG2 cells and reduced the growth of tumors in a HepG2 cell xenograft tumor model. In conclusion, these results suggest that miR-181a can inhibit autophagy in HCC by targeting Atg5, resulting in decreased apoptosis of HCC cells and increased tumor growth.

2. INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignancy of hepatocytes. It accounts for approximately 90% of primary liver cancers and is a leading cause of death from cancer worldwide (1). A defective autophagy response contributes to the development process of HCC (2). However, other researchers proposed that autophagic deficiency might promote HCC (3). Autophagy is a conserved intracellular degradation process for cell survival under conditions of starvation, stress, or infection (4). Thus, it is still necessary to elucidate the role of autophagy in HCC and how autophagy is regulated in HCC.

Interestingly, microRNA-181a (miR-181a) was found to inhibit autophagy in several tumors, such as breast cancer, gastric cancer, and neuroblastoma (5–7). One target of miR-181a in suppressing autophagy of tumor cells is Atg5 (5). Atg5 is a gene that encodes autophagy protein 5, which is necessary for autophagosome elongation in autophagy (8).

However, the role of miR-181a in HCC autophagy remains unclear. A recent study found that in HCC, miR-181a was up-regulated, which may be related to HCC pathogenesis (9). Thus, we will investigate the role of miR-181a in HCC autophagy in this study.

3. METHODS

3.1. Cell culture

Human hepatocellular cancer HepG2 cells were purchased from the China Center for Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone) supplemented with 10% fetal bovine serum (FBS, HyClone) and antibiotic solution (100 μg/ml streptomycin and 100 IU/ml penicillin), in a humidified incubator at 37°C with 5% CO_2. HepG2 cells were seeded into a 6-well plate. Cells from 80% confluent cultures were incubated with adenovirus-miR-181a-sponge to down-regulate miR-181a or with adenovirus-sponge controls (MOI=50) (Hanbio, China) for 2 h. The cells were cultured with new medium for 36 h and were then assessed by western blotting and flow cytometry.

3.2. Hepatocellular carcinoma tissue

Human HCC tissue was obtained at surgery, and control tissue was from subjects who underwent liver resection for hepatic hemangioma. Samples for quantitative real-time PCR and western blotting were washed in normal saline and stored at -80°C.

3.3. Quantitative real-time PCR

Total RNA was extracted from frozen tissue using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. cDNA was synthesized with reverse transcriptase (Fermentas, Glen Burnie, MD, United States).

The real-time PCR was performed using an Applied Biosystems 7900 (Thermo, USA) and SYBR Green real-time PCR master mix (Toyobo, Japan) according to supplier's recommendations. The 10 μl PCR reactions (each containing 5 μl of SYBR, 0.2 μl of forward and 0.2 μl of reverse primers, 4 μl of cDNA, and 0.6 μl of water) underwent 5 min at 95°C, 50 cycles of 10 s at 95°C and then 30 s at 60°C, followed by 10 s at 95°C, 10 s at 60°C and 30 s at 40°C. The primers were as follows: human miR-181a F, 5'- ACACTCCAGCTGGGAACATTCAACGCTGTCG -3' and R, 5'- CTCAACTGGTGTCGTGGAGTC GGCAATTCAGTTGAGACTCACCG -3'; β-actin F, 5'- CTCGCTTCGGCAGCACA -3' and R, 5'- AACGCTTCACGAATTTGCGT -3'. The threshold cycle number (Ct) was determined for all PCR reactions. Levels of mRNA expression for each gene were calculated using the $2^{-\Delta\Delta CT}$ method (10).

3.4. Western blotting

HCC tissue samples or HepG2 cells were directly lysed in RIPA buffer (Beyotime, China). Aliquots of protein extracts (50 μg) were separated by SDS-PAGE on a 20% gel. Subsequently, the protein was electrophoretically transferred onto a PVDF membrane (Millipore, USA) and was blocked with TBS-TWEEN 20 (TBST) containing 5% skim milk. The membranes were incubated with mouse anti-human P62 (Abcam, UK), rabbit anti-human LC3 (Sigma, USA), rabbit anti-human Atg5 (CST, USA), and rabbit anti-human GAPDH (CST, USA) overnight at 4°C, followed by horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (Sigma, USA) for 2 h. Finally, the blots were developed with a chemiluminescent reagent (Pierce Biotechnology, USA).

3.5. Luciferase assay

HepG2 cells were transfected with an adenovirus encoding miR-181a or with a control adenovirus. Human Atg5 with a wild-type (WT) or mutant (Mut) version of each potential miR-181a binding site was generated and fused to the luciferase reporter vector psi-CHECK-2 (Promega, USA). The full-length WT 3' untranslated region (UTR) containing the predicted miR-181a targeting site and the mutant (MUT) 3'-UTR of Atg5 were amplified and cloned into the psi-CHECK-2 vector (Promega, USA). HEK293T cells were seeded in a 6-well plate and cultured until 80% confluence. The luciferase reporter vectors containing WT or Mut 3'-UTR were cotransfected with miR-181a or control into HEK293T cells using Lipofectamine™ 2000 (Invitrogen, USA). The cells were lysed at 36 h after transfection, and the activity levels of firefly luciferase and renilla luciferase were measured by a dual-luciferase reporter system (Promega, E1910). The data shown are representative of three independent experiments.

3.6. Flow cytometry assay

The apoptosis of HepG2 cells was detected by flow cytometry. HepG2 cells were washed twice with PBS and then centrifuged (1000 rpm) for 5 min. These cells (2×10^5) were incubated with 100 μl of binding buffer and 5 μl of Annexin V-FITC for 20 min. Subsequently, 3 μl of propidium iodide was added, and the cells were incubated for 10 min in the dark at room temperature. After 400 μl of binding buffer was added in with the cells, they were analyzed on a flow cytometer (BD Biosciences, USA) with a 488-nm laser. The emissions were captured at 530 nm and 575 nm.

3.7. Nude mouse xenograft model

BALB/c nude mice aged 5–8 weeks and weighing 18–20 g were purchased from Shanghai SLAC

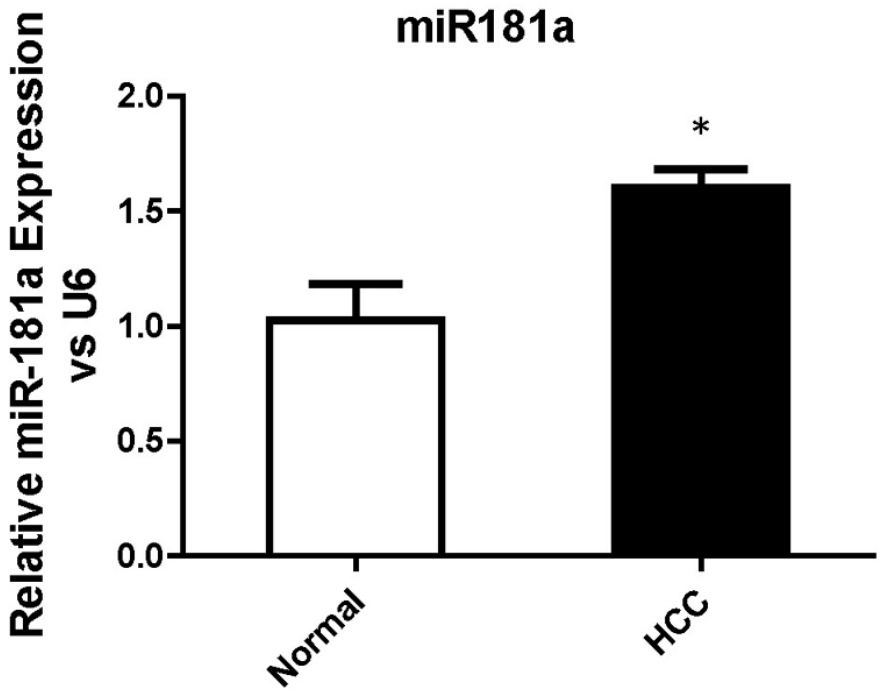

Figure 1. miR-181a mRNA expression is increased in human HCC tissue. The mRNA expression of miR-181a in human HCC tissue was significantly higher than that in control tissue.

Laboratory Animal Co., Ltd. (Shanghai, China). The mice were housed under pathogen-free conditions with free access to rodent chow and water at 20–22°C with a 12-hour light-dark cycle. Then, HepG2 cells ($1–5\times10^6$) were injected subcutaneously into each mouse. The tumor volume in each mouse was measured once a week. Tumor volume was calculated as follows: $V = 1/2\times a\times b^2$ (a= tumor length, b=tumor width). After 4 weeks, all mice were sacrificed by cervical dislocation under ether anesthesia, and then the tumors were weighed.

3.8. Statistical analysis

Data were expressed as the mean±standard deviation. Significant differences were evaluated by Student's t-test (two-tailed). P-values<0.05 were considered statistically significant. All statistical analyses were performed using IBM SPSS software, ver. 21.0 (IBM Co., Armonk, NY, USA).

4. RESULTS

4.1. miR-181a mRNA expression is increased in human HCC tissue

The mRNA expression of miR-181a in human HCC tissue is 1.6±0.1, which is significantly higher than the level in controls (1.0±0.2) (Figure 1). This suggests that miR-181a is increased in HCC compared with non-HCC tissue.

4.2. Autophagy is decreased in human HCC tissue

The protein expression of LC3, P62 and Atg5 in human HCC tissue was assessed by western blotting. The expression levels of LC3-II (0.48±0.01) and Atg5 (0.38±0.01) were lower than those in normal liver tissue (LC3-II=1.0±0.1, Atg5=1.0±0.0) (Figure 2). The expression of P62 (1.6±0.01) was higher than that in normal liver tissue (P62=1.0±0.00). Thus, autophagy is decreased in human HCC tissue compared with control tissue.

4.3. miR-181a inhibition up-regulates autophagy in HepG2 cells

In HepG2 cells transfected with an miR-181a sponge, the protein expression of LC3, P62 and Atg5 was assessed by western blotting. The expression levels of LC3-II (1.56±0.0.1) and Atg5 (2.18±0.01) were increased and that of P62 (0.51±0.01) was decreased compared with controls (LC3-II=1.0±0.01, Atg5=1.0±0.01, P62=1.0±0.1) (Figure 3). Thus, miR-181a inhibition up-regulates autophagy in HepG2 cells.

4.4. miR-181a binds to the 3'-UTR of Atg5

The targeting of miR-181a was validated by a luciferase assay. The activity of WT Atg5 in miR-181a-transfected cells was decreased (0.8±0.1) compared with control cells (3.5±0.1). In contrast, the activity

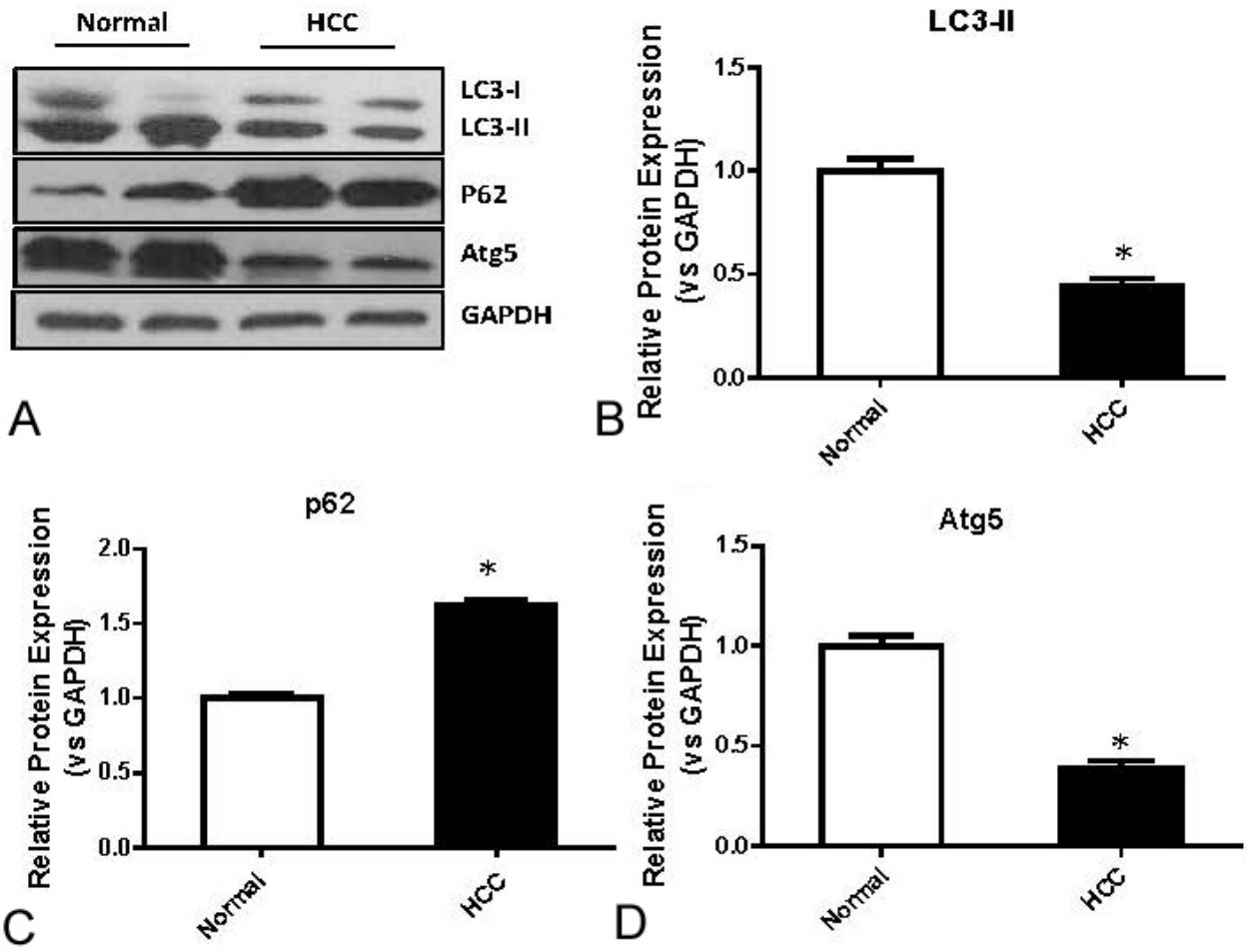

Figure 2. Autophagy is decreased in human HCC tissue. The protein expression levels of LC3 and Atg5 in human HCC tissue were lower, while that of P62 was higher, than those in normal liver tissue. *: P<0.05.

of mutant Atg5 in miR-181a-transfected cells was significantly increased compared with the WT group (4.1±0.5), which was similar to the control (3.8±0.2). This result suggests that miR-181a can bind to the 3'-UTR of Atg5 and that Atg5 is the target gene of miR-181a in HepG2 cells (Figure 4).

4.5. miR-181a inhibition promotes the apoptosis of HepG2 cells

The apoptosis of HepG2 cells was assessed by flow cytometry. In HepG2 cells transfected with an miR-181a sponge, apoptosis was significantly increased (8.9%±0.3%) compared with controls (3.1%±0.2%) (Figure 5). This result suggests that miR-181a inhibition could promote the apoptosis of HepG2 cells.

4.6. miR-181a inhibition inhibits tumor growth

In mice injected with miR-181a sponge-transfected HepG2 cells, the tumor volume (0.07±0.01 mm^3) was significantly smaller than that in controls (0.10±0.02 mm^3) 21 days after injection. This difference was stronger 28 days after HepG2 cell injection (miR-181a sponge-transfected group=0.12±0.02 mm^3; controls=0.21±0.03 mm^3) (Figure 6A).

Twenty-eight days after HepG2 cell injection, the mice were sacrificed and the tumor weight was measured. In mice injected with miR-181a-transfected HepG2 cells, the tumor weight was significantly smaller than that of controls (miR-181a-transfected group=1.5±0.1 g; controls=2.7±0.1 g) (Figure 6B). These results suggested that miR-181a inhibition could inhibit tumor growth.

5. DISCUSSION

In our study, we found that the protein expression levels of LC3 and Atg5 were lower, while that of P62 was higher, in human HCC tissue than in controls. Inhibition of miR-181a increased the expression of LC3 and Atg5. We next found that ATG is a target gene of miR-181a in HepG2 cells. We further demonstrated that inhibition of miR-181a could increase the apoptosis of HepG2 cells and reduce

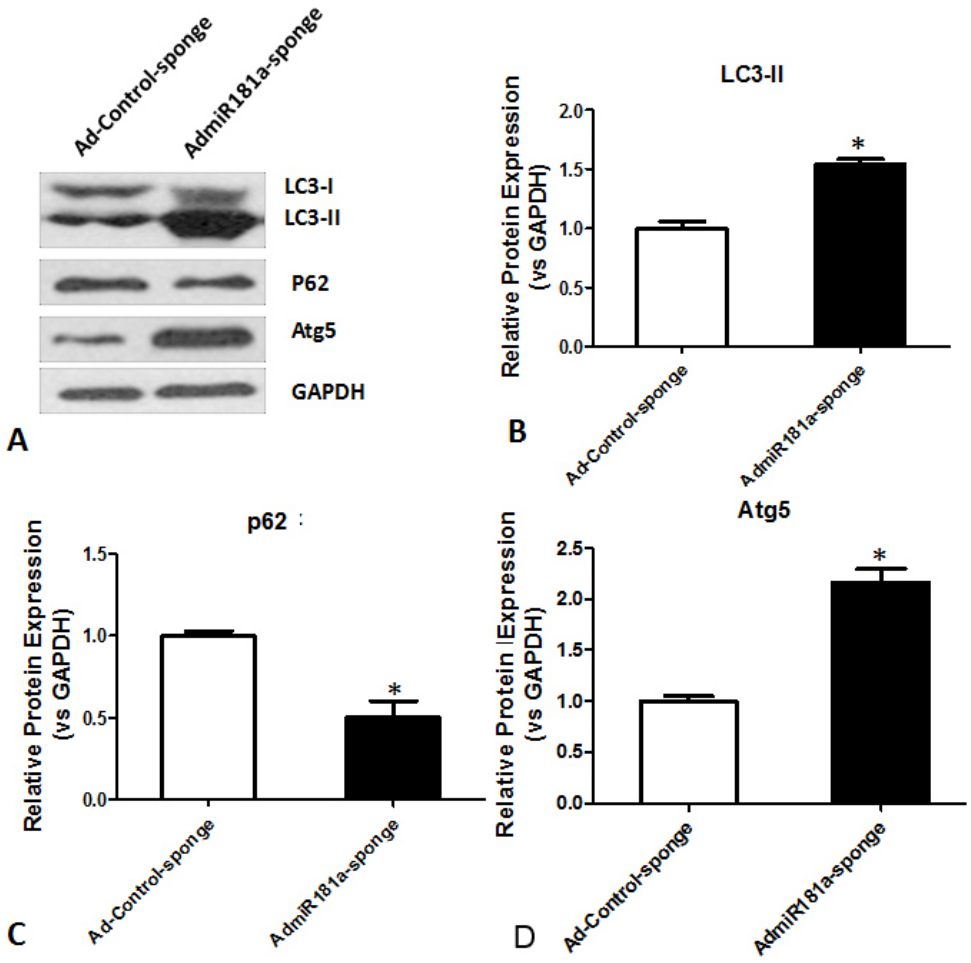

Figure 3. miR-181a inhibition up-regulates autophagy in HepG2 cells. In HepG2 cells transfected with an miR-181a sponge, the protein expression levels of LC3 and Atg5 were increased, but that of P62 was reduced, compared with controls. *: P<0.05. Ad-miR-181a-sponge: HepG2 cells transfected with an miR-181a sponge. Ad-Control-sponge: HepG2 cells transfected with a control sponge.

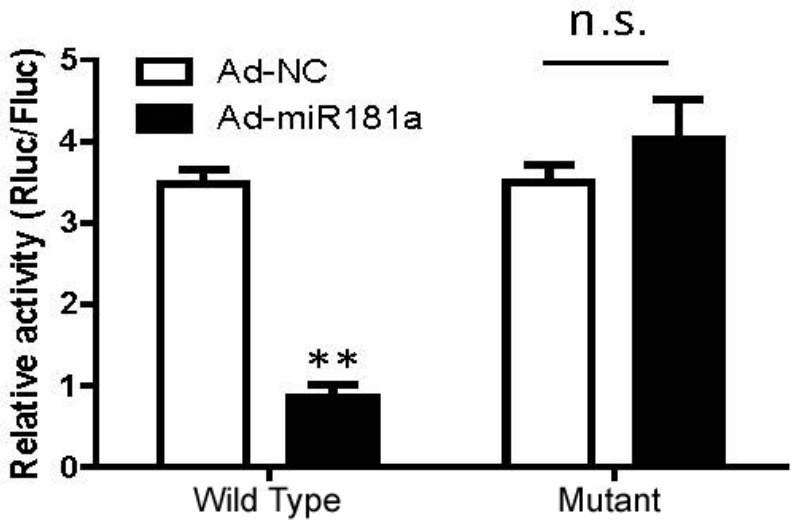

Figure 4. miR-181a binds to the 3'-UTR of Atg5. The activity of WT Atg5 in miR-181a-transfected cells was decreased compared with the control. In contrast, the activity of mutant Atg5 in miR-181a-transfected cells was significantly increased and was similar to the control. *: P<0.0.5. Ad-miR-181a: HepG2 cells transfected with miR-181a. Ad-NC: HepG2 cells transfected with a normal control. WT: Wild-type Atg5 gene with potential miR-181a binding sites. Mutant: Atg5 gene with mutations at potential miR-181a binding sites.

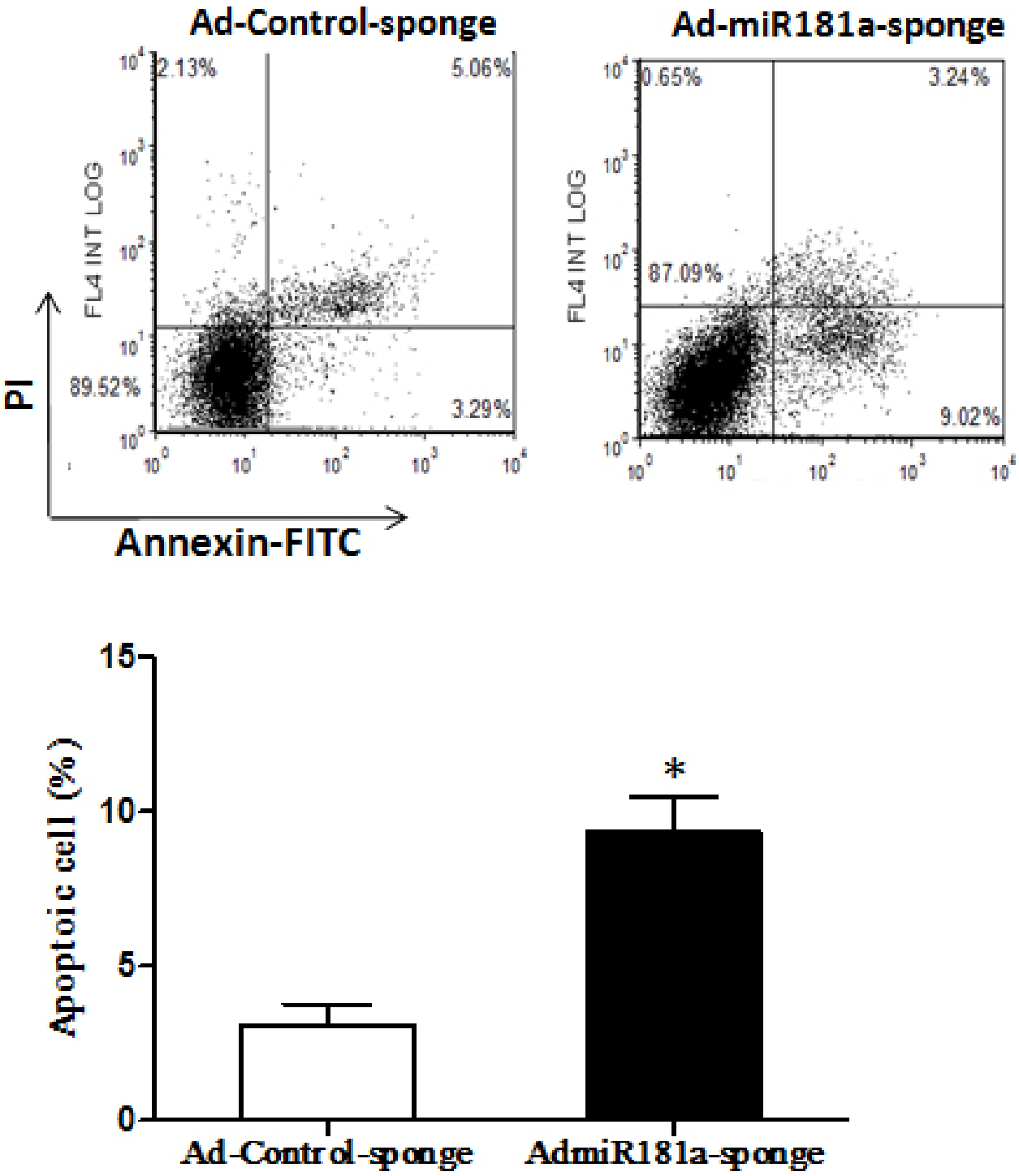

Figure 5. miR-181a inhibition promotes the apoptosis of HepG2 cells. The apoptosis of HepG2 cells was assessed by flow cytometry. In HepG2 cells transfected with an miR-181a sponge, apoptosis was significantly increased compared with controls. *: P<0.05. Ad-miR-181a-sponge: HepG2 cells transfected with an miR-181a sponge. Ad-Control-sponge: HepG2 cells transfected with a control sponge.

tumorigenesis in a nude mouse xenograft model. Our results suggest that miR-181a can promote HCC through inhibiting autophagy by targeting Atg5.

In our study, the protein expression levels of LC3 and Atg5 were decreased in human HCC tissue compared with control tissue. This suggests that autophagy is decreased in HCC. Our result is consistent with findings in a previous study that autophagy may be a suppressor mechanism in HCC (11). Thus, autophagic deficiency promotes tumor growth in HCC. In our study, we further explored the

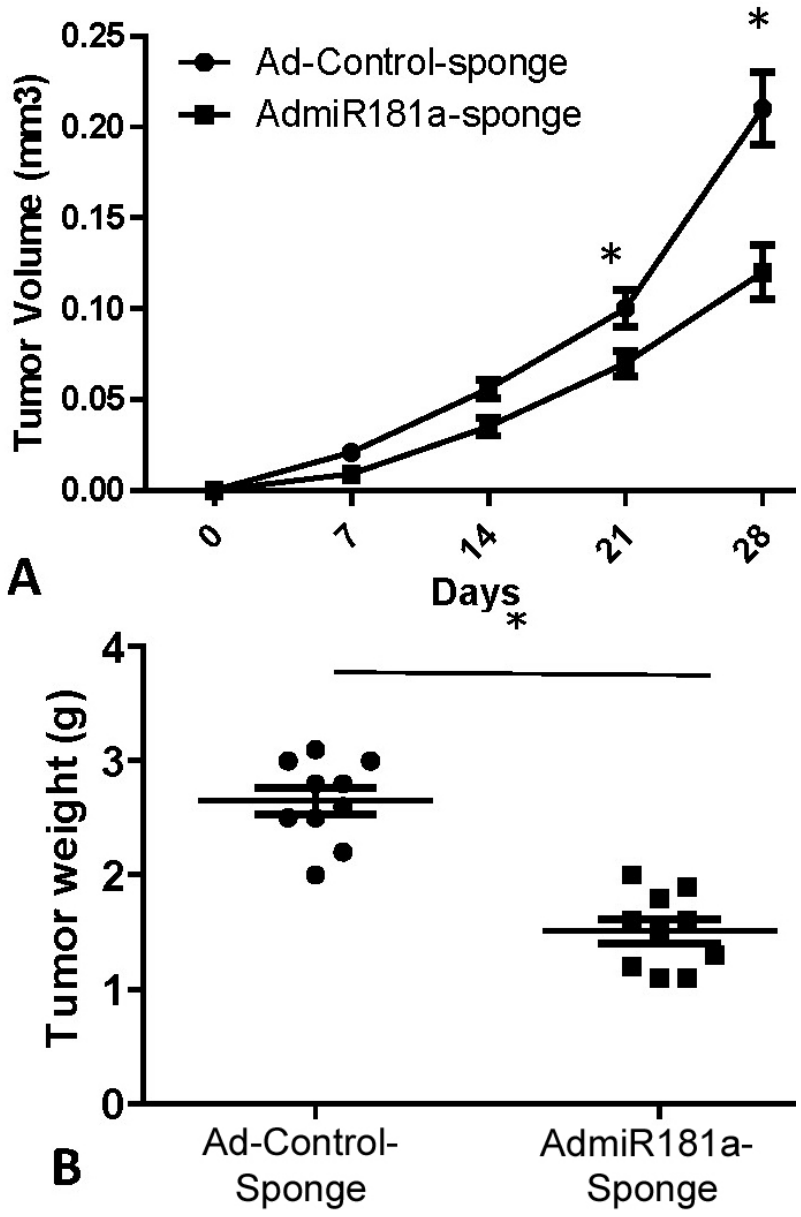

Figure 6. Down-regulation of miR-181a expression inhibits tumor growth. (A) Tumor volume. In mice injected with miR-181a sponge HepG2 cells, the tumor volume was significantly smaller than that in controls 21 days after injection. This difference was stronger 28 days after HepG2 cells injection. (B) Tumor weight. The mice were sacrificed 28 days after the injection of HepG2 cells, and the tumor weight was measured. In mice injected with miR-181a sponge HepG2 cells, the tumor weight was significantly smaller than that of controls. *: P<0.05. Ad-miR-181a-sponge: HepG2 cells transfected with a miR-181a sponge. Ad-Control-sponge: HepG2 cells transfected with a control sponge.

regulation of autophagy, which may provide further knowledge regarding appropriate targets for HCC treatment.

In our study, the expression of miR-181a was increased in HCC tissue compared with control tissue. This is consistent with findings in previous studies (9,12–14). Moreover, the present study found that inhibition of miR-181a could promote the expression of LC3 and Atg5 in HepG2 cells. This suggests that miR-181a may reduce autophagy in HCC cells. Moreover, the luciferase assay showed that miR-181a targets Atg5 in the cells. The Atg5 gene encodes autophagy protein 5, which is required for autophagosome elongation in autophagy (8). Studies by Takamura *et al.* show that deletion of either the Atg5 or Atg7 gene in mice results in the development of hepatomegaly and hepatocellular adenoma (15). This study suggested that Atg5 is essential not only in autophagy but also in preventing carcinogenesis.

It may suppress carcinogenesis through autophagy. Therefore, the results of our study suggest that miR-181a may promote HCC through autophagy inhibition by targeting Atg5 in HCC cells.

We further demonstrated that inhibition of miR-181a could enhance the apoptosis of HepG2 cells and reduce tumor volume and weight after 4 weeks in a tumor xenograft model. This suggests that miR-181a could promote HCC tumorigenesis. On one hand, the increased apoptosis of HepG2 cells may be related to the increased autophagy. A previous study found that autophagy may be related to an increase in apoptosis in HCC (16). Thus, we supposed that the decreased apoptosis of HCC may be due to decreased autophagy by miR-181a inhibition. On the other hand, both the volume and weight of xenograft tumors in the miR-181a inhibition group were smaller than those in the normal group. This suggests that miR-181a may promote HCC. Considering that miR-181a could inhibit autophagy and reduce apoptosis in HCC cells, this contribution of miR-181a to tumor growth may be due to its effect on autophagy and apoptosis.

In conclusion, our study found that miR-181a could inhibit HCC autophagy by targeting Atg5, resulting in decreased apoptosis of HCC cells and increased tumor growth in xenograft. These findings may provide a novel target for HCC treatment.

6. ACKNOWLEDGEMENTS

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Abbreviations: HCC, Hepatocellular cancer; miR-181a, microRNA-181a

Key Words: MicroRNA-181a, Autophagy, Atg5, Hepatocellular Carcinoma

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