Mir10a overexpression aggravates the renal ischemia-reperfusion injury associated with a decrease in PIK3CA

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Research article

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Abstract

Background: To investigate the effect of miR-10a on the renal tissues with ischemia-reperfusion (I/R) injury in rats and explore the underlying mechanisms of miR-10a in the HK-2 cells of hypoxia-reoxygenation.

Methods: The miR-10a level was measured in renal tissues with I/R rats by RT-PCR. In order to research the role of miR-10a in the renal tissues, miR-10 agonist and miR-10a antagonist were used to treat I/R rats. The levels of serum creatinine (Scr) and blood urea nitrogen (BUN) in serum, renal histopathology, apoptosis of cells in renal tissues were analyzed, separately. The phosphatidylinositol 3-kinase (PI3K)/Akt pathway related proteins were measured by Western blot. The HK-2 cell was cultured to study the mechanism of miR-10a in the model of hypoxia-reoxygenation. The dual luciferase reporter gene assay was used to confirm the PI3K p100 catalytic subunit α (PIK3CA) was a target gene of miR-10a.

Results: After renal I/R injury in rats, the miR-10a expression was significantly increased ($p<0.05$). Injection of miR-10a agonist significantly aggravated the injury of renal and raised the apoptosis of cells in renal in rats with renal I/R injury ($p<0.05$). However, administration of miR-10a antagonist obviously improved the injury of renal, decreased the renal cells apoptosis and inhibited the PI3K/Akt pathway activity ($p<0.05$). In vitro experiments, the negative relation between PIK3CA and miR-10a was confirmed. Further, overexpression of miR-10a significantly decreased the proliferation of HK-2 cells, and increased the cells apoptosis via up-regulating PI3K/Akt pathway ($p<0.05$).

Conclusion: miR-10a could aggravate the renal I/R injury associated with a decrease in PIK3CA/PI3K/Akt pathway.

Background

The renal ischemia-reperfusion (I/R) injury is commonly caused in certain renal surgeries, such as sepsis, ischemia and nephrotoxins damage [1]. When renal I/R injury happens, cell homeostasis is destroyed which leads to inflammation and apoptosis [2]. Furthermore, the renal I/R injury can also increase incidence rate of other diseases, such as myocardial infarction, and stroke [3].

Plenty of microRNAs (miRNAs) are known to play critical role in pathogenesis of rats with renal I/R injury [4]. As a member of miR-10 family, miR-10a played an essential role in the process of programmed cell death [5] and many diseases [6]. However, the potential mechanism of miR-10a in renal I/R injury has not been reported.

As known, phosphatidylinositol 3-kinase (PI3K)/Akt pathway takes part in the physiological progress of different biological reactions, for example inflammation and apoptosis [7, 8]. As a target gene of miR-10a, PI3K p100 catalytic subunit α (PIK3CA) plays a critical role in the cisplatin resistance of lung adenocarcinoma circulating tumor cells in PI3K/Akt pathway [9]. Many reports have found that hypoxia/reoxygenation (H/R) injury can activate the PI3K/Akt pathway to protect the renal tubular

epithelial cells [10-12]. However, the association between miR-10a and PIK3CA in renal I/R injury is not clarified.

In the present work, the role of miR-10a was observed in the rats with renal I/R injury and the HK-2 cells with H/R damage. And the role of miR-10a on PIK3CA/PI3K/Akt pathway was also performed to explore *in vitro*.

Methods

Experimental animals

Forty-eight male Sprague-Dawley rats, weighing 220±20g, were fed at 22-24 °C, humidity 50-60%, as well as under a 12-h dark/light cycle with free access to food and water. All experiments were strictly conformed with the National Institutes of Health (NIH Pub. No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of the Second Hospital of Shandong University (No. 20190103-002).

Establishment of renal I/R model

The rats were anesthetized by intraperitoneal injection of 3% sodium pentobarbital (50 mg/kg). According to previous reports [13,14], the renal pedicles were exposed and then the right kidney was removed. The left renal pedicle was clamped for 45 min followed with reperfusion for 24h. Rats in sham group were treated with similar operation except for the occlusion of renal pedicle. After operation, the rats were fed with free access to food and water.

Animal groups

The rats were randomly divided into 6 groups (n=6): 1) Sham group, in which the rats were injected normal saline through the tail vein injection, 1 h before surgery. 2) Renal ischemia-reperfusion group (I/R), in which the rats were subjected to renal ischemia. 3) miR-10a agonist group (miR-10a), in which the rats were administered 10 mg/kg miR-10a agonist (Ribio, Guangzhou, China) through tail vein injection, 1 h before I/R induction. 4) miR-10a agonist negative control group (miR-NC), in which the rats were administered 10 mg/kg miR-10a agonist negative control through tail vein injection, 1 h before I/R induction. 5) miR-10a antagonist group (anti-miR), in which the rats were administered 10 mg/kg miR-10a antagonist (Ribio, Guangzhou, China) through tail vein injection, 1 h before I/R induction. 6) miR-10a antagonist negative control group (anti-NC), in which the rats were administered 10 mg/kg miR-10a antagonist negative control through tail vein injection, 1 h before I/R induction.

Samples Collection

3% sodium pentobarbital (50 mg/kg) was treated via intraperitoneal injection to anesthetize and sacrificed after 24 h reperfusion. Blood samples (5 mL) were taken from the abdominal aorta. Partial

kidneys were stored at -80 °C and partially placed in 4% paraformaldehyde for 24 h, then embedded in paraffin.

RT-PCR

Total RNA samples were extracted according to TRIzol kit (ThermoFisher, Guangzhou, China). The cDNA was synthesised by the TaqMan^{TM} reverse transcription kit (4366596, ThermoFisher). Conditions: 95 ° C for 15 s, 60 ° C for 30 s, 72 ° C for 45 s (50 cycles). The $2^{-\Delta\Delta Ct}$ method was used to analyzed.

The following primers were used: miR-10a-5', CTGGAAAATTTCTGGGCCAA; miR-10a-3', CCAGACTGTCCTCATTCAGAAAAA; U6-5', GACCTCTATGCCAACACAGT; U6-3', AGTACTTGCGCTCAGGAGGA. PIK3CA-5', GCATACATTCGAAAGACC; PIK3CA-3', CTCAGTTATCTTTTCAG; GAPDH-5', TGACTTCAACAGCGACACCCA; GAPDH-3', CACCCTGTTGCTGTAGCCAAA.

Renal function test

The blood samples were collected and centrifuged at 800×g for 10 min. Serum creatinine (Scr) and blood urea nitrogen (BUN) in serum were analyzed by an automatic biochemical.

Hematoxylin-eosin (H&E) Staining

The renal sections (5 μm) were stained with H&E (Solarbio, Wuhan, China). Renal histopathological changes were observed at 400 magnification. The swelling, vacuolation and exfoliation of renal tubular epithelial cells were evaluated using a five-point quantitative scoring method [15]: 0, <10%; 1, 10- 25%; 2, 25-50%; 3, 50-75%; and 4, 75-100%.

Terminal Dexynucleotidyl Transferase (TdT)-mediated dUTP Nick End Labeling (TUNEL)

The cell apoptosis in renal tissues (5 μm) was detected by TUNEL apoptosis assay kit (Solarbio, Wuhan, China). The sections were randomly observed at 400 magnification. The apoptotic nuclei were stained yellow brown or dark brown. Apoptotic index (AI) = (number of apoptotic cells/total cells) × 100%.

Western blot

The concentration of protein sample was measured by a BCA protein quantification kit (Solarbio, Beijing, China). 40 μg samples were mixed with 10% SDS-PAGE electrophoresis before transferred to PVDF membrane (Millipore, Massachusetts, USA). The membranes were blocked with 5% degreased milk powder for 1 h. The primary antibody of each protein was diluted with 5% BSA. And the rabbit anti-rat Bax 1:800, orb224426, Biorbyt, Cambridge, UK Bcl-2 1:800, orb228150, Biorbyt caspase-3 1:800, orb10231, Biorbyt PIK3CA (1:800, orb228203, Biorbyt), PI3K (1:800, orb137259, Biorbyt), p-PI3K (1:800, orb338965, Biorbyt), AKT (1:800, orb213545, Biorbyt), p-AKT (1:800, orb222951, Biorbyt), β-actin (1:2000, orb178392, Biorbyt). All primary antibodies were reacted overnight at 4°C, then incubated with the second antibody goat anti-rabbit Ig G (1:1500, ab6721, Abcam, UK) for 1h.

Cell Culture

HK-2cells (Shanghai Institute of Cell Research), human renal tubular epithelial cells, were cultured in DMEM/F12 with 10% fetal bovine serum and 1% penicillin-streptomycin (GIBCO, Invitrogen, Beijing, China) at 37°C, 5% CO_2.

Establishment of H/R model

Hypoxia induced cell injury model was established as follows: HK-2 cells were cultured in anoxic environment (5% CO_2, 1% O_2, 94% N_2) for 24 h, then transferred to aerobic environment (5% CO_2, 21% O_2, 74% N_2) for 3h.

Cell transfection

Before transfection, the cells were planted into a six-well plate for 24 h. Lipofectamine TM2000 (Invitrogen, Carlsbad, CA) and plasmid (GenePharma, Shanghai, China) were added in a sterile centrifuge tube containing 200 μ l DMEM / F12 for 5 min, separately. The two solutions were mixed for 20 min. Finally, the cells were cultured in six well plates for 6 h. Six groups were structured according to different treatment.) Control group, in which cells were cultured under normal condition for 27 h.) Hypoxia/reoxygenation group (H/R), in which cells injury induced by hypoxia.) miR-10a mimic group (miR-10a), in which cells were transfected with miR-10a mimic (5'-CAAAUUCGGAUCUACAGGGUAUU-3' and anti-5'-UACCCUGUAGAUCCGAAUUUGUG-3) 6 h before H/R induction.) miR-10a mimic negative control group (miR-NC), in which cells were transfected with miR-10a mimic negative control (5'-UUCUCCGAACGUGUCACGUTT-3' and anti-5'-UACCCUGUAGAUCCGAAUUUGUG-3') 6 h before H/R induction.) miR-10a inhibitor group (anti-miR), in which cells were transfected with miR-10a inhibitor (5'-CACAAAUUCGGAUCUACAGGGUA-3') 6 h before H/R induction.) miR-10a inhibitor negative control group (anti-NC), in which cells were transfected with miR-10a inhibitor negative control (5'-CAGUACUUUUGUGUAGUACAA-3') 6 h before H/R induction.

CCK-8

Cells (2×10^4 cells/mL) were cultured at 37 °C, 5% CO_2 for 24 h, 48 h, 72 h, and 96 h. 10 μL CCK-8 solution (Beyotime, Wuhan, China) was added into each well for 4 h. The absorbance (OD) value measured at 450 nm.

Clone formation

2 mL Cells (250 cells/mL) were planted into six-well plate, then cultured at 37 °C, 5% CO_2 for 2 weeks. And changed the fresh medium every three days. Methanol was used to fix cells and 1mL Giemsa solution (Beyotime) was used to stain for 35 min, then washed twice with ultrapure water.

Flow cytometry

After cultured 24 h, the cells collected and washed with pre-cooled 1×PBS at 4°C. The cells were suspended with 200 μL of 1× Binding Buffer and then added into 5μL FITC labeled for 15 min at 37 °C. Before detection, 150μL of 1×Binding Buffer was added into 5μL propidium iodide (PI) staining. The cells were placed in a flow cytometry and analyzed by Cell Quest software.

Dual luciferase reporter assay

The correction of miR-10a and PIK3CA was predicted RegRNA 2.0. miR-10a mimics or control scrambled were transfected into HK-2 cells for 48 h using Lipofectamine 2000 at a 2:1 molar ratio. The activity of cells luciferase was tested by dual-luciferase assay (Solarbio, Wuhan, China).

Statistical Analysis

Data analysis was performed by SPSS 19.0, and the results were represented as mean ± standard deviation (ˆX ± SD). The t-test was used for two groups; the one-way variance analysia and Tukey test were used for multiple groups. $p < 0.05$ represented significant difference.

Results

miR-10a was overexpressed in renal I/R rats

As showed in Figure 1A, the expression of miR-10a was obviously increased in I/R group compared with sham group ($p<0.05$). RT-PCR was used to detect the expression of miR-10a in each group, the results showed in Figure 1B. The expression of miR-10a was the highest in miR-10a group and the lowest in sham group among groups ($p<0.05$). Further, the Scr and BUN levels were significantly increased after renal I/R injury when compared to sham group ($p<0.05$, Figure 1C,D). Compared with I/R group, the levels of Scr and BUN were obviously raised in miR-10a group, and notably decreased in anti-miR group ($p<0.05$, Figure 1C,D).

miR-10a overexpression aggravated renal injury in renal I/R rats

As showed in Figure 2A, the renal injury score significantly increased after renal I/R injury when contrasted to sham group ($p<0.05$). The renal injury was further exacerbated in miR-10a group compared with I/R group ($p<0.05$). However, the renal injury was obviously improved in anti-miR group compared to I/R group ($p<0.05$). The cell apoptosis of renal tissues in each group were showed in Figure 2B. Among groups, the apoptotic index (AI) was the highest in miR-10a group and the lowest in sham group ($p<0.05$). Compared with I/R group, the AI was notably decreased in anti-miR group ($p<0.05$). These data suggested that miR-10a overexpression aggravated renal injury in renal I/R rats.

miR-10a overexpression inhibited the PIK3CA/PI3K/Akt pathway

In Figure 3A, compared with sham group, the expression of Bax and Caspase-3 was significantly increased, the expression of Bcl-2 was obviously decreased after I/R injury ($p<0.05$). Compared with I/R

group, the Bax and Caspase-3 expression were further raised, the Bcl-2 expression was further declined in miR-10a group ($p<0.05$). However, the Bax and Caspase-3 expression were notably decreased, the Bcl-2 expression was significantly increased in anti-miR group when compared to I/R group ($p<0.05$). In Figure 3B, the PIK3CA, p-PI3K and p-Akt expression was obviously declined after renal I/R injury compared with sham group ($p<0.05$). In miR-10a group, the level of PIK3CA, p-PI3K and p-Akt was further declined. Nevertheless, the PIK3CA, p-PI3K and p-Akt expression was increased in anti-miR group contrasted to I/R group ($p<0.05$).

miR-10a overexpression inhibited H/R induced HK-2 cells proliferation

In order to confirm miR-10a overexpression could control renal cells proliferation, miR-10a overexpression and lowexpression were established in H/R induced HK-2 cells. RT-PCR was used to test the expression of miR-10a in each group (Figure 4A). The cells proliferation was showed in Figure 4B,C. Similar with results *in vivo*, the proliferation was significantly increased in miR-10a group compared with H/R group ($p<0.05$). However, the proliferation was significantly decreased in anti-miR group compared with H/R group ($p<0.05$).

miR-10a overexpression increased H/R induced HK-2 cells apoptosis

The HK-2 cells apoptosis were analyzed in Figure 5A. Among groups, the apoptosis was the highest in miR-10a group and the lowest in control group ($p<0.05$). Compared with H/R group, the apoptosis was notably decreased in anti-miR group ($p<0.05$). For apoptosis related proteins, the results were consistent with that *in vivo* (Figure 5B). Compared with control group, the expression of Bax and Caspase-3 was significantly increased, the expression of Bcl-2 was obviously decreased after H/R injury ($p<0.05$). Compared with H/R group, the Bax and Caspase-3 expression were further raised, the Bcl-2 expression was further declined in miR-10a group ($p<0.05$). However, the Bax and Caspase-3 expression were notably decreased, the Bcl-2 expression was significantly increased in anti-miR group when compared to H/R group ($p<0.05$).

miR-10a targeted PIK3CA to regulate PI3K/Akt pathway in H/R induced HK-2 cells

The PIK3CA/PI3K/Akt pathway related proteins in H/R induced HK-2 cells were also observed in Figure 6A. The expression of PIK3CA, p-PI3K and p-Akt was obviously declined after H/R injury compared with control group ($p<0.05$). In miR-10a group, the expression of PIK3CA, p-PI3K and p-Akt was further declined. But, the level of PIK3CA, p-PI3K and p-Akt was obviously increased in anti-miR group contrasted to H/R group ($p<0.05$). Furthermore, a dual luciferase reporter system confirmed miR-10a targeted PIK3CA (Figure 6B). These data suggested that PIK3CA was a target gene of miR-10a and miR-10a aggravated renal injury via regulating PIK3CA/PI3K/Akt pathway.

Discussion

At present, renal I/R injury is known as a primary reason of acute kidney failure and renal damage [16, 17]. In the present study, it was identified that miR-10a overexpression exacerbated renal I/R injury through promoting renal cells apoptosis and inhibiting PIK3CA/PI3K/AKT signaling pathway. The experiments *in vitro* were consistent with the results *in vivo*. The dual luciferase assay confirmed that PIK3CA was a target of miR-10a.

Increasing evidences suggest miRNAs play a crucial role in occurrence and development of diseases. For renal I/R injury, miRNAs play an important role in different aspects, such as apoptosis and inflammation [18, 19]. Xie et al reported that miR-128-3p improved the acute kidney injury via inhibiting cell apoptosis [18]. Previous study showed that miR-10awas played an important role in extracellular matrix accumulation in the kidney of diabetic mellitus in which the level of miR-10a decreased after high fat diet administration [20]. Another research reported that the level of urinary miR-10a was positively correlated with the degree of kidney injury induced by renal I/R. Furthermore, compared to healthy donors, the urinary miR-10a levels were substantially elevated in focal segmental glomerulosclerosis patients [21]. In this study, we found that miR-10a expression was remarkably up-regulated in renal tissues with renal I/R rats. miR-10a overexpression further aggravated the renal injury *in vivo* and *in vitro*. Furthermore, the levels of apoptosis related proteins Bax and Caspase3 were markedly increased. All the results suggested that miR-10a overexpression could promote the renal damage and apoptosis in renal I/R injury.

PI3K/Akt signaling pathway plays an important role in the regulation of cell proliferation and survival, and has been proved to be related to the protection of brain and kidney from I/R injury by reducing oxidative stress and inflammatory response [18, 22]. Moreover, the phosphorylation of PI3K and Akt was decreased in renal tissues by I/R, and activating of PI3K/Akt pathway could ameliorate renal I/R injury [23, 24]. Consistent with previous reports, the PI3K/Akt pathway was inhibited in renal I/R injury and H/R cells in this research. Furthermore, miR-10a overexpression was further inhibiting the PI3K/Akt pathway *in vivo* and *in vitro*. More importantly, miR-10a targeted PIK3CA to regulate PI3K/AKT signaling pathway in renal I/R injury.

Conclusions

In summary, the present work explored miR-10a was increasingly expressed in renal I/R injury. Overexpression of miR-10a aggravated renal injury and inhibited PIK3CA/PI3K/Akt pathway *in vivo* and *in vitro.* The results provide new insight into the interaction of miR-10a and renal I/R injury.

Abbreviations

I/R: ischemia-reperfusion ; Scr: serum creatinine (Scr); BUN: and blood urea nitrogen; PI3K: phosphatidylinositol 3-kinase; PIK3CA: PI3K p100 catalytic subunit; H/R: hypoxia/reoxygenation; AI: Apoptotic index

Declarations

Ethics approval and consent to participate

All animal experiments were conducted in accordance with the NIH guidelines (NIH Pub. No. 85-23, revised 1996) and have been reviewed and approved by the Animal Protection and Use Committee of the University of Qingdao Fuwai Cardiovascular Hospital.

Consent for publication

All co-authors have seen and agree to the manuscript for publication. We certify that the submission is original work and is not under review at any other publication.

Competing interests

The authors declare that they have no competing interests, and all authors confirm its accuracy.

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No.

Author Contributions

DX carried out the experimental work and the data collection and interpretation. WL participated in the design and coordination of experimental work, and acquisition of data. DX and GW carried out the study design, the analysis and interpretation of data and drafted the manuscript. TZ participated in the study design, data collection, analysis of data and preparation of the manuscript. All authors read and approved the final manuscript.

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None.

Availability of data and materials

The figures data used to support the findings of this study were supplied by T Zhang under license and so cannot be made freely available. Requests for access to these data should be made to T Zhang, bgpzopzk@163.com.

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Figures

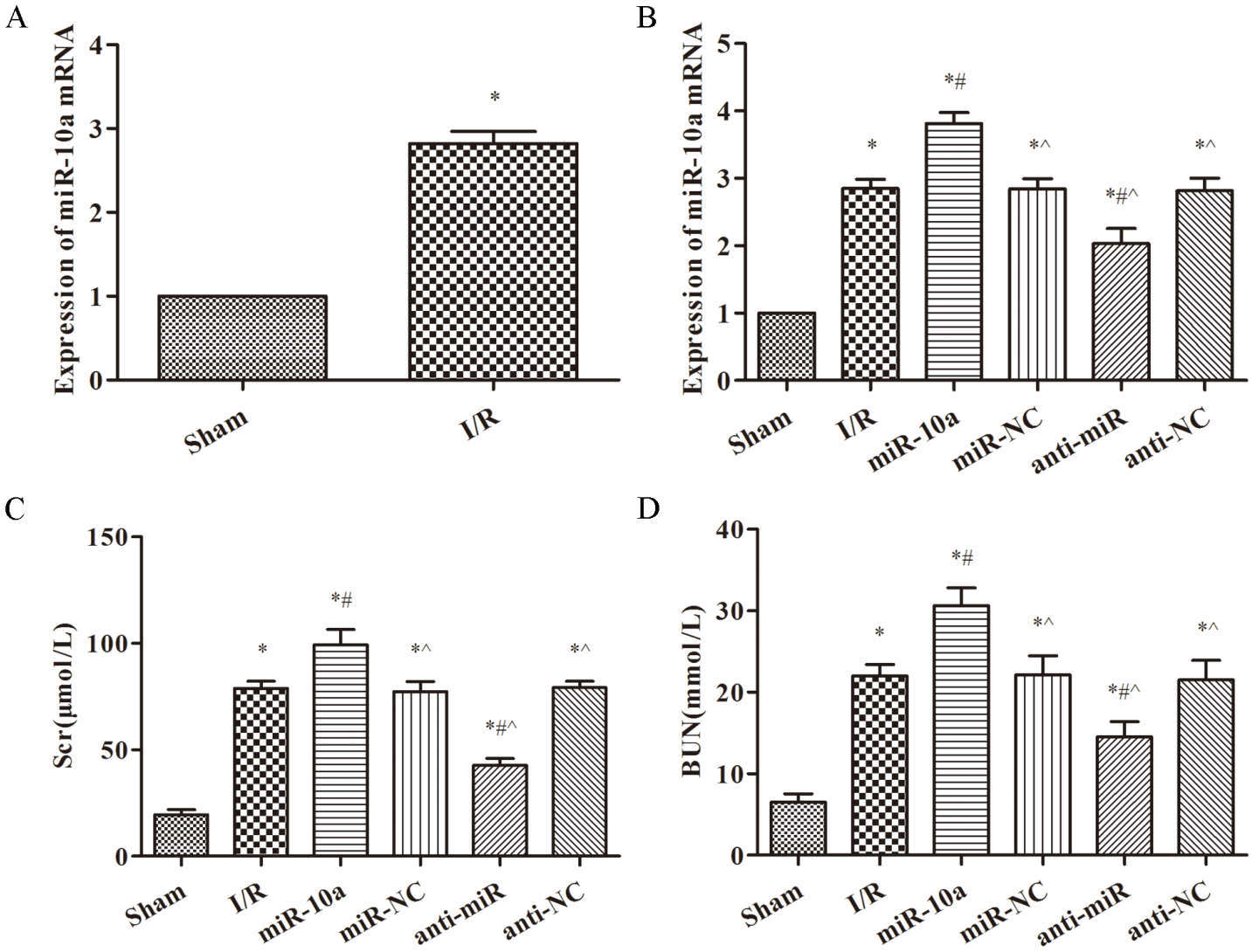

Figure 1

miR-10a expression was increased in kidney tissues with renal I/R injury rats and its overexpression aggravated the renal function in renal I/R rats. (A, B) RT-PCR was used to detect the miR-10a expression in kidney tissue; (C, D) Serum creatinine (Scr) and urea nitrogen (BUN) levels were measured in serum. Renal ischemia-reperfusion group (I/R), miR-10a agonist group (miR-10a), miR-10a agonist negative control group (miR-NC), miR-10a antagonist group (anti-miR), miR-10a antagonist negative control group (anti-NC). *p < 0.05 compared to Sham group; #p < 0.05 compared to I/R group; ^p < 0.05 compared to the miR-10a group (n=6).

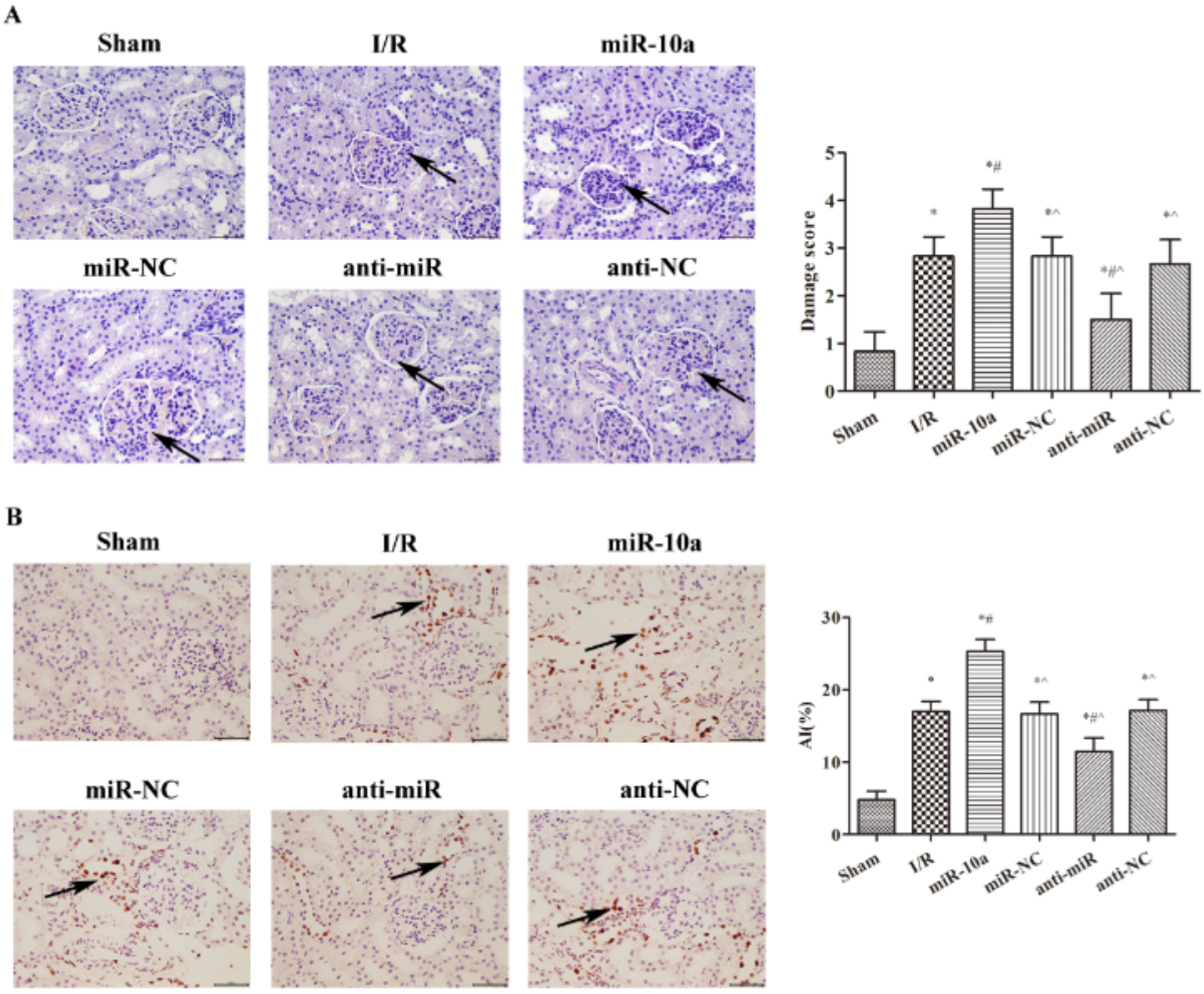

Figure 2

miR-10a overexpression aggravated renal tissue injury and increased cells apoptosis in renal tissues. (A) HE staining was used to observe the kidney tissue pathological changes. (B) TUNEL staining was used to observe the apoptosis of renal cells. *p < 0.05 compared to Sham group; #p < 0.05 compared to I/R group; ^p < 0.05 compared to the miR-10a group (n=6).

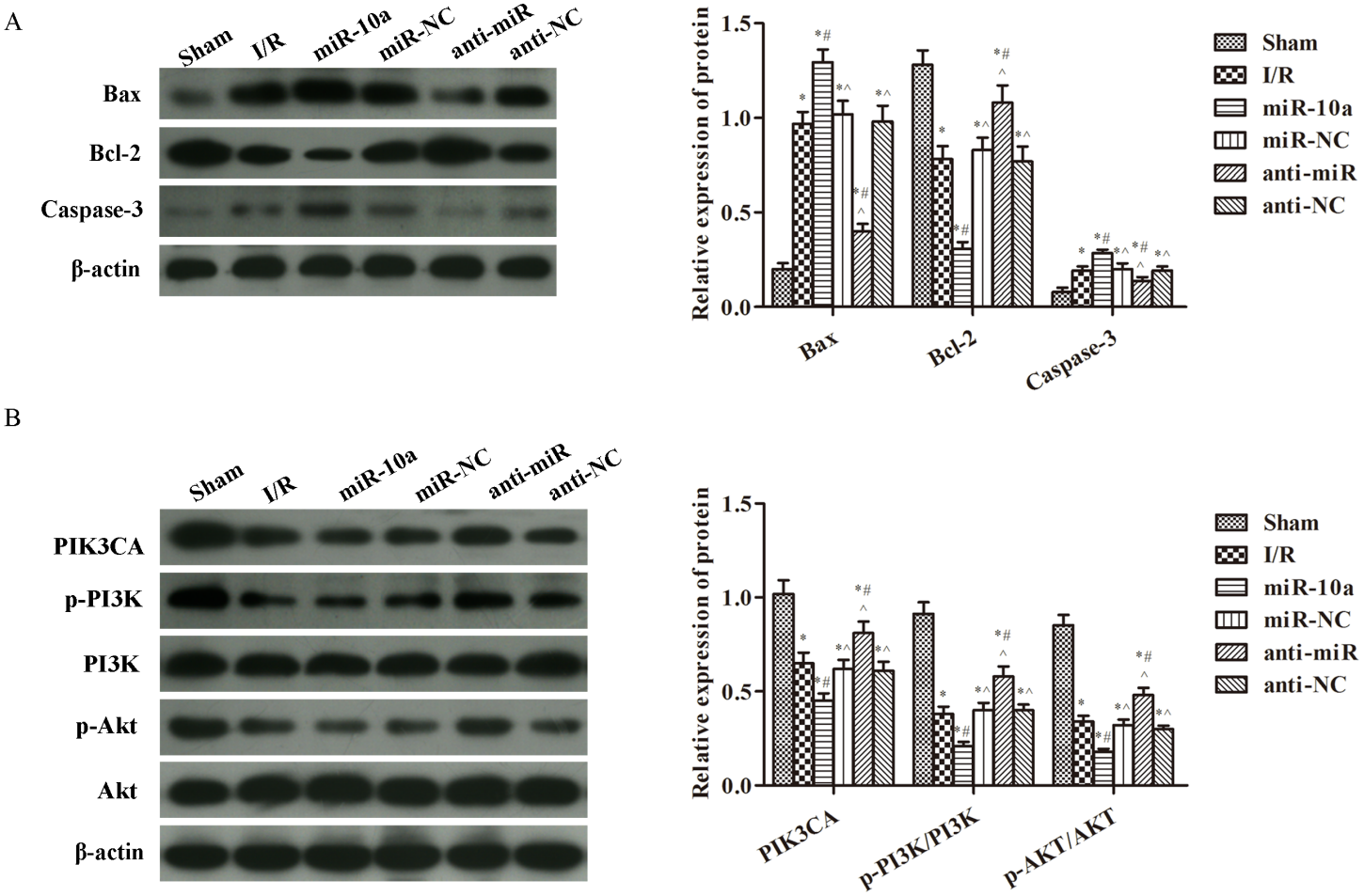

Figure 3

miR-10a overexpression increased apoptosis related proteins and inhibited PIK3CA/PI3K/Akt pathway. (A) Western blot was used to detect the expression of Bax, Bcl-2 and Caspase-3 in renal tissues; (B) Western blot was used to detect the expression of PIK3CA, p-PI3K/PI3K, p-Akt/Akt in renal tissues. *p < 0.05 compared to Sham group; #p < 0.05 compared to I/R group; ^p < 0.05 compared to the miR-10a group (n=6).

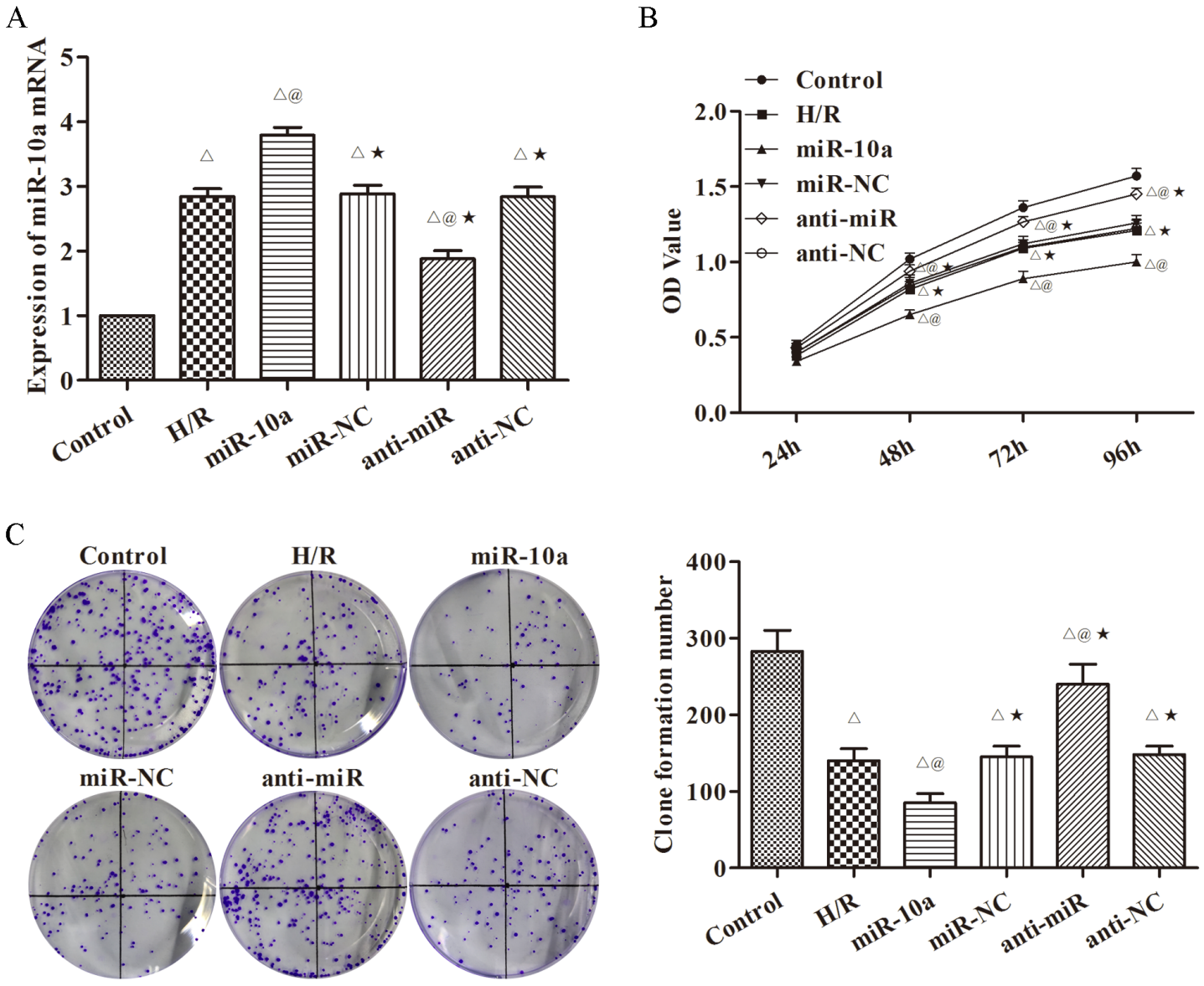

Figure 4

miR-10a overexpression inhibited the proliferation of H/R induced HK-2 cell. (A) RT-PCR was used to detect the expression of miR-10a in cells; (B,C) The proliferation of cells in each group was detected by CCK8 and cloning formation experiment. The more clone number, the stronger cell proliferation. Hypoxia/reoxygenation group (H/R), miR-10a mimic group (miR-10a), miR-10a mimic negative control group (miR-NC), miR-10a inhibitor group (anti-miR), miR-10a inhibitor negative control group (anti-NC). △p < 0.05 compared to control group; @p < 0.05 compared to H/R group; ★p<0.05, compared to the miR-10a group (n=6).

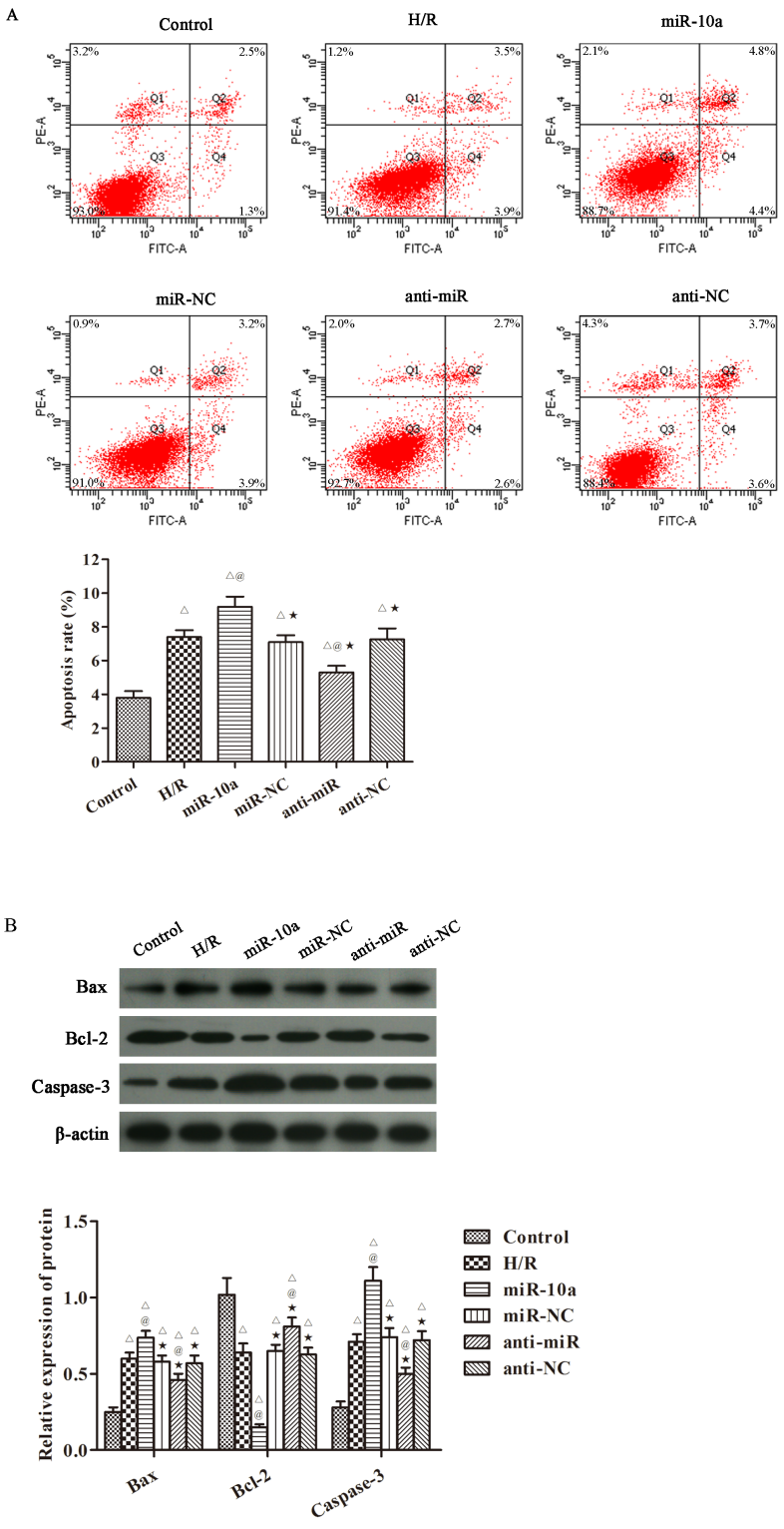

Figure 5

miR-10a overexpression increased cells apoptosis and apoptosis related proteins in H/R induced HK-2 cells. (A) The cells apoptosis was detected by flow cytometry; (B) Western blot was used to detect the expression of Bax, Bcl-2 and Caspase-3proteins. △p < 0.05 compared to control group; @p < 0.05 compared to H/R group; ★p<0.05, compared to the miR-10a group (n=6).

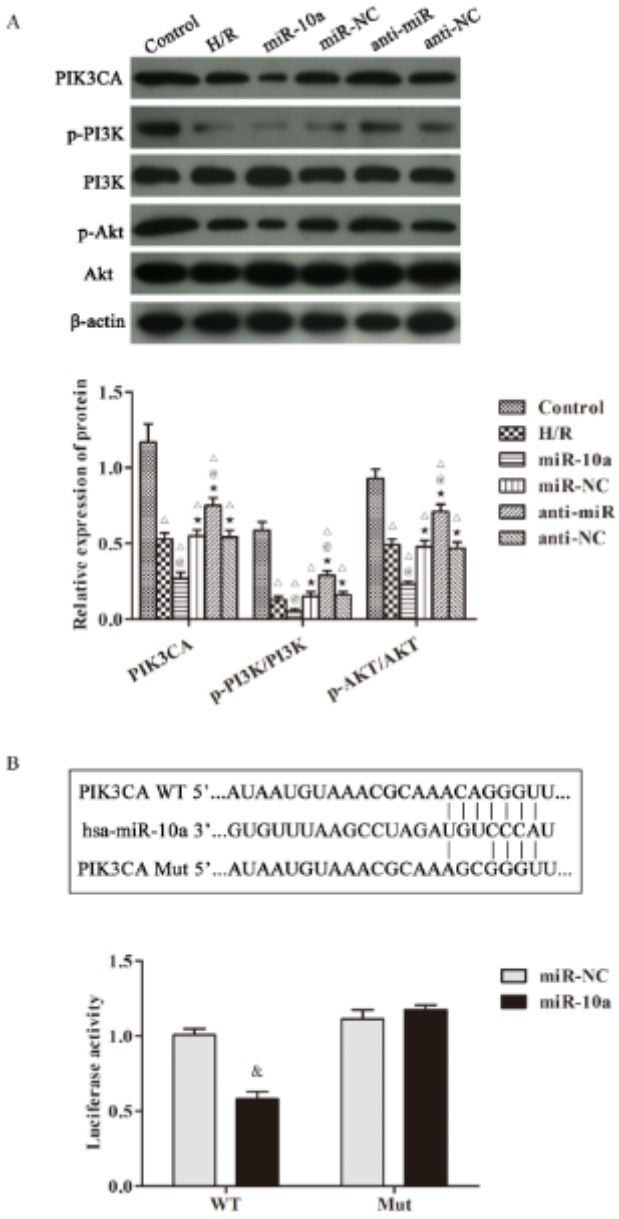

Figure 6

miR-10a overexpression inhibited PIK3CA/PI3K/Akt pathway in H/R induced HK-2 cells. (A) Western blot was used to detect the expression of PIK3CA, p-PI3K/PI3K, p-Akt/Akt in cells; (B) Double luciferase reporter result of recombinant vector of miR-10a and targeted gene PIK3CA. △p < 0.05 compared to control group; @p < 0.05 compared to H/R group; ▯p<0.05, compared to the miR-10a group (n=6). &p < 0.05 compared to the miR-NC group (n=6).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- NC3RsARRIVEGuidelinesChecklist2014.docx