

MiR-181a mediates Ang II-induced myocardial hypertrophy by mediating autophagy

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Abstract. – **OBJECTIVE:** To investigate the relationship between miR-181a and cardiac hypertrophy and autophagy in rats with myocardial hypertrophy, and whether miR-181a regulates the autophagy through ATG5, thereby participating in the occurrence and development of myocardial hypertrophy.

MATERIALS AND METHODS: The rat model of myocardial hypertrophy was established via the abdominal aortic coarctation. The expression of miR-181a in cardiac tissues was detected via reverse transcription-polymerase chain reaction (RT-PCR). The expressions of autophagy-related proteins, ATG5 and LC3II/LC3I, in cardiac tissues, were detected via Western blotting (WB). After the primary culture of myocardial cells in rats, they were stimulated via Angiotensin II (Ang II) to observe the effects of autophagy inhibitor 3-methyladenine (3-MA) and overexpression of ATG5 on the expression of hypertrophic genes in myocardial cells, respectively. The expressions of autophagy-related proteins ATG5 and LC3II/LC3I were detected via WB, the autophagic rate was observed via flow cytometry and the changes in autophagic vacuoles of myocardial cells were observed using the transmission electron microscope. The changes in mRNA and protein expressions of ATG in myocardial cells were observed after the overexpression of miR-181a or the inhibition of miR-181a activity. The changes in miR-181a and the expression of hypertrophic genes in myocardial cells after Ang II stimulation were observed via RT-PCR.

RESULTS: In rats with myocardial hypertrophy, the cardiac autophagy was increased and the expression of miR-181a in hypertrophic myocardium was downregulated. 3-MA inhibited the ATG5-induced autophagy and improved the Ang II-induced myocardial hypertrophy, while the overexpression of ATG5 enhanced the myocardial autophagy and the expression of hypertrophic genes. MiR-181a regulated the ATG5-induced myocardial autophagy, and its downregulation mediated the Ang II-induced myocardial hypertrophy.

CONCLUSIONS: The enhancement of ATG5-induced myocardial autophagy mediates the Ang II-induced myocardial hypertrophy. ATG5 is the target gene of miR-181a, it can regulate the myo-

cardial autophagy via ATG5, thus mediating the Ang II-induced myocardial hypertrophy.

Key Words:

MiR-181a, Ang II, Myocardial hypertrophy, Autophagy.

Abbreviations

Ang II = Angiotensin II; ANP = Atrial Natriuretic Peptide; BSA = Bovine serum albumin; cDNA = Complementary Deoxyribonucleic Acid; DMEM = Dulbecco's Modified Eagle Medium; EDTA = Ethylenediaminetetra-acetic acid; FBS = Fetal bovine serum; LC3 = Microtubule-associated protein 1 light chain 3; 3-MA = 3-Methyladenine; MDC = Monodansylcadaverine, β -MHC = Myosin Heavy Chain; PASW = Predictive Analytics Suite Workstation; PBS = Phosphate buffered saline, PCR = Polymerase chain reaction; RAS = Renin-angiotensin system; RT = Reverse transcriptase; TAAC = Transverse Abdominal Aortic Constriction; WB = Western blotting.

Introduction

In developed countries, the prevalence rate of heart failure in adults is about 1-2%, while it is up to more than 10% in population aged above 70 years old¹. The admission rate of patients with heart failure is high, consuming a lot of medical resources and seriously reducing the life quality of patients. At present, it is argued that the cardiac remodeling is the basic mechanism of the occurrence and development of heart failure^{2,3}. Under a variety of pathological stimuli, the substantial and interstitial structural changes occur in heart, such as cardiomyocyte hypertrophy and cardiac interstitial fibrosis, known as cardiac remodeling. Myocardial hypertrophy is an important link in cardiac remodeling, so further study on the mechanism of myocardial hypertrophy may provide us with new prevention and treatment targets for heart failure. Cardiomyocyte injury and loss play important roles in the myocardial hypertrophy and

cardiac remodeling^{4,5}, whereas cardiomyocyte autophagy plays a role in the cardiomyocyte injury and loss. Autophagy is an important pathway for the protein degradation of eukaryocytes. It is mainly to remove and degrade the damaged cell structure, aging organelles and useless biological macromolecules in cells, providing raw materials for the construction of intracellular organelles, namely the recycling of cell structure. Autophagy is an important regulatory mechanism for the eukaryotic cell growth, differentiation, executive function and death, which is related to many physiological and pathological processes of cells, including cancer, degenerative diseases and other human diseases. In recent years, the research has gradually been paid attention to the role of autophagy in cardiovascular diseases, because the pathological process of a variety of cardiovascular diseases is accompanied by the changes in autophagic activity of cardiovascular system. Autophagy has the effect of maintaining the normal physiological functions of cardiovascular system. The abnormal autophagy accelerates the occurrence of cardiovascular diseases. Besides, renin-angiotensin system (RAS) plays an important role in the development of cardiac remodeling, the most important bioactive substance of which is angiotensin II (Ang II), that can cause myocardial remodeling. The *in vivo* injection of Ang II can upregulate the cardiomyocyte autophagy. Moreover, it can also upregulate the autophagy of myocardial cells cultured *in vitro*⁶⁻⁸. Studies have shown that Ang II can cause myocardial remodeling through upregulating the cardiomyocyte autophagy.

microRNA (miRNA) is a kind of non-coding micro RNA that is highly conserved in the process of biological evolution. It regulates the expression of target genes by degrading the target mRNA or inhibiting its translation, thus affecting the physiological and pathological status of cells or organisms. Studies have found that miR-181a can inhibit the autophagy through regulating the mRNA and protein expressions of autophagy-related gene ATG5 (autophagy related 5) in breast cancer MCF-7, liver cancer Huh-7, and chronic myeloid leukemia K562 cells⁹⁻¹². Whether miR-181a regulates the cardiomyocyte autophagy and plays a role in cardiac hypertrophy is still unknown. In this study, the changes in miR-181a and autophagy in cardiac tissues of rats with cardiac hypertrophy and normal cardiac tissues were studied. The regulation of miR-181a for autophagy-associated protein ATG5 and the role of ATG5

in Ang II-induced myocardial hypertrophy, were also studied.

Materials and Methods

Preparation of SD Rat Model of Myocardial Hypertrophy

Specific pathogen free (SPF)-grade Sprague Dawley (SD) rats weighing 80-90 g were randomly divided into sham operation group (Sham) and operation group (TAAC). This study was approved by the Animal Ethics Committee of Anqiu People's Hospital Animal Center. At 8 h before modeling, rats were fed with water but no food. Under the anesthesia via intraperitoneal injection of 10% 0.3 mL/100 g chloral hydrate (Sigma-Aldrich, St. Louis, MO, USA), the median incision was made on the abdomen after routine disinfection to open and separate the abdominal cavity layer by layer; then, the abdominal aorta was narrowed below the celiac artery and under the anterior mesenteric artery to be 0.5 mm in the diameter, so as to establish the pressure-overload rat model. After operation, 5% 1 mL/kg/d ampicillin was intramuscularly injected for a total of 3 d to prevent infection. In Sham group, only the abdominal aorta was isolated without constrictive operation. After routine feeding to 4 weeks after surgery, the modeling was confirmed to be successful via cardiac ultrasound.

Classification and Culture of Primary Cardiomyocytes of Rats

After disinfection, the chest of newborn Sprague Dawley (SD) rats aged 1-3 days was cut in the xiphoid along the sternum to expose the heart; the apex of heart was cut and immediately washed in the cold phosphate buffered solution (PBS) for 3 times. The apical tissues were cut into 0.5-1.0 mm³ pieces and transferred into a 50 mL sterile flask with tap. 5-time-volume 0.25% ethylenediaminetetra-acetic acid (EDTA)-free trypsin was added and shaken in the water bath box at 37°C for 5 min. The supernatant was taken and added into the stop buffer containing serum and Dulbecco's modified eagle medium (DMEM), and the cells were mixed. The above steps were repeated for 15-20 times and the supernatant was collected. After filtration through the cell screen, the filtrate was centrifuged at 1200 rpm for 5 min. The supernatant was discarded and DMEM solution containing 15% fetal bovine serum (FBS) was added. After the full blowing and beating, the cell

suspension was inoculated into the culture bottle and placed in an incubator containing 5% CO₂ at 37°C. After 90 min, most fibroblasts were adhered, the suspension was taken and the cells were counted using the cell counting chamber. After the cell concentration was adjusted to be 3×10⁵ cells/mL, cells were paved onto the culture plate or dish. Double antibody and BrdU (final concentration of 0.1 mmol/L) were added and cultured with DMEM containing 15% FBS.

Intervention Program

10 mmol/L 3-methyladenine (3-MA), a kind of autophagy inhibitor, was used at 5 d to stimulate or transfect the ATG5 overexpression vector, followed by stimulation with 10⁻⁶ mol/L Ang II after half an hour. Lentivirus containing miR-181a mimics or inhibitors was given at 5 d [4×10⁵ cardiomyocytes were given 20 µL virus (10⁹ TU/mL) and polybrene (final concentration of 5 mg/mL)]. The sample was collected at 7 d for gene detection; the sample was collected at 8 d again for protein detection, flow cytometry and electron microscope observation.

Flow Cytometry

Monodansylcadaverine (MDC) was dissolved by DMEM to a concentration of 50 µM, and then added into the cells for incubation in a dark place at 37°C under 5% CO₂ for 60 min. After MDC was discarded, cells were washed with PBS at room temperature for 3 times. 500 µL trypsin containing 0.25% EDTA was added into each well (6-well plate) for incubation in a dark place at 37°C under 5% CO₂ for 5 min to detach cells. The digested cells were transferred into 167 µL newborn bovine serum to neutralize the trypsin, followed by centrifugation at 1200 rpm to remove the serum; then, cells were resuspended using PBS and sent for inspection.

RNA Extraction and RT-PCR

The total RNA was extracted from myocardial tissues and primary cardiomyocytes and quantified according to the standard procedures. The Complementary Deoxyribonucleic Acid (cDNA) was synthesized by reverse transcription according to the instructions of reverse transcription kit first, and amplified via polymerase chain reaction (PCR). Then, the PCR products were identified via agarose gel electrophoresis. 3 control wells were set for each sample, the fluorescence quantitative PCR detection was repeated, and the results were averaged.

Extraction of Total Protein and Western Blotting

The total protein was extracted from myocardial tissues and primary cardiomyocytes and quantified according to the standard procedures. After the membrane transfer of protein samples via electrophoresis, the primary antibody (Abcam, Cambridge, MA, USA) was incubated overnight, and the secondary antibody (Abcam, Cambridge, MA, USA) was incubated on the next day, followed by color development and analysis.

Transmission Electron Microscope

The primary cardiomyocytes were collected, fixed with 4% glutaraldehyde, and then fixed again with 2% osmic acid, followed by dehydration via alcohol, embedding via epoxy resin and ultra-thin section making. The transmission electron microscope was used for observation, photograph and record.

Statistical Analysis

Data were presented as mean ± standard deviation. Shapiro-Wilk test was used to determine whether the data satisfied the normal distribution, and Levene's test was used to determine whether the variance was homogeneous. If the normal distribution was satisfied, Student's *t*-test was used for the comparison of measurement data between the two groups; otherwise, Mann-Whitney U test was used. Analysis of variance was used for the intergroup comparison of multiple-sample measurement data with normal distribution and homogeneous variance. *p*<0.05 suggested that the difference was statistically significant. Predictive Analytics Suite Workstation (PASW) Statistics 18.0 (Armonk, NY, USA) statistical software was used for analysis.

Results

The Cardiac Autophagy was Enhanced and the Expression of miR-181a in Myocardial Tissues was Downregulated in Rats with Myocardial Hypertrophy

The mRNA level of autophagy-related gene ATG5 in cardiac tissues was detected. Compared with that in Sham group, the mRNA level of ATG5 in TAAC group was significantly upregulated (Figure 1A). The expression level of autophagy-related protein ATG5 in cardiac tissues was detected. Compared with those in Sham

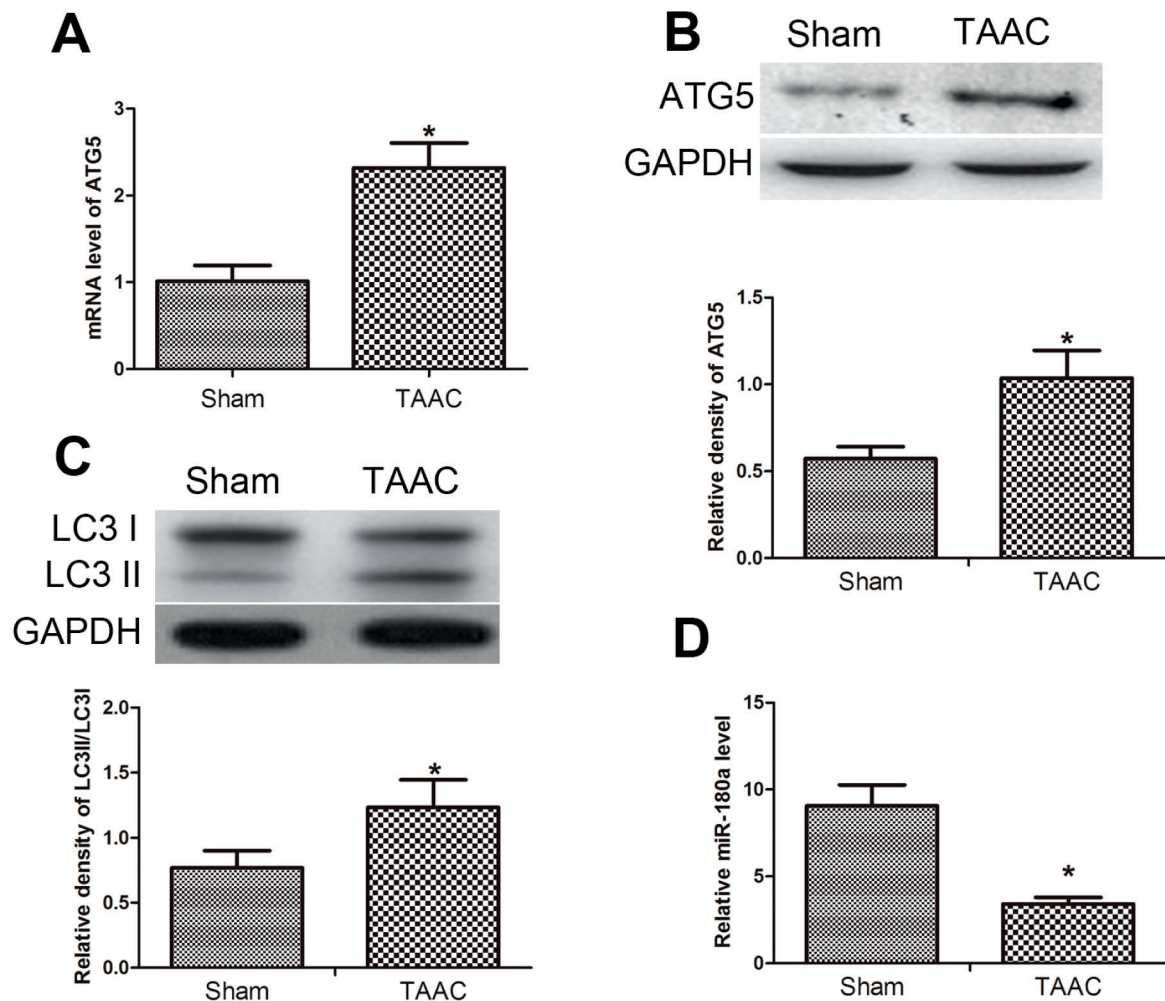


Figure 1. The cardiac autophagy was enhanced and the expression of miR-181a in myocardial tissues was downregulated in rats with myocardial hypertrophy. (A) Analysis of the mRNA level of ATG5. (B) Western blots analysis reveals the expression of ATG5. (C) Western blots analysis reveals the expression of LC3 II/LC3 I. (D) Analysis of the miR-181a level. * $p < 0.05$ vs. Sham group.

group, the protein expression level of ATG5 in TAAC group was upregulated and the expression levels of autophagy-related proteins LC3II/LC3I were also upregulated (Figure 1BC). The miR-181a expression in cardiac tissues in TAAC group was downregulated compared with that in Sham group (Figure 1D).

3-MA Inhibited the ATG5-Induced Autophagy and Improved the Ang II-Induced Myocardial Hypertrophy

The primary cardiomyocytes were cultured. Compared with those in Control group (Ctr group), the expression of autophagy-related gene ATG5 in myocardial cells in Ang II stimulation group (Ang II group) was significantly upregulated, and the expressions of hyper-

trophic genes Atrial Natriuretic Peptide (ANP) and Myosin Heavy Chain (β -MHC) were also upregulated. Compared with those in Ang II stimulation group, the expression of ATG5 in myocardial cells in 3-MA group was significantly downregulated, and the expressions of hypertrophic genes ANP and β -MHC were also significantly decreased. Ang II stimulated the up-regulation of ATG5 expression in myocardial cells, and the significant up-regulation of autophagy-related proteins ATG5 and LC3II/LC3I in myocardial cells. After the ATG5 expression was down-regulated by 3-MA, the expressions of autophagy-related proteins ATG5 and LC3II/LC3I in myocardial cells were significantly down-regulated, indicating that 3-MA can inhibit ATG5 in myocardial cells and

improve the Ang II-induced hypertrophic gene expression (Figure 2).

Overexpression of ATG5 Enhanced the Cardiomyocyte Autophagy and Hypertrophic Gene Expression

The primary cardiomyocytes were cultured. In addition to the overexpression of ATG5 (Over group), the expressions of hypertrophic genes ANP and β -MHC were also upregulated compared with those in empty vector group (Vec group) (Figure 3AB). With the overexpression of ATG5, the expressions of autophagy-related proteins ATG5 and LC3II/LC3I in myocardial cells were increased (Figure 3C). Autophagic vacuoles in myocardial cells were stained with monodansylcadaverine (MDC), and the autophagy rate of myocardial cells was detected using the flow cytometry: with the overexpression of ATG5, the autophagy rate of myocardial cells was significantly up-regulated (Figure 3D). Electron microscopy further confirmed that the number of autophagic vacuoles in myocardial cells increased with the overexpression of ATG5 (Figure 3E).

MiR-181a Regulated the ATG5-induced Myocardial Autophagy

Compared with that in negative control group (Nctr group), the mRNA expression of ATG5 in miR-181a overexpression group (Mim group) was decreased, while that in miR-181a inhibition group (Inh group) was increased (Figure 4A). Compared with that in Nctr group, the protein expression of ATG5 in miR-181a overexpression group was decreased, while that in miR-181a inhibition group was increased. Besides, compared with those in Nctr group, the protein expressions of LC3II/LC3I in miR-181a overexpression group were decreased, while those in miR-181a inhibition group were increased (Figure 4B). Autophagic vacuoles in myocardial cells were stained with MDC, and the autophagy rate of myocardial cells was detected using the flow cytometer. Compared with that in Nctr group, the autophagy rate in miR-181a overexpression group was significantly decreased; but it was significantly up-regulated after miR-181a was inhibited compared with that in Nctr group (Figure 4C). Moreover, the number of autophagic

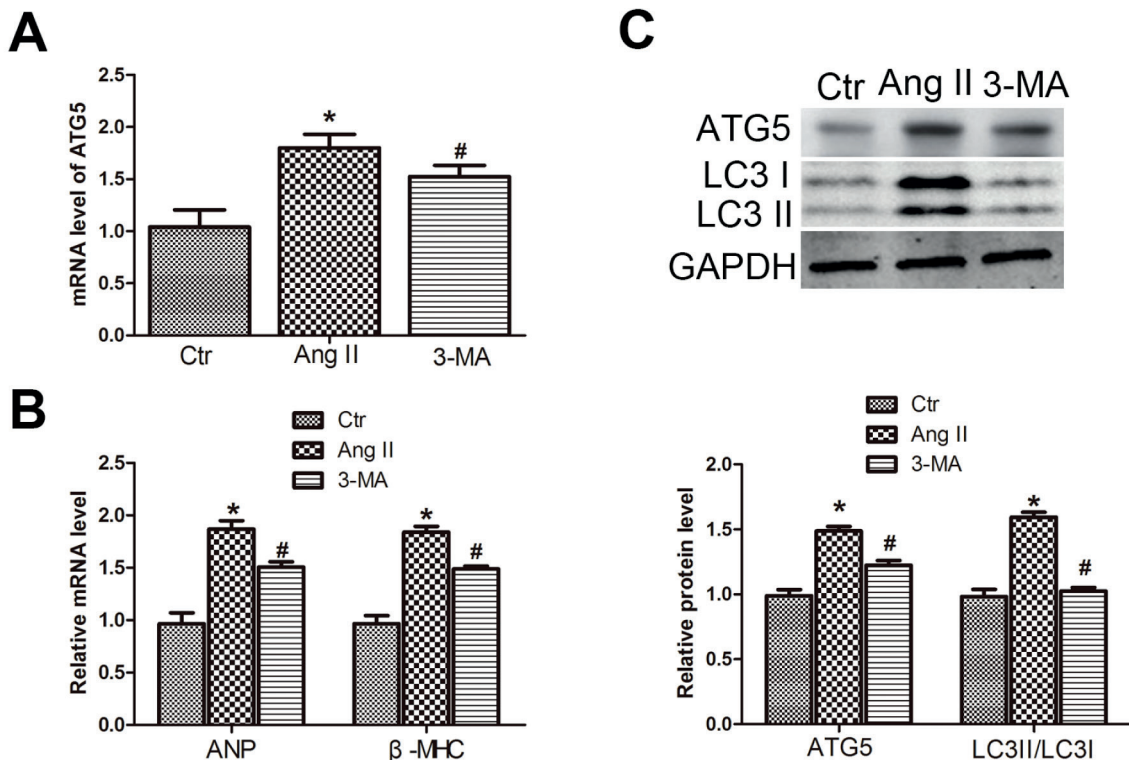


Figure 2. 3-MA inhibited the ATG5-induced autophagy and improved the Ang II-induced myocardial hypertrophy. (A) Analysis of the mRNA level of ATG5. (B) Analysis of the mRNA level of ANP and β -MHC. (C) Western blots analysis reveals the expression ATG5 and LC3 II/LC3 I. * $p < 0.05$ vs. Ctrl group, # $p < 0.05$ vs. Ang II group.

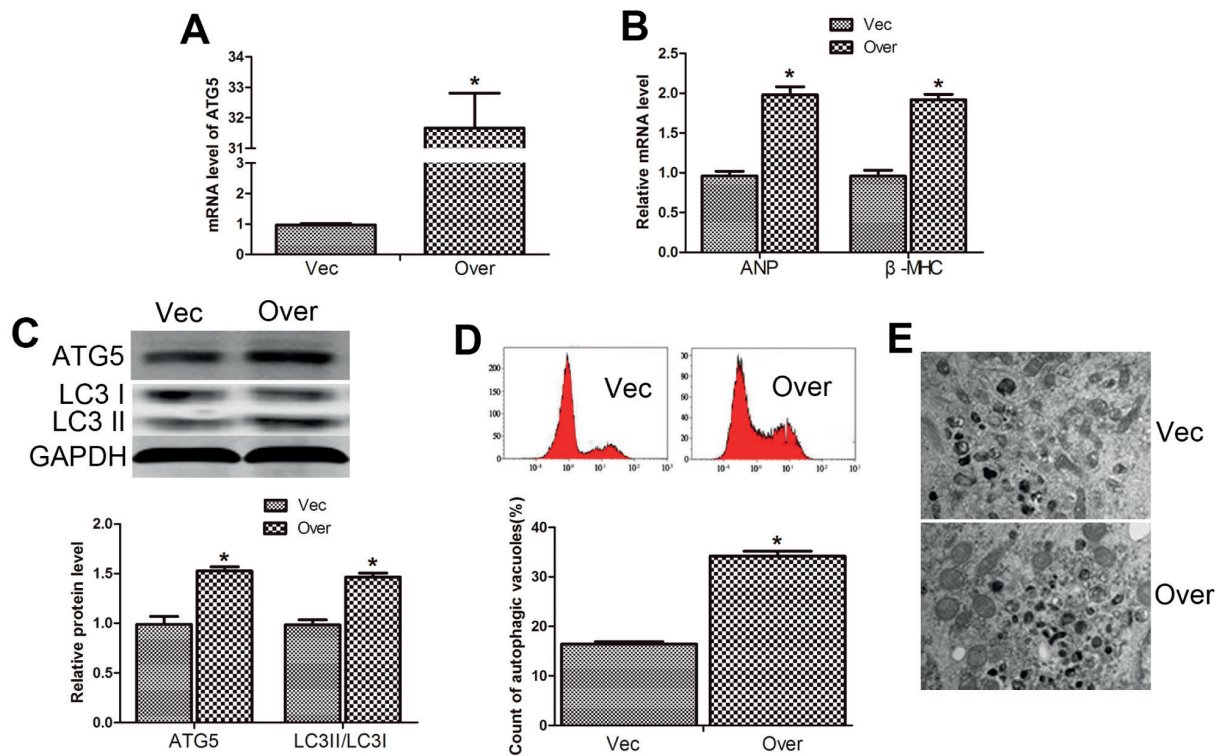


Figure 3. Overexpression of ATG5 enhanced the cardiomyocyte autophagy and hypertrophic gene expression. (A) Analysis of the mRNA level of ATG5. (B) Analysis of the mRNA level of ANP and β-MHC. (C) Western blots analysis reveals the expression ATG5 and LC3 II/LC3 I. (D) The count of autophagic vacuoles by flow cytometry. (E) Representative images of electron microscopy. * $p < 0.05$ vs. Vec group.

vacuoles in myocardial cells in each visual field under the electron microscope was significantly increased in miR-181a inhibition group compared with that in control group. Finally, compared with that in Ang II stimulation group, the number of autophagic vacuoles in myocardial cells in Ang II + miR-181a overexpression group was significantly decreased (Figure 4D).

Downregulation of miR-181a Mediated the Ang II-induced Myocardial Hypertrophy

The overexpression of miR-181a mimics was realized based on the cardiomyocyte hypertrophy stimulated by Ang II. The results showed that the expressions of hypertrophic genes ANP and β-MHC in miR-181a mimics group were decreased significantly. After the overexpression of miR-181a inhibitors in myocardial cells based on the cardiomyocyte hypertrophy stimulated by Ang II, the expressions of hypertrophic genes ANP and β-MHC in miR-181a inhibitor group were significantly increased (Figure 5).

Discussion

The rat model of cardiac hypertrophy was established using the abdominal aortic coarctation method (TAAC). At 4 weeks after surgery, the ventricular wall thickness was measured via ultrasonic cardiogram to confirm the successful modeling. The role of autophagy in cardiovascular disease has gradually been paid attention to, and the pathological processes of various cardiovascular diseases are accompanied by changes in the autophagy activity of cardiovascular system^{13,14}. It was found in this study that the expression of autophagy-related protein ATG5 in cardiac tissues of rats with myocardial hypertrophy in TAAC group was increased, and the expressions of LC3II/LC3I were also increased, indicating that the up-regulation of ATG5-induced autophagy may exist in cardiac tissues of rats with myocardial hypertrophy, which may be the result of abnormal response to hemodynamic stress. Persistent and long-term autophagy can excessively degrade the essential proteins and or-

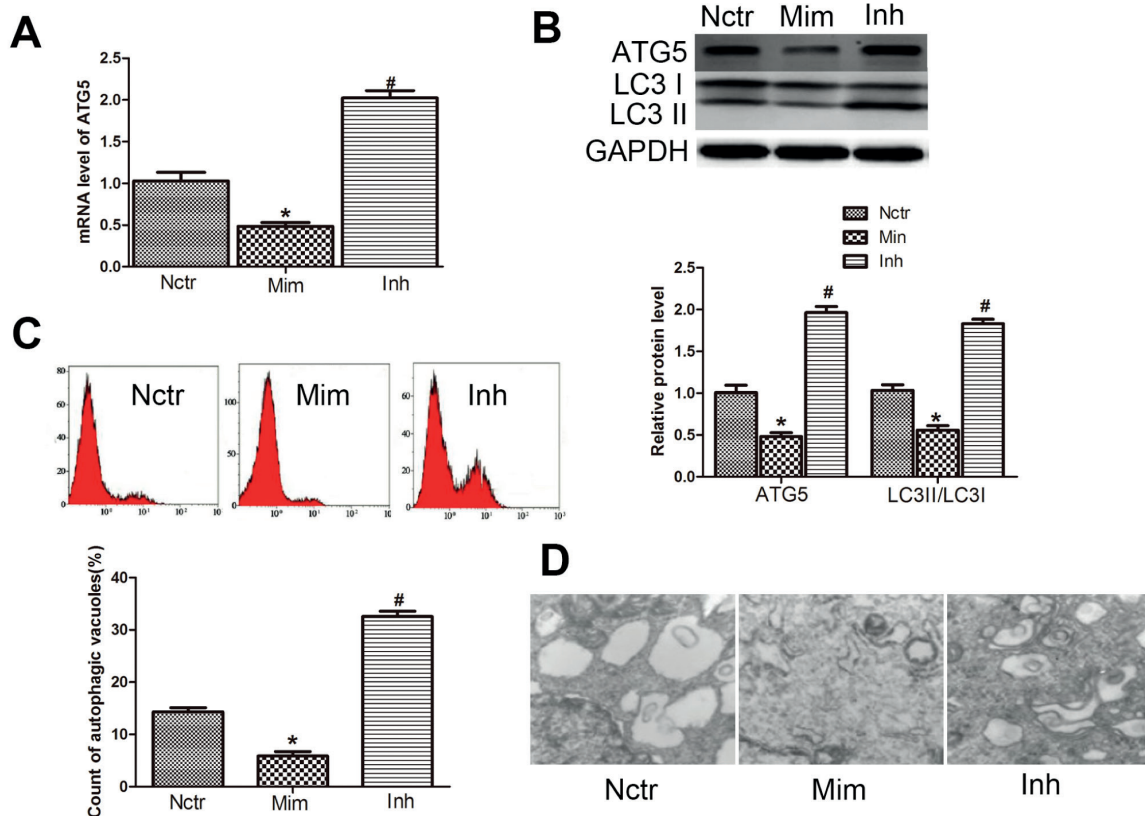


Figure 4. MiR-181a regulated the ATG5-induced myocardial autophagy. **(A)** Analysis of the mRNA level of ATG5. **(B)** Western blots analysis reveals the expression ATG5 and LC3 II/LC3 I. **(C)** The count of autophagic vacuoles by flow cytometry. **(D)** Representative images of electron microscopy. * $p < 0.05$ Mim vs. Nctr group, # $p < 0.05$ Inh vs. Nctr group.

ganelles, and the up-regulation of ATG5-induced autophagy is detrimental to the body. Real-time PCR showed that the expression of miR-181a in cardiac tissues of TAAC rats was significantly downregulated. Moreover, the animal experiments proved that there is a certain level of autophagy in normal heart, while the myocardial autophagy is significantly enhanced in hypertrophic heart. In contrast, miR-181a is expressed in normal myocardium, while it is significantly downregulated in hypertrophic myocardium.

Autophagy-related gene ATG5 mediates the Ang II-induced myocardial hypertrophy^{15,16}, and 3-MA is a commonly used autophagic inhibitor that inhibits autophagy via inhibiting PI3K^{17,18}. On the basis of Ang II stimulation, the expression of ATG5 in myocardial cells was downregulated and the expressions of hypertrophic genes in myocardial cells were improved after the application of 3-MA, which proved that ATG5 up-regulated the Ang II-induced myocardial hypertrophy. In this study, the myocardial cells were transfected with the ATG5-containing plasmid, and

the cardiomyocyte autophagy was enhanced and the expressions of hypertrophic genes in myocardial cells were increased after that. Thus, the experiment further confirmed that ATG5 up-regulation mediates the Ang II-induced myocardial hypertrophy. Ang II up-regulates the ATG5-induced autophagy, as well as the hypertrophic gene expression, and the inhibition of up-regulation of ATG5-induced autophagy can down-regulate the expression of hypertrophic genes. Therefore, it is concluded that the enhancement of ATG5-induced cardiomyocyte autophagy mediates the Ang II-induced myocardial hypertrophy. To demonstrate that the up-regulation of ATG5-induced myocardial autophagy mediates the Ang II-induced myocardial hypertrophy, only the hypertrophic genes ANP and β -MHC were used as the indexes of cardiomyocyte hypertrophy in this study. It will be more reasonable to fully evaluate the cardiomyocyte hypertrophy through detecting the morphological changes in cardiomyocyte via laser confocal microscopy and detecting the protein synthesis rate, etc. There are increasingly more

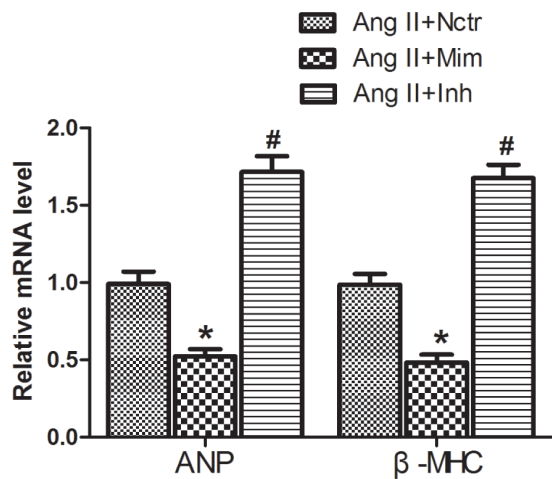


Figure 5. Down-regulation of miR-181a mediated the Ang II-induced myocardial hypertrophy. Analysis of the mRNA level of ANP and β -MHC.

reports on the miRNA regulation of autophagy in recent years. Studies have shown that miR-885-3p can regulate the autophagy caused by autophagy-related protein ULK2 (Unk-51 Like Autophagy Activating Kinase 2)¹⁹. It is reported that miR-181a can negatively regulate the autophagy of tumor cells caused by ATG5 protein⁹⁻¹¹. Recently, there are also reports on the miRNA regulation of myocardial hypertrophy. The expression of miR-199b in myocardial tissues with myocardial hypertrophy is up-regulated, and the inhibition of miR-199b expression can reverse the cardiomyocyte hypertrophy and fibrosis in mice²⁰. Like other miRNAs, miR-181a is associated with the tumor, nervous system²¹, reproductive system²², circulatory system^{23, 24}, digestive system, respiratory disease, adipogenesis²⁵, cell senescence, drug metabolism and cell differentiation. miRNA regulates gene expression at post-transcriptional level by complementing the 3'UTR of target gene mRNA, resulting in degradation or inhibition of translation. Besides, miRNA regulates the biological health and diseases, including cardiovascular disease. The targeted relationship between miR-181a and ATG5 was proved in myocardial cells. The overexpression of miR-181a in myocardial cells can inhibit the mRNA and protein expressions of endogenous ATG5 in myocardial cells, while inhibiting the level of miR-181a in myocardial cells can up-regulate the mRNA and protein expressions of endogenous ATG5. Subsequently, the data in this study showed that the overexpression of miR-181a in myocardial cells

is down-regulated the expressions of LC3II/LC3I in myocardial cells, and flow cytometry showed that the autophagy rate was reduced after MDC staining and the number of autophagic vacuoles under transmission electron microscope was also reduced. The inhibition of miR-181a in myocardial cells up-regulated the expressions of LC3II/LC3I in myocardial cells, and flow cytometry showed that the autophagy rate was increased after MDC staining and the number of autophagic vacuoles under transmission electron microscope was also increased. These results suggested that miR-181a can regulate the ATG5-induced myocardial autophagy. Moreover, the overexpression of miR-181a mimics in myocardial cells could relieve the expressions of hypertrophic genes in myocardial cells, while the inhibition of miR-181a activity in myocardial cells could increase the expressions of hypertrophic genes in myocardial cells.

Conclusions

The enhancement of ATG5-induced myocardial autophagy mediates the Ang II-induced myocardial hypertrophy. ATG5 is the target gene of miR-181a, it can regulate the myocardial autophagy via ATG5, thus mediating the Ang II-induced myocardial hypertrophy.

Conflict of interest

The authors declare no conflicts of interest.

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