

# Integrative microRNA and mRNA expression profiling in acute aristolochic acid nephropathy in mice

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**Abstract.** In acute aristolochic acid nephropathy (AAN), aristolochic acid (AA) induces renal injury and tubulointerstitial fibrosis. However, the roles of microRNAs (miRNAs/miRs) and mRNAs involved in AAN are not clearly understood. The aim of the present study was to examine AA-induced genome-wide differentially expressed (DE) miRNAs and DE mRNAs using deep sequencing in mouse kidneys, and to analyze their regulatory networks. In the present self-controlled study, mice were treated with 5 mg/kg/day AA for 5 days, following unilateral nephrectomy. AA-induced renal injury and tubulointerstitial fibrosis were detected using hematoxylin and eosin staining and Masson's trichrome staining in the mouse kidneys. A total of 82 DE miRNAs and 4,605 DE mRNAs were identified between the AA-treated group and the self-control group. Of these DE miRNAs and mRNAs, some were validated using reverse transcription-quantitative PCR. Expression levels of the profibrotic miR-21, miR-433 and miR-132 families were significantly increased, whereas expression levels of the anti-fibrotic miR-122-5p and let-7a-1-3p were significantly decreased. Functions and signaling pathways associated with the DE miRNAs and mRNAs were analyzed using Gene Ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG). A total of 767 DE pairs (in opposing directions) of miRNAs and their mRNA targets were identified. Among these, regulatory networks of miRNAs and mRNAs were analyzed using KEGG to identify enriched signaling pathways and extracellular matrix-associated pathways.

In conclusion, the present study identified genome-wide DE miRNAs and mRNAs in the kidneys of AA-treated mice, as well as their regulatory pairs and signaling networks. The present results may improve the understanding of the role of DE miRNAs and their mRNA targets in the pathophysiology of acute AAN.

## Introduction

Aristolochic acid (AA) is found in plants of the genera *Aristolochia* and *Asarum* (1). Plants of the *Aristolochia* genus are commonly used as herbal medicines in Asia, Central America and other countries (2). DNA damage and/or carcinogenicity is caused by AA derived-DNA adducts after AA exposure, and death or apoptosis of renal tubular cells is caused by AA-induced DNA damage (3,4). AA can cause aristolochic acid nephropathy (AAN), leading to acute kidney injury and/or chronic kidney disease (CKD), accompanied by tubulointerstitial fibrosis (5,6). Additionally, AA is a carcinogen associated with urothelial tumorigenesis following sufficient long-term exposure, as carcinogenicity is caused by AA derived-DNA adducts after AA exposure (6).

In acute AAN, AA induces renal injury and tubulointerstitial fibrosis (5). Fibrosis is one common final outcome of several kidney diseases (7). Renal tubulointerstitial fibrosis is a complex biological process involved in multiple cellular and signaling pathways. Moreover, fibrosis shares common pathways in several different organs (8). The progression of renal fibrosis primarily includes renal injury, recruitment and activation of immune cells, activation of fibroblasts, epithelial-to-mesenchymal transition (EMT), extracellular matrix (ECM) production and deposition, and tubular injury and atrophy (7). Pro-fibrosis factors, such as transforming growth factor- $\beta$  1 (TGF- $\beta$ 1), are key molecular mediators of renal fibrosis (9,10).

MicroRNAs (miRNAs/miRs) are a class of small, endogenous, non-coding RNAs that silence target mRNAs (11). miRNAs bind to their target mRNAs through complementary nucleotide sequences, and the target gene is suppressed by a miRNA through degradation and/or translation inhibition of its target mRNA (12). It is suggested that miRNAs

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serve a key role in renal fibrosis (13). For example, miRNAs regulate the effects of pro-fibrosis factors, EMT and ECM production (14).

To the best of our knowledge, the expression profiling of miRNAs and mRNAs, and their regulatory pairs or networks has not been reported in acute AAN. To identify the expression profiles and regulatory networks of miRNAs and mRNAs in acute AAN, the present study examined renal genome-wide differentially expressed (DE) miRNAs and DE mRNAs using deep sequencing in mouse kidneys induced by a short-term exposure to AA.

## Materials and methods

The flow chart of the experimental design of the present study is shown in Fig. 1.

**Animal treatment.** A total of four kidney samples were used as the self-control group, and were obtained through single nephrectomy from four 8-week-old male C57BL/6 mice (specific pathogen free; weight, ~20 g; Experimental Animal Center of Soochow University). The mice were raised in a specific pathogen free animal house with a constant temperature (24°C), humidity (50%), specific pathogen free filtered atmosphere and 12 h light/dark cycles. All the mice had free access and were fed with radiation sterilization food and water sterilized with high temperature and high pressure. The mice were anesthetized using chloral hydrate (400 mg/kg intraperitoneal injection) before single nephrectomy. After 4 weeks, these four mice were treated with an intraperitoneal injection of AA I (cat. no. A5512; Sigma-Aldrich; Merck KGaA) at a dosage of 5 mg/kg/day for 5 days. A total of 100 mg AA I was diluted with 5 ml DMSO (final concentration, 5%) and 95 ml saline. The final concentration of the injected AA I was 1 mg/ml. All mice were euthanized by cervical dislocation. The death of mice was verified by the stopping of both breathing and the heartbeat. The remaining four kidney samples were obtained when these four mice were sacrificed on the 6th day. All kidney samples were divided into two parts, one of which was fixed in 10% formalin solution for 5 days at room temperature, while the other was frozen at -80°C in TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). The present study was approved by the Ethics Committee of the Children's Hospital of Soochow University (Suzhou, China; approval no. 2020-CHSU-012).

**Histological analysis.** After fixing in 10% formalin solution for ≥3 days at 26°C, all kidney samples were dehydrated and transparent as follows: 70% alcohol for 1 h, 80% ethanol for 1 h, 90% ethanol for 1 h, 95% ethanol for 1 h, anhydrous ethanol twice for 1 h, anhydrous ethanol and xylene solution (1:1) for 1 h and xylene twice for 1 h. The kidney samples were embedded in paraffin and sectioned into 5-μm-thick slices. The slices were placed in at 60°C for 30 min, dewaxed after being immersed in xylene twice for 10 min each time and then immersed in ethanol (100, 95, 80 and 70%) and deionized water for 5 min. Sections of the kidney samples were stained with hematoxylin and eosin (HE) or Masson's trichrome. HE staining were performed as follow: Hematoxylin dye for 10 min at 26°C, rinsed with water and 0.7% hydrochloric acid

ethanol for 5 sec. Cells were then washed with water until the cell nuclei were blue, along with 95% ethanol for 30 sec, alcoholic eosin dye for 30 sec at 26°C, 95% ethanol twice for 30 sec, 100% ethanol twice for 5 min, carbolic Acid and xylene (1:4) for 5 min, xylene twice for 5 min and the slices were sealed with neutral balsam.

Masson's trichrome staining was performed as follow: Staining with Regaud's hematoxylin dye for 10 min at 26°C, rinsed with unstained color with water, 0.7% hydrochloric acid ethanol for 5 sec, washed with water for 8 min, ponceaux and acid fuchsin (0.7% ponceaux, 0.3% acid fuchsin and 1% acetic acid) for 8 min, 2% acetic acid solution for 45 sec, 1% phosphomolybdic acid for 4 min, aniline blue for 5 min and 0.2% acetic acid until no obvious blue color escaped from the slices. Subsequently, slices were rinsed with 95% ethanol twice for 30 sec, 100% ethanol twice for 5 min and xylene twice for 5 min, and then sealed with neutral balsam. The slices were observed and captured at x400 magnification under a light microscope.

**RNA extraction.** Total RNA was extracted from the kidney samples using TRIzol® reagent according to the manufacturer's protocol. Total RNA was qualified and quantified using NanoDrop and an Agilent 2100 bioanalyzer (both from Thermo Fisher Scientific, Inc.).

**Sequencing of miRNAs.** Small RNAs from the total RNA were obtained from the 18-30 nt region following 15% urea polyacrylamide gel electrophoresis (urea-PAGE) gel electrophoresis. After the addition of 3' and 5' adaptors (MGIEasy Small RNA Library Prep Kit V2.0; BGI Genomics), the cDNA of small RNAs was synthesized and enriched by reverse transcription and PCR amplification. The reverse transcription was performed using the First Strand Master Mix and Superscript II Reverse Transcriptase (SuperScript™ First-Strand Synthesis System; Invitrogen; Thermo Fisher Scientific, Inc.) at 42°C for 1 h and 70°C for 15 min. PCR was performed using PCR Primer Cocktail and PCR Master Mix (MGIEasy Small RNA Library Prep Kit V2.0; BGI Genomics) to enrich the cDNA fragments, under the following conditions: Initial denaturation at 95°C for 3 min, followed by 18 cycles at 98°C for 20 sec, 56°C for 15 sec and 72°C for 15 sec, with a final extension at 72°C for 10 min. The library of small RNAs was constructed from the PCR products in the 100-120 bp region, separated by agarose gel electrophoresis, eliminating primer-dimers and other potential byproducts. The library of small RNAs was denatured by heat (94°C), and the single-stranded DNA was cyclized. The library of cyclized small RNAs was sequenced using a BGISEQ-500 platform (BGI Genomics). The clean data was filtered from the raw data. The clean reads of miRNAs were mapped to the mouse genome and other sRNAs database with annotation using the software Anchor Alignment-Based Small RNA Annotation version 1.0 (University of Nevada) (15), except Rfam version 12.0, which was performed using cmsearch version 1.1 (16).

**Sequencing of mRNAs.** mRNA was selected using poly-T oligo-attached magnetic beads, and ribosomal RNA was depleted. mRNA was fragmented and reverse transcribed into double-stranded cDNA using N6 random primers.

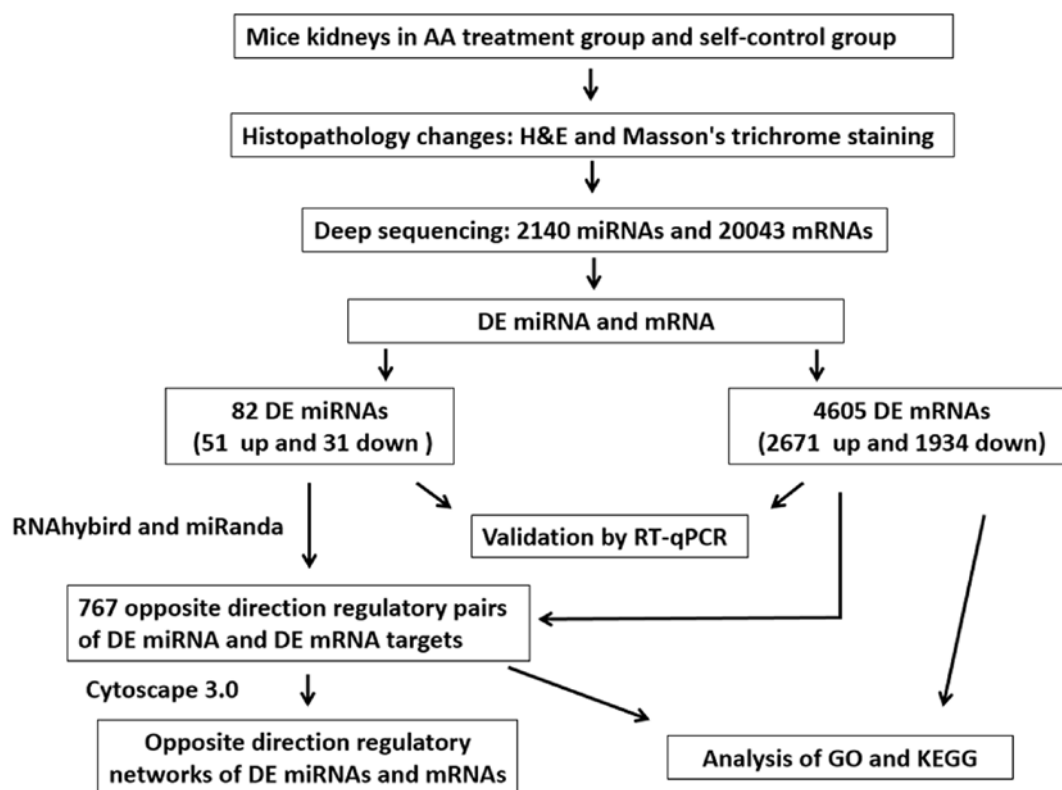


Figure 1. Flow chart of the experimental design. AA, aristolochic acid; H&E, hematoxylin and eosin; miRNA, microRNA; DE, differentially expressed; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

The reverse transcription was performed using the First Strand Master Mix, N6 random primers and Superscript II Reverse Transcriptase (SuperScript™ First-Strand Synthesis System; Invitrogen; Thermo Fisher Scientific, Inc.) at 42°C for 1 h and 70°C for 15 min. The dsDNA was subjected to end-repair and 3'-adenylation. A bubble adapter was ligated to the double ends of the dsDNA. After purifying, the ligated products were amplified by PCR. PCR was performed using PCR Primer Cocktail and PCR Master Mix (MGIEasy RNA Library Prep Kit V3.0; BGI Genomics) to enrich the cDNA fragments, under the following conditions: Initial denaturation at 95°C for 3 min, followed by 18 cycles at 98°C for 20 sec, 56°C for 15 sec and 72°C for 15 sec; with a final extension at 72°C for 10 min. The PCR products were denatured by heat (94°C) and the single-stranded DNA was cyclized using splint oligo and DNA ligase. The cyclized mRNA was sequenced using the BGISEQ-500 platform. The clean mRNA data were filtered from the raw data and mapped to the mouse genome with annotation using HISAT version 2.0.4 (Center for Computational Biology; Johns Hopkins University) (17). A detailed explanation of the methods of miRNA and mRNA sequencing and analysis is provided in Data S1.

**Functional analysis of the DE miRNAs and DE mRNAs.** Hierarchical clustering analysis was performed between all 8 kidney samples using function `hclust` of R software version 3.5.2 (R Core Team). Principal component analysis (PCA) was performed with all 8 kidney samples using function `princomp` of R software version 3.5.2 (R Core Team). Between the AA-treated group and the self-control group, DE miRNAs

were defined using a cut-off with read count >50, adjusted P-values <0.001 and  $\log_2$  fold changes (AA/control) >1.5 or <-1.5. DE mRNAs were defined using a cut-off with read count >50, adjusted P-values <0.001 and  $\log_2$  fold changes (AA/control) >1.0 or <-1.0. These cut-off points were used to assist in identifying the key DE miRNAs and DE mRNAs enriched in biological processes and pathways, which may be involved in the pathophysiology of acute AAN.

Target genes of miRNAs were identified based on the similarity of the sequences with target genes, predicted using RNAhybrid version 2.2 (18) and miRanda (version released in 2010) (19). The functions and pathways analysis of both DE mRNAs and the target genes of DE miRNAs were performed using Gene Ontology (GO) (20) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (21). Networks of DE miRNAs and DE mRNAs were drawn using Cytoscape version 3.0 (National Institute of General Medical Sciences).

**Reverse transcription-quantitative (RT-q) PCR.** To validate the DE miRNA and DE mRNA results, RT-qPCR was performed on reference DE miRNAs and DE mRNAs from the total RNA of all 8 kidney samples extracted as aforementioned. RT-qPCR assays were performed using a Light Cycler 480 II real-time PCR system (Roche Diagnostics). U6 and GAPDH were used as the internal controls for miRNA and mRNA, respectively. The M-MLV Reverse Transcriptase kit (Promega Corp.) was used for the synthesis of cDNA, using the following conditions: 42°C for 1 h and 70°C for 10 min. The cDNA products of mRNA were amplified using SYBR

Premix Ex Taq kit (Takara Bio, Inc.) using the following thermocycling conditions: 95°C for 30 sec; followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec, and a final cycle of 95°C for 15 sec, 60°C for 30 sec and 95°C for 15 sec. All the primers (Shanghai GeneChem Co., Ltd.) for detection of mRNAs are listed in Table I. The RT-qPCR reactions for all miRNAs were performed using a miDETECTA Track™ miRNA qRT-PCR Starter kit (Guangzhou RiboBio Co., Ltd.) according to the manufacturer's protocol. RT-qPCR was performed in triplicate. The expression levels of the selected miRNAs and mRNAs were normalized to those of U6 and GAPDH, respectively. The changes in expression of the assessed miRNAs and mRNAs between the AA treatment group and the self-control group were calculated using the  $2^{-\Delta\Delta C_q}$  method (22).

**Statistical analysis.** Data are presented as the mean  $\pm$  standard error of the mean. Statistical analysis was performed using GraphPad Prism version 8.0 (GraphPad Software, Inc.). Statistical differences were calculated using a paired Student's t-test.  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Renal histopathological alterations induced by AA.** Histopathological changes in the kidney samples were detected using H&E and Masson's trichrome staining. There were markedly widened tubular lumens and flattened tubular cells in the AA-treated group compared with in the self-control group (Fig. 2A-D). Tubulointerstitial fibrosis was induced by AA treatment, as shown by the observable increase in collagen based on the Masson's trichrome staining in the AA-treated group compared with in the self-control group (Fig. 2C and D).

**Renal DE mRNAs induced by AA.** A total of 20,043 mRNAs were detected using deep sequencing in the 8 kidney samples. The mRNA expression patterns were significantly different between the AA-treated group and the self-control group based on hierarchical clustering analysis and PCA (Fig. 3).

Among the total detected mRNAs, 4,605 were considered to be significantly DE between the AA-treated group and the self-control group. Of these, 2,671 DE mRNAs were upregulated and 1,934 were downregulated.

AA treatment induced extensive alterations in cellular function and processes based on the results of GO and KEGG pathway analysis on DE mRNAs. GO analysis of DE mRNAs identified 'single-organism process' and 'cellular process' as the most altered biological process, 'cell' and 'cell part' as the most altered cellular component, and 'binding' and 'catalytic activity' as the most altered molecular function (Fig. 4A). KEGG analysis identified 'signal transduction', 'global and overview maps', 'cancers: Overview', 'immune system' and 'endocrine system' as the most altered pathways (Fig. 4B). Furthermore, 'amino acid metabolism', 'fatty acid degradation', 'complement and coagulation cascades' and 'ECM-receptor interaction' were the most enriched pathways (Fig. 4C). The 'amino acid metabolism' included all types of amino acid metabolism, such as 'Arginine and proline

metabolism', 'Alanine, Aspartic acid and glutamate metabolism', 'glycine, serine and threonine metabolism' and 'Valine, leucine and isoleucine degradation'.

**Renal DE miRNAs induced by AA.** A total of 2,140 miRNAs were detected by deep sequencing. Among these, 82 DE miRNAs were identified between the AA-treated and the self-control group (Table SI). Of these, 51 DE miRNAs were upregulated in the AA treatment group compared with in the self-control group, and 31 DE miRNAs were downregulated.

The top 10 upregulated miRNAs in this study were miR-124-5p, miR-122-3p, miR-124-3p, miR-344d-3p, miR-409-5p, miR-129-2-3p, miR-496a-3p, miR-147-3p, miR-5128 and miR-7649-3p (Table SI). Among the 51 upregulated DE miRNAs, miRNAs with the highest expression levels in the AA treatment group were miR-21a-5p, miR-31-5p, miR-21a-3p, miR-146b-5p, miR-212-3p, miR-34b-3p, miR-132-3p, miR-34c-3p and miR-34c-5p. The 10 most downregulated miRNAs were miR-3073a-5p, miR-3073b-3p, miR-3073b-5p, miR-1948-5p, miR-92a-2-5p, miR-3073a-3p, miR-669c-5p, miR-7085-5p, miR-1968-5p and miR-1948-3p (Table SI). Among the 31 downregulated DE miRNAs, the highest expressed miRNAs in the self-control group were miR-187-3p, miR-190a-5p, miR-122-5p, miR-486a-5p and let-7a-1-3p.

The profibrotic miR-21, miR-433 and miR-132 families, and the oncogenic miR-34 family were significantly increased, while the anti-fibrotic miR-122-5p and let-7a-1-3p were significantly decreased following AA treatment.

**Validation of DE miRNAs and DE mRNAs.** To validate the DE miRNAs and DE mRNAs, RT-qPCR was performed on miR-21a-5p, miR-324-3p, miR-132-3p, miR-1968-5p, TIMP metalloproteinase inhibitor 1 (Timp1), serpin family E member 1 (Serpin1), interleukin (IL)-11 and TGF- $\beta$ 1. These miRNA and mRNA were chosen as they were among the most altered mRNAs and miRNAs, which also had high expression levels (high read counts). Moreover, the chosen mRNAs are pro-fibrosis factors (23,24). As shown in Fig. 5, changes in expression levels of the assessed miRNAs and mRNAs based on RT-qPCR were consistent with the results of deep sequencing (Table SI). Relative expression levels of miR-21a-5p ( $3.90 \pm 0.90$  vs.  $1.00 \pm 0.08$ ), miR-324-3p ( $2.87 \pm 0.62$  vs.  $1.00 \pm 0.13$ ) and miR-132-3p ( $3.81 \pm 0.77$  vs.  $1.00 \pm 0.25$ ) were significantly increased in the AA-treated group compared with that in the self-control group, respectively, and the expression level of miR-1968-5p ( $0.23 \pm 0.02$  vs.  $1.00 \pm 0.07$ ) was significantly decreased (Fig. 5A-D). Relative mRNA levels of Timp1 ( $304.24 \pm 88.65$  vs.  $1.00 \pm 0.56$ ), Serpin1 ( $92.07 \pm 44.17$  vs.  $1.00 \pm 0.56$ ), IL-11 ( $23.25 \pm 1.48$  vs.  $1.00 \pm 0.47$ ) and transforming growth factor (TGF)- $\beta$ 1 ( $3.85 \pm 0.62$  vs.  $1.00 \pm 0.16$ ) were significantly increased in the AA-treated group compared with that in the self-control group (Fig. 5E-H).

**Integrated analysis of DE miRNA-DE mRNA target pairs.** Targets of DE miRNAs were predicted using both RNAhybrid and miRanda. A total of 8,230 pairs of DE miRNA-mRNA targets were found. Compared with the DE mRNA data in the present study, 1,569 pairs of DE miRNA-DE mRNA targets were found. Among these, there was a total of 767 opposite direction regulatory pairs of DE miRNA-DE mRNA targets,

Table I. Sequences of primers used for reverse transcription-quantitative PCR.

mRNAs	Forward (5'-3')	Reverse (3'-5')
GAPDH	TGGTGAAGGTCGGTGTGAAC	GCTCCTGGAAGATGGTGATGG
TGF- $\beta$ 1	AGCCTGCCTCTTGAGTCCCT	CTCCCAAGGAAAGGTAGGTGAT
IL-11	GCTGGGACATTGGGATCTTTG	GAGCTGTAAACGGCGGAGTAG
Timp1	CCCCAGAAATCAACGAGACC	GTACGCCAGGGAACCAAGAA
Serpine1	GACACCCTCAGCATGTTTCATC	TTGGTCGGAAAGACTTGTGAA
U6	CTCGCTTCGGCAGCACA	AACGCTTCACGAATTTGCGT
miR-21a-5p	GUACUUAUCAGACUGAUGUUGA	UCAACAUCAGUCUGAUAAGUUA
miR-124-3p	TCTTTAAGGCACGCGGTG	TATGGTTTTGACGACTGTGTGAT
miR-132-3p	GCGCGCGTAACAGTCTACAGG	GTCGTATCCAGTGCAGGGTCC
miR-1968-5P	TGCAGCTGTTAAGGATGGTGGACT	GCGAGCACAGAATTAATACGAC

IL-11, interleukin 11; TGF- $\beta$ 1, transforming growth factor  $\beta$ 1; miR, microRNA; Timp1, TIMP metalloproteinase inhibitor 1; Serpine1, serpin family E member 1.

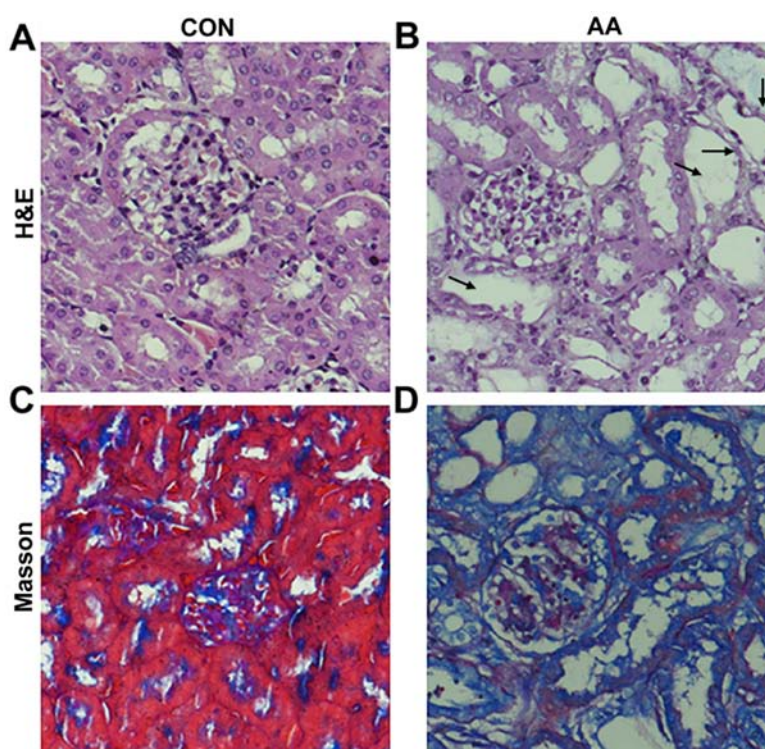


Figure 2. Histopathological changes induced by AA treatment analyzed by H&E and Masson's trichrome staining in kidney samples. Magnification, x400. Compared with (A) the self-control group, notably widened tubular lumens and flattened tubular cells were observed in (B) the AA-treated group by H&E staining. The arrows pointed out to the representative widened tubular lumens or flattened tubular cells. Compared with (C) the self-control group, a notable increase in collagen, as shown by an increase in Masson's trichrome staining, was observed in (D) the AA-treated group. AA, aristolochic acid; H&E, hematoxylin and eosin; CON, control.

including 416 pairs of upregulated DE miRNAs and down-regulated DE mRNAs, and 351 pairs of downregulated DE miRNAs and upregulated DE mRNAs (Data S2).

These 767 opposite direction regulatory pairs of DE miRNAs and DE mRNAs included 82 DE miRNAs and 624 DE mRNAs. Using GO analysis on the 624 DE mRNAs, it was identified that 'cellular process', 'biological regulation' and 'regulation of biological process' were the most notably altered biological processes, 'binding' was the most changed

molecular function, and 'cell' and 'cell part' were the most highly altered cellular components (Fig. 6A).

KEGG analysis on the aforementioned 624 DE mRNAs identified 'signal transduction', 'global and overview maps', 'cancers', 'endocrine system', 'cellular community-eukaryotes' and 'immune system' as the main altered pathways (Fig. 6B). The results were similar to that of total DE mRNAs. The most enriched KEGG pathways were identified as signaling pathways, ECM-associated pathways, 'metabolic pathways' and 'pathways



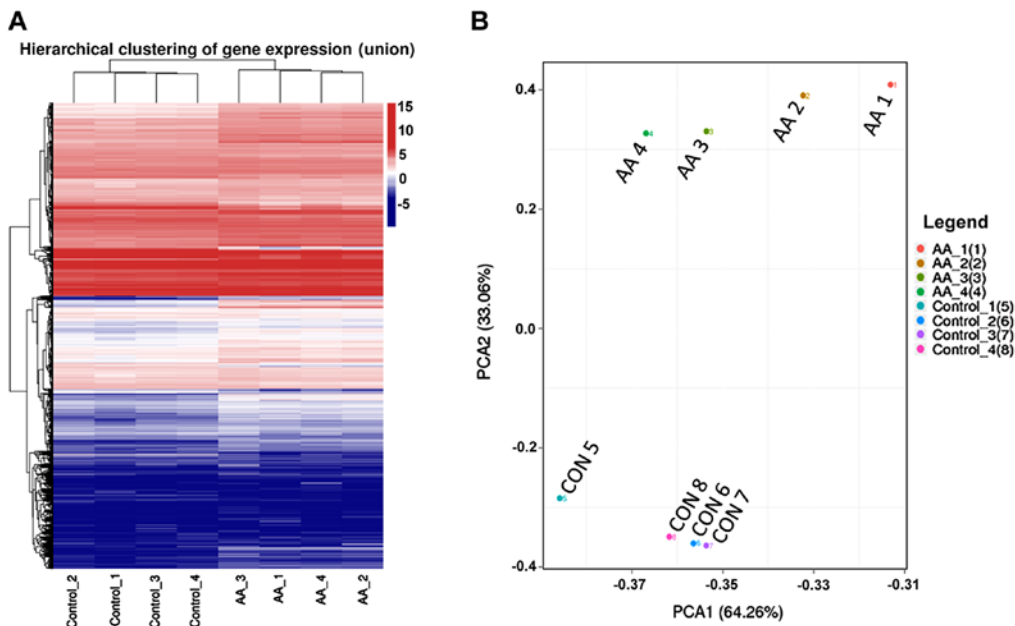


Figure 3. HCA and PCA of mRNA expression patterns. HCA and PCA were used to separate all 8 samples into different groups or clusters. Each group or cluster had similar mRNA expression patterns. (A) All 8 samples were separated into two groups, consistent with the treatment groups and the self-control groups, by HCA of mRNA expression levels. Each column represents an individual sample. Each row represents a differentially expressed mRNA. Different colors represent different expression levels of mRNA. The intensity of the red color is associated with higher expression levels. By contrast, the intensity of the blue color is associated with lower expression levels. (B) PCA separated all 8 samples into the AA-treated and the self-control clusters. PCA decreased a large amount of mRNA expression information in samples to a few independent variables (principal components) to compare the samples and identify clusters with high similarities in mRNA expression levels. The separate points represent separate samples. The x- and y-axes represent the contributor rate of the first component and second component, respectively. HCA, hierarchical clustering analysis; PCA, principal component analysis; AA, aristolochic acid.

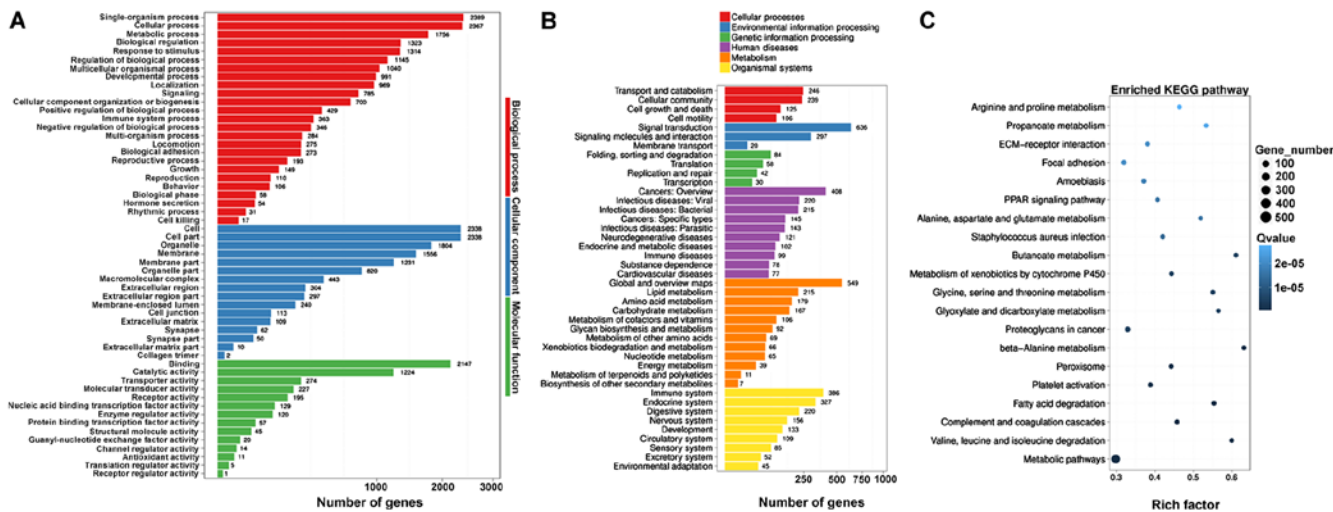


Figure 4. Analysis of GO and KEGG pathways of differentially expressed mRNAs. (A) GO analysis. (B) KEGG analysis of pathway enrichment. (C) Enriched KEGG pathways. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

in cancer' (Fig. 6C). Signal transduction was the most changed pathway, and signaling pathways were the most enriched pathways. A total of 40 signaling pathways and 6 ECM-associated pathways were identified by KEGG analysis. The most altered signaling pathways included 'Hedgehog signaling pathway', 'ABC transporters', 'ErbB signaling pathway', 'cAMP signaling pathway', 'Relaxin signaling pathway', 'Ras signaling pathway' and 'PI3K-Akt signaling pathway' (Fig. 6C). The most altered ECM-associated pathways included 'ECM-receptor interaction' and 'focal adhesion' (Fig. 6C).

*Regulatory networks of DE miRNAs on DE mRNAs involved in signaling pathways and ECM-associated pathways following AA treatment.* Signaling pathways were the most enriched KEGG pathways. Additionally, ECM-associated pathways were enriched pathways and closely associated with renal fibrosis. A total of 63 DE miRNAs and their 107 DE mRNA targets were found from the aforementioned 40 signaling pathways and 6 ECM-associated pathways. As shown in Fig. 7, opposing direction regulatory networks of DE miRNAs-DE mRNAs included 134 DE miRNA-DE mRNA pairs (Table SII). Regulatory

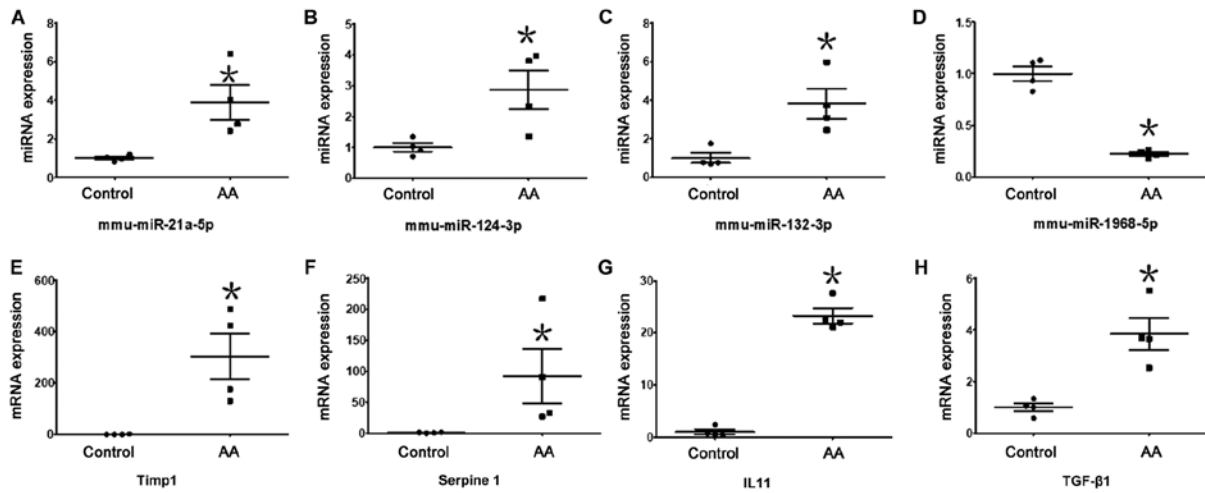


Figure 5. Expression levels of DE miRNAs and DE mRNAs validated by RT-qPCR. Expression levels of 4 DE miRNAs and 4 DE mRNAs were measured using RT-qPCR and normalized to U6 or GAPDH, respectively. Relative expression levels of (A) miR-124-3p (P=0.0398), (B) miR-21a-5p (P=0.0391) and (C) miR-132-3p (P=0.0399) were significantly increased in the AA group compared with in the self-control group. Expression levels of (D) miR-1968-5p (P=0.0014) were significantly decreased in the AA group compared with in the self-control group. Relative mRNA levels of (E) Timp1 (P=0.0416), (F) Serpine1 (P=0.0475), (G) IL11 (P=0.0038) and (H) TGF-β1 (P=0.0146) were significantly increased in the AA group compared with in the self-control group. \*P<0.05. AA, aristolochic acid; DE, differentially expressed; miRNA/miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; AA, aristolochic acid; IL11, interleukin 11; TGF-β1, transforming growth factor β1; mmu, *Mus musculus*.

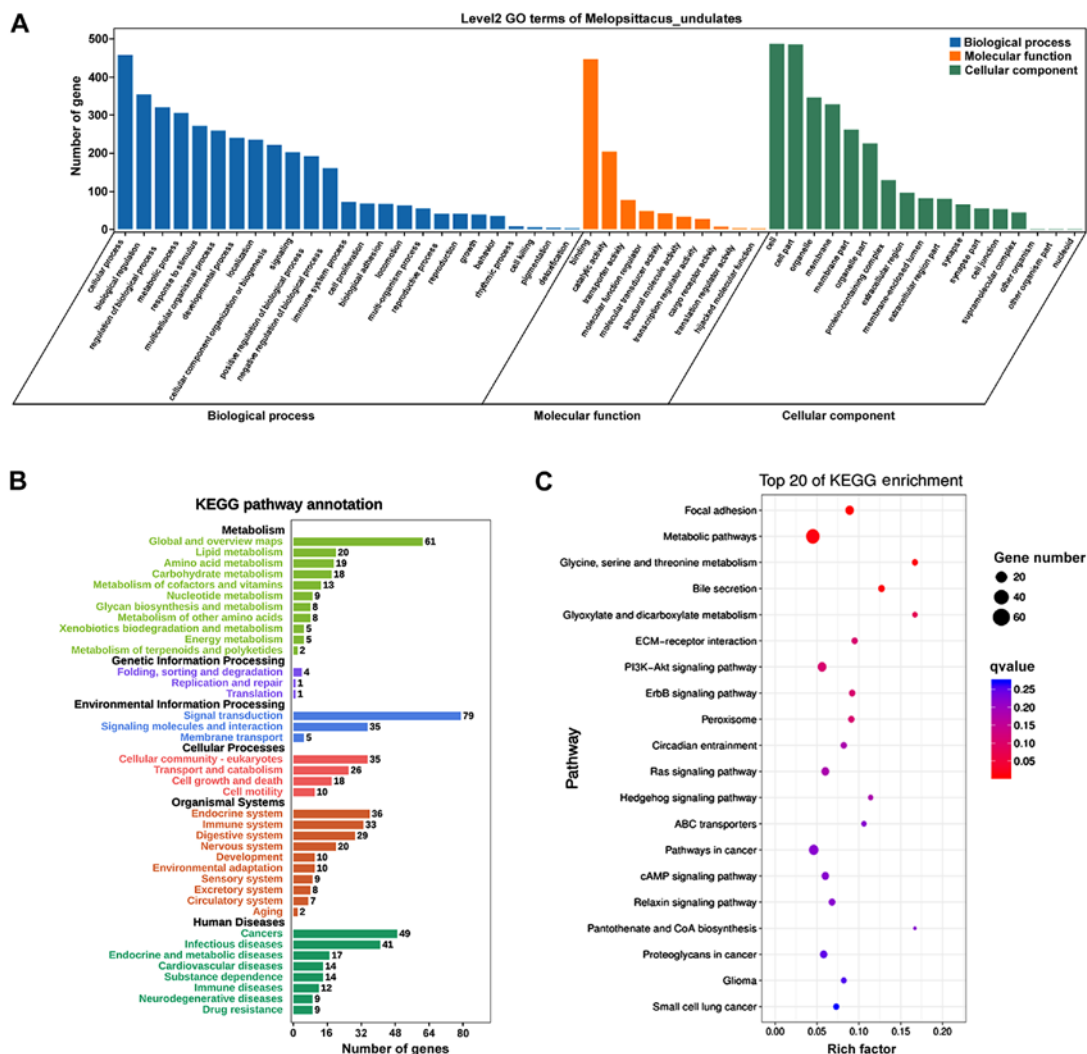
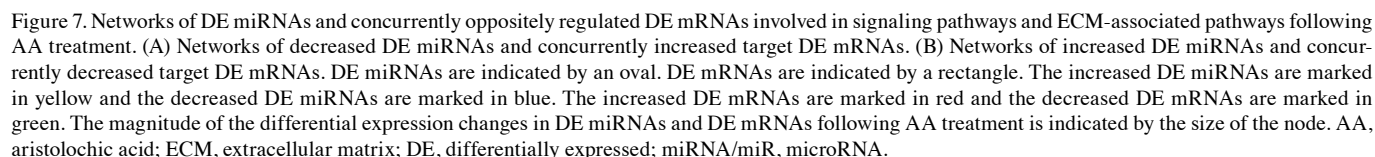


Figure 6. GO and KEGG pathway analysis of DE miRNAs and concurrently oppositely regulated DE mRNAs. (A) GO analysis. (B) KEGG analysis of pathway enrichment. (C) Enriched KEGG pathways. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DE, differentially expressed; miRNA, microRNA.



## Discussion

There was notable dysregulation of the miRNA expression profile following treatment with AA. Specifically, 82 DE miRNAs were identified following AA treatment, the



most notable alterations of which occurred in the profibrotic miR-21, miR-433 and miR-132 families, and the oncogenic miR-34 family, which were significantly increased, and in the anti-fibrotic miR-122-5p and let-7a-1-3p, which were significantly decreased. Similar increases in the miR-21 and miR-34 families following long-term exposure to a carcinogenic dose (10 mg/kg/day for 12 weeks) of AA in rats have been previously reported in rats (25,26). Activity of the TGF- $\beta$ /Smad3 signaling pathway is increased in renal fibrosis by miR-21 (25,26) via Smad7, PTEN, miR-433 (27) and Azin1. Silencing of miR-132 was reported to reduce renal fibrosis (28,29). Significant downregulation of let-7a was reported to be associated with renal fibrosis in chronic kidney disease (30).

Global mRNA profiling was notably altered following AA treatment. Amino acid metabolism, fatty acid metabolism, 'complement and coagulation cascades', 'ECM-receptor interaction' and 'focal adhesion' were the most significantly enriched pathways, based on KEGG analysis of DE mRNAs. The pathways of 'complement and coagulation cascades', 'ECM-receptor interaction' and 'focal adhesion' directly participate in the process of tubulointerstitial fibrosis (23,31). Amino acid metabolism pathways are involved in the production of matrix collagen during fibrosis, which is enriched in glycine, hydroxyproline, and hydroxylysine, and other amino acid-derived compounds (32).

Additionally, GO and KEGG analysis were used to analyze the 624 DE mRNAs regulated by DE miRNAs. The renal tubular injury was largely the result of death or apoptosis of tubular epithelial cells (33,34). The following tubulointerstitial fibrosis was induced by infiltration of immune cells, production and secretion of pro-fibrosis factors, downregulation and inhibition of anti-fibrosis factors, myofibroblasts activation, EMT, ECM production, tubular atrophy and others (7). In this process, the most altered biological process in GO analysis may serve an important role, which included 'biological regulation', 'metabolic process' and 'response to stimulus' in the biological process category, 'binding' in the molecular function category, and 'organelle' and 'membrane part' in the cellular component category. This process was also affected by the most altered pathways in KEGG analysis, which included 'signal transduction', metabolism, 'endocrine system', 'cellular community - eukaryotes' and 'immune system'. Additionally, 'cancers' were one of the top changed pathways, as AA is also a carcinogen, and EMT in fibrosis is an important pathway in cancer.

In KEGG, similar to the results obtained from analysis of the total DE miRNAs, ECM-associated pathways, metabolic pathways and cancer-associated pathways were the most enriched KEGG pathways. However, unlike the results of total DE miRNAs, signaling pathways were identified as the most enriched KEGG pathways regulated by DE miRNAs. A total of 40 signaling pathways were identified. In the most significantly altered signaling pathway in this study, fibrosis was reported in previous studies to be involved in the 'Hedgehog signaling pathway' (35-37), 'ABC transporters' (38), 'ErbB signaling pathway' (39,40), 'cAMP signaling pathway' (41,42), 'Relaxin signaling pathway' (43,44), 'Ras signaling pathway' (45,46) and 'PI3K-Akt signaling pathway' (47,48). Additionally, six ECM-associated pathways were directly associated with renal fibrosis.

It has been suggested that DE miRNAs serve an important role in renal injury and tubulointerstitial fibrosis by regulating the DE mRNAs enriched in the signaling pathways and ECM-associated pathways. In the aforementioned 40 enriched signaling pathways and 6 ECM-associated pathways, the opposite direction regulatory networks of 63 DE miRNAs and their 107 DE mRNA targets were drawn. A total of 134 opposite direction regulatory pairs of DE miRNAs and DE mRNAs were included in these regulatory networks. The majority of the 63 DE miRNAs had >1 DE mRNA target. For example, the increased DE miRNAs with the largest numbers of targets included the miR-21 family (miR-21a-5p and miR-21a-3p), the miR-124 family (miR-124-5p and miR-124-3p), miR-129-2-3p and miR-34b-3p. The decreased DE miRNAs with the largest numbers of targets included miR-122-5p, the miR-3073 family (miR-3073a-5p, miR-3073a-3p and miR-3073b-5p), the miR-1948 family (miR-1948-5p and miR-1948-3p), miR-669c-5p and let7a-1-3p. Among these, fibrosis was reported to be regulated by the increase in expression of the miR-21 family (49-54), miR-124 family (55,56) and miR-34b-3p (57,58), and the decrease in miR-122-5p (59-61) and let7a-1-3p (62). The present study was the first to report that AAN was associated with an increase in miR-129-2-3p expression and a decrease in the expression levels of the miR-3073 family (miR-3073a-5p, miR-3073a-3p and miR-3073b-5p), miR-1948 family (miR-1948-5p and miR-1948-3p) and miR-669c-5p.

In the present self-controlled study, mice were treated with AA, following unilateral nephrectomy. This self-control design may help increase the sensitivity and accuracy of results by decreasing the interference of individual variations. However, there may be other influencing factors, including anesthesia, hyper-perfusion and residual renal function following unilateral nephrectomy. These factors were likely less influencing than the AA-induced acute renal injury. Although these factors often had a notable effect under the background of hypertension, diabetes, genetic defect, toxic drugs or other harmful conditions, no significant changes were reported in the control mice group given only unilateral nephrectomy (63-65). The nature and severity of the renal response to unilateral nephrectomy were reported to be strain-dependent in mice (66). It has been reported that the unilateral nephrectomy did not induce sclerosis or fibrosis in the glomerulus, tubulointerstitium or vasculature after 8 weeks in the C57 strain mice (66).

Acute AAN in humans results in AA-induced acute kidney injury and may progress to chronic kidney disease (CKD, as necroinflammation, an auto amplification loop between tubular cell death/apoptosis and interstitial inflammation, results in continued renal injury and tubulointerstitial fibrosis (67-69). Halting the progression of CKD following AA exposure by inhibition or even resolution of AA-induced renal tubular injury and tubulointerstitial fibrosis should be considered as a potential therapeutic approach. The present study identified DE miRNAs, their mRNA targets and the enriched pathways of the mRNA targets, which may assist in improving the understanding of the underlying mechanisms. The DE miRNAs and their regulated mRNAs represent potential targets that may protect the kidney from continued tubular injury and tubulointerstitial fibrosis following AA exposure, and may assist in the development of novel clinical therapies for treatment of CKD following AA exposure.

In conclusion, in the present self-controlled study, renal injury and tubulointerstitial fibrosis were induced by short-term treatment with AA in mice, and genome-wide DE miRNAs and DE mRNAs, and their regulated pairs and networks were identified. The present results may assist in improving the understanding of the role of the DE miRNAs and their mRNA targets in the pathophysiology of renal injury and tubulointerstitial fibrosis in acute AAN.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

XL designed this study. ZZ participated in the design and writing of the study, the major experiments and data analysis. XX, YS and YZ participated in the *in vivo* experiments. XX, WQ and FW participated in the *in vitro* experiments. XZ, CB, HH and SL analyzed the data from the deep sequencing results of miRNA and mRNA. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the Children's Hospital of Soochow University (approval no. 2020-CHSU-012).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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