miR-664a-5p promotes experimental membranous nephropathy progression through HIPK2/Calpain1/GSα-mediated autophagy inhibition

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Research Article

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Abstract

[Background] We previously found that miR-664a-5p is specifically expressed in urinary exosomes of idiopathic membranous nephropathy (IMN) patients, but its function and mechanism in MN progression are unclear.

[Objective] To investigate the function and mechanism of miR-664a-5p in MN.

[Methods] The miR-664a-5p expression in HK-2 cells, exosomes, human podocytes and renal tissues were studied, as well as the activity and apoptosis of these cells, the binding of miR-664a-5p to HIPK2 mRNA, the levels of several relative proteins and autophagy, several relative characteristics of exosomes. The MN progression in MN mice model was also studied.

[Results] Albumin increased the miR-664a-5p content and apoptosis of HK-2 cells, which was blocked by miR-664a-5p antagomir. miR-664a-5p bound to the 3' UTR of HIPK2 mRNA and reduced its expression. miR-664a-5p antagomir restored albumin-mediated Calpain1 up-regulation, GSα shear and autophagy decline. Autophagy inhibitor CQ blocked the protective effect of miR-664a-5p antagomir, HIPK2 overexpression, and Calpain inhibitor SJA6017 on albumin-mediated injury. The miR-664a-5p level increased in exosomes from albumin-treated HK-2 cells, and it could be horizontally transported to podocytes through exosomes. In MN mice, exosomes from albumin-treated HK-2 cells promoted the pathological MN symptoms, and AAV-Anti-miR-664-5p (mouse homology miRNA) could improve them.

[Conclusion] Albumin increases the miR-664a-5p level and causes changes in the HIPK2/Calpain1/GSα pathway, which leads to autophagy inhibition and apoptosis up-regulation of renal tubular epithelial cells. miR-664a-5p can horizontally enter podocytes through exosomes. Targeted inhibition of miR-664a-5p can reduce the apoptosis of renal tubule cells and podocytes, and may improve the MN progression.

Introduction

Membranous nephropathy (MN) is a type of autoimmune disease characterized by diffuse deposition of immune complex under glomerular basal membrane epithelial cells accompanied by diffuse thickening of the basal membrane. MN has been enlisted as the most common causes of nephrotic syndrome in adults [1, 2], and the main clinical manifestations include macroalbuminuria, hypoproteinemia, severe edema and hyperlipemia. 30–40% of patients with MN will progress to end-stage renal failure after 10–15 years [3]. Even though the current nonspecific immunosuppressive therapies are usually effective in treating MN, the cure rate remains poor and have many persistent adverse effects [4]. MN is mainly divided into primary MN and secondary MN, and the etiology of the primary MN is unknown. The secondary MN occurs because of various conditions such as infection, malignancy, systemic autoimmune disease, or drug use [5]. To reveal the pathogenesis of MN, especially primary MN, is important for the early diagnosis and treatment of MN.
MicroRNAs (miRNAs) are a class of endogenous non-coding RNA about 20 ~ 25 nucleotides long. Studies have pointed out that their expression is disordered in kidney tissues of MN patients, including let-7b-5p [6], miR-186 [7], and microRNA-192 [8], etc. Exosomes are disk-like vesicles with a diameter of 30-150nm secreted by cells [9], which are rich in various proteins, lipids and non-coding RNAs, including miRNAs. Studies show that exosomal miRNAs have multiple biological functions and can regulate the progression of MN [8], renal fibrosis [10], chronic nephropathy [11], acute kidney injury [12], diabetic nephropathy [13] and other nephropathy. Additionally, exosomes can also enter the urine, which has potential in diagnosis [8, 14]. We previously found that compared with healthy volunteers, patients with IMN have unbalanced expression profiles of miRNAs in urinary exosomes and miR-664a-5p was the IMN-specific urinary exosomal miRNA with the largest range of change [15]. However, whether miR-664a-5p is important in the progression of MN remains unclear.

After injection of cationic serum albumin (cBSA), mice can show a series of MN or nephrotic syndrome symptoms, such as proteinuria, serum cholesterol levels up-regulation, serum albumin content down-regulation and so on [16, 17]. It is reported that albumin treatment can directly induce injury of renal tubular epithelial cells, which closely relates to renal re-absorption and excretion [17–21], as well as injury of podocytes [22], an important component of the glomerular filtration barrier. Additionally, studies show that the content of miR-199a-5p of exosomes secreted by HK-2 cells increased and can enter the urine, resulting in abnormal miR-199a-5p content in the urinary exosome of diabetic nephropathy patients [23]. These results indicate that renal tubular epithelial cells and podocytes are the main target cells in MN model induced by cBSA. It is suggested that the imbalance of urinary exosomal miRNA expression in MN patients may be related to the alteration of miRNA expression profiles in renal tubular epithelial cells.

Herein, the effect of albumin on the expression of miR-664a-5p in HK-2 cells and podocytes were explored, and the role of miR-664a-5p in HK-2 cell injury and in MN mice was clarified. Mechanically, we found that miR-664a-5p decreased Homeodomain Interacting Protein Kinase 2 (HIPK2) expression by targeting HIPK2 mRNA, resulting in Calpain1/GSα-mediated autophagy inhibition, which led to the apoptosis of renal tubular cells. Meanwhile, we found that miR-664a-5p could transfer from HK-2 cells into podocyte through exosomes and could induce podocytes damage through HIPK2/Calpain1/GSα-mediated autophagy down-regulation.

[Methods] The content of miR-664a-5p in renal tubular epithelial cells HK-2, exosomes, human podocytes and renal tissues was detected by qRT-P

**Methods and materials**

**Cell culture and treatment**

HK-2 cells (procell, CL-0109, Wuhan, CN), human podocytes (procell, CP-H075, Wuhan, CN) and HEK293T cells (procell, CL-0005, Wuhan, CN) were purchased from Wuhan Punocai Life Technology Co., LTD. HK-2 cells were cultured in MEM medium (procell, PM150410, Wuhan, CN) containing 10% FBS (procell,
164210-500, Wuhan, CN) and 1% penicillin/streptomycin (procell, PB180120, Wuhan, CN) under 37°C and 5% CO2. Podocytes were cultured on Type I collagen (Gibco, 17100-017, Shanghai, USA)-coated dishes and cultured in RPMI-1640 medium (procell, PM150110, Wuhan, CN) containing 20 U/mL IFN-γ (procell, PCK062, Wuhan, CN) and 10% FBS at 33°C for amplification, and differentiated into mature podocytes after 14 days of culture at 37°C with RPMI-1640 medium with 5% FBS but without IFN-γ. After that, podocytes were synchronized with RPMI-1640 medium containing 0.2% FBS for 24 h. HEK293T were cultured in DMEM medium (procell, PM150210, Wuhan, CN) containing 10% FBS and 1% penicillin/streptomycin under 37°C and 5% CO2.

**qRT-PCR detection**

RNAs were extracted from cells or exosomes by miRNeasy Mini kit (Qiagen, 217004, Dusseldorf, DE) and reverse transcribed into cDNAs by RevertAid First Strand cDNA Synthesis Kit (Thermo, K1622, Shanghai, CN), then cDNAs were measured by qRT-PCR with SYBR Green qPCR Master (Roche, 4943914001-SR, Shanghai, CN), and the amount was calculated by $2^{-\Delta\Delta Ct}$. The primers were as follows: 5’-TGCGCAGTGGCTAGGAAATGAT-3’ (forward) and 5’-CCAGGTGCAGGGTCCGAGGTATT-3’ (reverse) for hsa-miR-664a-5p, 5’-TGGCGATATTCATTACCTTACTCCCCA-3’ (forward) and 5’-CCAGGTGCAGGGTCCGAGGTATT-3’ (reverse) for mmu-miR-664-5p and 5’-CGCTTGCGCAGCACATATAC-3’ (forward) and 5’-AAATGAACGCTTTCACGA-3’ (reverse) for U6.

**CCK-8 detection**

The operation followed the kit manual. Briefly, cells were transfected with miR-664a-5p antagonir (ribobio, miR30005948-4-5,Guangzhou, CN) or Anta NC (ribobio, miR3N0000001-4-5,Guangzhou, CN), combined with or without 100 mg/mL albumin [18] (sigma, A9080, Shanghai, CN) or PBS, or HIPK2 siRNA (5’-GGUGGAUCCAUCUAGACAA-3’) or siRNA NC (5’-CACUGAUUU CAAAUGGCGUAU-3’), or HIPK2 overexpression vector (abmgood, 232980110000, Richmond, Canada) or vector, or 20 µM chloroquine [24] (CQ, macklin, C843545, Shanghai, CN), or 100 µM SJA6017 [25] (MedChemExpress, HY-118933, Shanghai, CN). After 72h, CCK8 (MCE, HY-K0301, Shanghai, CN) solution was added and the OD 450nm was determined by the plate reader (Molecular Devices Co., Sunnyvale, CA, USA).

**Flow cytometry detection**

After cell treatment was completed, cells were collected, rinsed twice with PBS, and centrifuged at 1000 rpm for 5 min. Then operation followed the instructions of ANNEXINV-FITC/PI cell apoptosis detection kit (JianGSu Keygen Biotech Corp., Ltd., KGA108, Shanghai, CN). 500 µL Binding Buffer mixed with 5 µL AnnexinV-FITC and 5 µL PI was added. After the reaction at room temperature for 10 min, cells were detected by flow cytometry (CytoFLEX, Beckman, USA).

**Double-luciferase reporter gene assay**

The wild type and mutated HIPK2 3’-untranslated regions (3’-UTR) sequence were respectively cloned into pmirGLO Dual-luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA) to construct HIPK2 3’-UTRWT and HIPK2 3’-UTRMT. A mixture of Lipofectamine 2000 (Invitrogen, 11668019, Shanghai,
CN), double-luciferase reporter plasmid, and miRNA antagonir NC or miR-664a-5p antagonir were transfected into HEK293T cells. After 24 h, the luciferase activity was analyzed using a double luciferase reporting system (Beyotime, RG027, Shanghai, CN).

**WB detection**

The operation followed the standard protocol. Briefly, protein were separated by electrophoresis and transferred on PVDF membrane; PVDF membrane was consecutively treated with primary antibodies (HIPK2, Affinity, DF7982, 1:1000, Wuhan, CN; Calpain1, Affinity, DF6306, 1:1000, Wuhan, CN; GSc, sigma, 06-237, 1: 1000, Shanghai, CN; LC3, Affinity, AF5402, 1: 1000, Wuhan, CN; P62, Affinity, AF5384, 1: 1000, Wuhan, CN; GAPDH, Xianzhi, AB-P-R001, 1: 1000, Wuhan, CN; CD9, Proteintech, 20597-1-AP, 1:1000, Wuhan, CN; TSG101, Affinity, DF8427, 1:1000, Wuhan, CN; CD63, Affinity, AF5117, 1:1000, Wuhan, CN; calnexin, Affinity, AF5362, 1:1000, Wuhan, CN; HRP labeled secondary antibody (HRP labeled secondary antibody of sheep and rabbit, Boster, BA1054, 1:10000, Wuhan, CN) and ECL reagents. Finally, the film was analyzed using Image J software (National Institutes of Health, USA).

**Exosome extraction**

The operation followed the standard protocol. Briefly, HK-2 cells were treated with 100 mg/mL albumin. The supernatant was centrifuged. ECS reagent (exosome extraction and purification kit, umibio, UR52121, Shanghai, CN) was added and the mixture was centrifuged. The precipitation was evenly dispersed with PBS, and the suspension was centrifuged, and the supernatant, rich in exosome particles, was retained. The crude exosomes were transferred into the upper chamber of the EPF column and centrifuged. The liquid at the bottom of the EPF column was collected, which was the purified exosome particles and stored at -80℃.

**Electron microscope detection**

The operation followed the standard protocol. Briefly, samples, ultra-pure water and PTA dye liquid (sigma, P4006, Shanghai, CN) were dropped on the copper mesh. After cooling, the copper mesh was dried naturally and observed under electron microscope (FEI, Tecnai G20 TWIN, Hillsboro, USA).

**Protein concentration detection**

The operation followed the standard protocol. Briefly, BCA protein concentration assay kit (Biyuntian, P0012S, Shanghai, CN) was used to determine exosomal protein concentration. The concentrations were determined by measuring the OD562nm.

**Autophagy flux detection**

10 µL Ad-Mcherry-GFP-LC3B virus stock solution (Beyotime, C3011, Shanghai, CN) was added to HK-2 cells. After the culture at 37℃ for 24h, fresh culture-medium was added and the cells were cleaned with PBS. Autophagy flux was observed under confocal laser fluorescence microscope (Olympus, FV3000, Tokyo, Japan) 48h later.
**Exosome uptake detection**

The operation followed the standard protocol. Briefly, miR-664a-5p with or without Cy3-labeled was transfected into exosome from HK-2 cell treated with PBS (Exo\textsuperscript{ctrl}), and then 10µL exosomes was added to human podocytes. 24h later, drops of 4% paraformaldehyde (macklin, P885233, Shanghai, CN) were added. The slide was dried, and drops of DAPI (sigma, D9542, Shanghai, CN) dye solution was added, and the slide was set at room temperature for 5min and sealed and images were observed under a laser confocal fluorescence microscope.

**Murine model of MN**

The female BALB/c mice were acquired from Yichang University (CN), fed in a 12 h light/dark cycle chamber at 24 ± 0.5 °C. All experimental protocols were approved by the Hospital Institutional Animal Care and Use Committee of Zhejiang Provincial Institute of Traditional Chinese Medicine (Approval No.IACUC-A202208001) and comply with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. 30 female BALB/c mice aged 4–6 weeks were randomly divided into 6 groups with 5 mice in each group. The model group was treated with complete Freund's adjuvant (CFA, Sigma, F5881, Shanghai, CN) containing 0.2 mg cationic BSA (cBSA, Chondrex, 9058, Woodinville, USA) for 2 weeks, and then was given intravenous 100µg cBSA three times a week for 6 weeks [17]. Mice were treated with intravenous injection of PBS or 100µg Exo\textsuperscript{ctrl} or exosomes from albumin-treated HK-2 cells (Exo\textsuperscript{Albumin}) once a week at the beginning of modeling. Meanwhile, mice were also injected with 1.5×10<sup>9</sup> pfu adeno-associated virus (AAV-Anti NC or AAV-Anti-miR-664-5p) intravasously every 4 weeks.

**Urine protein, serum cholesterol and albumin content detection**

The operation followed the kit manual. Briefly, 24h urine was collected and urine protein was determined by urine protein quantitative test kit (Nanjing Jiancheng, C035-2-1, Nanjing, China) according to OD595nm. Serum cholesterol content was detected by the total cholesterol detection kit (mlbio, ml094953, Shanghai, CN) according to OD550nm. And serum albumin content was detected using the albumin detection kit (mlbio, ml095005, Shanghai, CN) according to OD603nm.

**IgG deposition test**

The operation followed the standard protocol. Briefly, the sample was dehydrated, and xylene was added to make the tissue transparent, then the tissue was embedded, sliced, and the slices were attached to the slides and baked 60°C. Then, it was successively dewaxed and repaired. Then the primary antibody (IgG, CST, 4418, 1:100, Shanghai, China) and the second antibody (Goat Anti-Rat, BIOSS, bs-0293G-CY3,1:100, Beijing, CN) were added, then DAPI chromogenic solution was added. The slide was sealed, and the images were observed under a fluorescence microscope (Olympus, IX51, Tokyo, Japan).

**Periodic Acid Schiff (PAS) staining**
The operation followed the standard protocol. Briefly, the sample was dehydrated, and xylene was added to make the tissue transparent, then the tissue was embedded, sliced, and the slices were attached to the slides and baked 60°C. Then, it was dewaxed and the slices were soaked in 1% periodic acid solution (Sinopharm, 80098516, Shanghai, China) and washed with distilled water. Schiff’s reagent (Sinopharm, 71019654, Shanghai, China) was added, and the slide was incubated at room temperature for 20min, and the slices were rinsed with water. The slide was treated by Mayer Hematoxylin (sigma, H9627, Shanghai, China) and rinsed until the nuclei turned blue. The slide was dehydrated, made transparent with xylene, air dried, sealed, and observed with microscope (Olympus, BX53, Tokyo, Japan).

**Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay (TUNEL assay)**

The operation followed the standard protocol. Briefly, TUNEL cell apoptosis assay kit (promega, G7360, Beijing, CN) was used. The slices were treated with protease K working solution. 100 µL Equilibration Buffer and TdT enzyme reaction solution was added dropwise successively. The slices were put into the staining cylinder with 2×SSC solution to terminate the reaction, and placed at room temperature for 15 min. 100 µL Streptavidin HRP (streptavidin horseradish peroxidase) solution was added to the slices, and then 100 µL DAB chromogenic solution was added. The slide was sealed after Mayer hematoxylin counterstaining. The slide was observed under a microscope.

**IHC detection**

Slices were repaired with 0.01 M citric acid buffer (pH 6.0), and then 3% hydrogen peroxide was used to block endogenous peroxidase. Then the diluted primary antibody (HIPK2, NOVUS, NBP1-89462, 1:200, Guangzhou, CN) was added and the slices were incubated in wet box overnight. The secondary antibody (Goat Anti-Rabbit IgG (H + L) HRP, Affinity, S0001, 1:200, Wuhan,CN) was added and the slices were incubated at 37°C for 30 min. Then the freshly prepared DAB chromogenic solution (biosharp, BL732A, Hefei, CN) was dripped on and the slices were observed under the microscope. The positive signal is brownish yellow or brownish brown. After re-staining with Harris hematoxylin, the slide was sealed and observed under the microscope.

**Data analysis**

Statistical software Prism9.0 was used for data analysis, and all data were expressed as Mean ± SD. One-way analysis of variance or student’s t-test was used for statistical analysis between groups. p < 0.05 was considered statistically significant.

**Results**

**Albumin induces cell injury by changing the miR-664a-5p content in renal tubular epithelial cells**
HK-2 cells and podocytes were treated with albumin to construct MN cell models. CCK-8 assay and flow cytometry showed that albumin (100mg/mL) could reduce the activity of HK-2 cells and increase the apoptosis (Fig. 1B-1C). Subsequently, qRT-PCR experiment showed that albumin treatment increased the content of miR-664a-5p in HK-2 cells (Fig. 1A). However, the miR-664a-5p content didn’t change in podocytes treated with albumin, which could induce podocytes injury (Supplementary Fig. 1).

Next, we used miR-664a-5p antagonir and albumin to co-treat HK-2 cells. qRT-PCR results showed that miR-664a-5p antagonir decreased the content of miR-664a-5p in HK-2 cells compared with those cells treated with miRNA antagonir NC. The promoting effect of albumin on miR-664a-5p content in HK-2 cells was reversed by miR-664a-5p antagonir (Fig. 1A). The results of CCK-8 assay and flow cytometry showed that miR-664a-5p antagonir treatment increased the cell activity and decreased the apoptosis of HK-2 cells (Fig. 1B-1C). Moreover, miR-664a-5p antagonir inhibited the effect of albumin on HK-2 cell activity and apoptosis level (Fig. 1B-1C). It demonstrated that albumin could damage renal tubular epithelial cells, probably depending on miR-664a-5p up-regulation.

The promoting effect of miR-664a-5p on albumin-mediated renal tubular epithelial cell injury depends on the decreased expression of HIPK2

Binding loci between miR-664a-5p and HIPK2 mRNA 3’UTR were predicted with targetscan and the double-luciferase reporter containing the wild type sequence or mutated sequence was constructed (Fig. 2A). Compared with miRNA agomir NC treatment group, miR-664a-5p agomir treatment could reduce the luciferase activity of HIPK2 3’-UTR\textsuperscript{WT}, but could not change the luciferase activity of HIPK2 3’-UTR\textsuperscript{MT} (Fig. 2B). Western Blot assay results showed that miR-664a-5p antagonir increased HIPK2 protein content in HK-2 cells (Fig. 2C). Additionally, albumin reduced HIPK2 content in HK-2 cells, and this effect was reversed by miR-664a-5p antagonir (Fig. 2C).

To clarify the effects of HIPK2 on the cell injury inhibition of miR-664a-5p antagonir-mediated HK-2, HIPK2 siRNA was constructed. Western Blot results showed that HIPK2 siRNA-2 had the strongest inhibitory effects on the expression of HIPK2 in HK-2 cells (Supplementary Fig. 2). Subsequently, HK-2 cells were treated with HIPK2 siRNA and miR-664a-5p antagonir combined with albumin. WB results confirmed that HIPK2 siRNA could aggravate albumin-mediated HIPK2 expression inhibition and prevent miR-664a-5p antagonir from raising the HIPK2 protein content in albumin-treated HK-2 cells (Fig. 3A). In CCK-8 and flow cytometry experiments, HIPK2 siRNA decreased the activity of HK-2 cells and increased the apoptosis regardless of albumin. MiR-664a-5p antagonir blocked albumin-mediated activity decrease and apoptosis increase of HK-2 cells, but these effects were inhibited by HIPK2 siRNA (Fig. 3B-3C). In addition, HIPK2 overexpression could increase the expression of HIPK2 (Supplementary Fig. 2), the cell activity and inhibit the cell apoptosis of HK-2 cells (Group3 vs Group1, Fig. 5B-5C). Moreover, albumin-mediated cell activity inhibition and apoptosis induction could be blocked by HIPK2 overexpression (Group9 vs Group5, Fig. 5B-5C). It suggested that HIPK2 expression down-regulation caused by miR-664a-5p was involved in albumin-mediated renal tubular epithelial cell injury.
miR-664a-5p is involved in albumin-mediated renal tubular epithelial cell injury by regulating Calpain1/GSα/autophagy signaling pathway through HIPK2

It was reported that HIPK2 can promote autophagy, which relates to the the expression decrease of Calpain1 [26]. Calpain1 can shear and activate the heterotrimer G-protein subunit GSα, causing autophagy inhibition in Drosophila [27, 28]. Decreased autophagy is important for the renal tubular cell injury [29]. We investigated the effects of albumin, miR-664a-5p antagonir and HIPK2 on the expression of Calpain1 and GSα shear in HK-2 cells. The data showed that albumin increased the Calpain1 content in HK-2 cells and promoted GSα shear, which were blocked by miR-664a-5p antagonir (Fig. 4A-4B). Additionally, HIPK2 knock-down increased Calpain1 expression and GSα shear in HK-2 cells in the presence or absence of albumin and reversed the inhibition of miR-664a-5p antagonir on Calpain1 expression and GSα shear in HK-2 cells induced by albumin treatment (Fig. 4A-4B). Furthermore, the stimulative effect of albumin on GSα shear was also inhibited by the Calpain inhibitor SJA6017 (Supplementary Fig. 3). It indicated that albumin stimulation could activate Calpain1/GSα pathway depending on the miR-664a-5p-mediated HIPK2 expression inhibition in renal tubular epithelial cells.

WB and confocal laser microscope results showed that albumin could reduce the LC3II/I proportion, increase the p62 expression and reduce the number of intracellular autophagosome and autophagosome in HK-2 cells, which was blocked by miR-664a-5p antagonir (Fig. 4C-4D). The effects of miR-664a-5p antagonir on LC3II/I ratio, p62 expression, intracellular autophagosome and autophagosome in HK-2 cells treated with albumin were blocked by HIPK2 siRNA. Additionally, calpain inhibitor SJA6017 enhanced the ratio of LC3II/I and decreased the expression of p62 and blocked the albumin-mediated change of LC3II/I and p62 protein (Fig. 5A and Supplementary Fig. 4A). It demonstrated that miR-664a-5p could inhibit autophagy level through HIPK2/Calpain1/GSα signal pathway in renal tubular epithelial cells treated with albumin.

To confirm that autophagy inhibition by miR-664a-5p/HIPK2/Calpain1/GSα pathway was related to renal tubular epithelial cell injury, HK-2 cells were treated with albumin, miR-664a-5p antagonir or HIPK2 overexpression or Calpain inhibitor SJA6017 combined with autophagy inhibitor CQ. CQ inhibited autophagy by blocking autophagic lysosome formation, leading to the accumulation of autophagic selective substrate p62 [30]. WB showed that CQ could increase LC3II/I proportion and p62 expression, suggesting that autophagy was inhibited. Moreover, the autophagy caused by miR-664a-5p antagonir, HIPK2 overexpression and SJA6017 in HK-2 cells could be reversed by CQ (Fig. 5A and Supplementary Fig. 4A). The results of CCK-8 assay and flow cytometry showed that CQ reduced cell activity and enhanced apoptosis in HK-2 cells treated with or without albumin and miR-664a-5p antagonir, HIPK2 overexpression, or SJA6017 showed opposite effects, which were blocked by CQ (Fig. 5B-5C). It suggested that miR-664a-5p/HIPK2/Calpain1/GSα signal pathway-mediated autophagy inhibition was involved in renal tubular epithelial cell injury induced by albumin.

miR-664a-5p is transported from renal tubular epithelial cells to podocytes via exosomes
Intracellular high-abundance non-coding RNA can be secreted into exosomes, and when the exosomes are taken up by target cells, non-coding RNA in exosomes can be transferred to target cells, achieving horizontal transport of non-coding RNA [13, 23, 31]. Podocytes, the epithelial cells of the renal capsule attached to the lateral glomerular basement membrane (GBM), constitute the glomerular hemofiltration barrier together with vascular endothelial cells and the glomerular basement membrane. It was reported that glomerular endothelial cell-derived exosomal miR-192-5p can reduce the expression of nephronectin in podocytes and participate in the regulation of IMN [8].

To investigate the exosomal miR-664a-5p transport, exosomes from HK-2 cells was extracted and confirmed by transmission electron microscope and WB (Supplementary Fig. 5). Subsequently, qRT-PCR assay was used to detect miR-664a-5p content in exosomes from HK-2 cells treated with or without albumin. Data showed that compared with Exo\textsuperscript{Ctrl}, the content of miR-664a-5p in Exo\textsuperscript{Albumin} increased (more than 4 folds, Fig. 6A). Then, Cy3-labeled miR-664a-5p agomir was transfected into Exo\textsuperscript{Ctrl} and incubated with podocytes. Under laser confocal microscopy, Cy3 was observed in podocytes (Fig. 6B), indicating that miR-664a-5p in exosomes could be taken up by podocytes. Further, qRT-PCR showed that compared with podocytes without exosome treatment, the miR-664a-5p content in podocytes treated with Exo\textsuperscript{Ctrl} didn't change and that in podocytes treated with Exo\textsuperscript{Albumin} treatment increased (Fig. 6C). These data showed that miR-664a-5p from renal tubular epithelial cells treated with albumin could be transported into podocytes via exosomes.

Renal tubular epithelial cells-derived exosomal miR-664a-5p can alter HIPK2/Calpain1/GS\alpha/ autophagy pathway and apoptosis of podocytes

To determine whether renal tubular epithelial cells-derived exosomes can induce podocyte injury through miR-664a-5p/HIPK2/Calpain1/GS\alpha/autophagy pathway, podocytes were treated with miR-664a-5p antagonir, HIPK2 overexpression or calpain inhibitor combined with exosomes. WB showed that Exo\textsuperscript{Albumin} reduced the HIPK2 expression and this effect was eliminated by miR-664a-5p antagonir (Supplementary Fig. 6). Compared with Exo\textsuperscript{Ctrl}, Exo\textsuperscript{Albumin} treatment induced GS\alpha shear in podocytes (Fig. 7A and Supplementary Fig. 4B). miR-664a-5p antagonir, HIPK2 overexpression plasmid, and calpain inhibitor SJA6017, all blocked the intracellular GS\alpha shear induced by Exo\textsuperscript{Albumin} (Fig. 7A and Supplementary Fig. 4B). Additionally, compared with Exo\textsuperscript{Ctrl}-treated podocytes, the LC3II/I ratio decreased and p62 content increased in Exo\textsuperscript{Albumin}-treated podocytes. This effect was blocked by miR-664a-5p antagonir, HIPK2 overexpression, and caplain inhibitor (Fig. 7B and Supplementary Fig. 4B). Then the apoptosis of podocytes was analyzed. Data showed a significant increase in podocyte apoptosis after Exo\textsuperscript{Albumin} treatment compared with Exo\textsuperscript{Ctrl}-treated cells(Fig. 7C). Both miR-664a-5p antagonir and SJA6017 reduced the apoptosis of podocytes in the Exo\textsuperscript{Ctrl} group, and blocked the increased apoptosis induced by Exo\textsuperscript{Albumin} (Fig. 7C). Additionally, although HIPK2 overexpression could not change the apoptosis of podocytes treated with Exo\textsuperscript{Ctrl}, it reduced the apoptosis of podocytes treated with Exo\textsuperscript{Albumin}
(Fig. 7C). It demonstrated that exosomes from albumin-treated renal tubular epithelial cells could inhibit autophagy level and induce apoptosis of podocytes, depending on miR-664a-5p mediated HIPK2/Calpain1/GSα signal pathway inhibition.

**Renal tubular epithelial cells-derived exosomes enhance MN progression through miR-664a-5p**

In order to explore the effects of renal tubular epithelial cells-derived exosomes on MN progression, we constructed a mouse model of experimental MN and treated it with intravenous injection of normal saline or Exo\(^{Ctrl}\) or Exo\(^{Albumin}\). It was found that compared with control mice, MN mice showed proteinuria and serum cholesterol up-regulation accompanied with serum albumin down-regulation and IgG deposition in glomerular of kidney tissues (Fig. 8). It indicated that MN mice were established. Data showed that compared with AAV-Anti-NC injection, AAV-Anti-miR-664-5p treatment could ameliorate 24h urine protein and serum cholesterol content, and inhibit the down-regulation of serum albumin caused by MN induction (Fig. 8A-8C). Furthermore, AAV-Anti-miR-664-5p treatment could inhibit IgG deposition in glomerular of kidney tissues in MN mice (Fig. 8D). And qRT-PCR assay confirmed that compared with control mice, the expression of miR-664a-5p increased in kidney tissues of MN mice, which could be blocked by injection of AAV-Anti-miR-664-5p (Fig. 8E). Additionally, AAV-Anti-miR-664-5p injection could improve renal tubular injury and apoptosis in tubular and glomerular and reverse the expression inhibition of HIPK2 in tubular and glomerular, GSα shear up-regulation and autophagy down-regulation of kidney tissues in MN mice (Fig. 9 and Fig. 10). It indicated that AAV-Anti-miR-664-5p could ameliorate MN progression and HIPK2/Calpain1/GSα signal pathway-mediated autophagy inhibition by decreasing the expression of miR-664a-5p in kidney tissues.

We also found that Exo\(^{Ctrl}\) didn't change 24h urinary protein content, serum cholesterol and albumin level and IgG deposition in glomerular of kidney tissues in MN mice. While Exo\(^{Albumin}\) increased albuminuria and serum cholesterol content and IgG fluorescence intensity in glomerular of kidney tissues, and decreased the content of serum albumin in MN mice, which could be inhibited by AAV-Anti-miR-664-5p treatment (Fig. 8A-8D). And qRT-PCR results showed that the expression of miR-664a-5p in kidney tissues from MN mice could be enhanced by Exo\(^{Albumin}\) treatment, which was blocked by injection of AAV-Anti-miR-664-5p (Fig. 8E). PAS staining and TUNEL assay results showed that the structural damage of renal tubules in MN mice, including tubular atrophy, dilatation and vacuolation and the thickening of basement membrane in the glomeruli and cell apoptosis in tubular and in glomerular, could be enhanced by Exo\(^{Albumin}\) but not Exo\(^{Ctrl}\) treatment, which were blocked by AAV-Anti-miR-664-5p (Fig. 9). The results of IHC test showed that the HIPK2 content in renal tubule and glomerular of kidney of MN mice treated with Exo\(^{Ctrl}\) didn't change compared with that of PBS-treated MN mice. However, Exo\(^{Albumin}\) exacerbated the HIPK2 expression reduction in renal tubule and glomerular of kidney of MN mice, which could be blocked by AAV-Anti-miR-664-5p (Fig. 10A). Moreover, Exo\(^{Albumin}\) treatment increased GSα shear and autophagy downregulation, which could be reversed by AAV-Anti-miR-664-5p (Fig. 10B). It indicated that exosomes from albumin-treated renal tubular epithelial cells, could induce cells injury of renal tubular epithelial cells
and podocytes and induce HIPK2/Calpain1/GSα signal pathway-mediated autophagy inhibition dependently of miR-664a-5p, leading to the progression of MN.

**Discussion**

We previously found that hsa-miR-664a-5p, hsa-miR-378d and hsa-miR-23b-5p were specifically expressed in the urine exosomes of IMN patients but not in those of healthy volunteers. Among them, hsa-miR-664a-5p is most abundant [15]. It is reported that miR-664-5p can regulate vascular smooth muscle cell homeostasis [32]. miR-664a-5p can promote the osteogenic differentiation ability of bone marrow mesenchymal stem cells [33]. The homolog of hsa-miR-664a-5p, namely mmu-miR-664-5p, was expressed in mice. It is reported that miR-664-5p can affect differentiation of mouse myoblasts [34]. Moreover, mouse bone marrow mesenchymal stem cells can improve ovarian granulocell apoptosis through exosomal miR-664-5p [35]. However, there are few reports on the correlation between miR-664a-5p or miR-664-5p and kidney. Here, we found that the expression of miR-664-5p or miR-664a-5p increased in the kidney of experimental MN mice and in renal tubular epithelial cells exposed to albumin. But albumin couldn’t change miR-664a-5p level in podocytes. It was speculated that the increased miR-664a-5p content in kidney of MN may relate to the up-regulation of miR-664a-5p in renal tubular epithelial cells. In cell experiments, albumin-mediated renal tubular epithelial cell apoptosis was blocked by miR-664a-5p antagonim. In vivo, AAV-Anti-miR-664-5p improved renal tubular injury and MN progression in mice. These results indicated that miR-664a-5p could promote MN progression by inducing apoptosis of renal tubular epithelial cells. Targeted inhibition of miR-664a-5p improved MN process, which suggested that miR-664a-5p is an important target to improve MN progression.

miRNA guides the silencing complex to degrade mRNA or hinder its translation by pairing with the target mRNA. It is reported that miR-664a-5p or miR-664-5p can bind to the mRNA of OPA1 [32], HMGA2 [33], p53 [35] and other genes to regulate their expression. Here, we found that miR-664a-5p could bind to the 3’ UTR of HIPK2 mRNA, thereby reducing the HIPK2 protein content in renal tubular epithelial cells, which could result in HIPK2 expression inhibition of renal tubules in MN mice. Although there are few reports on the role of HIPK2 in MN, there are reports that HIPK2 closely relates to other nephropathy, especially renal fibrosis. For example, HIPK2 promotes the EMT process of renal tubular epithelial cells and renal fibrosis by activating the Smad pathway, Notch pathway, NF-kB pathway and Wnt/β-catenin pathway, while inhibiting the HIPK2 expression can improve the renal fibrosis process [36–38]. Interestingly, we found that HIPK2 knockdown could block the improving function of miR-664a-5p antagonim on albumin-induced tubular epithelial cell injury, while HIPK2 overexpression could inhibit albumin-induced tubular epithelial cell injury. It is reported that overexpression of HIPK2 can improve myocardial apoptosis induced by hypoxia reoxygenation [39]. It was speculated that HIPK2 plays a protective role in the apoptosis of renal tubular epithelial cells induced by albumin, but whether HIPK2 plays a different role in the fibrosis process of renal tubular epithelial cells induced by albumin remains to be investigated.

Autophagy plays a protective role in albumin-induced renal tubular epithelial cell injury [29]. We focused on the role of autophagy in nephropathy for a long time, and many reports have been published, which
revealed the protective effects of autophagy in diabetic nephropathy [13], MN [40], IgA nephropathy [41] and puromycin amino nucleoside induced nephropathy [24]. Here, we found that autophagy level decreased in albumin-exposed renal tubular epithelial cells and kidney tissues of MN mice, which was induced by miR-664a-5p or miR-664-5p up-regulation. Inhibition of autophagy induced apoptosis of renal tubular epithelial cells, and blocked the protective function of miR-664a-5p antagomir on renal tubular epithelial cell injury induced by albumin. These results indicated that decreased autophagy mediated by miR-664a-5p was closely related to MN. It is reported that HIPK2 enhances autophagy in hepatocellular carcinoma cells [42], primary hepatocytes [26], and spinal cord injury tissues [43]. HIPK2 reduces the expression of Calpain1, which can shear and activate GSα, resulting in autophagy inhibition in vivo [27, 28]. We also found that HIPK2 knock-down could block the protection of miR-664a-5p antagomir on Calpain1 expression, GSα shear and autophagy level in albumin-exposed renal tubular epithelial cells. HIPK2 overexpression and Calpain inhibitor SJA6017 could retard autophagy inhibition and apoptosis of renal tubular epithelial cells induced by albumin stimulation, which could be reversed by CQ. It demonstrated that miR-664a-5p/HIPK2/Calpain1/GSα signal pathway-mediated autophagy inhibition was involved in albumin-exposed renal tubular epithelial cell injury. Blocking miR-664a-5p/HIPK2/Calpain1/GSα/autophagy signaling cascade might improve MN.

Exosomes play an important role in cell paracrine function. It is reported that exosomes secreted by renal tubular epithelial cells can be taken up by macrophages [23]. There are few reports on whether renal tubular epithelial cell-derived exosomes can be taken up by podocytes. However, studies show that exosomes released by injured podocytes can promote apoptosis of renal tubular epithelial cells through miRNA-424 and miR-149 [44], and podocyte-derived exosomal miR-221 can also mediate dedifferentiation of renal tubular epithelial cells in diabetic nephropathy [45]. Here, we found that renal tubular epithelial cell-derived exosomal miR-664a-5p could be taken up by podocytes, leading to an increase to the expression of miR-664a-5p and cell apoptosis of podocytes. We also found that intravenous injection of ExoAlbumin increased MN progression and the apoptosis of podocytes and HIPK2 expression in glomerular cells, which could be blocked by AAV-Anti-miR-664-5p. And ExoAlbumin could promote GSα shear and decrease autophagy level and induce cells apoptosis of podocytes, which could be reversed by miR-664a-5p antagomir, HIPK2 overexpression and Calpain inhibition. Thus, exosomes from albumin-exposed renal tubular epithelial cells could aggravate MN progression through damaging podocytes, probably dependently of miR-664a-5p/HIPK2/Calpain/GSα signal pathway-mediated autophagy inhibition. Furthermore, it was found that exosomes from albumin-exposed renal tubular epithelial cells could enhance the cell apoptosis and decrease the expression of HIPK2 in tubular from MN mice. Studies have indicated that exosomes secreted by renal tubular epithelial cells after hypoxia treatment can be taken up by other renal tubular epithelial cells, thus playing a protective function [46]. Whether exosomes from albumin-exposed renal tubular epithelial cells could be uptaken by adjacent renal tubular epithelial cells and aggravate renal tubular injury, was unknown, which needs further investigation.
Herein, the role of miR-664a-5p in MN and the mechanism by which miR-664a-5p induce apoptosis of renal tubular epithelial cells and podocytes were revealed. However, there are still some limitations in this study. Firstly, it hasn't been confirmed that whether the imbalance of miR-664a-5p/HIPK2/Calpain1/GSα/autophagy signaling cascade existed in the kidney tissues of MN patients. Secondly, it is reported that renal tubular epithelial cell-derived exosomal miR-199a-5p can enter the urine, resulting in abnormal levels of urinary exosomal miR-199a-5p in diabetic nephropathy patients [23]. Moreover, we did not compare the content difference of urinary exosomes of MN mice and control mice. But results from our previous study showed that the urinary exosomal miR-664a-5p content of MN patients was higher than that of healthy volunteers [15], suggesting that renal tubular epithelial cell-derived exosomal miR-664a-5p may enter the urine and is a potential biomarker for the diagnosis of MN, which needs to be verified by clinical trials with large-sample size samples.

Conclusion

In this investigation, albumin could increase the content of miR-664a-5p in renal tubular epithelial cells, resulting in renal tubular epithelial cell injury and triggering MN progression through HIPK2/Calpain1/GSα signaling pathway-mediated autophagy inhibition. Meanwhile, we found that miR-664a-5p in renal tubular epithelial cells can be transferred horizontally into podocytes through exosomes, leading to the imbalance of miR-664a-5p/HIPK2/Calpain1/GSα/autophagy signaling cascade in podocytes, inducing podocytes apoptosis and aggravating MN progression. Inhibition of miR-664a-5p/HIPK2/Calpain1/GSα/autophagy signaling cascade can reverse the above process and improve the MN process, thus becoming a potential therapeutic target for MN.

Declarations

Ethical Approval

All experimental protocols were approved by the Hospital Institutional Animal Care and Use Committee of Zhejiang Provincial Institute of Traditional Chinese Medicine(Approval No.IACUC-A202208001) and comply with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Competing interests

The authors declare no competing interests.

Authors' contributions

Conceptualization, Z.S. and Z.Z.; Methodology, Z.S., Z.Z., P.R.; Software, P.R., L.Z.; Validation, L.Z., D.Z. and W.C.; Formal Analysis, D.Z.; Investigation, Z.S., Z.Z., L.Z.; Resources, D.Z.; Data Curation, L.Z.; Writing – Original Draft Preparation, Z.S., Z.Z.; Writing – Review & Editing, J.J., W.C.; Visualization, J.J. and W.C.; Supervision, J.J.; Project Administration, J.J. and W.C.; Funding Acquisition, J.J.. All authors have read and agreed to the published version of the manuscript.
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Availability of data and materials

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

References


Figures
Figure 1

Effects of miR-664a-5p antagomir and albumin on HK-2 cell damage. (A) The content of miR-664a-5p in HK-2 cells after 72h treatment with miRNA antagomir NC or miR-664a-5p antagomir combined with solvent or albumin (100mg/mL) was detected with qRT-PCR, and U6 was used as the internal reference. (B) HK-2 cells were treated with a solvent or albumin (100mg/mL) and transfected with miRNA antagomir NC or miR-664a-5p antagomir. After 72h, CCK-8 assay kit was used to detect cell activity in each group. (C) HK-2 cells were treated with miRNA antagomir NC or miR-664a-5p antagomir in combination with solvent or albumin (100mg/mL) for 72h, and ow cytometry with Annexin V-FITC/PI dye was used to detect apoptosis. Data were expressed as Mean±SD. One-way analysis of variance was used for statistical analysis between groups, with ** indicating p<0.01.
Figure 2

Binding detection of miR-664a-5p and the 3’UTR of HIPK2 mRNA and effects of miR-664a-5p and albumin on HIPK2 expression in HK-2 cells. (A) The predicted binding site and the mutation sequence of the binding site between miR-664a-5p and the HIPK2 mRNA 3’UTR. (B) HEK293T cells were transfected with the luciferase reporter plasmid HIPK2 3’-UTR<sup>WT</sup> or HIPK2 3’-UTR<sup>MT</sup> together with miRNA agomir NC or miR-664a-5p agomir, and then the intracellular luciferase activity was detected. (C) Western Blot assay was used to detect HIPK2 protein expression in HK-2 cells treated with solvent or albumin (100mg/mL) and transfected with miRNA antagonir NC or miR-664a-5p antagonir for 72h. GAPDH was the internal parameter (left panel). The statistical results of gray value of WB strip are shown on the right. Data were expressed as Mean±SD. One-way analysis of variance was used for statistical analysis between groups, with ** indicating p<0.01 and ns indicating p>0.05.
Figure 3

Effects of HIPK2 knockdown on HIPK2 expression and damage in HK-2 cells treated with miR-664a-5p antagonim and albumin. (A) HK-2 cells were treated with solvent or albumin (100mg/mL), combined with miRNA antagonim NC or miR-664a-5p antagonim together with NC siRNA or HIPK2 siRNA. 72h later, the intracellular HIPK2 protein content was detected by Western Blot, with GAPDH as the internal reference (left panel). The statistical results of gray value of WB strip are shown on the right. (B) CCK-8 kit was used to detect the activity of HK-2 cells in each group after 72h of different stimulation conditions. (C) HK-2 cells were treated with solvent or Albumin (100mg/mL), combined with miRNA antagonim NC or miR-664a-5p antagonim together with NC siRNA or HIPK2 siRNA. 72h later, apoptosis was detected by flow cytometry with Annexin V-FITC/PI dye. Data were expressed as Mean±SD. One-way analysis of variance was used for statistical analysis between groups, with ** indicating p<0.01.
Figure 4

Effects of HIPK2 and miR-664a-5p on Calpain1 expression, GSα shear and autophagy in HK-2 cells treated with albumin. (A-C) HK-2 cells treated with solvent or albumin (100mg/mL) and combined with miRNA antagomir NC or miR-664a-5p antagomir. After transfection with NC siRNA or HIPK2 siRNA for 72h, intracellular Calpain1 (A), GSα shear (B), LC3II/I ratio and p62 protein content (C) were detected by Western Blot, using GAPDH as reference (left panel). The statistical results of gray value of WB strip are...
shown on the right. Data were expressed as Mean±SD. One-way analysis of variance was used for statistical analysis between groups, with ** indicating p<0.01. (D) HK-2 cells were infected with RFP-GFP-LC3 adenovirus and treated differently. After 72h, the changes of autophagy flux in each group were observed under laser confocal microscope. The white arrow indicates autophagic lysosome that has fused with lysosome (GFP$^+$RFP$^+$ punctate aggregate) and the blue arrow indicates autophagosome that has not fused with lysosome (GFP$^+$RFP$^+$ punctate aggregate). Scale =20µm.

Figure 5

Effects of chloroquine on the intracellular autophagy level and injury of HK-2 cells treated with miR-664a-5p antagonir, HIPK2 overexpression and Calpain inhibitor combined with albumin. (A) HK-2 cells were
treated with solvent or albumin (100mg/mL), and treated with miR-664a-5p antagomir or HIPK2 overexpression or Calpain inhibitor SJA6017 combined with autophagy inhibitor chloroquine meanwhile. 72h later, LC3II/I ratio and p62 protein content in each group were detected by Western Blot, using GAPDH as the internal reference. (B) CCK-8 kit was used to detect HK-2 cell activity after 72h of different treatments. (C) HK-2 cells were treated with different stimuli for 72h and flow cytometry with Annexin V-FITC/PI dye was used to detect cell apoptosis in each group. Data were expressed as Mean±SD. One-way analysis of variance was used for statistical analysis between groups, with ** indicating p<0.01.

Figure 6

Uptake capacity of renal tubular epithelial cell-derived exosomal miR-664a-5p by podocytes and effects of exosomal miR-664a-5p on the expression of miR-664a-5p in podocytes. (A) HK-2 cells were treated with exosomes-free FBS, which was produced by high-speed centrifugation. Then cells were treated with solvents or albumin (100mg/mL), respectively. Exosomes in the supernatant of cells were collected by exosomes extraction kit, and the content of miR-664a-5p in different exosomes were detected by qRT-
PCR, with U6 as the internal reference. (B) Cy3-labeled or unlabeled miR-664a-5p agomir was transfected into solvents-treated HK-2 cell-derived exosomes, and then co-incubated with human podocytes for 24h. The distribution of Cy3 in podocytes of each group was observed under laser confocal microscope. Scale =20μm. (C) Effects of exosomes from HK-2 cells on the content of miR-664a-5p in human podocytes were detected by qRT-PCR, with U6 as the internal reference. Exo$^{\text{Ctrl}}$ refers to exosomes isolated from HK-2 cells after 72h solvent treatment, and Exo$^{\text{Albumin}}$ refers to exosomes isolated from HK-2 cells after 72h albumin treatment (100mg/mL). The data were presented by Mean±SD, and one-way analysis of variance was used for statistical methods, with ns indicating p>0.05 and ** indicating p<0.01.
Figure 7

Effects of exosomes and miR-664a-5p antagonomir, HIPK2 overexpression, and Calpain inhibitor effect on GSα shear, autophagy level, and apoptosis in podocytes. (A-B) Human podocytes were treated with ExoCtrl or ExoAlbumin combined with miR-664a-5p antagonomir or HIPK2 overexpression or Calpain inhibitor, respectively. 72h later, GSα shear, LC3II/I ratio and p62 protein content in each group were detected by Western Blot, using GAPDH as the internal reference. (C) After 72h of different stimulation, the apoptosis
level of podocytes in each group was detected by flow cytometry with Annexin V-FITC/PI dye. Data were expressed as Mean±SD. One-way analysis of variance was used for statistical analysis between groups, with ** indicating p<0.01.

Figure 8

Effects of exosomes and miR-664-5p on MN progression in experimental MN mice. (A) After AAV-Anti NC or AAV-Anti-miR-664-5p together with exosomes were injected into control mice or MN mice, 24h urine protein content was detected by urine protein quantitative test box. (B-C) Serum cholesterol and albumin contents of mice in each group were detected by kits. (D) Kidney tissues of control mice and MN mice were taken after different treatments, and IgG deposition was detected by IF method. Scale =20μm. (E) The content of miR-664-5p in kidney tissues of mice in each group was detected by qRT-PCR, with U6 as the internal reference. Data were expressed as Mean±SD. One-way analysis of variance was used for statistical analysis between groups, with ** indicating p<0.01.
Figure 9

Effects of exosomes and miR-664-5p on renal structural lesions in experimental MN mice. (A) The kidney tissues of control mice or MN mice treated with AAV-Anti NC or AAV-Anti-miR-664-5p together with different exosomes treatments were stained with PAS method to observe the pathological changes of kidney tissues. Scale = 50 μm. (B) Kidney cell apoptosis was detected by TUNEL kit. Scale = 20 μm. Data were expressed as Mean±SD. One-way analysis of variance was used for statistical analysis between groups, with ** indicating p<0.01.
Figure 10

Effects of exosomes and miR-664a-5p on HIPK2, GSα shear and autophagy in kidney tissues of experimental MN mice. (A) After injection of solvent or Exo\textsuperscript{Ctrl} or Exo\textsuperscript{Albumin} together with AAV-Anti NC or AAV-Anti-miR-664-5p, IHC method was used to detect HIPK2 expression in kidney tissues of control mice or MN mice. Scale =50μm. (B) GSα shear, LC3II/I ratio and p62 protein content in kidney tissues of mice in each group were detected by Western Blot, with GAPDH as the internal reference (left panel). The statistical results of gray value of WB strip are shown on the right. Data were expressed as Mean±SD. One-way analysis of variance was used for statistical analysis between groups, with ** indicating p<0.01.

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