Original Article
Effect of miR-200 on autosomal polycystic nephropathy in rats

Kebo Bi, Xiaoling Deng, Weihong Yu, Chunlian Yu, Xinglei Cui, Meiyan Yu, Tao Zhang

Department of Nephrology, Weihai Central Hospital, Weihai, Shandong, China

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Abstract: Autosomal polycystic nephropathy (APN) is the most common genetic disorder in kidney, and has an incidence over 0.1% in whole population. Strong positive staining of polycystin1 has been identified in APN patients, as it can induce the differentiation of tubular formation cells by interaction between tubular epithelial cells and extracellular matrix. Over-expression of polycystin-1 causes under-differentiation of tubular cells. MicroRNA (miR) can regulate protein expression. MiR-200 has been reported to inhibit PKD1 gene, which encodes polycystin1. This study thus investigated the role of miR-200 in protecting against APN by inhibiting PKD1. The mechanism of miR-200 was observed in animal models and at cellular level. Hans: SRPD rats including APN and wild type groups were given miR-200 mimics to observe Blood urea nitrogen (BUN) and serum creatinine (Scr) levels. MiR-200 level was detected by PCR, which also quantify pdk1 gene level. Western blotting was used to test the expression of polycystin1 expression. Compared to negative control group, miR-200 significantly improved the kidney function of Hans: SPRD rats, which had smaller kidney size and fewer cysts. MiR-200 treatment also suppressed Pkd1 gene expression level, and decreased polycystin1 expression. MiR-200 can inhibit Pdk1 gene and its protein product polycystin1 in Hans: SPRD rats, suggesting the improvement of renal function in APN rats by miR-200 mimics, thus suggesting its potential treatment effect on human APN.

Keywords: Autosomal polycystic nephropathy, microRNA-200, PKD-1, polycystin1

Introduction
Autosomal polycystic nephropathy (APN) is the most common genetic disorder in kidney, and has an incidence over 0.1% in whole population [1]. APN is mainly manifested as progressive dilation of renal tubular cysts, accompanied with degeneration of normal renal tubular structure to form multiple aqueous cyst, leading to renal failure eventually [2].

Study has found strong expression of polycystin1 in APN patients [3]. In normal group, polycystin1 induces the interaction between renal tubular epithelial cells and extracellular matrix to facilitate the normal differentiation of tubular formation cells [4]. While in APN patients, polycystin1 interrupts the modulatory mechanism for the inter-dependence between epithelial cells and extracellular matrix, causing abnormal components of matrix and under-differentiation of epithelial cells, further aggravating disease conditions [5].

MicroRNA (miR) can regulate the expression of certain proteins at genetic level. Recent study has revealed the critical role of miR in pathogenesis and progression of multiple diseases [6]. MiR is one type of small non-coding single stranded RNA with 18-24 bp length, and can bind with 3’-untranslated region (3’-UTR) of target mRNA by complete/incomplete complementary binding. The newly-formed double stranded RNA will be degraded by RNA exonuclease for specific inhibition of target mRNA, thus inhibiting or activating downstream genes. MiR mainly regulates downstream gene regulation by inhibiting mRNA transcription. Meanwhile, such modulation on mRNA degradation and activation may further regulate protein levels to further affect cellular functions or oncogene activation [7]. Previous report indicated that miR-200 down-regulation might increase polycystin1 coding gene PKD1 level, and interrupted tubular formation of renal tubular epithelial cells [8]. However, if up-regulation of miR-200 had treatment effect on APN still remains unclear yet.
In this study, we employed Han: SPRD heterozygous rat model, which is one commonly used APN animal model. MiR-200 mimics or control reagents were applied. By observing rat blood urea nitrogen (BUN) and serum creatinine (Scr) level, and miR-200 and PDK1 gene expression level, in addition to polycystin1 expression profile, the protective effect of miR-200 on renal function of APN was evaluated.

Materials and methods

Reagents

Anti-rabbit polycystin1 antibody (ab203240) was purchased from Abcam (China). β-actin internal reference antibody was a product of Kangcheng Bio (China). Rabbit anti-mouse IgG (H+L) was purchased from Proteintech (China). SYBR Green PCR Master Mix was produced by Toyobo (China). MiR-200 inhibitor mimics and negative control RNA were synthesized by Gimma (China). Primers pkdrFwrRAT and pkdrRevRAT were produced by Ruibo Biotech (China). PCR primers were synthesized by Toyobo (China).

Animals

Hans: SPRD rats were purchased from Mayo Clinic (US) and were bred in SPF-grade animal facility of Shandong University. Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Weihai Central Hospital.

Genotyping

Tail tissues cubes were collected from ~3 weeks rats. Each tissue cube was mixed with 0.5 mL lysis buffer containing 10 μL proteinase K. After incubation in warm incubator, tissue mixture was centrifuged at 12,000 rpm for 15 min. Supernatants were saved for extracting DNA using phenol/chloroform. After 75% ethanol rinsing, genomic DNA was re-suspended in 20 μL DEPC water. PCR reaction was performed using pkdrFwrRAT and pkdrRevRAT primers in a 50 μL system under following conditions: 50°C for 30 min, 95°C pre-denature for 5 min, followed by 45 cycles each containing 95°C denature for 15 sec, 60°C annealing for 30 sec and 72°C elongation for 45 sec. After amplification,MspI endonuclease was used to digest products, which were then separated in agarose gel electrophoresis. Those rats with heterozygosity (3 DNA bands) were selected for further experiments.

Animal treatment

Heterozygous Hans: SPRD rats (N=30) were randomly divided into three groups (N=10 each): (1) Model group received no treatment; (2) Control group received intravenous injection of miR-200 negative control drugs (5 μM/kg weekly via tail veins); (3) Treatment group received 5 μM/kg miR-200 mimics weekly via tail vein injection. Food and water were provided ad libitum for all rats. After 3 months, rats were sacrificed to collect blood samples from abdominal aorta and renal tissues.

Blood urea nitrogen (BUN) and serum creatinine (Scr) assay

2 mL blood samples were collected from abdominal aorta in each rat. Blood samples were immediately centrifuged at 1,500 rpm for 20 min at 4°C. Supernatants were saved for analyzing BUN and Scr levels on an automatic biochemical analyzer.

Real-time quantitative PCR

MiR-200 primer sequences were obtained from previous literatures [9] and were synthesized by Sigma (US). Primer sequences were: miR-200-Forward, 5'-TGCCT GCTGA TGGAT GTCTT A-3'; miR-200-Reverse, 5'-TGGAC GGCAT TACCA GACAG-3'; U6-Forward, 5'-CTCGC TTCGG CAGCA CA-3'; U6-Reverse, 5'-AACGC TTCAC GAATT TGCGT-3'; PDK-1-Forward [10], 5'-TCCTT AGAGG GCTAC GGGAC-3'; PDK-1-Reverse, 5'-CCATG TGCAG TTACG AGCTT C-3'; GAPDH-Forward, 5'-GGGAA ACTGT GGCGT GAT-3'; GAPDH-Reverse, 5'-GAGTG GGTGT CGCTG TTGA-3'. PCR reaction was performed in a 50 μL system under following conditions: 50°C for 30 min, 95°C pre-denature for 5 min, followed by 40 cycles each containing 95°C denature for 30 sec, 55°C annealing for 30 sec and 72°C elongation for 50 sec, and ended with 72°C elongation for 5 min. Real-time PCR amplification curve and dissolving curve were confirmed. Relative expression level was determined by comparing Ct values of target gene and internal reference gene using 2−ΔΔCt method.

Western blotting

100 mg renal tissues were added with RIPA lysis buffer (1 mM in PMSF) and mixed. Lysis was performed at 4°C for 15 min, following by 14,000 rpm centrifugation for 15 min. Supernatants were saved in a new tube to prepare
full protein solution. After BCA quantification, equal concentration of proteins were denatured in boiling water for 5 min, and were separated by 10% SDS-PAGE. Proteins were then transferred to PVDF membrane under 300 mA electrical field for 1 hour. Anti-rabbit polycystin1 antibody (1:1,000) was used for hybridization at 4°C overnight. On the next day TTBS was used to rinse the membrane, followed by the addition of secondary antibody (1:1,000) for 37°C incubation for 2 hours. Chemiluminescence method was then used to develop the membrane.

Statistical analysis

All experiments were carried at least in triplicates. SPSS19.0 software was used to perform statistical analysis and present results as mean ± standard deviation. P<0.05 indicated significant difference. Student t-test was used to compare means across two groups, while comparison over two groups was performed by analysis of variance (ANOVA).

Results

BUN and Scr levels in Hans: SPRD rats

After 4-week treatment of miR-200, kidney function indexes of miR-200 were significantly improved compared to control or model group (P<0.05, Figures 1, 2).

MiR-200 level after treatment

After 12-week of miR-200 mimics injection via tail veins, renal tissues were extracted for PCR amplification to detect miR-200 expression level. As shown in Figure 3, miR-200 level was significantly elevated in treatment group compared to control and model group (P<0.05).

PKD1 expression level

After treating with miR-200 mimics, we found higher level of miR-200 in renal tissues. Its effect on PDK1 gene, however, was still unclear. We thus investigated mRNA level of PDK1 gene. As shown in Figure 4, compared to model or control group, miR-200 mimics treatment group had significantly depressed mRNA level of PKD1 (P<0.05).

Polycystin 1 protein expression level

As shown in Figure 5, after treating with miR-200 mimics, the expression level of polycystin1 was significantly depressed compared to model or control group (P<0.05).

Discussion

As the most common hereditary disease of kidneys, APN has no effective treatment to block
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disease progression so far [11]. Due to its involvement of multiple organs, it may cause severe consequences [12]. Current countermeasures mainly include the surgical decompression of kidney cortex by removal cysts or conservative treatment to protect kidney functions [13]. Surgery, however, may further aggravate renal injury, thus making it not an optimal choice when comparing to the prevention of renal infection. Therefore the establishment of effective treatment to protect kidney function of APN patients is of critical importance.

Clinical study has revealed that APN was caused by mutation of two genes, namely PKD1 and PKD2, which occupied 75%~85% and 15% of total cases, respectively. Therefore, the expression of PKD1 gene plays a critical role in polycystic nephropathy. The protein product of PKD1 and PKD2 genes were called polycystin1 and polycystin2, respectively. They form polycystic protein complex on kidney cell. Under over-expression of those proteins, the physical stimulus of urination can bend cilia, thus inducing intracellular Ca²⁺ peak via polycystin2 to participate in the modulation of cell cycle and mitosis. These observations all pointed to the important role of PKD1 gene and its product, polycystin1, in the pathogenesis of APN. Hans: SPRD rats are a type of mature APN model with natural mutation [14]. It is featured with enlargement of bilateral kidneys, which contain large amounts of cysts having unequal sizes, most of which locate near proximal tubules. The disease aggravates with increased number of cysts. Animals have an average age at 17 months and mostly die from kidney failure, thus mimicking the disease progression of human APN [15].

Recent studies found important roles of miRNA-directed post-transcriptional regulatory mechanism in various diseases such as tumors and some metabolic disorders. The occurrence of APN is similar with the over-proliferation of tumor cells, and is also related with miRNA abnormality [16]. The expression of polycystin is closely correlated with the occurrence of APN. Under normal conditions, polycystin is only expressed in cortical renal epithelial cells and vascular endothelial cells. It is expressed in medulla adjacent renal tissues only under tissue injury. Therefore polycystin is abundantly expressed in APN. The inhibition of polycystin had critical roles in protecting kidney function in APN [17]. Previous study has shown that miR-200 could inhibit PKD1 gene expression, to down-regulate its product polycystin1. However, whether miR-200 had roles in protecting kidney functions in APN individuals via such pathway is unclear. Other reports showed that the occurrence of APN was related with over-proliferation of cells. miR-200 in human colorectal carcinoma and gastric cancer was displayed as inhibiting tumor cell invasion and migration for further facilitating the apoptosis [18, 19]. Similarly, in human cervical cancer, PDK1, PKCζ and caspase-3 can form a pro-apoptotic complex to accelerate the programmed death of cervical cancer cells [12]. The effect of miR-200 on facilitating cell apoptosis and improving renal function still requires further illustrations.

As one type of chemically synthesized miRNA, miR mimics can simulate the function of endog-

**Figure 4.** PDK1 gene expression level in rat kidney. **, P<0.05 compared to model group.

**Figure 5.** Polycystin1 level in rats. **, P<0.05 compared to model group.
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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Tao Zhang, Department of Nephrology, Weihai Central Hospital, 602 West Building, 3-56, Weihai 264400, Shandong, China. Tel: +86-631-8474007; Fax: +86-631-8474007; E-mail: zhagntaoo@sina.com

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