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Inhibition of MiR-199a-5p Reduced Cell Proliferation in Autosomal Dominant Polycystic Kidney Disease through Targeting CDKN1C

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: With a prevalence of about 1:500 to 1:1,000, autosomal dominant polycystic kidney disease (ADPKD) often causes renal failure, with many serious complications. However, there is no Food and Drug Administration (FDA) approved therapy available.

Material/Methods: MiR-199a-5p level in ADPKD patient samples, rat model, and cell lines were determined with Realtime PCR assay. After miR-199a-5p inhibitor was transfected, we detected the cell proliferation and apoptosis using an MTT assay and an Annexin V-FITC staining kit, respectively. Finally, TargetScan version 5.1 was used to predict the miRNA target and the target gene of miR-199a-5p was proved by a Luciferase assay.

Results: We identified a dramatically up-regulated microRNA, miR-199a-5p, in ADPKD tissues and cell lines. Our data show that inhibition of miR-199a-5p suppressed cyst cells proliferation and induced cell apoptosis. We found that miR-199a-5p might exert this effect through targeting CDKN1C/p57.

Conclusions: Up-regulation of miR-199a-5p in ADPKD tissues might promote cell proliferation through suppressing CDKN1C, suggesting miR-199a-5p as a novel target for ADPKD treatment.

MeSH Keywords: Cell Proliferation • Cyclin-Dependent Kinase Inhibitor p57 • MicroRNAs • Polycystic Kidney, Autosomal Dominant

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Background

Polycystic kidney disease (PKD) is a serious human kidney disease characterized by many fluid-filled cysts in the renal parenchyma. It is a monogenetic renal disease and can be classified into autosomal dominant PKD (ADPKD) and autosomal recessive PKD (ARPKD) [1–4]. With a prevalence of about 1:500 to 1:1000, ADPKD is quite common and is attributed to mutations of PKD1 and PKD2, with severe clinical manifestations that include abdominal mass, chronic flank, or back pain, gross hematuria, urinary tract infection, and urolithiasis [5–7]. In addition, cysts can cause renal failure, with many serious complications. However, no FDA approved drugs or therapy are available for ADPKD patients and even the underlying mechanism of ADPKD is still unclear.

MicroRNAs (miRNAs, miRs) are a class of endogenous noncoding RNAs with a length of around 22nt RNAs, and can regulate the expressions of the target gene in a post-transcriptional manner [8,9]. MiRNA has been reported to participate in many biological processes, including cell proliferation, cell apoptosis, and carcinogenesis [10–13]. The most important features in ADPKD are the high proliferation of cyst epithelial cells and the secretion of fluid, which lead to cyst expansion [14]. Recently, Patel et al. reported that miRNA 17-92 cluster plays a role in several models of PKD [13]. In the present study, we demonstrate that miR-199a-5p is up-regulated in ADPKD tissues and plays a critical role in the proliferation of cyst epithelial cells, suggesting miR-199a-5p as a potential target for treatment.

Material and Methods

Ethics statement

The entire study was approved by the Ethics Committee of Second Military Medical University, Shanghai, China. After signing informed consent, human cystic kidney tissues were obtained from ADPKD patients who receive nephrectomies. Normal renal cortical tissues were obtained from kidneys that were removed for circumscribed tumors. Histological examination of these kidney samples did not show any renal pathology.

Animal and treatment

An established animal model of ADPKD heterozygous (Cy/+) and normal littermate control (+/+) Han: SPRD rats were used as described in our previous study [15]. All the experiments were approved by the Second Military Medical University, China in accordance with the Guide for Care and Use of Laboratory Animals published by the US NIH (publication No. 96-01). Rats had free access to tap water and standard rat diet [15].

Cell culture and transfection

Human ADPKD cystic lining epithelial cells OX161 (a kind gift from Sheffield Kidney Institute, Division of Clinical Sciences (North), University of Sheffield, UK), human ADPKD cell lines WT9-12 (a gift given by prof. Jing Zhou of Harvard University), human renal tubular epithelial UCL93, RCTEC and HK2 cells, human mesangial cell HMCL, and mouse mesangial cell RCM cell lines, maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (PAA, Austria) at 37°C. After being used for checking the level of miR-199a-5p, OX161 cells were used for transfection. Firstly, cells were seeded in 24-well plates at a 6×10^4 cells per well and transfected with miR-199a-5p inhibitor and normal control inhibitor (Genema Technology, Shanghai, China) by using Lipofectamine 2000 (Invitrogen, Canada) transfection reagent according to the manufacturer's instructions. Cells were used for further experiments 48 h later.

RNA extraction and real time PCR

Paired ADPKD tissues and adjacent normal tissues of renal cancer resected surgically used for qRT-PCR were collected from 5 ADPKD patients during surgery at Changzheng Hospital (Shanghai, China) [16]. RNA from human renal tissues, rat renal tissues, and cells were homogenized in Trizol (Invitrogen) and isolated according to the manufacturer's instructions. The miR-199a-5p were determined with Roche technology, as described in a previous study [17].

Cell proliferation assay

After being seeded into 96-well plates at 4000 cells per well, cells were transfected with miR-199a-5p inhibitor/normal control inhibitor. Cell viability was determined by an MTT assay at Days 1–5 after transfection, as described in previous studies [18–20].

Apoptosis

Cell apoptosis were detected by using the Apoptosis Detecting Kit (Invitrogen) and analyzed by flow cytometry [17]. Briefly, after different treatments, cells were labeled with annexin V-FITC and propidium iodide (PI) following the manufacturer's instructions. Samples were determined by flow cytometry and the results were analyzed using CellQuest software (Becton Dickinson, San Jose, CA) as described previously.

Plasmids

The luciferase-3' UTR reporter constructs were generated by introducing the wild type and mutated CDKN1C/p57 3'-UTR into a pGL3 promoter vector (Promega) by the Xba1 site (Generay, Shanghai, China) in a method described previously [17,21,22]. All PCR products were verified by DNA sequencing.

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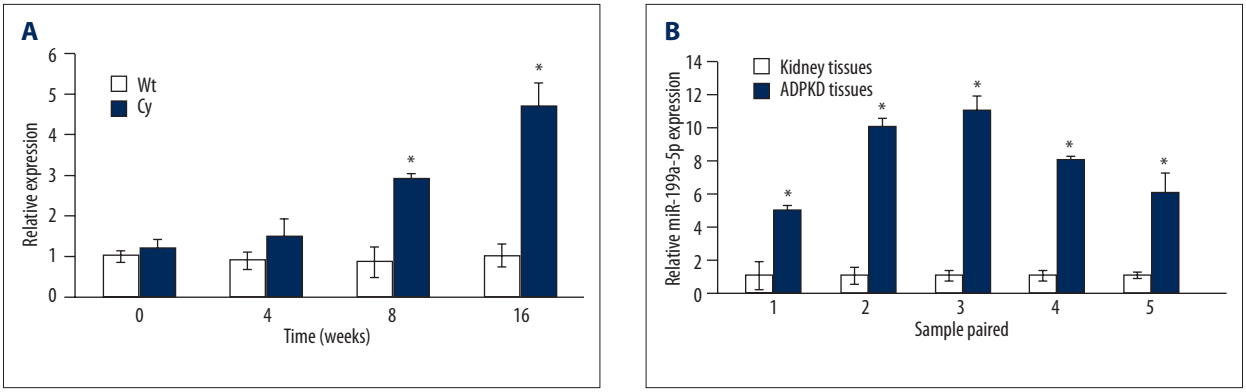

Figure 1. Increase of miR-199a-5p in animal models and tissue in patients with ADPKD. Bar graphs of increased levels of miR-199a-5p in AKPKD rat models (**A**) and in tissues of patients with ADPKD (**B**). * P<0.05 *vs.* the control group.

Western blot assay

Proteins from normal and ADPKD tissues were obtained using ProteoJET™ Mammalian Cell Lysis Reagent (Fermentas) according to manufacturer's protocol, and then analyzed by Western blotting to detect CDKN1C (Cell Signaling Technology, 1:1000) and β-actin (Cell Signaling Technology, 1:1000). The secondary antibody was purchased from Sigma.

miRNA-target prediction and luciferase assays

TargetScan version 5.1 (*http://www.targetscan.org/index.html*) was used to find potential targets for miR-199a-5p. Luciferase assays were performed by using a luciferase assay kit (E1910, Promega) according to the manufacturer's instructions. TK Renilla was used for normalization in Dual Luciferase assays.

Statistical analysis

Data are expressed as means ±SEM for each experiment. For single comparisons, we performed an unpaired 2-tailed t-test; for multiple comparisons, we used an analysis of variance (ANOVA) followed by Student-Newman-Keuls post hoc test. We performed experiments for quantification in a blinded fashion. P<0.05 was considered to be significantly different.

Result

MiR-199a-5p level was higher in human ADPKD tissues and rat model

MiR-199a-5p was detected in Han: SPRD rat model and human tissues by using a realtime PCR method. We found that the expression of miR-199a-5p increased with the disease progression in Han: SPRD cystic rats (Figure 1A). We also found that miR-199a-5p level increased in 5 paired human ADPKD tissues compared with the adjacent normal control tissues (Figure 1B).

MiR-199a-5p inhibitor inhibited the proliferation rate of human renal ox161 cells

The expression level of miR-199a-5p was determined in 7 kinds of human renal cells and we found miR-199a-5p was dramatically higher than that in other cells (Figure 2A). The expression is similar to that in cyst tissues, and ox161 was selected for the following experiments. We found that our inhibitor effectively inhibited the level of miR-199a-5p in ox161 cells (Figure 2B). By using an MTT assay (Figure 2C) and a colony assay (Figure 2D), we found that miR-199a-5p inhibitor significantly inhibited ox161 cell proliferation.

Inhibition of miR-199a-5p induced cell apoptosis

After transfection, apoptosis in ox161 cells was determined by Annexin V and PI staining method. We found that miR-199a-5p inhibitor induced cells apoptosis (Figure 3A, 3B). These data indicated that miR-199a-5p plays a role in cell apoptosis regulation.

miR-199a-5p directly targeted CDKN1C/p57

By using a TargetScan method, wild type of human CDKN1C 3'UTR was found to contain a putative miR-199a-5p binding site, but the mutated mouse BMI1 3' UTR was unable to bind (Figure 4B). We also demonstrated that CDKN1C level is down-regulated in ADPKD tissues (Figure 4A). We also found that miR-199a-5p significantly reduced the luciferase activity of the wild type CDKN1C 3'UTR (Figure 4C) but had no effect on the luciferase activity of the mutated CDKN1C 3'UTR (Figure 4D).

Discussion

Previous studies have demonstrated that miR-199a-5p has important roles in cancer cell proliferation, fibrosis, cardiac hypertrophy, and angiogenesis [23–27]. In the present study, we reported the up-regulation of miR-199a-5p in ADPKD animal

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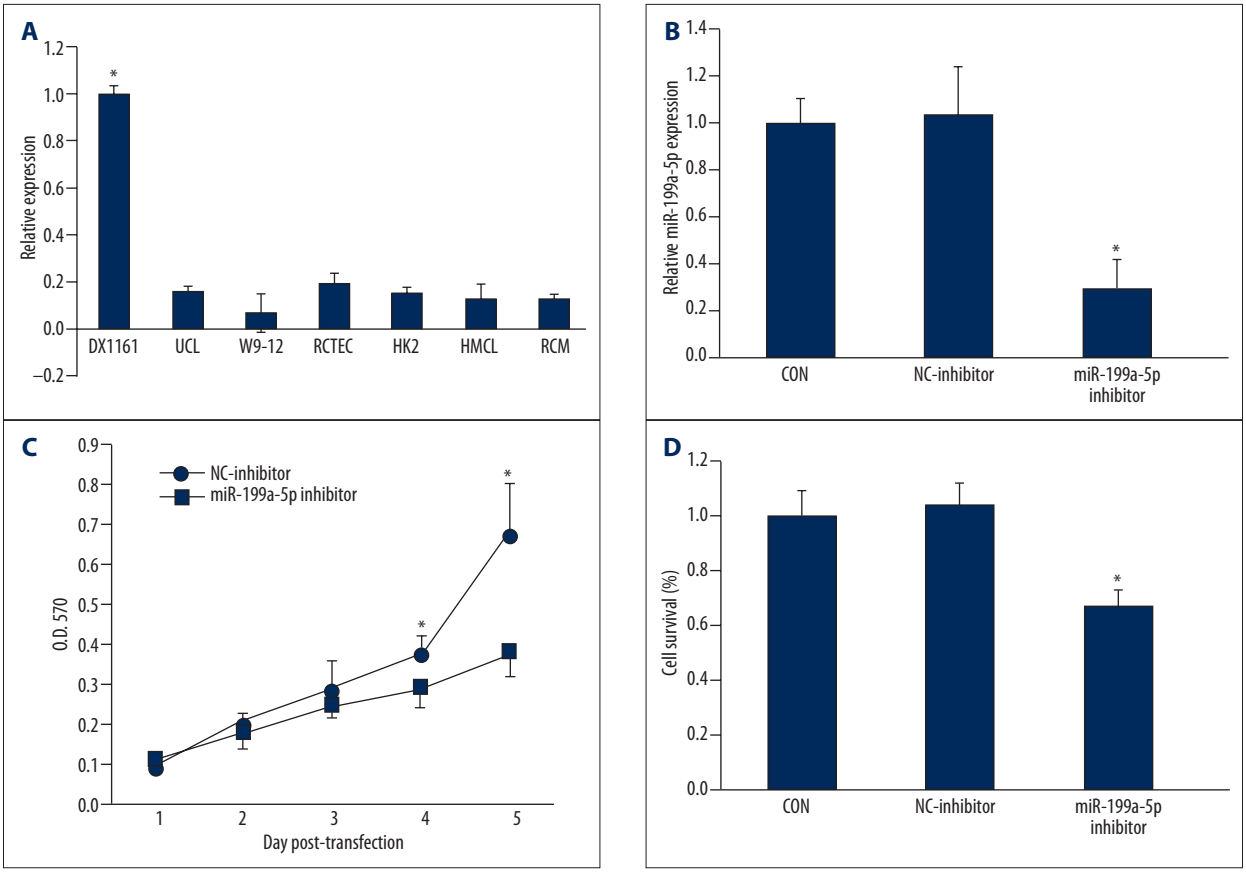

Figure 2. miR-199a-5p inhibitor inhibited the proliferation of human PKD1 cystic ox161 cell line. A: Bar graphs of expression of miR-199a-5p in many human renal cell lines (**A**) and cells treated with miR-199a-5p inhibitor (**B**). Cell proliferation and survival in different treated groups were conducted with MTT assay (**C**) and colony formation assay (**D**), respectively. * P<0.05 *vs.* the control group.

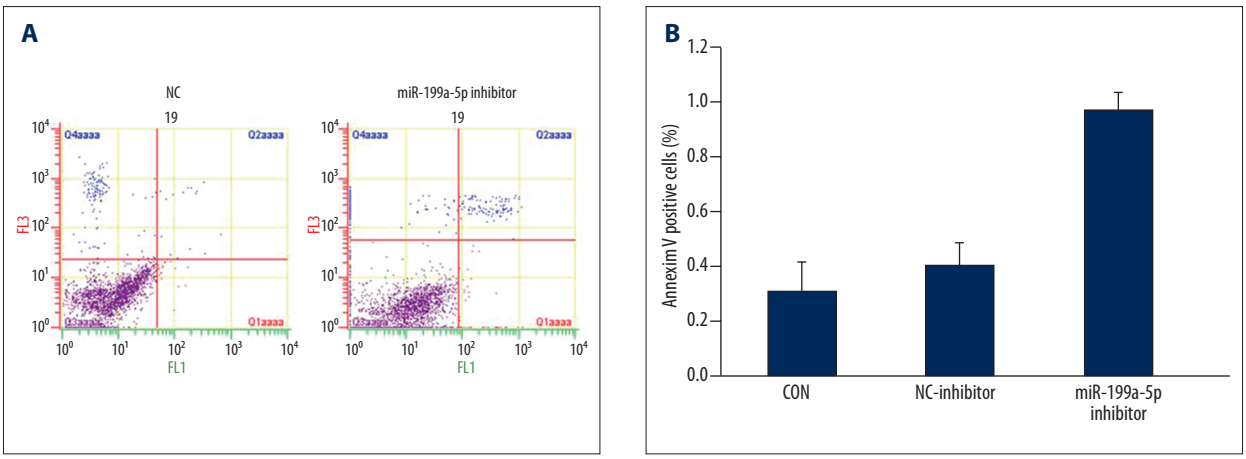

Figure 3. Inhibition of miR-199a-5p induced apoptosis in ox161 cells. (**A**) Representative images of apoptosis in ox161 cells after transfection of miR-199a-5p inhibitor. (**B**) a bar graph of Annexin V positive cells in different groups. * P<0.05 *vs.* the control group.

models and human tissues. Inhibition of miR-199a-5p also reduced the proliferation of cyst cells and induced apoptosis and cell cycle arrest. Finally, we identified CDKN1C/p57 as a direct target of miR-199a-5p.

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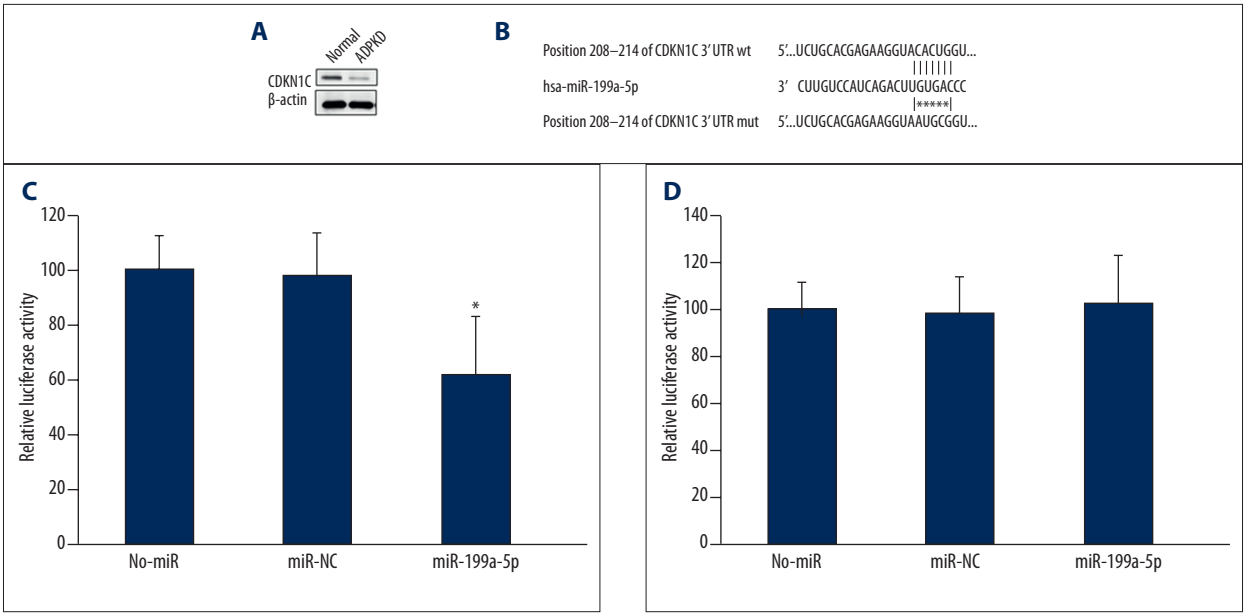

Figure 4. miR-199a-5p directly targeted CDKN1C/p57. (**A**) Western blot assay of CDKN1C in ADPKD tissues and normal tissues. (**B**) miR-199a-5p targets CDKN1C 3'UTR site predicted by TargetScan. Bar graphs of firefly luciferase activities in HEK293 cells transfected with wild type PGL3-CDKN1C (**C**) or mutated PGL3-CDKN1C (**D**) vector and miR-NC or miR-199a-5p. * P<0.05 *vs.* the control group.

MiRNAs participate in many physiological and pathological processes through a mechanism of post-transcriptionally suppressing target genes [28–30]. Numerous miRs have been found to be up-regulated and down-regulated in many diseases [31], indicating that miRs act as a very critical network *in vivo* and provide important clues for therapy target identification. In our study, we found that miR-199a-5p increased dramatically in animal models and in human ADPKD tissues, which provides important information that the abnormal expression of miR-199a-5p exerts critical roles in the genesis and progression of ADPKD. Next, we found that this miR was extremely high in a human cyst cell line, OX161, compared to other renal cell lines. This prompted us to study the effects of miR-199a-5p inhibitor on cyst cell proliferation and the progression of ADPKD. Our data showed that miR-199a-5p inhibitor suppressed the proliferation of OX161 cells, suggesting a role of miR-199a-5p in the high rates of proliferation of cyst epithelial cells. At the same time, WT9-12 is also an ADPKD-related cell line, which was also expected to have a high level of miR-199a-5p; however, we did not see a huge difference in miR-199a-5p level between WT9-12 and other renal cell lines. The underlying mechanism still needs to be clarified.

Cell proliferation suppression has 2 possible causes: apoptosis and cell cycle arrest. We demonstrated that miR-199a-5p inhibition induced apoptosis in OX161 cells. Increased cell apoptosis is an important feature of ADPKD [32,33], and we found that miR-199a-5p inhibitor induced cell apoptosis, which is not consistent with the increase of apoptosis in the disease model. It might be that apoptosis induction is not dominant in this process, but the underlying mechanism still needs further studies. Then we predicted potential targets for miR-199a-5p with a bioinformatics tool, TargetScan 5.1, and examined by a dual reporter gene, we confirmed CDKN1C as a directly target of miR-199a-5p. Down-regulation of miR-199a-5p increased the CDKN1C/p57 gene expression. CDKN1C/p57 is very important in cell cycle regulation, and cells deficient in this gene exhibit high rates of cell growth and decreased differentiation [34,35]. CDKN1C/p57 was down-regulated in many proliferation diseases, including cancer [36,37]. In the miR-199a-5p inhibitor group, CDKN1C expression is obviously up-regulated, suggesting CDKN1C as a mediator of the proliferation inhibitory effect of miR-199a-5p inhibitor and as a target of this miR.

Conclusions

In conclusion, we demonstrated the up-regulation of miR-199a-5p and found that inhibition of miR-199a-5p suppressed the cyst cells proliferation and induced cell apoptosis. Finally, we found miR-199a-5p can exert a proliferation inhibitory effect through targeting CDKN1C/p57, which is also lower in AKPKD tissues compared with normal tissues. Our study provides some clues for a novel target in the clinical treatment of ADPKD.

Conflicts of interest

The authors have no conflicts of interest to declare.

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