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Absence of Long Noncoding RNA H19 Promotes Childhood Nephrotic Syndrome through Inhibiting ADCK4 Signal

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCDEFG **Jinwen Xu**
B **Tingting Ge**
C **Hongxia Zhou**
CE **Lin Zhang**
ABCDEFG **Liping Zhao**

Department of Pediatrics and Nephrology, Wuxi Children's Hospital Affiliated to Nanjing Medical University, Wuxi, Jiangsu, P.R. China

Corresponding Authors: Liping Zhao, e-mail: kew-2000@126.com, Jinwen Xu, e-mail: xujinwen_jean@163.com

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Background: Nephrotic syndrome (NS) is a common chronic kidney disease in children characterized by a group of clinical symptoms such as massive proteinuria, hypoproteinemia, high edema, and hyperlipidemia. Despite the tremendous efforts already made, the diagnosis for nephrotic syndrome still remains poor in children.





Material/Methods: The blood samples from 30 healthy children and 30 children with nephrotic syndrome were collected. The expression of H19 and ADCK4 (which are genes recently identified to play key roles in the development of nephrotic syndrome) in peripheral blood mononuclear cells (PBMCs), were detected by real-time quantitative polymerase chain reaction (RT-qPCR). The expression of ADCK4 was also detected by RT-qPCR or western blot when H19 was overexpressed or knocked down in human primary renal podocytes. Luciferase activity analysis was performed to measure whether H19 could regulate the promoter activity of ADCK4. RNA pull-down. In addition, mass spectrometry assay was used to find the transcription factor which could bind with H19, and RNA immunoprecipitation assay (RIPA) analysis was done to further confirm the interaction between H19 and candidate transcription factor.

Results: Long noncoding RNA H19 (lncRNA H19) expression was downregulated in PBMCs of children with nephrotic syndrome. ADCK4 was also downregulated. In human primary renal podocytes, overexpression of H19 promoted the expression of ADCK4, while H19 knockdown inhibited it. Furthermore, our study demonstrated that H19 could regulate the promoter activity of ADCK4. Using RNA pull-down and mass spectrometry technology, we found the transcription factor-THAP1 could bind with H19, and the interaction between them was further confirmed by RIPA analysis.

Conclusions: H19 expression in blood samples may be a novel marker of the diagnosis of nephrotic syndrome in children.

MeSH Keywords: **Adult Children • Nephrotic Syndrome • RNA, Long Noncoding**

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Background

Nephrotic syndrome (NS) is a chronic kidney disease with increased glomerular basement membrane permeability, which is characterized by a group of clinical symptoms such as massive proteinuria, hypoproteinemia, high edema and hyperlipidemia [1]. NS is very common in children aged 3 to 6 years, with an incidence of 2 to 7 cases per 100 000 children [2]. The etiology of nephrotic syndrome is still unknown. Podocytes are highly differentiated cells that play a critical role in maintaining the renal glomerular filtration barrier. Dysfunction of podocytes, such as aberrant apoptosis or reduced migration capacity, could destroy the barrier and lead to proteinuria and glomerulosclerosis [3,4]. The aarF domain containing kinase 4 (ADCK4) is highly expressed in glomerular podocytes and ADCK4 mutations significantly promote NS which is steroid-resistant [5]. Yang et al. reported that ADCK4 mutation was associated with glomerulopathy [6].

Long noncoding RNA (lncRNA) is a class of RNAs with lengths greater than 200 nucleotides and a lack of protein-coding potential. Although initially thought to be transcriptional, recent studies have shown that lncRNA plays an important role in modulating small RNA and protein function, guiding epigenetic modifications and acting as enhancer RNA [7]. The aberrant expression of lncRNA is closely associated with the occurrence and development of many human diseases [8]. Several lncRNAs have been reported to be involved in the pathogenesis of kidney diseases [9]. More than 20 additional lncRNAs such as PVT1, MALAT1, TUG1, and NEAT1 have been linked to diabetic nephropathy, which is a chronic loss of kidney function occurring in those with diabetes mellitus [10–13]. Shen et al. reported that TapSAKI promotes urine derived sepsis-induced kidney injury through triggering inflammation [13]. Hu et al. found that lncRNA LOC105374325 gives rise to podocyte injury through increasing Bax and Bak levels by competitive binding miR-34c or miR-196a/b [14]. Che et al. reported that lncRNA MEG3 could promote podocyte injury through inhibiting Wnt/ β -catenin signaling [15]. Liu et al. found that lncRNA PVT1 promote podocyte damage and apoptosis in diabetic nephropathy [16].

In this study, we detected the expression of lncRNA H19 and ADCK4 in peripheral blood mononuclear cells (PBMC) of 30 healthy children and 30 children with NS, and we found that both lncRNA H19 and ADCK4 expression was downregulated in NS children. In human primary renal podocytes, overexpression of H19 promoted the expression of ADCK4, while H19 knockdown inhibited it. Further study demonstrated that H19 could regulate the promoter activity of ADCK4. Using RNA pull-down and mass spectrometry technology, we found a transcription factor-THAP1 could bind with H19, and the interaction between them was further confirmed by RIPA analysis.

H19 expression in blood sample may be a novel marker for the diagnosis of NS in children.

Material and Methods

RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR) analysis

The blood samples from 30 healthy children and 30 children with NS were collected. The collection and detection of blood samples of humans were approved by the Medical Ethics Committee of Wuxi Children's Hospital. PBMCs were isolated by Ficoll isolation. RNA was extracted from PBMC with TRIzol (TianGen, Beijing, China). cDNA was synthesized with HiScript® II Reverse Transcriptase (Vazyme, Nanjing, China) according to manufacturer's instructions. Real-time quantitative polymerase chain reaction (RT-qPCR) was performed with specific primers, and the house-keeping gene GAPDH was used as an internal control. The PCR primers were as follows:

H19 forward: 5'-AGCGGGTCTGTTTCTTACTT-3',

H19 reverse: 5'-AGCTGGGTAGCACCATTTC-3';

ADCK4 forward: 5'-TCCGATTCATGCAGACTGAC-3';

ADCK4 reverse: 5'-CTGTGAACTCTGTCCCAAAC-3';

GAPDH forward: 5'-CAGGGCTGCTTTAACTCTGGTAA-3',

GAPDH reverse: 5'-GGGTGGAATCATATTGGAACATGT-3'.

Cell culture and transfection

The human renal podocytes were purchase from GuangZhou Jennio Biotechnology Company (GuangZhou, China) and cultured in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 ug/mL streptomycin at 37°C in 5% CO₂ atmosphere. H19, sh-H19 and corresponding control plasmids were constructed by Nanjing Dongji Biotechnology (Nanjing, China). Cells were seeded in 12-well culture plates with a concentration of 2×10⁵ cells/well. Plasmids were transfected using commercial transfection agents Lipofectamine 2000 (Invitrogen, USA) under the manufacturer's instruction. For "knock down" assay, cells seeded in 12-well culture plate were transfected with sh-H19 or sh-NC plasmid. Sh-H19: 5'-ATGGTGTCTTTGATGTTGGG-3'.

Western blot

Proteins were extracted from cells using RNA immunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Beijing, China), and quantified by bicinchoninic acid (BCA) Protein Assay Kit. Then, 40 μ g protein samples were separated on 8% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and transferred to polyvinylidene difluoride membrane (Millipore, Billerica, USA) for 3 hours. Then 5% non-fat dry milk was used to block the membranes, and primary antibodies

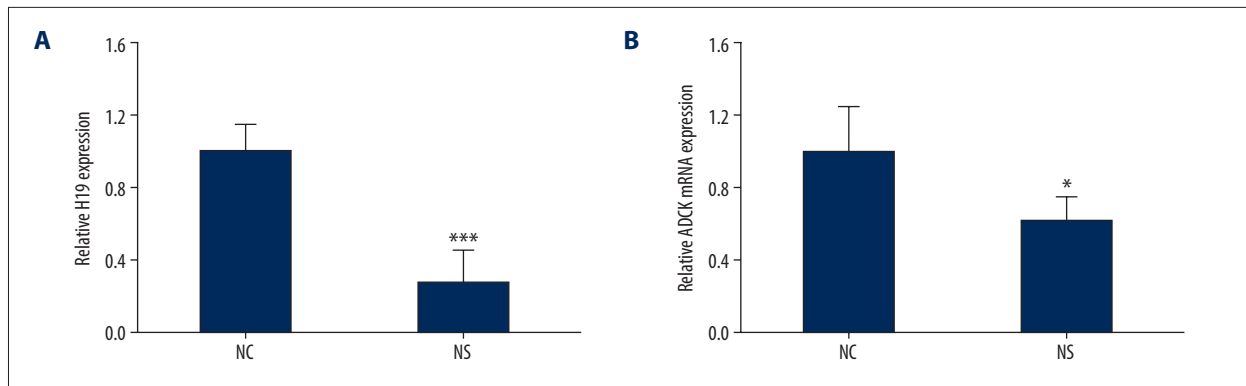


Figure 1. (A, B) H19 and ADCK4 expression was decreased in PBMCs of NS children. The expression of H19 and ADCK4 in PBMCs of 30 healthy children and 30 NS children was detected by RT-qPCR. PBMCs – peripheral blood mononuclear cells; NC – healthy children; NS – nephrotic syndrome; RT-qPCR – real-time quantitative polymerase chain reaction. *** $P < 0.001$, * $P < 0.05$.

were incubated at 4°C overnight, including ADCK4 (1: 1000, ab97647, Abcam) and GAPDH (1: 2000, 2118S, CST). Then the membranes were incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibody separately. Finally, bands were imaged using luminous fluid (Beyotime Biotechnology, Beijing, China).

RIPA

Human renal podocytes were transfected with plasmids. Then RIP analysis was done with the Magna RIP Kit (Millipore, Billerica, MA, USA) with the THAP-1 antibody (Abnova, H00055145-PW2, USA). Subsequently, RNA was extracted, and RT-qPCR was completed to quantify the expression of H19.

RNA pull-down and mass spectrometry analysis

H19 was transcribed *in vitro* with MEGAscript™ T7 Transcription Kit (AM1334, ThermoFisher Scientific), and labeled with biotin using Biotin RNA Labelling Mix (11685597910). The biotinylated RNA was then incubated with streptavidin-linked magnetic beads at 4°C overnight. The obtained mix was washed and incubated with the cell lysis of human renal podocytes. The beads-RNA-proteins were then washed and retrieved. Finally, the retrieved proteins were analyzed by mass spectrometry.

Luciferase reporter assay

The human renal podocytes were plated in 24-well culture plates, then transfected with pmirGLO-ADCK4 plasmids and NC, H19, sh-NC, and sh-H19 plasmids for 24 hours. Then cells were harvested, and the luciferase activity was assayed with Dual-Luciferase Reporter Assay System (Promega Corp, Madison, WI, USA).

Statistical analysis

Statistical analysis was completed with GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA) with Student's *t*-test to evaluate the significant group differences. All results are expressed as the mean ± SEM (standard error of mean), while *P*-values < 0.05 were considered significant.

Results

H19 and ADCK4 expression was decreased in PBMCs of NS children

We analyze the expression of H19 and ADCK4 in PBMCs of 30 NS children and 30 healthy children. The specifications of human samples including age, NS type, 24-hour urine protein, body weight, and creatinine clearance were listed in Supplementary Table 1. As Figure 1A and 1B show, both H19 and ADCK4 expression was decreased in NS children.

H19 promoted the expression of ADCK4 in human primary renal podocytes

We next determined whether H19 could regulate ADCK4 expression. H19 was overexpressed or knocked down in human primary renal podocytes through transfecting H19 or sh-H19 plasmids and the expression level of ADCK4 was detected by RT-qPCR or western blot. As Figure 2A shows, the expression of H19 was upregulated when H19 plasmid was transfected while its expression was inhibited when sh-H19 plasmid was transfected. Consistent with H19 expression, the mRNA expression of ADCK4 was upregulated when H19 was overexpressed while its expression was inhibited when H19 was knocked down (Figure 2B). What is more, we detected the expression of ADCK4 with western blot and found the same trend (Figure 2C, 2D). In order to determine whether H19

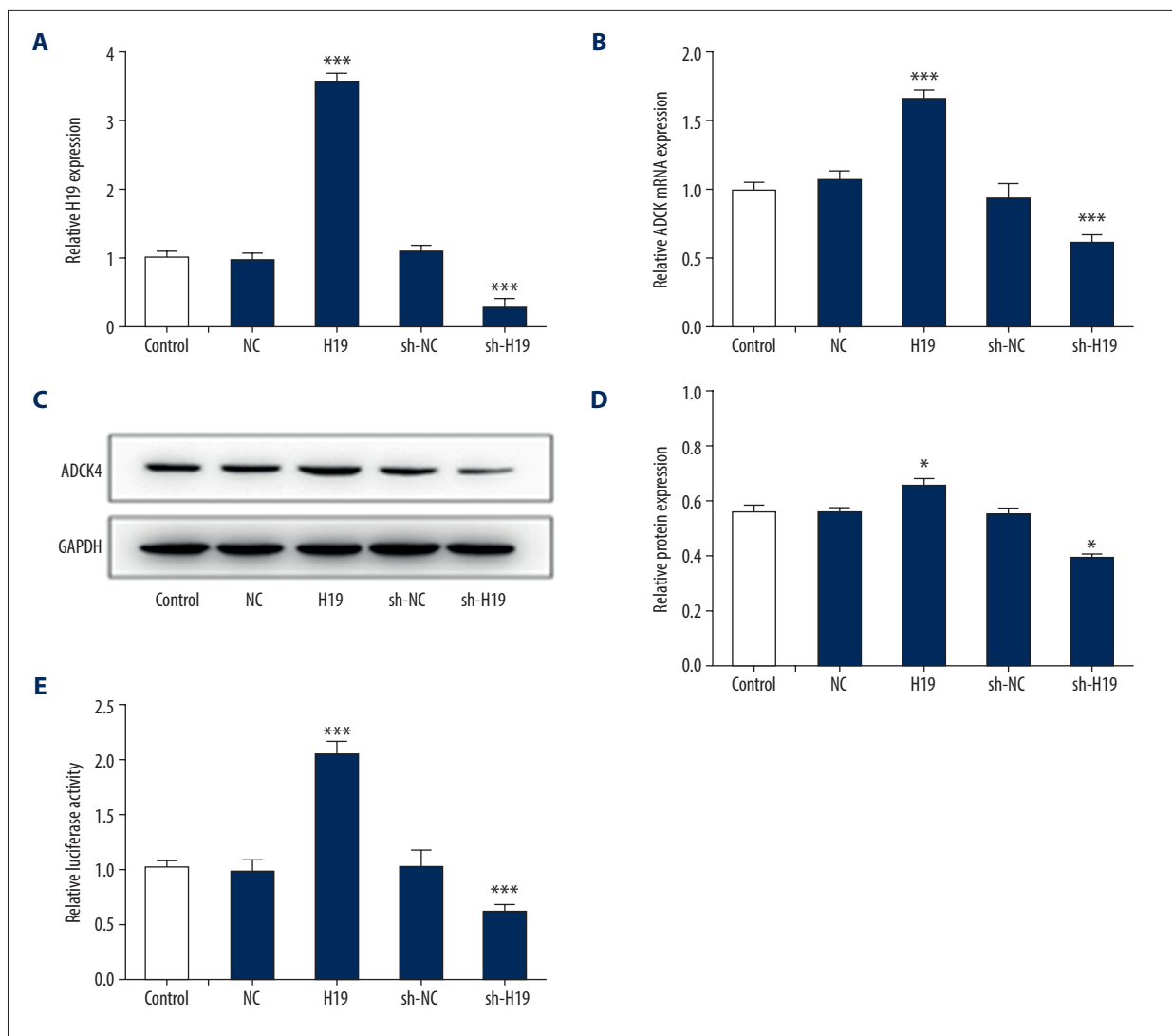


Figure 2. H19 promote the expression of ADCK4 in human primary renal podocytes. Human primary renal podocytes were transfected with NC, H19, sh-NC, and sh-H19 plasmids. 48 hours later, the expression of H19 and ADCK4 was detected by RT-qPCR and western-blotting. **(A)** The mRNA expression of H19 was detected with RT-qPCR. **(B)** The mRNA expression of ADCK4 was analyzed by RT-qPCR. **(C)** The protein level of ADCK4 was detected by western blotting. **(D)** The quantification of ADCK4 expression. **(E)** Luciferase activity analysis was performed to measure luciferase activity in human primary renal podocytes transfected with pmirGLO-ADCK4 and NC, H19, sh-NC, and sh-H19 plasmids. *** $P < 0.001$. NC – healthy children; RT-qPCR – real-time quantitative polymerase chain reaction.

could regulate the promoter activity of ADCK4, luciferase activity analysis was performed. As Figure 2E shows, transfection with H19 plasmid significantly promoted the luciferase expression while transfection with sh-H19 decreased the expression. While the binding sites of H19 on ADCK4 promoter were mutated, H19 did not affect the activity of luciferase reporter (Supplementary Figure 1). These results confirmed that H19 could regulate the expression of ADCK4.

H19 could bind with THAP1

Transcription factors are very important for gene transcription in eukaryotic cells. Some lncRNAs can act as ligands, bind with some transcription factors to control gene transcription activity [17]. We performed RNA pull-down and mass spectrometry assay to find the transcription factors binding with H19 (Supplementary Table 2). As Figure 3A shows, there were totally 318 proteins which could bind with H19, and 7 proteins among them were associated with transcription regulator activity. We chose the transcription factor THAP1 for further analysis.

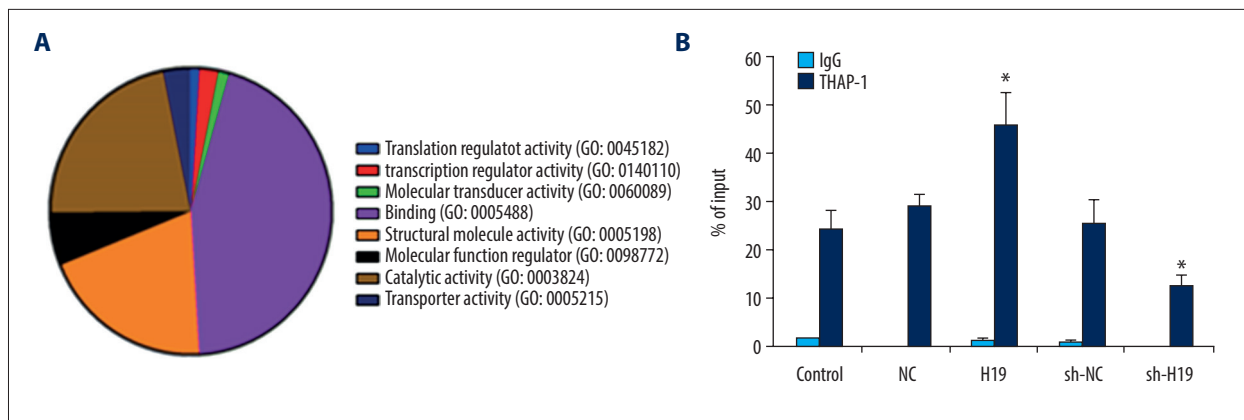


Figure 3. H19 could bind with THAP1. **(A)** RNA pull-down assay was used to detect transcription factors binding to H19, and specific transcription factors were identified by mass spectrometry. THAP1 was one of the 7 transcription regulator factors which were significantly associated with H19. **(B)** RIPA analysis was used to analyze the enrichment degree of H19 and THAP1 by THAP1 antibody. * $P < 0.05$.

The data including the THAP1 peptides obtained after mass spectrometry analysis was shown in Supplementary Table 3. THAP1 is a transcription factor which contains highly conserved DNA-binding and protein-interacting regions, playing important roles in a variety of physiological or pathological processes including dystonia and embryonic stem cell survival and differentiation [18,19]. RIPA analysis was used to assess the enrichment degree of H19 and THAP1. As Figure 3B shows, THAP1 antibody could pull down more H19 when H19 was overexpressed. We detected the THAP1 level in PBMCs of 30 healthy children and 30 NS children by RT-q-PCR and found that THAP1 level was increased (Supplementary Figure 2). Further study revealed that THAP1 promoted the expression of ADCK4 (Supplementary Figure 3). Luciferase reporter assay demonstrated that THAP1 overexpression promoted the activity of the luciferase reporter containing ADCK4 promoter (Supplementary Figure 4). These studies indicated that H19 may regulate ADCK4 through binding with the transcription factor THAP1.

Discussion

The diagnoses of nephrotic syndrome (NS) are commonly delayed and incorrect [20]. It is urgent to find new biomarkers for helping to make correct diagnoses. In this study, we detected the expression of H19 in PBMCs of NS and healthy children and found that H19 is downregulated in NS children, which

may be a potent diagnostic indicator for NS. H19 is the first discovered lncRNA, locating on the telomere region of human chromosome 11p15.5 [21]. H19 has been extensively studied in many diseases such as cancers, acute myocardial infarction, non-alcoholic fatty liver [22–24]. As for kidney diseases, Fan et al. revealed that H19 took part in diabetic nephropathy through regulating the expression of vitamin D receptor [25].

Increasing evidences reveal that ADCK4 mutation plays a key role in NS [26]. However, the regulation mechanism of ADCK4 still remains unclear. In this study, we found that the expression of H19 was positively correlated with ADCK4 in PBMCs of children. Furthermore, we founded that H19 could regulate the expression of ADCK4 in human primary renal podocytes. Therefore, H19 may be a novel target for developing new therapeutic strategies. Using RNA pull-down and RIPA analysis, we verified that H19 could bind with THAP-1. The regulatory mechanism THAP-1 to ADCK4 is worth further exploring.

Conclusions

In conclusion, we clarified that H19 may be a diagnosis marker and therapeutic targets for childhood NS.

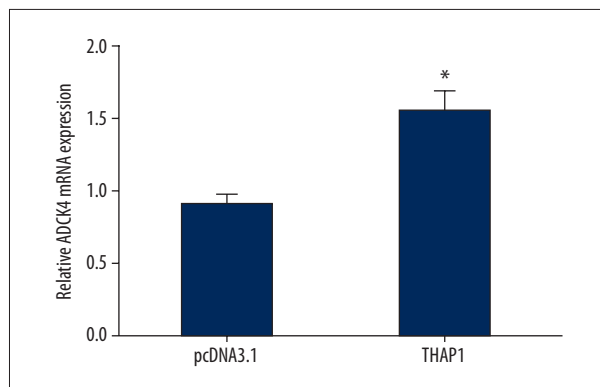
Conflict of interests

None.

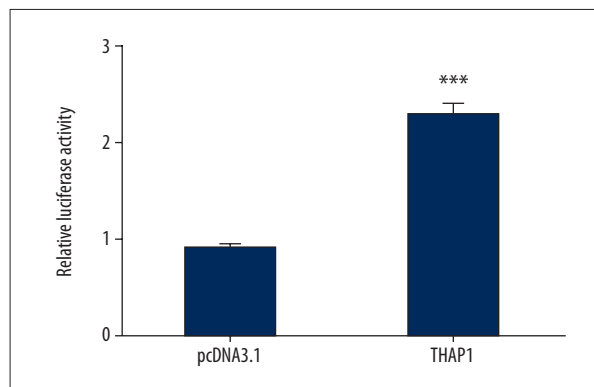
Supplementary Data

Supplementary Tables 1–3.

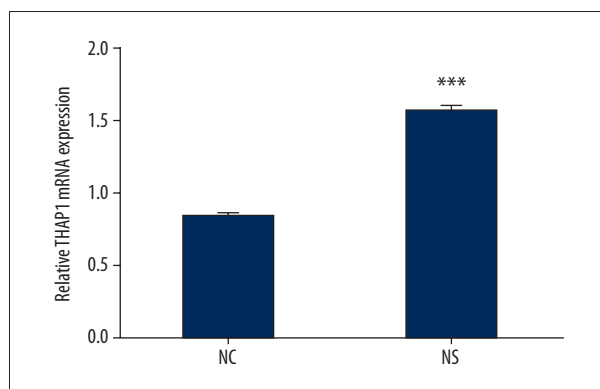
Supplementary Tables data available from the corresponding author on request.



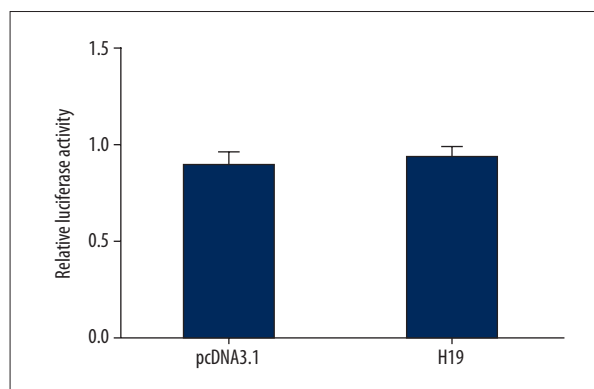
Supplementary Figure 1. H19 did not affect the activity of luciferase reporter containing mutant binding site of H19 on ADCK4 promoter.



Supplementary Figure 3. THAP1 promoted the expression of ADCK4.



Supplementary Figure 2. THAP1 expression was increased in PBMCs of NS children. PBMCs – peripheral blood mononuclear cells; NS – nephrotic syndrome.



Supplementary Figure 4. THAP1 promoted the activity of the luciferase reporter containing ADCK4 promoter.

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