

Hsa_circ_0001461 as a novel biomarker in patients with primary focal segmental glomerulosclerosis

Type

Research paper

Keywords

nephrotic syndrome, minimal change disease, Circular RNAs, focal segmental glomerulosclerosis

Abstract

Introduction

Circular RNAs (circRNAs), a novel class of non-coding RNAs, have been implicated in various diseases and are considered to play an important role in physiological and pathological processes. We aimed to profile renal circRNA in children with primary focal segmental glomerulosclerosis (FSGS) and predict the potential mechanism underlying the pathogenesis of FSGS.

Material and methods

A global circRNA expression analysis was performed in renal tissues from patients with FSGS (n=3) and controls (n=3). Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted to validate the expression levels of three circRNAs in a cohort of 44 patients with FSGS, 44 age-matched healthy controls, and 15 patients with minimal change disease (MCD).

Results

A total of 152 circRNAs were differentially expressed in patients with FSGS compared with controls. Hsa_circ_0001461 and hsa_circ_0000605 were significantly increased in patients with FSGS compared with controls, as determined by qRT-PCR ($p < 0.001$). The receiver operating characteristic (ROC) curve areas of hsa_circ_0001461 and hsa_circ_0000605 were 0.948 and 0.934, respectively. Hsa_circ_0001461 was significantly elevated in patients with FSGS compared with those with MCD and was correlated with 24 h urinary protein in patients with FSGS ($r = 0.732$, $p < 0.001$). Bioinformatics analysis predicted mir-30a interaction with hsa_circ_0001461. Mir-30a was negatively correlated with the renal hsa_circ_0001461 level ($r = -0.90$, $p < 0.001$).

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Key words circular RNAs, focal segmental glomerulosclerosis, minimal change disease, nephrotic syndrome

Introduction

Primary focal segmental glomerulosclerosis (FSGS), resulting from podocyte injury, is a clinicopathological entity that manifests as massive proteinuria, edema, and hypoalbuminemia, accounting for 10–20% of idiopathic nephrotic syndrome (NS) in children [1]. FSGS often shows steroid resistance, therapeutic dilemma, and a tendency to progress to end-stage renal disease (ESRD) [2]. Due to the profound renal morbidity of FSGS, it has become the subject of intensive basic scientific and clinical research over the past sixty years. To our knowledge, the precise pathogenesis of FSGS remains unclear.

FSGS and MCD have a similar clinical presentation and diverse prognosis. The relationship between MCD and primary FSGS remains controversial. The diagnosis of these two entities is a challenge for the nephropathologists owing to the bias of sampling under some circumstances. CD80 [3], uPAR [4] and miRNAs [5] have been reported as biomarkers to differentiate FSGS from MCD. the research based on different circRNAs between FSGS and MCD is still blank.

Circular RNAs (circRNAs) are a large class of non-coding RNAs that are produced

by backsplicing [6]. Because of the absence of the 5' cap and 3' end, circRNAs are resistant to exonuclease and stable in tissues [7-8]. circRNAs show tissue-specific and cell-specific expression patterns [9-10] and are implicated in multiple diseases such as cancers [11], cardiovascular disorders [12], and diabetes mellitus [13]. They regulate gene expression in various ways, including acting as miRNA sponges [7, 14]. RNA binding protein sponges [15] and protein templates [16-18]. Recently, hsa_circ_007500 (circHLA-C) was found to be significantly elevated in kidney tissues from patients with lupus nephritis (LN) and was proposed to play a vital role in the pathogenesis of LN by sponging mir-150 [19]. The profiling of circRNAs in peripheral blood has been reported in acute kidney injury and membranous nephropathy. circLRP6 could regulate mesangial cell proliferation, oxidative stress, extracellular matrix accumulation, and inflammation by sponging miR-205, upregulating HMGB1, and activating the TLR4/NF- κ B pathway in diabetic nephropathy [20]. circRNA_15698 was significantly upregulated in diabetic mice and promoted the synthesis of extracellular matrix-related proteins through miR-185/TGF- β 1 [21]. To date, the circRNA expression profile of primary FSGS in children remains unknown.

microRNAs (miRNAs), endogenous non-coding RNAs, have been intensively explored in the pathogenesis of renal diseases. Mir-30a is essential for the development of the kidney [22]. Serum mir-30a was upregulated in children with nephrotic syndrome and correlated with proteinuria [23]. The level of serum mir-30a was associated with the activity of FSGS [24]. Multiple studies indicated that mir-30a plays a vital role in the process of podocyte injury and renal fibrosis [25]. However, the

regulatory mechanism between circRNAs and miRNAs has not yet been elucidated.

In the present study, we first investigated the expression profile of circRNAs in renal tissues from individuals with primary FSGS by high-throughput sequencing. Second, we compared candidate circRNAs in the whole cohort of our study by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Finally, we conducted a bioinformatics analysis to predict the competing endogenous RNA (ceRNA) of candidate circRNAs.

Materials and methods

Patients and specimens

A total of 109 participants were enrolled in the current study from the Guangzhou Women and Children's Medical Center between July 2018 and January 2020. Patients with FSGS (n=47, 3 for genome-wide expression analysis, 44 for circRNAs validation) and MCD (n=15) were consecutively recruited from the Department of Nephrology of the Guangzhou Women and Children's Medical Center. Renal biopsies were performed in children with NS, who had initial or secondary steroid resistance, reduced serum complement, or impaired renal function. The inclusion criteria for FSGS/MCD were as follows: age between 1 and 18 y; diagnosed with NS according to the ISKDC criteria; patients in recurrence at the time of kidney biopsy; and renal pathological changes confirmed by an expert. Renal pathological diagnosis was made by light microscopy (LM), immunofluorescence and transmission electron microscopy (TEM). FSGS was diagnosed morphologically by the presence of focal and segmental

sclerosis by LM with foot process effacement as seen by TEM. MCD was diagnosed by the presence of morphological **features** of foot process effacement in TEM, while no glomerular lesions **were** captured by LM. Both FSGS and MCD showed negative or low-intensity mesangial IgM (sometimes accompanied by C3 or C1q) staining detected in immunofluorescence. The exclusion criteria for FSGS/MCD were as follows: genetic mutations contributing to steroid-resistant nephrotic syndrome, lupus nephritis, IgA nephropathy, Henoch-Schonlein purpura nephritis, hepatitis B-related nephritis, hepatitis C-related nephritis, and other secondary nephritis, and estimated glomerular filtration rate (eGFR) lower than 30 ml/min/1.73m² at least lasting for three months. In addition, 47 children (3 for genome-wide expression analysis, 44 for circRNAs validation) with renal mass (histologically confirmed as nephroblastoma or clear cell carcinoma), regarded as controls, were enrolled from the surgical oncology department of the Guangzhou Women and Children's Medical Center. Renal tissues as controls were obtained from renal parenchyma at least 5 cm from the edge of the renal mass. Percutaneous renopuncture biopsy was performed to obtain three or four pieces of renal tissues (1.0-1.5cm in length) from each patient with FSGS/MCD. One piece of renal tissue was used for histologic diagnosis, the remaining pieces were all used for research. The design strategy is demonstrated in Figure 1. The present study was approved by the Institutional Review Board of the Guangzhou Women and Children's Medical Center. **An estimated total sample size of 54 (allocation ratio = 1) would be needed to provide 80.41% Power to reject the null hypothesis of equal means with a significance level of 0.05 using a two-sample Wilcoxon-Mann-Whitney test by G**

Power. The written informed consents were obtained from legal representatives of all participants before the percutaneous renal biopsy or surgery in Guangzhou Women and Children's Medical Center.

Profiling

Total RNA was extracted from kidney tissues from patients with FSGS (n=3) and controls (n=3) using TRIzol Reagent (Invitrogen) according to the manufacturer's protocols. The concentration of the RNA was qualified and quantified using NanoDrop-ND1000. The integrity of the RNA was analyzed by 1% agarose gel electrophoresis. Isolated RNA was stored at -80 °C until further analysis.

RNA library construction and circRNA sequencing were performed by Ribo Biotech Inc. (Guangzhou, China). A Ribo-Zero rRNA removal kit and Rnase-R were used to remove rRNA and linear RNA from the total RNA, respectively. Then, the rRNA-depleted RNA was processed with a TruSeq Stranded RNA Library Prep Kit (Illumina, USA) to establish the RNA library. The library was qualified and quantified using the BioAnalyzer 2100 system (Agilent Technologies, USA). The RNA libraries were denatured as single-stranded DNA, captured on Illumin Flow cells, amplified in situ as clusters, and then sequenced on an Illumina Hi-Seq sequencing instrument (PE 150 mode). The effective reads were eligible for circRNA analysis following 3 adapter trimming and the removal of poor-quality reads.

Hierarchical clustering analysis was performed to distinguish the expression levels of circRNAs between the FSGS and controls. CircRNAs with fold changes > 2.0 and p-values < 0.05 were defined as significant differential expressions.

Validation of candidate circRNAs and miRNAs

The total RNA of kidney tissues was reverse transcribed into cDNA with dNTP Mix (HyTest Ltd), RNase Inhibitor (Epicenter, Illumina, Inc.), and SuperScript III (Thermo Fisher Scientific, Inc.). Due to the low abundance of circRNAs and limited renal tissue through renopuncture biopsy, circRNAs with high expression levels were selected as candidate circRNAs. Candidate circRNAs were validated by real-time polymerase chain reaction (PCR) on an ABI ViiA 7 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The divergent junction-spanning primers for specific detection of circRNAs are listed in Table I. β -actin was used as an endogenous control gene for circRNAs. The mir-30a expression level in the kidney was also assessed using a TaqMan PCR kit (Applied Biosystems, Foster City, CA, USA) on an ABI ViiA 7 Real-time PCR System (Applied Biosystems, Foster City, CA, USA), and let-7 was chosen as an internal control. Relative expression was analyzed by the $\Delta\Delta C_t$ analysis.

Statistical analysis

Statistical analysis was performed using SPSS 18.0 (Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, CA, USA). Baseline statistics were represented as the mean \pm SD or median (P_{25} , P_{75}) for quantitative data and numbers for categorical data. The Mann-Whitney U test or Student's t-test was used to compare differences in circRNA expression levels between groups. The chi-square test or Fisher's exact test was performed between two categorical variables. Receiver operating characteristic (ROC) curves were obtained, and the specificity and sensitivity of predictive power were assessed by area under the curve (AUC). Correlations

between circRNAs and clinical parameters or miRNA levels were analyzed using Pearson's correlation analysis. Statistical significance was determined at $p < 0.05$.

Results

CircRNA expression profile in renal tissues

To analyze the profiling of circRNAs in FSGS, we conducted RNA-deep sequencing of RNA extracted from the kidney tissues of three patients with FSGS and three normal kidney tissues. The results revealed that a total of 6322 circRNAs were detected in the kidney, of which 152 circRNAs were differentially expressed in patients with FSGS compared with controls (fold changes ≥ 2.0 , p -values < 0.05). Among these abnormally expressed circRNAs, 79 circRNAs were upregulated, and 73 were downregulated, as shown on the cluster heatmap and volcano plots (Figure 2A and B). The top ten differentially expressed circRNAs between FSGS patients and controls are shown in Table II.

Validation of circRNAs by qRT-PCR

We selected the candidate circRNAs with high expression levels in both groups to ensure stable detection as well as remarkably differential expression. Subsequently, hsa_circ_0001461, hsa_circ_0000605, and hsa_circ_0001849 were verified in kidney tissues from a subset of FSGS patients ($n = 30$) and controls ($n = 30$) by qRT-PCR. The demographic and baseline characteristics of FSGS patients and controls are summarized in Table III. The results indicated that hsa_circ_0001461 and hsa_circ_0000605 were both significantly increased in patients with FSGS ($p < 0.001$,

$p < 0.001$, respectively) (Figure 3A and B); however, no significant difference in the hsa_circ_0001849 level was found between the two groups ($p > 0.05$) (Figure 3C).

ROC curves were obtained to validate the potential diagnostic value of hsa_circ_0001461 and hsa_circ_0000605 for FSGS. The ROC curves of candidate circRNAs indicated that hsa_circ_0001461 (AUC 0.948, 95% CI 0.898–0.997, $p < 0.001$) and hsa_circ_0000605 (AUC 0.934, 95% CI 0.876–0.993, $p < 0.001$) were capable of differentiating patients with FSGS from controls (Figure 4A and B).

Correlation of circRNA fold changes with laboratory parameters in FSGS

Subsequently, we analyzed the correlation between the expression fold changes of hsa_circ_0001461/hsa_circ_0000605 and laboratory parameters (24 h urinary protein, serum creatinine, eGFR) in the FSGS group. We found that hsa_circ_0001461 was positively correlated with 24 h urinary protein in patients with FSGS ($r = 0.732$, $p < 0.001$) (Figure 4C). However, there was no significant relationship between hsa_circ_0001461 and other laboratory indexes, and hsa_circ_0000605 was not correlated with laboratory parameters in patients with FSGS.

Comparison of circRNAs among FSGS, MCD, and controls

We next compared the concentrations of hsa_circ_0001461 and hsa_circ_0000605 in a cohort of patients with FSGS ($n = 14$), patients with MCD ($n = 15$), and age-matched controls ($n = 14$). The demographic and baseline characteristics of the patients are summarized in Table IV. No significant differences were observed in age, sex, serum albumin levels, 24 h urine protein, and eGFR between the FSGS and MCD groups. Hsa_circ_0001461 was expressed much higher in kidney samples

from patients with FSGS than in those with MCD and controls ($p < 0.001$) (Figure 5A). No significant difference was found between patients with MCD and controls ($p > 0.05$). In addition, patients with FSGS had higher concentrations of hsa_circ_0000605 than controls ($p < 0.001$) (Figure 5B). However, there was no significant difference between patients with FSGS and MCD ($p > 0.05$). This suggests that hsa_circ_0001461 might be a potential novel biomarker to distinguish FSGS from MCD.

Prediction of the ceRNA for hsa_circ_0001461

Hsa_circ_0001461, an extronic circular RNA, contains several binding sites for AGO2, which is essential for the microRNA sponge function. Renal hsa_circ_0001461 had the greatest fold change in patients with FSGS compared with those with MCD and controls. Renal hsa_circ_0001461 was significantly associated with the level of proteinuria in FSGS. Mir-30a was predicted to interact with hsa_circ_0001461 by TargetScan and miRanda (Figure 6A). Hsa_circ_0001461 contained eight binding sites for mir-30a. We found that mir-30a was negatively correlated with the renal level of hsa_circ_0001461 ($r = -0.90$, $p < 0.001$) (Figure 6B).

Discussion

In this study, we identified 152 aberrantly expressed circRNAs in kidney tissues by next-generation sequencing and then validated three circRNAs (hsa_circ_0001461, hsa_circ_0000605, and hsa_circ_0001849) with high transcript levels and obvious differences in patients with FSGS and controls in a larger cohort. Hsa_circ_0001461 and hsa_circ_0000605 were confirmed to be significantly upregulated in patients with

FSGS by qRT-PCR. Patients with FSGS had higher levels of hsa_circ_0001461 compared with patients with MCD and controls. In addition, hsa_circ_0001461 was positively associated with proteinuria in FSGS. Hence, we considered hsa_circ_0001461 as a potential novel biomarker for FSGS. The mir-30a was predicted to be a potential binding target of hsa_circ_0001461, and mir-30a displayed a negative correlation with hsa_circ_0001461, which indicated that hsa_circ_0001461 might function as a miRNA sponge to participate in the pathogenesis of FSGS.

Almost 90% of the human genome is transcribed into non-coding RNA transcripts, of which long non-coding RNAs have been extensively studied. Thousands of circRNAs have been discovered in eukaryotes with the rapid development of high-throughput RNA sequencing [8, 26]. In recent decades, many studies concerned with circRNAs have been found that might serve as potential biomarkers due to their stability and tissue-specific expression pattern [9-10]. HHLA2 and circ_ABCB10 were shown to be significantly increased in renal cell carcinoma and correlated with short overall survival [27-28]. A study found that serum circR_126 was upregulated in patients with acute kidney injury (AKI) and considered it as a probable biomarker and predictor of mortality in AKI [29]. In another study, circ_101319 in the peripheral blood was significantly higher and exhibited promising diagnostic value in patients with idiopathic membranous nephropathy (IMN) [30]. MUC3A in exosomes from serum and urine was considered a potential biomarker for IMN [31]. Plasma circRNA_002453 was shown to be a novel biomarker in the diagnosis of lupus nephritis [32]. In the current study, hsa_circ_0001461 and hsa_circ_0000605 were significantly increased in patients with

FSGS compared to controls. ROC curve analysis showed that hsa_circ_0001461 and hsa_circ_0000605 could distinguish patients with FSGS from controls.

There are controversies regarding whether FSGS and MCD are the same disease or distinct entities. Some researchers believe that although both are classified as podocytopathy, they are supposed to have different development mechanisms [33]. Differentiation between FSGS and MCD depends on the renal biopsy and the experience of the nephropathologist. The search for reliable biomarkers to distinguish FSGS from MCD is still **underway**. Previous studies have shown that serum mir-192, mir-205 [5] and urinary/renal uPAR [4, 34] have the potential to differentiate FSGS from MCD. In our study, hsa_circ_0001461 was expressed at higher levels in patients with FSGS than in those with MCD and controls.

Hsa_circ_0001461 has been reported in a small **number** of studies related to **the** tumor. Hsa_circ_0001461 was found upregulated and prompted osteosarcoma progression and metastasis by sponging mir-181b [35]. Hsa_circ_0001461 participated in the progression of osteosarcoma by sponging miR-375 to upregulate the expression of Yes-associated protein 1 [36]. In addition, hsa_circ_0001461 prompted the tumor proliferation and migration via inhibiting the mir-873 expression and increasing the ZEB1 expression in papillary thyroid cancer [37]. On the other hand, circ_0001461 was decreased in gastric cancer and inhibited the tumor progression through regulating RUNX1 and YBP-1 [38]. The role of circ_0001461 in nephropathy needs intensive exploration in the future.

Hsa_circ_0001461, an extronic circular RNA, contains several binding sites for

AGO2, which is essential for microRNA sponge function. The mir-30a was predicted as potential targets of hsa_circ_0001461 through bioinformatics analysis. Mir-30a were verified to be abundant in podocytes and tubular epithelial cells and play a vital role in the physiological and pathological processes in the kidney [39-40]. Mir-30a regulates the function of podocytes through calcium/calcineurin signaling by targeting TRPC6, PPP3CB, and NFATC3 [41]. In addition, mir-30a inhibits apoptosis in podocytes by targeting Notch1 and P53 through Notch signaling and p53 signaling pathways [42]. Hsa_circ_0001461 is thought to regulate podocyte function as the upstream molecule of mir-30a.

However, there are some limitations in our work. First, our study is single-centered with a limited sample size. Larger independent cohorts are warranted to validate our findings. Second, the precise mechanism of hsa_circ_0001461 in the kidney has not been elucidated, and in-depth experiments are needed to reveal the function of circ. Finally, whether the differential circRNAs are specific to FSGS is uncertain, comparison among other renal diseases is desirable in future studies.

In summary, we have defined a distinctive circRNAs signature from kidney samples in children with FSGS. Renal circ_0001461 is identified as a potential biomarker for FSGS. This study provides a promising method to investigate the molecular pathogenesis of FSGS. Further investigations are warranted to reveal the functional mechanism of circRNAs in vivo and vitro.

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Conflicts of interest The authors declare that they have no conflict of interest.

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Table I . Primer pairs

RNA transcript	Primer Pairs
Hsa_circ_0001461	Fwd: CTCTGGCGTTGGTGTTTTCA Rev: ACTGTCGGGAATCTGTCTCT
Hsa_circ_0000605	Fwd:ACCTTTCTAACAGCCCAAGTAAA Rev: TCTCCACTCAACTCATCTGCT
Hsa_circ_0001849	Fwd: CTCAAAGGGGCTTGGAAGAA Rev: TGCCAGTTATCTTCAGTCCAGT
Beta-actin, ActB	Fwd: CCTCGCCTTTGCCGATCC Rev: CTTCTGACCCATGCCACC

Table II. Top 10 deregulated circRNAs in kidney tissues between FSGS patients and normal controls

Up-regulated circRNAs	Log2(Fold change)			Down-regulated circRNAs	Log2(Fold change)		
	FSGS	vs.	NC		FSGS	vs.	NC
hsa_circ_0001461	3.613202			hsa_circ_0000586	-4.36883		
hsa_circ_0000605	3.570701			hsa_circ_0008838	-4.336300		
hsa_circ_0001849	3.329014			hsa_circ_0001850	-4.293221		
hsa_circ_0006010	3.303928			hsa_circ_0017348	-3.862200		
hsa_circ_0003261	2.992817			hsa_circ_0044638	-3.770316		
hsa_circ_0007248	2.987162			hsa_circ_0008659	-3.737470		
hsa_circ_0012460	2.8172672			hsa_circ_0000475	-3.588270		
hsa_circ_0005523	2.7819287			hsa_circ_0019985	-3.498271		
hsa_circ_0041267	2.7771908			hsa_circ_0002470	-3.327182		
hsa_circ_0020670	2.7761928			hsa_circ_0006706	-3.309516		

FSGS, focal segmental glomerulosclerosis; NC, normal control

Table III. The demographic and baseline characteristics of FSGS patients and NC

Characteristic	FSGS	NC	P value
No.	30	30	
Gender(M/F)	22/8	20/10	0.573
Age of onset (year)	3.31(2.00, 3.48)	3.29(1.95, 4.50)	0.806
Serum albumin(g/l)	14.18±3.93	35.12±4.67	<0.001
Serum creatinine (μmol/l)	31.47±10.78	30.33±12.18	0.704
eGFR (ml/min/1.73m ²)	138.04±24.41	148.11±32.22	0.178
24-hour Proteinuria (mg/kg.d)	340.39±145.07	-	-

FSGS, focal segmental glomerulosclerosis; NC, normal control; M, Male; F, female; eGFR, estimated glomerular filtration rate

Table IV. The demographic and baseline characteristics of patients with FSGS and MCD

Characteristic	FSGS	MCD	P value
No.	14	15	
Gender(M/F)	12/2	13/2	1.00
Age of onset (year)	3.15(1.90, 6.05)	4.00(2.80,5.50)	0.512
Serum albumin(g/l)	14.56±4.41	13.75±4.32	0.624
Serum creatinine (μmol/l)	28.71 ±10.74	30.80±14.09	0.659
eGFR(ml/min/1.73m ²)	156.72±31.61	136.67±31.09	0.097
24-hour Proteinuria (mg/kg.d)	197.71±121.80	183.00±66.45	0.687

FSGS, focal segmental glomerulosclerosis; MCD, minimal change disease; M, male; F, female; eGFR, estimated glomerular filtration rate

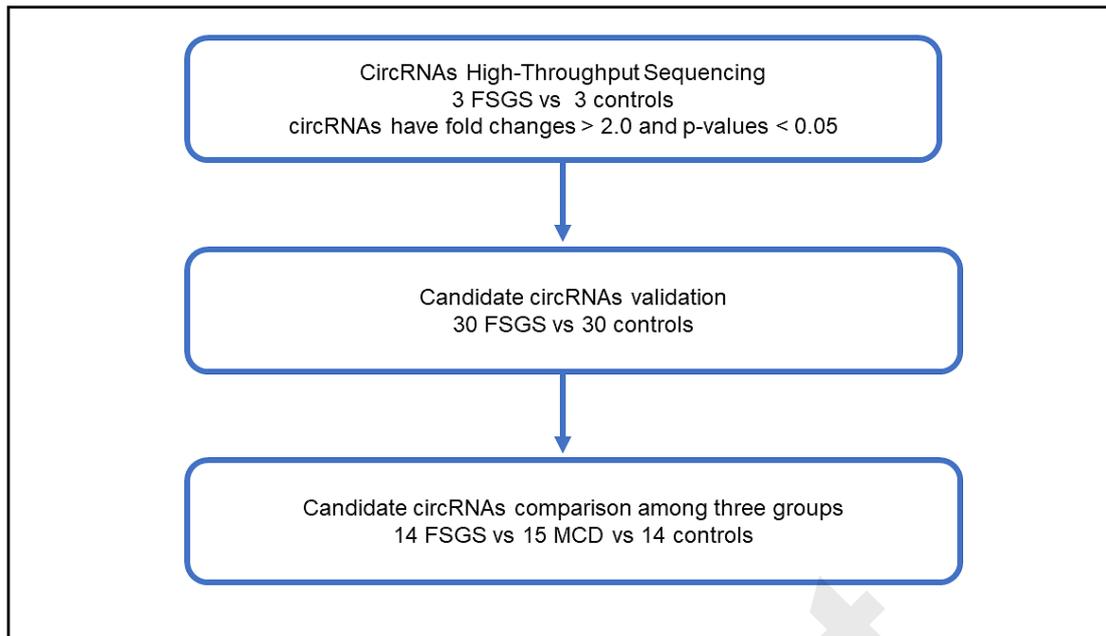


Figure 1. An overview of the design strategy.

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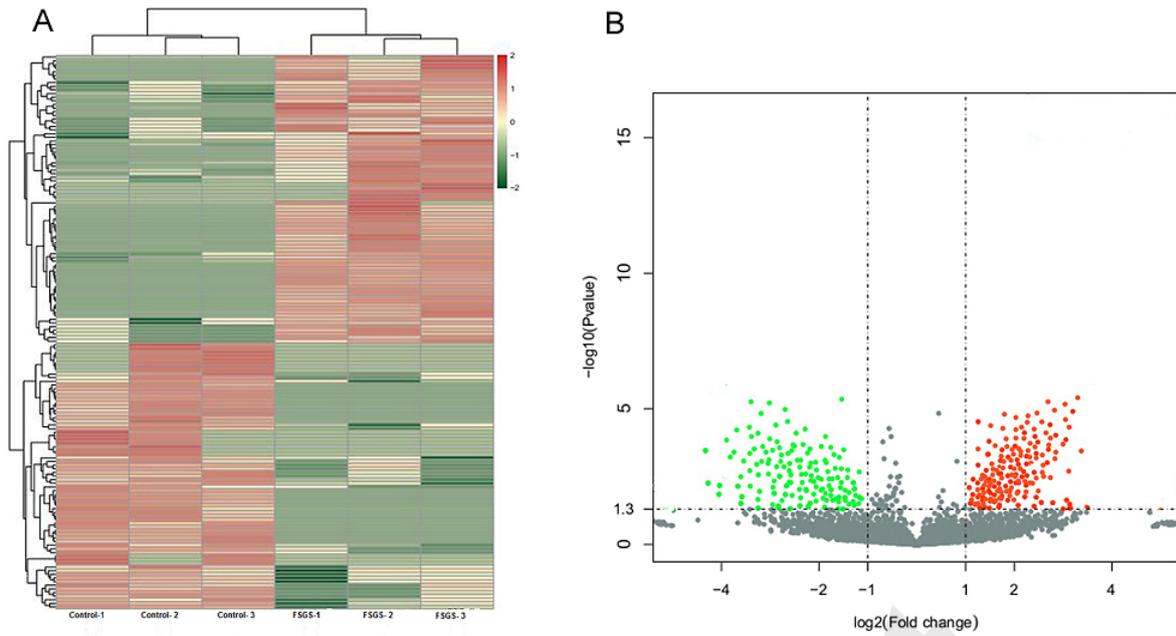


Figure 2. (A) The cluster heatmap shows the difference of circRNAs expression pattern among the samples. The color of red and green indicates high and low expression levels between two groups. (B) Volcano plot shows differentially expressed circRNAs in renal tissues of patients with FSGS and controls. FSGS, focal segmental glomerulosclerosis.

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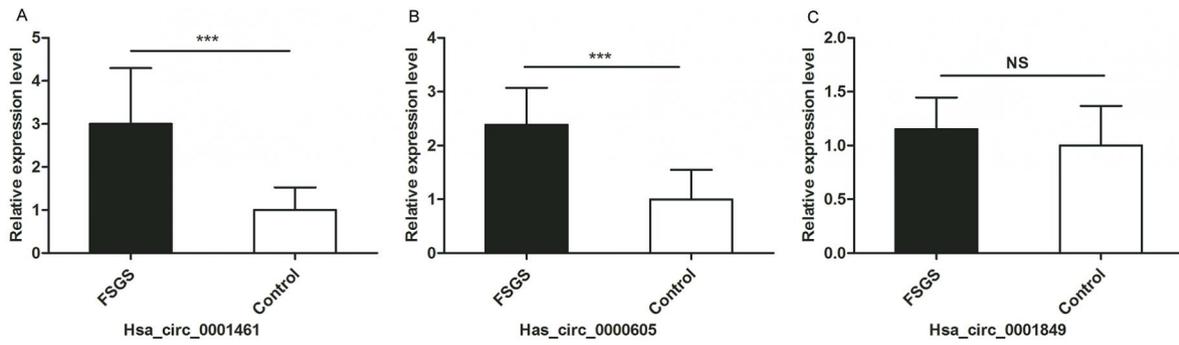


Figure 3. The expression levels of hsa_circ_0001461 (A), hsa_circ_0000605 (B) and has_circ_0001849 (C) in renal tissues of patients with FSGS compared to controls. *** P < 0.001; NS, non-significant; FSGS, focal segmental glomerulosclerosis.

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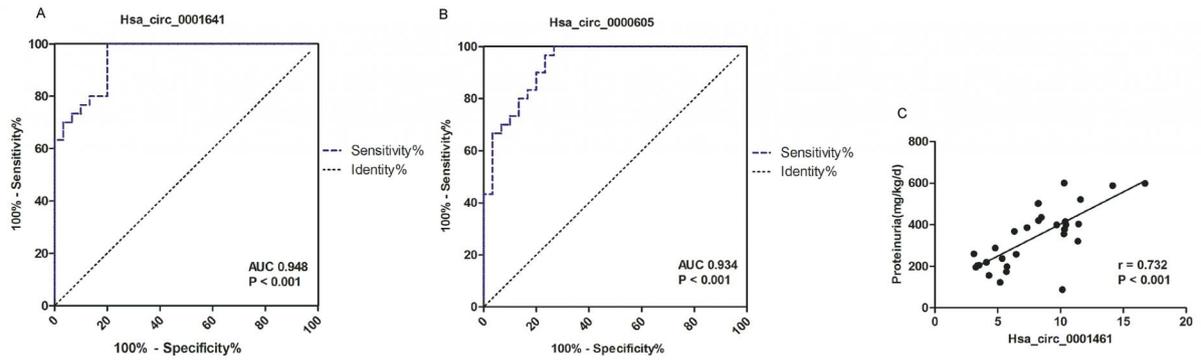


Figure 4. Receiver operating characteristic (ROC) analysis of patients with FSGS compared with controls regarding hsa_circ_0001461 (A) and hsa_circ_0000605 (B). (C) Hsa_circ_0001461 was positively correlated with 24 h urinary protein in patients with FSGS ($r = 0.732$, $p < 0.001$). FSGS, focal segmental glomerulosclerosis.

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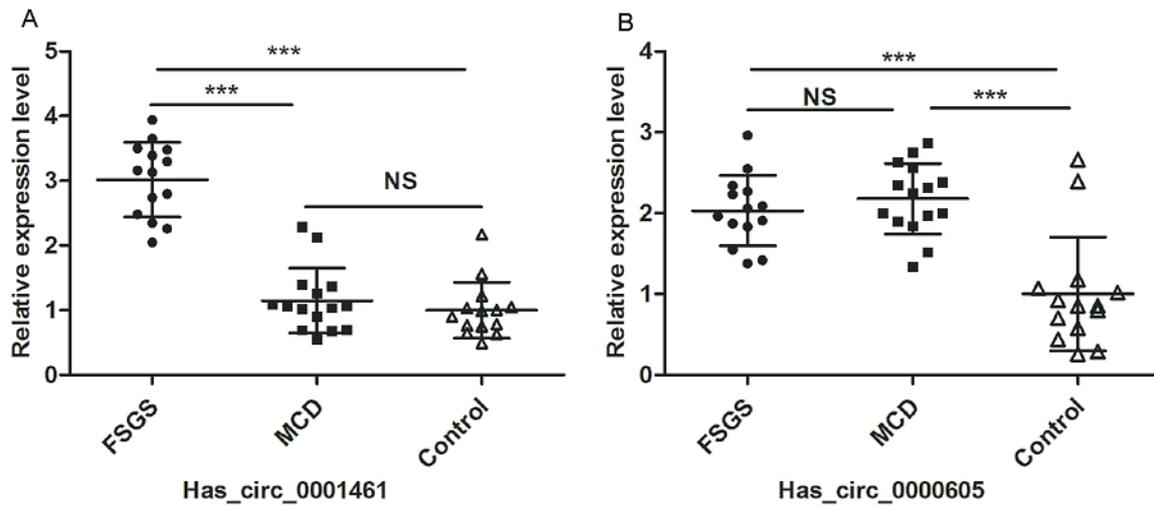
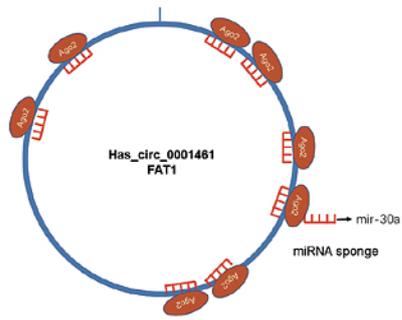


Figure 5. The expression levels of hsa_circ_0001461 (A) and hsa_circ_0000605 (B) in patients with FSGS compared with those with MCD and controls. *** $P < 0.001$; NS, non-significant; FSGS, focal segmental glomerulosclerosis; MCD, minimal change disease.

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A



B

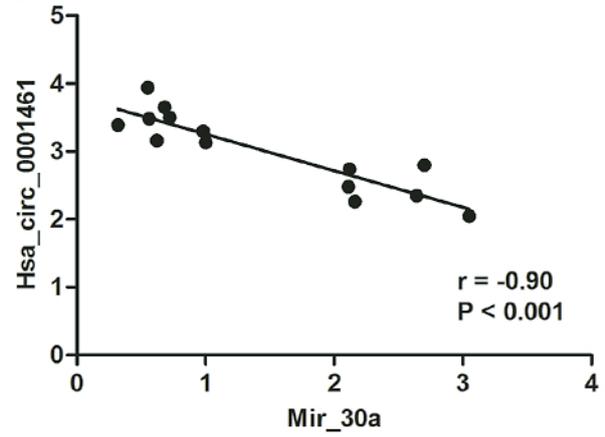


Figure 6. The interaction between renal hsa_circ_0001461 and mir-30 in FSGS. (A) Schematic cartoon of hsa_circ_0001461 in the pathogenesis of FSGS by sponging mir-30a. (B) Mir-30a was negatively correlated with the renal level of hsa_circ_0001461 ($r = -0.90$, $p < 0.001$). FSGS, focal segmental glomerulosclerosis.