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Original Research

MicroRNA-599-Regulated Susceptibility to Acute Kidney Injury in Patients with Cirrhosis is Mediated by the Sirtuin 1 (*SIRT1*) rs4746720 Single Nucleotide Polymorphism

Fangfang Zhou[1,†], Yixin Chen[1,†], Youjun Xu[1], Qun Luo[1,*]

[1]Department of Nephrology, Ningbo No.2 Hospital, 315010 Ningbo, Zhejiang, China
*Correspondence: nbeyluoqun@126.com (Qun Luo)
[†]These authors contributed equally.

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Abstract

Objective: The aim of this case-control study was to analyze the association between sirtuin 1 (*SIRT1*) single nucleotide polymorphism (SNP) and the risk of acute kidney injury (AKI) in Han Chinese patients with cirrhosis and to explore its potential mechanism. **Methods**: Twenty-nine AKI patients with cirrhosis (AKI group) and 87 non-AKI patients with cirrhosis (control group) were recruited from a Han Chinese population. SNaPshot sequencing technology was used for the detection of SNPs. Dual luciferase reporter vectors were constructed and co-transfected into HK-2 human proximal tubular epithelial cells. *SIRT1*-overexpressing recombinant plasmids were constructed and co-transfected into HK-2 cells. The expression of microRNA-599 (*miR-599*) and *SIRT1*/peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*PGC-1*α)/nuclear respiratory factor 1 (*NRF1*)/mitochondrial transcription factor A (*TFAM*) was detected by the quantitative polymerase chain reaction, and the expression of the corresponding proteins was detected by Western blotting. **Results**: There were no statistically significant between-group differences in the genotype and allele frequencies of *SIRT1* rs4746720. In the subgroup of patients with hepatic encephalopathy, the *SIRT1* rs4746720 SNP was significantly associated with the development of AKI, and the risk of AKI in patients with the T allele was six times higher than in those with the C allele. The results of the *in vitro* experiments demonstrated that the T allele of *SIRT1* rs4746720 increased the binding of *miR-599* to the rs4746720 locus within the 3′-UTR of *SIRT1* ($p < 0.001$). The results of the *SIRT1*-overexpressing recombinant plasmid experiments confirmed that the T allele of *SIRT1* rs4746720 mediated the binding of *miR-599*, leading to decreased *SIRT1* and *PGC-1*α, *NRF1*, and *TFAM* ($p < 0.05$). **Conclusions**: The *SIRT1* rs4746720 SNP might be linked with AKI in cirrhotic patients, and the T allele increased the risk of AKI in those with hepatic encephalopathy. The rs4746720 SNP in the *SIRT1* 3′-UTR is linked to the development of AKI in cirrhotic patients with hepatic encephalopathy, potentially by mediating the binding of *miR-599*.

Keywords: acute kidney injury; cirrhosis; sirtuin 1; single-nucleotide SNP; microRNA-599

1. Introduction

Acute kidney injury (AKI) is a common complication in patients with cirrhosis. In fact, in hospitalized patients with cirrhosis, the incidence of AKI can be as high as 20–40% [1–3]. The occurrence of AKI in patients with cirrhosis prolongs the hospital stay and increases their risk of multiple organ failure, such that their 30-day and 1-year mortality rates are 10- and 8-fold higher, respectively, than those of cirrhotic patients without AKI [4–6]. Thus, early detection of and interventions for AKI are important measures to improve patient outcomes [3,7]. There are several risk factors for AKI patients with cirrhosis, including advanced age, diabetes, and infection [8–11]. Moreover, candidate gene studies have suggested that some patients have a genetic predisposition to developing AKI in cirrhosis, such as the endothelial nitric oxide synthase gene (*eNOS*) G894T single nucleotide polymorphism (SNP), the vasopressin 1a receptor gene (*AVPR1A*) promoter region rs113481894 SNP, or an angiotensin-converting enzyme gene insertion or deletion, as these are associated with the risk of hepatorenal syndrome [12–14].

Silent mating type information regulator 2 homolog 1 (*SIRT1*) is a highly conserved nicotinamide adenine dinucleotide-dependent protein deacetylase [15], which has been shown to have nephroprotective effects in AKI models induced by multiple etiologies such as ischemia-reperfusion injury, sepsis, and nephrotoxic drugs [16–20]. For example, we showed that in the early stages of a rat model of AKI in cirrhosis, the expression of *SIRT1* and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (*PGC-1*α) in the renal tissues of rats was significantly reduced, suggesting that the *SIRT1/PGC-1*α signaling pathway may be involved in the mechanism of cirrhosis.

Currently, the role of *SIRT1* SNPs in AKI patients with cirrhosis remains unknown. Accordingly, in this study, we examined the function of *SIRT1*, as it is a candidate susceptibility gene for AKI in cirrhosis. Our aim was to develop a sound theoretical basis for the pathogenesis of AKI in cirrhosis that can be used for early diagnosis and the identification of therapeutic targets.

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2. Materials and Methods

2.1 Participants

This study enrolled patients with liver cirrhosis who were admitted to the Department of Hepatology, Ningbo No.2 Hospital (Zhejiang, China) from October 2020 to January 2021. The inclusion criteria were being Han Chinese, aged $\geq$18 years old, and being hospitalized for a diagnosis of liver cirrhosis. The exclusion criteria had organic kidney disease, having received renal replacement therapy, intending to undergo or have undergone kidney transplantation or liver transplantation, having liver cancer and other malignant tumors, having a confirmed pregnancy, or having a life expectancy $<$3 days. Those meeting the inclusion criteria were divided into an AKI group and a non-AKI group. The research protocol was approved by the Medical Ethics Committee of the hospital, and all of the participants provided signed informed consent. The diagnostic criteria for AKI were based on the 2015 International Ascites Club guidelines: a serum creatinine (SCr) concentration $>$0.3 mg/dL (26.5 µmol/L) from baseline within 48 h of admission, or $>$50% from baseline within 7 days of admission.

2.2 Clinical Data Collection

The data collected on the general condition of the participants comprised their age, sex, primary disease, comorbidities, medical history, family history, having complications of cirrhosis (infection/spontaneous peritonitis, gastrointestinal (GI) bleeding, ascites, and hepatic encephalopathy), body mass index (BMI), systolic blood pressure, and diastolic blood pressure. The laboratory indicators of the participants that were recorded included SCr concentration (baseline SCr concentration, i.e., at admission, and highest SCr concentration), blood urea nitrogen (BUN) concentration, albumin concentration, total bilirubin concentration, alanine aminotransferase (ALT) concentration, aspartate aminotransferase (AST) concentration, glucose concentration, total cholesterol concentration, low-density lipoprotein concentration, triglyceride concentration, blood sodium concentration, C-reactive protein (CRP) concentration, hemoglobin concentration, platelet count, prothrombin time, and international normalized ratio. The simplified Modification of Diet in Renal Disease formula was used to determine the estimated glomerular filtration rate (eGFR).

2.3 Specimen Collection and Genomic DNA Extraction

A sample (0.5 mL) of peripheral venous blood was collected from the patients in the morning while they were at rest and had an empty stomachs. The sample was treated with an anticoagulant (ethylenediaminetetraacetic acid) and then stored at –80 °C. After all of the blood samples had been collected, their DNA was extracted using a blood genomic DNA extraction kit and then stored at –20 °C for later use.

Table 1. Primer sequence of *SIRT1* SNP.

SNP site	Polymorphism	Sequence 5′-3′
rs4746720	T/C	F: CCAAAGAATGGTATTTTCACTT
		R: AAGTTAGCTGCCACAGTT

Note: F, Forward primer; R, Reverse primer.

2.4 Primer Design and Synthesis

The SNP locus information of *SIRT1* was obtained, revealing that the functional *SNP* site of *SIRT1* was rs4746720. Thus, the primer sequence was designed as shown in Table 1.

2.5 Cell Culture

The kidney proximal tubular epithelial cell line Human Kidney 2 (HK2, cat. ZB188) was purchased from the Shanghai Zhibei Biotechnology Co., Ltd., which has been validated by short tandem repeat profiling and was negative for mycoplasma contamination. Dulbecco's modified Eagle's medium/F-12 modification containing 10% fetal bovine serum was added to recovered human proximal tubular epithelial (HK-2) cells, which were then cultured in an incubator at 37 °C under an atmosphere of 5% CO_2. Cells were passaged at a density of 5×10^5 cells/mL.

2.6 Dual Luciferase Reporter Gene Assay

LipofectamineTM3000 liposomal transfection reagent was used to co-transfect the pmir-GLO-*SIRT1*-3′-UTR-T and pmir-GLO-*SIRT1*-3′-UTR-C luciferase reporter plasmids with an *miR-599* mimic and negative control (NC), respectively, into HK-2 cells. A luciferase kit was used to detect the relative luciferase activity of cells after 72 h.

2.7 Quantitative Polymerase Chain Reaction (qPCR)

Overexpressed recombinant plasmids pcDNA3.1-*SIRT1*-T and pcDNA3.1-*SIRT1*-C bearing different alleles of *SIRT1* rs4746720 were constructed, and HK-2 cells were co-transfected with an *miR-599* mimic, an *miR-599* inhibitor, or an NC, respectively. Total cellular RNA was extracted using TRIzol reagent, and the concentration and purity of the extracted RNA were determined. RNA was reverse transcribed into complementary DNA (cDNA), which was detected by qPCR using an *miR-599* upstream primer (5′-GCACGGCAGTGTGTGTCAGTGTTTA-3′) and downstream primer (5′-TATGGTTCTTCACACGACTCCTTCAC-3′). The upstream primer of the internal reference U6 was forward 5′-CTCGCTTCGGCAGCACA-3′, and the downstream primer was 5′-AACGCTTCACGAATTTGCG-3′. The PCR reaction system comprised 2$\times$ SYBR Color qPCR Master Mix (10 µL), upstream and downstream primers (0.6 µL), cDNA (8.8 µL), and double-distilled H_2O (20 µL). The reaction conditions were 40 cycles comprising pre-denaturation at 95 °C for 30 s, denaturation at 95 °C

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Table 2. Clinical data of study population.

	AKI group (n = 29)	Control group (n = 87)	*p*
Age (year)	59.0 ± 14.8	60.1 ± 11.2	0.677
Male (n, %)	20 (69.0)	58 (66.7)	0.819
BMI (Kg/m^2)	23.7 ± 3.7	23.9 ± 4.0	0.805
Mean arterial pressure (mmHg)	87.9 ± 17.0	89.9 ± 12.9	0.577
Diabetes (n, %)	5 (17.2)	29 (33.3)	0.099
Hypertension (n, %)	6 (20.7)	20 (23.0)	0.797
Infection (n, %)	18 (62.1)	23 (26.4)	0.001*
Ascites (n, %)	22 (75.9)	50 (57.5)	0.077
Hepatic Encephalopathy (n, %)	9 (31.0)	21 (24.1)	0.463
Gastrointestinal Bleeding (n, %)	9 (31.0)	19 (21.8)	0.316
Albumin (g/L)	28.8 ± 5.5	30.2 ± 6.7	0.297
Total bilirubin (µmol/L)	50.8 (30.1, 168.9)	37.0 (21.9, 72.1)	0.037*
ALT (U/L)	43.0 (23.0, 103.0)	32.0 (19.0, 46.0)	0.038*
AST (U/L)	79.0 (48.5, 204.5)	56.0 (32.0, 81.0)	0.004*
Serum sodium (mmol/L)	136.6 ± 5.4	137.2 ± 5.2	0.614
CRP (mg/L)	18.1 (7.5, 45.1)	4.8 (1.9, 17.5)	<0.001*
PT (s)	17.9 (15.9, 21.4)	17.1 (14.6, 19.6)	0.152
INR	1.5 (1.4, 2.0)	1.5 (1.3, 1.7)	0.118
Baseline SCr (µmol/L)	66 (52.3, 80.7)	60.8 (52.5, 75.0)	0.296
eGFR (mL/min/1.73 m^2)	56.0 ± 23.1	101.9 ± 28.9	<0.001*
MELD score	20.8 ± 9.3	11.9 ± 6.1	<0.001*

Note: BMI, Body Mass Index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; PT, prothrombin time; INR, International Normalized Ratio; SCr, serum creatinine; eGFR, estimation of glomerular filtration rate; MELD, Model of end-stage liver disease; AKI, acute kidney injury; *$p < 0.05$.

for 10 s, and annealing at 60 °C for 30 s. The relative gene expression was calculated using the $2^{-\Delta\Delta CT}$ method.

2.8 Western Blot Analysis

Phenylmethylsulfonyl fluoride was added to lyse and extract total proteins, and the bicinchoninic acid assay was used to determine the total protein concentration. Subsequently, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrotransferred to a polyvinylidene difluoride membrane. Then the membrane was blocked in blocking solution for 1 h, followed by incubation overnight at 4 °C with primary antibodies against *SIRT1*, *PGC-1*α, nuclear respiratory factor 1 (*NRF1*), mitochondrial transcription factor A (*TFAM*) (1:1000), and glyceraldehyde 3-phosphate dehydrogenase (1:5000). Next, the membrane was washed and then incubated for 1 h at room temperature with secondary antibody (1:5000). Finally, enhanced chemiluminescence was used for protein detection, and ImageJ software (v1.42; National Institutes of Health, Bethesda, MD, USA) was used to quantify the bands.

2.9 Statistical Analysis

Statistical Package for Social Sciences (SPSS) version 26.0 (IBM SPSS Statistics, Armonk, NY, USA) was used for the data analysis. The normally distributed measurements are expressed as the means ± standard deviations, the *t*-test was used for comparisons between groups, and analysis of variance was used for comparisons between multiple groups. The non-normally distributed measures are expressed as medians (interquartile intervals), and the Mann–Whitney *U* test was used for comparisons between groups. Counts are expressed as cases and percentages, and comparisons between groups were made using a goodness-of-fit chi-square (χ^2) Hardy–Weinberg equilibrium (HWE) test. Logistic regression was used to analyze the degree of association in terms of an odds ratio (OR) with a 95% confidence interval (CI). $p < 0.05$ was considered statistically significant.

3. Results

3.1 Comparison of the General Data of Study Participants

Of the 116 participants, 29 (20 males) were assigned to the AKI group and had an average age of 59.0 ± 14.8 years, and 87 (58 males) were assigned to the control group and had an average age of 60.1 ± 11.2 years. There were no significant between-group differences in age, sex, BMI, mean arterial pressure, diabetes mellitus, hypertension, ascites, hepatic encephalopathy, GI bleeding, albumin concentration, blood sodium concentration, coagulation function, or baseline SCr concentration ($p > 0.05$). The infection rate, total bilirubin concentration, ALT concentration, AST concentration, CRP concentration, and model for end-stage liver disease (MELD) scores of the AKI group were

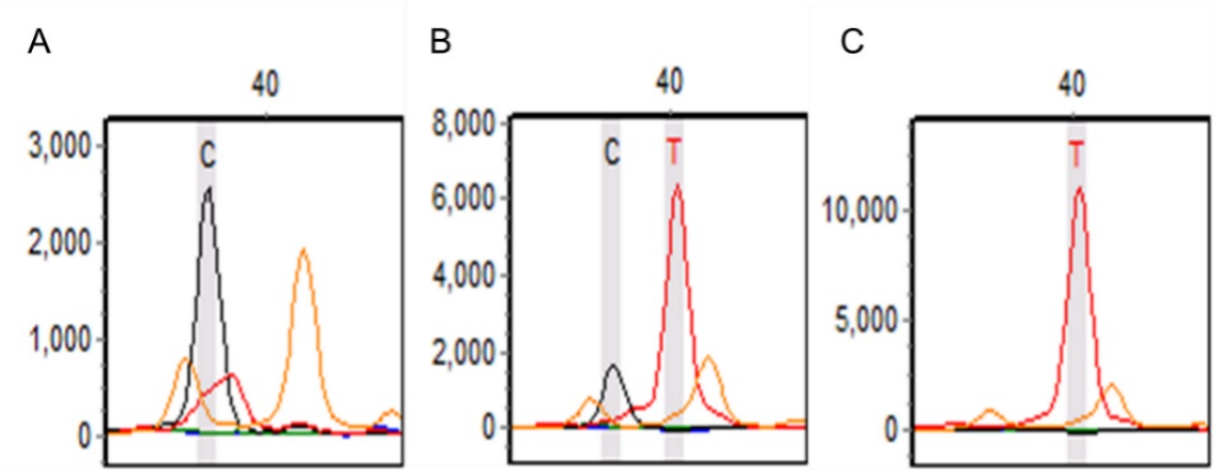

Fig. 1. The genotyping results of *SIRT1* rs4746720 polymorphism in the AKI group and the control group. (A) CC homozygous. (B) CT heterozygotes. (C) TT homozygous. Note: *SIRT1* rs4746720 extends forward, and the product is consistent with polymorphism.

Table 3. HWE test of *SIRT1* SNP genotype.

SNP site	Genotype	Observed value	Expected value	χ^2	p_{HWE}
rs4746720	TT	30 (34.5)	27 (31.0)	1.66	0.437
	CT	37 (42.5)	17 (19.5)		
	CC	20 (23.0)	43 (49.4)		

Note: p_{HWE}: the control group tested the p-value.

significantly higher than those of the control group ($p < 0.05$). The eGFR of the AKI group was significantly lower than that of the control group ($p < 0.001$; Table 2).

3.2 SIRT1 Genotyping

SNaPshot gene sequencing technology was used for *SIRT1* genotyping. This revealed that *SIRT1* rs4746720 sites exhibited CC, CT, or TT genotypes (Fig. 1).

3.3 Correlation Analysis of SIRT1 Gene SNPs and AKI in Cirrhosis

3.3.1 HWE Test

The goodness-of-fit χ^2 test showed that the frequency distribution of *SIRT1* rs4746720 and rs2273773 genotypes in the control group was consistent with the HWE ($p_{HWE} > 0.05$), indicating that the participants were representative of the population (Table 3).

3.3.2 Association Analysis of AKI in Cirrhosis

There was no significant between-group difference in the frequency distribution of the *SIRT1* rs4746720 TT, CT, and CC genotypes ($\chi^2 = 0.448$, $p = 0.799$). The frequency of the T and C alleles was 35 (60.3%) and 23 (39.7%) in the AKI group and 97 (55.7%) and 77 (44.3%) in the control group, respectively, indicating that there was no significant between-group difference in the frequency distribution of these alleles ($\chi^2 = 0.37$, $p = 0.540$). Logistic regression analysis showed that after adjustment for age and sex, the *SIRT1* rs4746720 genetic models (co-dominant: TT *vs.* CC, CT *vs.* CC; isotopic: T *vs.* C; dominant: TT+CT *vs.* CC; recessive: TT *vs.* CC+CT; hypersensitive: TT+CC *vs.* CT) were not significantly correlated with AKI susceptibility ($p > 0.05$; Table 4).

3.3.3 Stratified Analyses

Stratified analyses based on age, sex, and complications of cirrhosis (infection, ascites, GI bleeding, and hepatic encephalopathy) were conducted to study the effect of genetic factors on the risk of AKI in patients with cirrhosis. In the subgroup with hepatic encephalopathy, *SIRT1* rs4746720 SNPs were significantly linked with the development of AKI, and patients with the T allele had a six times greater risk of AKI than those with the C allele (OR = 6.00, 95% CI = 1.22–29.48; $p = 0.027$). However, in subgroups based on other factors, *SIRT1* rs4746720 SNPs were not significantly associated with AKI in cirrhosis ($p > 0.05$; Table 5).

3.3.4 Relationship Between Genotype Distribution and Liver and Kidney Function

The relationship between genotype and liver and kidney function indexes was analyzed in each group. In the AKI group, patients with the *SIRT1* rs4746720 TT genotype had significantly higher SCr and BUN concentrations and significantly lower eGFRs than those with the CC+CT genotypes ($p < 0.05$). However, there were no significant between-genotype differences in other indexes ($p > 0.05$). In the control group, there was no significant between-genotype difference in liver or kidney function ($p > 0.05$; Table 6).

Table 4. Association analysis between *SIRT1* rs4746720 polymorphism and AKI risk in cirrhosis.

Heredity model	Genotype/allele	AKI group (n = 29)	Control group (n = 87)	OR (95% CI)	*p*
Co-dominant	TT	12 (41.4)	30 (34.5)	1.34 (0.43–4.21)	0.611
	CT	11 (37.9)	37 (42.5)	0.98 (0.32–3.07)	0.978
	CC	6 (20.7)	20 (23.0)	1 (reference)	
Allele	T	35 (60.3)	97 (55.7)	1.21 (0.66–2.23)	0.534
	C	23 (39.7)	77 (44.3)	1 (reference)	
Dominant genes	TT+ CT	23 (79.3)	67 (77.0)	1.14 (0.41–3.21)	0.802
	CC	6 (20.7)	20 (23.0)	1 (reference)	
Recessive genes	TT	12 (41.4)	30 (34.5)	1.36 (0.57–3.24)	0.490
	CC+ CT	17 (58.6)	57 (65.5)	1 (reference)	
Hyperdominant genes	TT+CC	18 (62.1)	50 (57.5)	1.22 (0.52–2.91)	0.647
	CT	11 (37.9)	37 (42.5)	1 (reference)	

Note: OR and *p* are parameters after adjusting age and gender.

3.4 SIRT1 rs4746720 Mediates the Effects of miR-599 on the Double Luciferase Reporter Gene

The bioinformatics software programs TargetScan and miRanda were used to predict the target gene of microRNA-599 (*miRNA-599*). To verify the effect of *SIRT1* rs4746720 SNP binding with *miR-599*, whole-gene *SIRT1*–3′-UTR-T/C sequences were synthesized, and the pmirGLO-*SIRT1*–3′-UTR-T/C double luciferase reporter gene plasmid was constructed. This was used to co-transfect HK-2 cells with NC and *miR-599* mimics, respectively, and the changes in luciferase activity were compared. The results showed that the luciferase activity in the *SIRT1*-3′-UTR-T+*miR-599* mimic group was significantly lower than that in the *SIRT1*-3′-UTR-T+NC group and the *SIRT1*-3′-UTR-C+*miR-599* mimic group ($p < 0.001$), whereas there was no significant difference in luciferase activity between the *SIRT1*-3′-UTR-C+*miR-599* mimic group and the *SIRT1*-3′-UTR-C+NC group ($p > 0.05$). The above-described results showed that the expression of the T allele of *SIRT1* rs4746720 was regulated by *miR-599*, resulting in significant inhibition of the expression of luciferase by *miR-599*, whereas the C allele of *SIRT1* rs4746720 was not regulated by *miR-599*, resulting in no inhibition of the expression of luciferase by *miR-599* (Fig. 2).

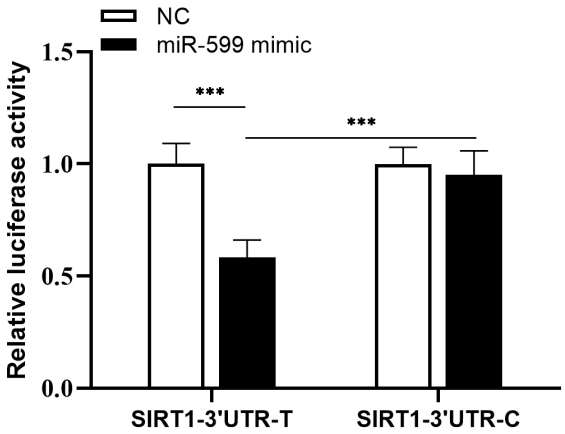

Fig. 2. Results of dual luciferase reporter genes interaction between *SIRT1* rs4746720 and *miR-599* ($^{***}p < 0.001$).

3.5 SIRT1 rs4746720 Mediates the Effect of miR-599 on SIRT1 Expression

To further study the effect of the *SIRT1* rs4746720-mediated effect of *miR-599* on *SIRT1* expression, overexpression plasmids pcDNA3.1-*SIRT1*-T/C, which contained different alleles (i.e., T or C) of *SIRT1* rs4746720, were constructed and verified by gene sequencing. Next, pcDNA3.1-*SIRT1*-T/C plasmids and pcDNA3.1 (empty plasmid) were used to transfect HK-2 cells with NC, an *miR-599* mimic, or an *miR-599* inhibitor, respectively. Subsequently, the relative expression of *miR-599*, *SIRT1* mRNA, and protein in cells was determined by qPCR and Western blotting, respectively. The results were as follows: (1) Compared with transfection with NC, transfection with the *miR-599* mimic caused the expression of *miR-599* in the *SIRT1*-T overexpression group and the empty plasmid group to increase by 41% and 58%, respectively ($p < 0.01$), whereas transfection with the *miR-599* inhibitor caused the expression of *miR-599* in the *SIRT1*-T overexpression group, *SIRT1*-C overexpression group, and empty plasmid group to decrease by 39%, 34%, and 39%, respectively ($p < 0.05$). These results confirmed the effectiveness of transfection with the *miR-599* mimic and *miR-599* inhibitor, respectively (Fig. 3A). (2) Compared with the empty plasmid+NC group, in the *SIRT1*-T overexpression+NC group and the *SIRT1*-C overexpression+NC group, the mRNA and protein expression of *SIRT1* was significantly upregulated ($p < 0.05$). This confirmed the functioning of the *SIRT1*-T/C overexpression plasmids. (3) Compared with the NC group, in the *SIRT1*-T overexpression group, co-transfection with the *miR-599* mimic significantly reduced the expression of *SIRT1* mRNA ($p < 0.01$). Protein expression of *SIRT1* decreased while with no significance ($p > 0.05$). Co-transfection with the *miR-599* inhibitor increased the expression of *SIRT1* mRNA and *SIRT1*, but this difference was not statistically significant. (4) In the

Table 5. Stratified analysis of *SIRT1* rs4746720 polymorphism with AKI risk in cirrhosis.

Variable		Genotype/allele	AKI group (n = 29)	Control group (n = 87)	OR (95% CI)	*p*
Age (years)	<60	TT *vs.* CC	8/3	13/12	2.46 (0.53–11.50)	0.252
		CT *vs.* CC	5/3	20/12	1.00 (0.20–4.96)	1.000
		T *vs.* C	21/11	46/44	1.83 (0.79–4.22)	0.156
	≥60	TT *vs.* CC	4/3	17/8	0.63 (0.11–3.49)	0.595
		CT *vs.* CC	6/3	17/8	0.94 (0.19–4.76)	0.942
		T *vs.* C	14/12	51/33	0.76 (0.31–1.83)	0.534
Gender	male	TT *vs.* CC	8/4	17/15	1.77 (0.44–7.06)	0.422
		CT *vs.* CC	8/4	26/15	1.15 (0.30–4.49)	0.836
		T *vs.* C	24/16	60/56	1.40 (0.68–2.91)	0.366
	female	TT *vs.* CC	4/2	13/5	0.77 (0.11–5.61)	0.796
		CT *vs.* CC	3/2	11/5	0.68 (0.09–5.45)	0.718
		T *vs.* C	11/7	37/21	0.89 (0.30–2.65)	0.837
Infection	no	TT *vs.* CC	4/3	20/15	1.00 (0.19–5.15)	1.000
		CT *vs.* CC	4/3	29/15	0.69 (0.14–3.49)	0.653
		T *vs.* C	12/10	69/59	1.03 (0.41–2.55)	0.956
	yes	TT *vs.* CC	8/3	10/5	1.33 (0.24–7.35)	0.741
		CT *vs.* CC	7/3	8/5	1.46 (0.25–8.43)	0.673
		T *vs.* C	23/13	28/18	1.14 (0.46–2.80)	0.780
Ascites	no	TT *vs.* CC	3/1	15/9	1.80 (0.16–20.03)	0.633
		CT *vs.* CC	3/1	13/9	2.08 (0.19–23.30)	0.553
		T *vs.* C	9/5	43/31	1.30 (0.40–4.25)	0.666
	yes	TT *vs.* CC	9/5	15/11	1.32 (0.35–5.05)	0.685
		CT *vs.* CC	8/5	24/11	0.73 (0.20–2.76)	0.647
		T *vs.* C	26/18	54/46	1.23 (0.60–2.52)	0.571
Hepatic encephalopathy	no	TT *vs.* CC	5/6	22/15	0.57 (0.15–2.21)	0.414
		CT *vs.* CC	9/6	29/15	0.78 (0.23–2.59)	0.680
		T *vs.* C	19/21	73/59	0.73 (0.36–1.49)	0.387
	yes	TT *vs.* CC	7/0	8/5	-	-
		CT *vs.* CC	2/0	8/5	-	-
		T *vs.* C	16/2	24/18	6.00 (1.22–29.48)	0.027*
Gastrointestinal bleeding	no	TT *vs.* CC	9/4	26/13	1.13 (0.29–4.35)	0.865
		CT *vs.* CC	7/4	29/13	0.78 (0.20–3.16)	0.732
		T *vs.* C	25/15	81/55	1.13 (0.55–2.34)	0.738
	yes	TT *vs.* CC	3/2	4/7	2.63 (0.30–23.00)	0.383
		CT *vs.* CC	4/2	8/7	1.75 (0.24–12.64)	0.579
		T *vs.* C	10/8	16/22	1.72 (0.56–5.33)	0.348

Note: $^{*}p < 0.05$.

SIRT1-C overexpression group and empty plasmid groups, *SIRT1* mRNA and *SIRT1* expression were not significantly changed by transfection with the *miR-599* mimic or the *miR-599* inhibitor ($p > 0.05$). Taken together, these results indicate that the T allele of *SIRT1* rs4746720 may mediate the interaction of *miR-599* with *SIRT1*, resulting in significant inhibition of the mRNA expression of *SIRT1*, leading to a downward trend in the expression of *SIRT1* (Fig. 3B,C).

3.6 SIRT1 rs4746720 Mediates the Effect of miR-599 on Downstream Pathways

The effect of the *SIRT1* rs4746720 SNP-mediated interaction of *miR-599* with *SIRT1* on the *PGC-1α*/*NRF1*/*TFAM* signaling pathway downstream of *SIRT1* was further investigated by determining the relative mRNA and protein expression of *PGC-1α*, *NRF1*, and *TFAM* with qPCR and Western blot analysis, respectively. The following results were obtained: (1) Compared with the empty plasmid+NC group; there was a significantly higher abundance of *PGC-1α*, *NRF1*, and *TFAM* mRNA and protein in the *SIRT1*-T overexpression+NC group and *SIRT1*-C overexpression+NC group ($p < 0.001$). (2) Compared with the NC group, in the *SIRT1*-T overexpression group, transfection with the *miR-599* mimic caused a significant reduction in the mRNA and protein expression of *PGC-1α*, *NRF1*, and *TFAM* ($p < 0.01$), whereas co-transfection with an *miR-599* inhibitor caused a significant increase in their levels ($p < 0.05$). (3) In the *SIRT1*-C overexpression group, co-transfection with the *miR-599* inhibitor caused a significant reduction in

Table 6. Liver and kidney function level in different genotypes of *SIRT1* rs4746720.

Variable	AKI group		*p*	Control group		*p*
	TT	CC+CT		TT	CC+CT	
SCr (μmol/L)	171.5 ± 88.1	111.8 ± 48.4	0.026*	68.0 ± 16.3	72.0 ± 15.1	0.255
eGFR (mL/min/1.73 m^2)	43.1 ± 20.7	65.1 ± 20.5	0.009*	104.4 ± 33.8	100.6 ± 26.2	0.564
BUN (mmol/L)	15.7 ± 6.8	9.5 ± 5.9	0.015*	5.2 ± 2.3	6.0 ± 3.7	0.256
Albumin (g/L)	27.6 ± 5.6	29.6 ± 5.4	0.350	30.5 ± 5.8	30.0 ± 7.1	0.748
Total bilirubin (μmol/L)	138.7 ± 191.3	117.7 ± 124.2	0.723	75.2 ± 85.3	56.0 ± 60.9	0.229
ALT (U/L)	48.7 ± 40.8	88.8 ± 97.2	0.191	59.6 ± 83.5	47.5 ± 64.2	0.454
AST (U/L)	79.0 ± 53.5	225.1 ± 309.7	0.073	84.9 ± 84.0	65.0 ± 55.0	0.189
MELD Score	23.5 ± 12.0	18.8 ± 6.6	0.193	12.2 ± 6.1	11.7 ± 6.1	0.685

Note: SCr, serum creatinine; eGFR, estimation of glomerular filtration rate; BUN, urea nitrogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase; MELD, Model of end-stage liver disease; $^*p < 0.05$.

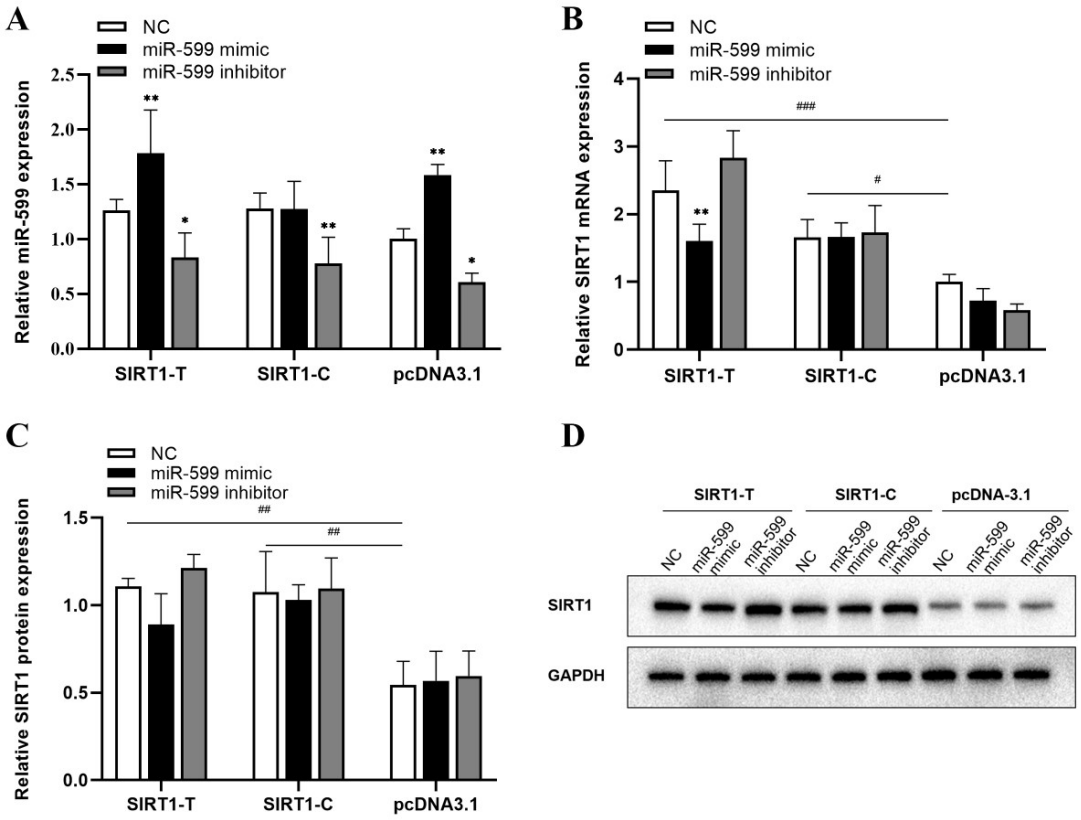

Fig. 3. Expression of *miR-599* and *SIRT1* in HK-2 cells after co-transfection with overexpression plasmid and *miR-599*. (A) Relative expression of *miR-599*. (B) Relative expression of *SIRT1* mRNA. (C) Relative expression of *SIRT1*; $^*p < 0.05$, $^{**}p < 0.01$, compared with the NC group; $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$, compared with the empty plasmid group. (D) *SIRT1* protein detected by western blot.

the mRNA expression of *TFAM* ($p < 0.001$) but not in the protein expression of *TFAM* ($p > 0.05$), as well as a significant reduction in the mRNA levels of *NRF1* and *TFAM* ($p < 0.001$) but not in the protein expression of *NRF1* and *TFAM* ($p > 0.05$). (4) In the empty plasmid group, co-transfection with the *miR-599* mimic and the *miR-599* inhibitor, respectively, did not cause significant changes in the mRNA or protein levels of *PGC-1α*, *NRF1*, and *TFAM* ($p > 0.05$). Taken together, these results suggest that the T allele of *SIRT1* rs4746720 may mediate the ability of *miR-599* to significantly inhibit the activity of the *PGC-1α/NRF1/TFAM* pathway (Fig. 4).

4. Discussion

The rapid development of genomics technology has enabled research revealing the important role of genetic factors in the occurrence and development of AKI [21–24]. However, there have been few studies on patients with cirrhosis and genetic susceptibility to develop AKI. Seckin *et al.* [12] reported that the GT/TT genotypes and T mutant

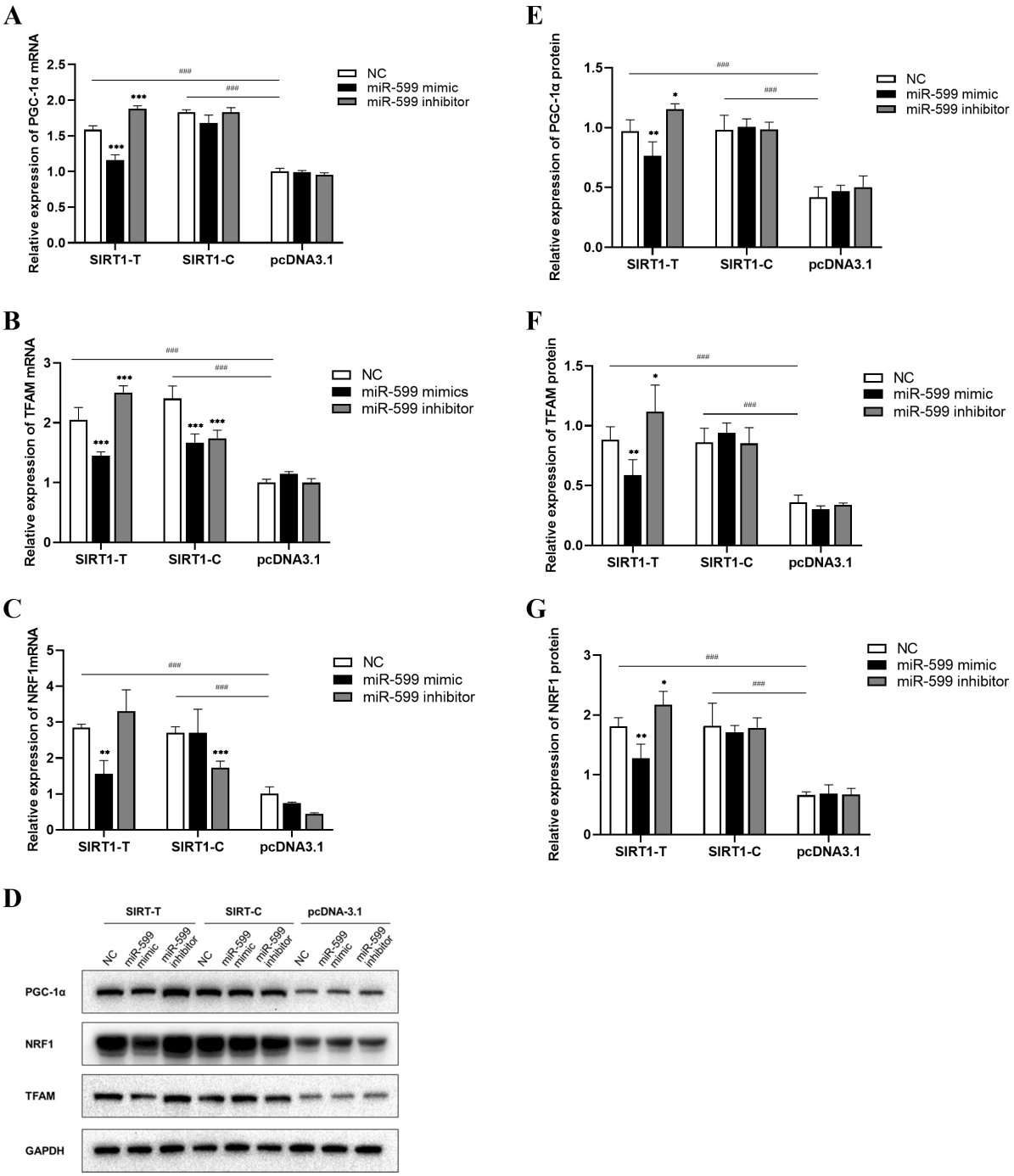

Fig. 4. Expression of *PGC-1α/NRF1/TFAM* in HK-2 cells after co-transfection with overexpression plasmid and *miR-599*. (A–C) *PGC-1α/NRF1/TFAM* mRNA relative expression. (D–G) *PGC-1α/NRF1/TFAM* protein relative expression; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, compared with NC; ###$p < 0.001$, compared to empty plasmid.

allele of the *eNOs* G894T SNP might be risk factors for the development of hepatorenal syndrome, as patients with the GT or TT genotype had a 5.0 or 5.8 times greater risk, respectively, of developing hepatorenal syndrome than those with the GG genotype. Wang *et al.* [13] studied 60 patients with cirrhosis and found that those with the T allele of the promoter of *AVPR1A* rs113481894 had a 2.23 times higher risk of developing type I hepatorenal syndrome than those with the C allele of the promoter of *AVPR1A* rs113481894. These candidate genes code for proteins involved in classical splanchnic-vasodilation-related processes. In recent years, further research has increased our understanding of the pathogenesis of AKI in cirrhosis [25,26]. In addition to traditional hemodynamic mechanisms, non-hemodynamic microvascular toxicities (caused by, for example, endotoxins or bile acids) may be directly related to the development of AKI [10,27]. The synergistic effect of toxic factors and microvascular dysfunction increases damage to proxi-

mal tubular epithelial cells, mediates the downregulation of mitochondrial function, and triggers intrarenal activation of the renin-angiotensin-aldosterone system, resulting in a decreased GFR [28].

4.1 Association of SIRT1 SNPs with AKI in Cirrhosis

SIRT1 is a highly conserved protein deacetylase that is widely expressed in renal tissue. In ischemia-reperfusion injury, sepsis, and AKI caused by cisplatin, *SIRT1* acts on many transcription factors to regulate members of downstream pathways, such as *PGC-1*α, forkhead box O, nuclear factor kappa B, and p53, resulting in nephroprotective effects [15–17]. Specifically, *SIRT1* mediates the deacetylation of *PGC-1*α, activates *NRF1* and *TFAM*, promotes mitochondrial biosynthesis and respiratory recovery, and drives proximal tubule repair, which may be a key pathway for renal protection [15,29,30]. In our early research using a rat model of AKI in cirrhosis, we found that the expression of *SIRT1* and *PGC-1*α in the renal tissue of rats decreased in the early stages of AKI, and the *SIRT1*/*PGC-1*α signaling pathway may be involved in the mechanism of AKI in cirrhotic patients. Chou *et al*. [31] showed that knockout of *SIRT1* increased tumor necrosis factor alpha-mediated renal insufficiency. Thus, *SIRT1* and the downstream pathways that it affects play an important role in protecting the kidney against damage with various etiologies. *SIRT1* SNPs are associated with aging, cardiovascular disease, alcoholic fatty liver disease, diabetic nephropathy, cardiorenal syndrome type 1, and many other diseases [32–35]. The current study represents the first examination of the association between *SIRT1* SNPs and AKI in cirrhosis. Our data analysis using Haploview 4.2 software (Broad Institute, Cambridge, MA,USA) and functional annotation screened out one functional SNP locus: the rs4746720 locus in the 3′-UTR region of *SIRT1*.

4.2 Association of SIRT1 rs4746720 with AKI in Cirrhosis

SIRT1 rs4746720 is associated with aging, type 2 diabetes, diabetic nephropathy, and other diseases [36–39]. In the current study, we found that the TT genotype and T allele frequencies of the *SIRT1* rs4746720 locus in the AKI group were higher than those in the control group (41.4% *vs.* 34.5%, 60.3% *vs.* 55.7%), and the risk of AKI in cirrhosis was increased in patients with the T allele; however, the latter difference did not reach statistical significance (p = 0.534). Stratified analyses revealed that the T allele led to a greater risk of developing AKI in cirrhosis with hepatic encephalopathy. Analyses of the liver and kidney function indicators of the AKI group with respect to genotype demonstrated that the SCr and BUN concentrations of patients with the TT genotype were significantly higher, and their eGFRs were significantly lower than those with the CC+CT genotype ($p < 0.05$), indicating that among AKI patients with cirrhosis, those who had the TT genotype had significantly worse renal function than those who had the CC+CT genotype. Similarly, Tang *et al*. [36] found that compared with other genotypes, the *SIRT1* rs4746720 TT genotype led to more severe diabetic nephropathy in Han Chinese patients with diabetes. Sarumaru *et al*. [40] showed that in Japanese patients with autoimmune thyroid disease, the thyroid autoantibody titer in patients with the *SIRT1* rs4746720 T allele (i.e., the TT+TC genotype) was significantly higher than that in patients with the CC genotype, which may affect the expression of *SIRT1* in inflammatory lesions of target tissues and increase susceptibility to disease. Zhang *et al*. [41] studied the relationship between *SIRT1* functional loci and healthy aging in Han Chinese older adults and found that the participants exhibited a significantly higher frequency of the *SIRT1* rs4746720 C allele than of the *SIRT1* rs4746720 T allele, a higher frequency of the CC genotype than of the CT+TT genotypes and that those with the C allele may have a higher risk of unhealthy aging than those with the T allele. In addition, in the current study, *SIRT1* rs4746720 was found to be most associated with AKI susceptibility in the hepatic encephalopathy subgroup, suggesting that gene-risk factors may jointly increase susceptibility to disease. MELD score, serum sodium concentrations, and hepatic encephalopathy are independent predictors of the risk of renal failure in cirrhosis cohorts, and hepatic encephalopathy is significantly associated with prognosis in patients with cirrhosis complicated by renal failure [42]. However, due to the small sample size of this study, further research is needed to confirm these findings.

4.3 SIRT1 rs4746720 may Mediate the Binding of miR-599 to SIRT1 to Affect the SIRT1/PGC-1α *Pathway*

Bioinformatics software was used to predict the miRNAs that may bind to rs4746720 in the 3′-UTR region of *SIRT1*, and this revealed that *miR-599* should be capable of performing this binding. Given that the site bound by *miR-599* is in the 3′-UTR, it is speculated that when the site has a T allele, it can be bound by *miR-599*, resulting in the inhibition of *SIRT1* expression, whereas when the site has a C allele (due to a T-to-C mutation), it cannot be bound by *miR-599*, resulting in no effect on *SIRT1* expression. The construction and use of a double luciferase reporter plasmid revealed that *miR-599* could significantly inhibit the expression of luciferase by the T allele of rs4746720 but could not inhibit the expression of luciferase by the C allele of rs4746720, confirming that the polymorphic site of *SIRT1* rs4746720 was likely to be the site where *miR-599* acts. The recombinant overexpression plasmid of *SIRT1* rs4746720 was constructed, and its use in experiments showed that the T allele of rs4746720 may mediate *miR-599*'s inhibition of the expression of *SIRT1* mRNA and its downstream effectors *PGC-1*α, *NRF1*, and *TFAM*.

The above-described results, combined with those from other studies, indicate that the T allele of *SIRT1* rs4746720 may mediate the ability of *miR-599* to target and inhibit the expression of *SIRT1* and its downstream *PGC-1*α/*NRF1*/*TFAM* signaling pathway, and may be involved

in the pathogenesis of AKI in cirrhosis. The pathogenesis of AKI in cirrhosis involves the regulation of multiple genes, proteins, and signaling pathways. This is the first study to show the regulatory relationship between *SIRT1* rs4746720 and *miR-599*. Current research on *miR-599* has mainly focused on its involvement in the pathogenesis of lung cancer, liver cancer, kidney cancer, and other cancer-related pathogenesis, as *miR-599* plays different roles in different tumor tissues. For example, in liver cancer, the expression of *miR-599* is downregulated; this is manifested by changes in the abundance of tumor suppressor factors, which affect tumor production. By contrast, in lung cancer, the expression of *miR-599* is upregulated, which promotes proliferation and invasion [43,44]. However, the role of *miR-599* in AKI has not been fully described, so further research is needed.

5. Limitations

This was a single-center retrospective case-control study with a small sample size that only included a Han Chinese population with cirrhosis. No relationship between *SIRT1* rs4746720 and the development of AKI was observed in cirrhotic patients. Thus, our conclusions need to be validated in future centralized studies with larger sample sizes. Moreover, in the current study, the serum levels of *SIRT1* and *miR-599* in patients in the AKI and control groups were considered to fully represent the effect of *SIRT1* rs4746720 SNP-mediated *miR-599* on AKI patients with cirrhosis at the population level. Furthermore, no *in vivo* functional verification experiments were performed in this study. Therefore, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 technology could be used to construct a knock-in *SIRT1* rs4746720 mutant mouse model of AKI in cirrhosis. This would enable verification of the mechanism of the susceptibility of *SIRT1* and its downstream pathways to *SIRT1* rs4746720 SNP-mediated *miR-599* control.

6. Conclusions

The *SIRT1* rs4746720 SNP might be linked with AKI in cirrhotic patients, and the T allele increased the risk of AKI in those with hepatic encephalopathy. In AKI patients with cirrhosis, the renal function of patients with the TT genotype was significantly lower than that of patients with the CC+CT genotype. The *SIRT1* rs4746720 SNP mediated *miR-599* binding to *SIRT1*, thereby affecting the expression of *SIRT1* and its downstream effectors, *PGC-1α/NRF1/TFAM*, and may be involved in the mechanism of AKI in cirrhotic patients.

Availability of Data and Materials

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

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by FZ, YC, YX, and QL. The first draft of the manuscript was written by FZ and YC, and it was revised by QL. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The research protocol was approved by the Medical Ethics Committee of Ningbo NO.2 hospital (YJ-KYSB-NBEY-2018-002-01), and all of the participants provided signed informed consent.

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Conflict of Interest

The authors declare no conflict of interest.

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