

RESEARCH ARTICLE

Lower expression of Hsa_circRNA_102682 in diabetic hyperhomocysteinemia negatively related to creatinemia is associated with TGF- β and CTGF

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Abstract

Background: Diabetic nephropathy is a kidney disease caused by long-term hyperglycemia. Hsa_circRNA_102682 is related to the pathogenesis of preeclampsia. Preeclampsia is related to hypertension and proteinuria, and diabetic nephropathy is mainly manifested by hypertension and proteinuria. The main pathological change in diabetic nephropathy is glomerular fibrosis.

Methods: This study used serum samples of patients treated at Li Huili Eastern Hospital, Ningbo, China, from July 10, 2018 to February 15, 2019. We included 73 patients with diabetes and divided them into a normal-homocysteine group and a high-homocysteine group. We selected used quantitative reverse transcriptase-polymerase chain reaction to measure Hsa_circRNA_102682 concentration in the serum. Serum transforming growth factor-beta and connective tissue growth factor levels were tested using ELISA. The Pearson correlation test was used to assess the correlations between Hsa_circRNA_102682, transforming growth factor-beta, connective tissue growth factor, homocysteine, and creatinine.

Result: Hsa_circRNA_102682 was significantly lower in diabetic patients with high levels of homocysteine than in those with normal levels of homocysteine, whereas transforming growth factor-beta and connective tissue growth factor levels were higher in diabetic patients with hyperhomocysteinemia. Hsa_circRNA_102682 was negatively correlated with the levels of transforming growth factor-beta, connective tissue growth factor, homocysteine, and creatinine. Transforming growth factor-beta and connective tissue growth factor were both positively correlated with homocysteine and creatinine.

Conclusion: Low Hsa_circRNA_102682 was associated with high levels of transforming growth factor-beta and connective tissue growth factor as well as homocysteine and creatinine. These results suggest that Hsa_circRNA_102682 might be related to the pathogenesis of hyperhomocysteinemia in diabetic nephropathy.

KEYWORDS

circular RNA, CTGF, diabetic nephropathy, Hsa_circRNA_102682, TGF- β

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1 | INTRODUCTION

Diabetic nephropathy (DN) is the main cause of end-stage renal disease, and with the rising incidence of diabetes, there are more and more patients with DN.¹ Although the main cause of DN is hyperglycemia-induced vascular dysfunction, its progression is also driven by a series of pathological mechanisms including intrarenal oxidative stress, inflammation, and fibrosis.² In the past, DN was considered to be a simple vascular disease, but DN is now recognized as a multidimensional and multicellular disease. DN is a major microvascular complication of diabetes. It is characterized by persistent proteinuria (urine albumin to creatinine ratio ≥ 30 mg/g) and decreased glomerular filtration rate (eGFR < 60 ml/min/1.73 m²).³ Serum homocysteine (Hcy) is an amino acid formed during the conversion of methionine into cystine in the body. The normal range of Hcy in blood is 5–15 μ mol/L. An excess of homocysteine in the blood or urine is known as hyperhomocysteinemia,⁴ which is a risk factor of diabetic nephropathy.⁵

As a major chronic complication of type 2 diabetes and the most frequent form of chronic kidney disease, main renal injury of kidney is glomerular fibrosis. Transforming growth factor-beta (TGF β) is a key player in tissue fibrogenesis and is proposed to be the major regulator in inducing epithelial-to-mesenchymal transition (EMT). In addition to extracellular matrix (ECM) protein synthesis, TGF- β is also involved in hypertrophy, proliferation, and apoptosis of renal cells.⁶ TGF- β /Smad2/3 signaling is activated in kidney tissues of DN rats and high glucose-treated NRK-52E cells.^{7,8} Connective tissue growth factor (CTGF) is another major fibrotic factor; it participates in cell proliferation, migration, and differentiation and mediates profibrotic activity.⁹ CTGF also has the potential to modulate factors such as vascular endothelial growth factor (VEGF) and bone morphogenic proteins.¹⁰ Renal CTGF may serve as an early marker for progression to dysfunction in DN.^{11,12}

Circular RNAs (circRNAs) have no 5' or 3' end and no polyA tail.^{13,14} CircRNA has a relatively stable structure, with highly tissue-specific expression in eukaryotic transcriptomes.¹⁵ There has been evidence that thousands of endogenous circRNAs exist in mammalian cells.¹⁶ Similarly, there is increasing evidence that circRNAs play a role in diseases. For example, circular RNA ciRS-7 may represent a useful target in the development of therapeutic strategies for Alzheimer's disease,¹⁷ and linear and circular expression of INK4/ARF-associated non-coding RNA is associated with atherosclerotic risk,¹⁸ Hsa_circ_0054633 in peripheral blood can be used as a diagnostic biomarker for pre-diabetes and type 2 diabetes mellitus,¹⁹ and Hsa_circ_002059 may be a potential novel and stable biomarker for the diagnosis of gastric carcinoma.²⁰ Although Hsa_circRNA_102682 has not been tested for its association with diabetes,¹⁹ there have been reports of its relation to the pathogenesis of preeclampsia.²¹ Here, we sought to study whether Hsa_circRNA_102682 can be used as a potential biomarker for DN.

2 | MATERIALS AND METHODS

2.1 | Subjects

This study was performed at Li Huili Eastern Hospital, Ningbo University Medical Center, Zhejiang Province, China. The subjects were patients with diabetes who came to the hospital for examination from July 10, 2018–February 15, 2019. We excluded patients with comorbidities such as hypertension, liver or kidney diseases, or cancer. The study was approved by the Ethics Committee of Ningbo University School of Medicine. All participants provided written informed consent. The diagnostic criteria for diabetes were one of the following three conditions as follows: typical symptoms of diabetes (thirst, increased urine, excess food and, weight loss) plus random blood glucose ≥ 11.1 mmol/L, FPG ≥ 7.0 mmol/L (125 mg/dl), or OGTT (oral glucose tolerance test) 2 h PG ≥ 11.1 mmol/L (200 mg/dl). Blood biochemical indicators BP, TG, TC, LDL-L, HDL-L, FPG, HBAIC, creatinine, Hcy, and BMI were also recorded.

2.2 | Specimen collection and preservation

The patient serum samples were collected from Li Huili Eastern Hospital, Ningbo University Medical Center, on July 10, 2018 and February 15, 2019. Peripheral venous blood (3 ml) was collected and centrifuged (at 3000 rpm for 10 min at room temperature). The serum was then immediately collected and stored at -80°C until use.

2.3 | Total RNA extraction and cDNA synthesis

According to the manufacturer's instructions, total RNA was isolated from the serum using TRIzol LS reagent (Invitrogen, Karlsruhe, Germany). The purity of the extracted RNA was measured with an ultraviolet spectrophotometer and samples with an absorbance ratio for 260/280 nm of 1.8–2.1 were used for further analysis. RNA was reverse-transcribed to cDNA using the HiFi-MMLV cDNA first-strand synthesis kit (CW BIO) at 42°C for 50 min, 85°C for 5 min, and immediately stored at -80°C until use.

2.4 | Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

For quantitative purposes, real-time PCR analysis was performed using LightCycler 480 SYBR Green I Master kit on LightCycler 480 II (Roche). qRT-PCR was performed under the following conditions: an initial denaturation step for 5 min at 95°C , followed by 45 cycles of 94°C for 10 s and primer-specific annealing temperature for 20 s, and 72°C for 30 s. The annealing temperature of Hsa_circRNA_102682 was 59°C and that of β -actin was 56°C . The threshold cycling method was used to calculate the relative fold changes,

and β -actin was used as an internal standardized control. The experiment was carried out three times independently.

2.5 | ELISA assays

Serum TGF- β and CTGF were measured by ELISA following the manufacturer instructions. TGF- β (BMS249-4) kit was purchased from Invitrogen, CA, US, and CTGF kit (DRE10422) was purchased from QiaoDu Biotec Company, Shanghai, China.

2.6 | Statistical analyses

Statistical analysis of the data was performed using GraphPad Prism 8.0 for Windows and SPSS version 18.0. Variables are expressed as mean \pm standard deviation, percentage, or median (quartile). All data were first tested using the Kolmogorov-Smirnov test to determine whether the values were normally distributed. For data that followed the normal distribution, the homogeneity test of variance was carried out. When the normal distribution and homogeneity of variance were both satisfied, the independent sample t test was used $p < 0.05$ was considered statistically significant. Pearson's correlation test was used to analyze the correlation between two variables.

3 | RESULT

3.1 | Participant characteristics

Diabetic patients were divided into two groups based on Hcy levels: a normal-Hcy group (serum Hcy in the normal range of 5–15 $\mu\text{mol/L}$) and a high-Hcy group (serum Hcy $>15 \mu\text{mol/L}$). Table 1 shows the clinical characteristics of these two groups of patients. Age, body mass index (BMI), fasting blood glucose (FPG), blood pressure (BP), glycated hemoglobin (HbA1c), triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL-L), high-density lipoprotein (HDL-L), insulin, or C peptide were not statistically different between the two groups ($p > 0.05$), but creatinine (Cr) values were significantly higher in the high-Hcy group than in the normal-Hcy group ($p < 0.0001$).

3.2 | Hsa_circRNA_102682 expression is low in patients with hyperhomocysteinemia, and there is a correlation between Hsa_circRNA_102682 and Hcy values.

Figure 1 compares the expression of Hsa_circRNA_102682 in the two groups. Hsa_circRNA_102682 was highly expressed in diabetic patients with normal homocysteine, and its expression is significantly lower in patients with hyperhomocysteinemia ($p < 0.05$).

TABLE 1 Clinical characteristics of the study population (normal serum homocysteine and high serum homocysteine)

	Normal (n = 30)	High (n = 43)	p^a
Age, year	60.13 \pm 1.73	62.93 \pm 2.10	0.34
Medical history, year	10.00 \pm 0.90	12.47 \pm 1.08	0.10
BMI, Kg/m ²	24.92 \pm 0.30	24.22 \pm 0.24	0.07
BP, mmHg	145.0 \pm 2.30	147.6 \pm 2.35	0.44
	75.80 \pm 1.44	74.23 \pm 1.05	0.37
FPG, mmol/L	9.07 \pm 0.47	9.81 \pm 0.46	0.28
HbA1c, %	9.21 \pm 0.01	9.37 \pm 0.01	0.74
TG, mmol/L	2.58 \pm 0.33	2.01 \pm 0.16	0.09
TC, mmol/L	4.95 \pm 0.16	4.89 \pm 0.16	0.80
HDL-L, mmol/L	1.16 \pm 0.04	1.23 \pm 0.06	0.44
LDL-L, mmol/L	2.84 \pm 0.14	2.89 \pm 0.13	0.79
C peptide, nmol/L	0.70 \pm 0.06	0.74 \pm 0.04	0.59
Insulin, mIU/L	17.19 \pm 2.71	21.59 \pm 1.92	0.17
Creatinine, $\mu\text{mol/L}$	75.56 \pm 2.46	105.0 \pm 3.14	<0.0001

Abbreviations: BMI, body mass index; BP, blood pressure; FPG, fasting blood glucose; HbA1c, glycated hemoglobin; HDL-L, high density lipoprotein; LDL-L, low density lipoprotein; TC, total cholesterol; TG, triglyceride.

^aAll variables were compared using an independent samples t test or nonparametric test

Next, we analyzed the correlation between high-Hcy values and Hsa_circRNA_102682. As shown in Figure 1B, there was a negative correlation between the Hcy and Hsa_circRNA_102682 levels ($r = -0.7289$, $p < 0.0001$).

3.3 | There is a negative correlation between Hsa_circRNA_102682 and creatinine

Further analysis was performed to determine whether there was a correlation between creatinine and Hsa_circRNA_102682. The results are shown in Figure 1C, and there was a negative correlation ($r = -0.4761$, $p = 0.0002$) between Hsa_circRNA_102682 and creatinine.

3.4 | Fibrotic factors TGF- β and CTGF were high in the serum of diabetic patients with hyperhomocysteinemia, and they were negatively correlated with Hsa_circRNA_102682 and positively correlated with homocysteine and creatinine.

Next, we tested TGF- β and CTGF levels in the serum of diabetic patients with and without hyperhomocysteinemia by ELISA. As shown in Figure 2A, TGF- β level was significantly higher in the high-Hcy group than in the normal-Hcy group ($p < 0.0001$). Correlation analysis showed that TGF- β was negatively correlated

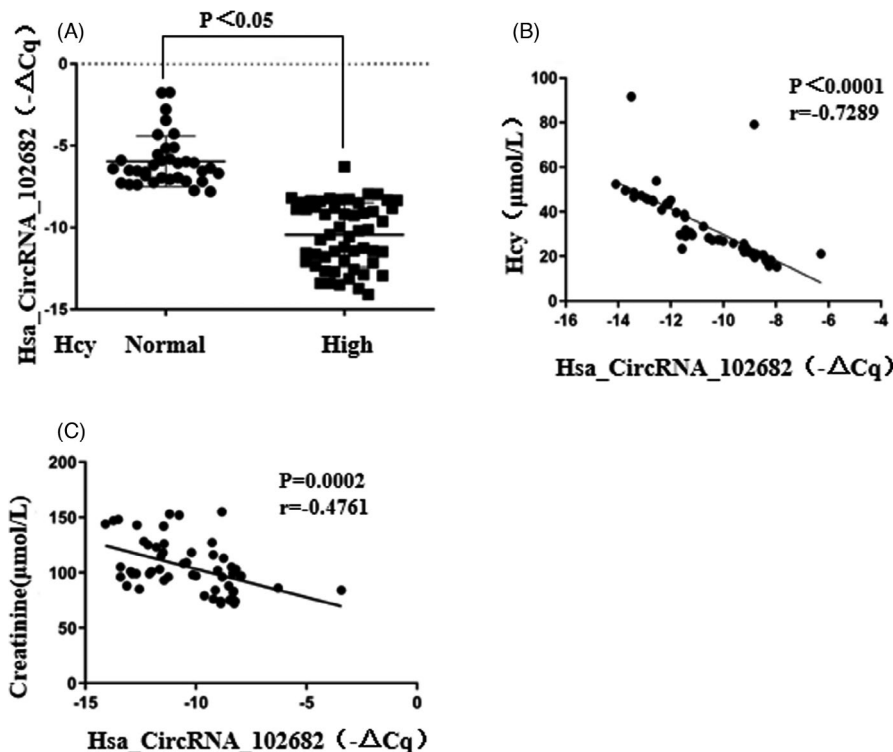


FIGURE 1 Hsa_circRNA_102682 in diabetic patients with hyperhomocysteinemia. (A) Serum levels of Hsa_circRNA_102682 in diabetic patients with normal Hcy (Normal; $n = 30$) and high Hcy (High; $n = 43$). (B) Correlation analysis of the serum Hsa_circRNA_102682 level with Hcy. (C) Correlation analysis of the serum Hsa_circRNA_102682 level with creatinine

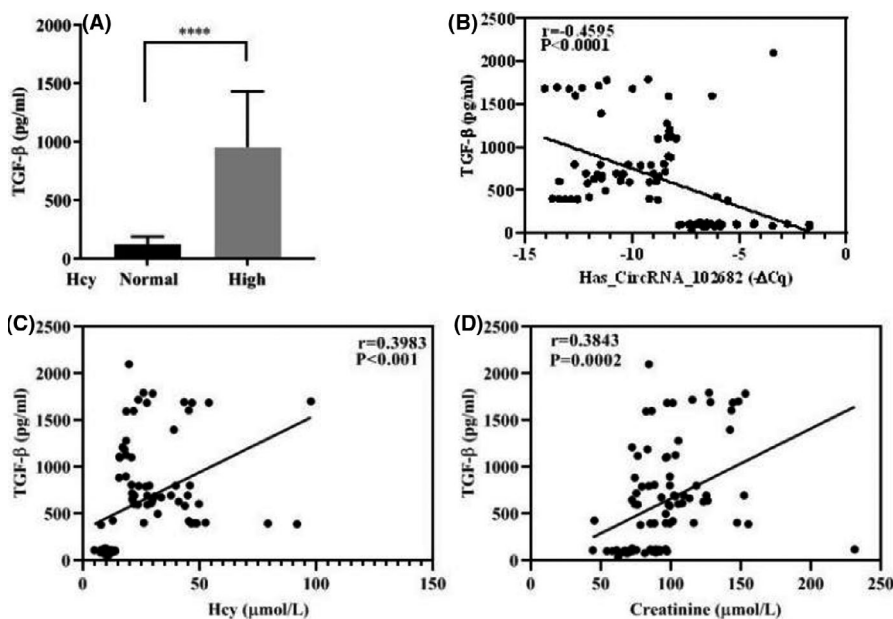


FIGURE 2 TGF- β was correlated with Hsa_circRNA_102682, Hcy, and creatinine. (A) TGF- β level in diabetic patients with hyperhomocysteinemia. (B) TGF- β was negatively correlated with Hsa_circRNA_102682. (C) TGF- β was positively correlated with Hcy. D. TGF- β was positively correlated with creatinine

with Hsa_circRNA_102682 ($r = -0.4595$, $p < 0.0001$, Figure 2B) and was positively correlated with homocysteine ($r = 0.03983$, $p < 0.001$, Figure 2C) and creatinine ($r = 0.3843$, $p < 0.0001$, Figure 2D). Similarly to TGF- β , CTGF level was much higher in the high-Hcy group than in the normal-Hcy group ($p < 0.0001$, Figure 3A). Correlation analysis showed that CTGF was negatively correlated with Hsa_circRNA_102682 ($r = -0.6615$, $p < 0.0001$, Figure 3B) and positively correlated with homocysteine ($r = 0.6287$, $p < 0.0001$, Figure 3C) and creatinine ($r = 0.4171$, $p < 0.0001$, Figure 3D).

3.5 | Potential diagnostic values of Hsa_circRNA_102682 and creatinine

Next, we explored the potential diagnostic value of Hsa_circRNA_102682 and creatinine. The ROC curve analysis was conducted, and the area under the ROC curve (AUC) was 0.9735, 0.8460, and 0.9730 for Hsa_circRNA_102682, serum creatinine, and their combination (Figure 4). Hsa_circRNA_102682 has a slightly higher diagnostic value than creatinine and their combination.

FIGURE 3 CTGF was correlated with Hsa_circRNA_102682, Hcy, and creatinine. (A) CTGF level in diabetic patients with hyperhomocysteinemia; (B) CTGF was negatively correlated with Hsa_circRNA_102682. (C) CTGF was positively correlated with Hcy. (D) CTGF was positively correlated with creatinine

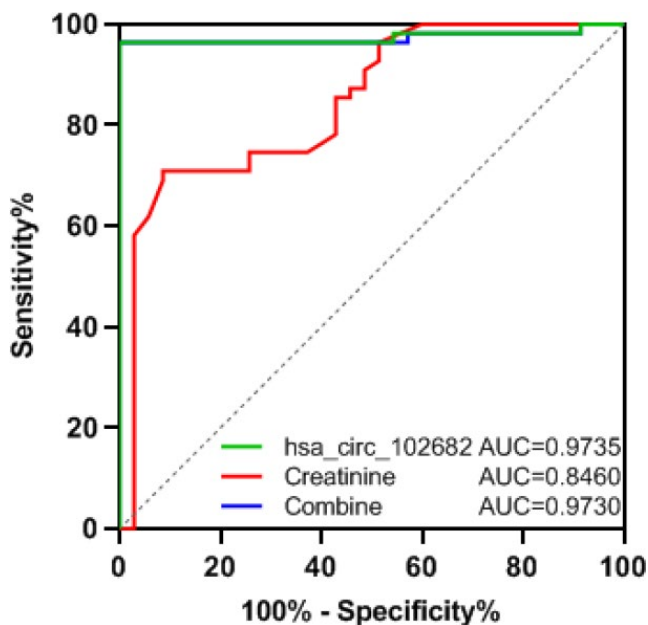
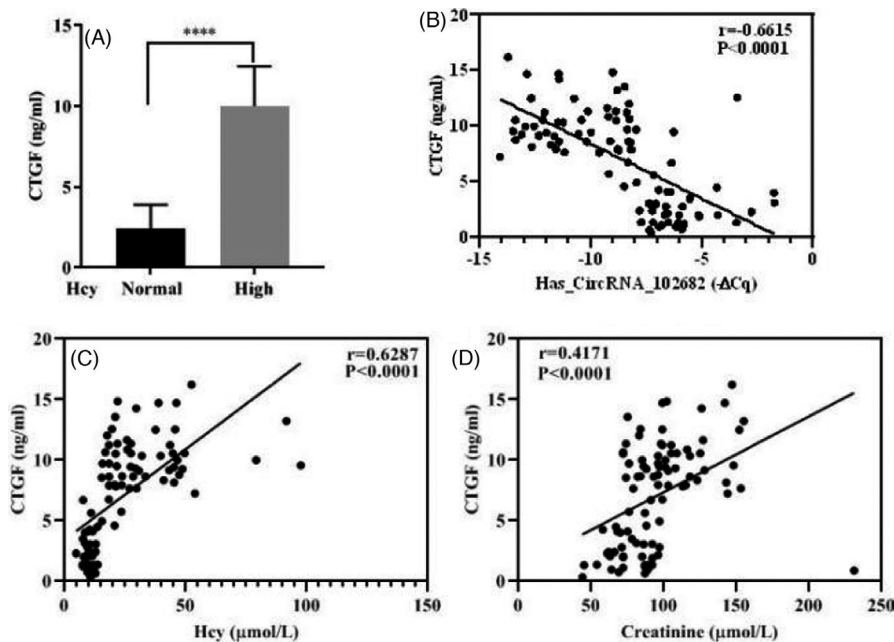


FIGURE 4 ROC analysis of the expression of Hsa_circRNA_102682 and creatinine for the diagnosis of DN. The ROC curve analysis was conducted, and the area under ROC curve was 0.9735, 0.8460, and 0.9730 for Hsa_circRNA_102682, serum creatinine, and their combination. AUC: area under the curve

4 | DISCUSSION

In this study, we collected clinical data of diabetes patients who were divided into two groups based on Hcy values. In the past, many studies have shown that circRNAs can be used as diagnostic markers for diseases. For example, Hsa_circ_0054633 in the peripheral blood can be used as a diagnostic biomarker for pre-diabetes and type 2 diabetes,¹⁹ and Hsa_circ_0001649 can be used as a potential

new biomarker for hepatocellular carcinoma.²² The circRNA Hsa_circ_0067934 is up-regulated in esophageal squamous cell carcinoma and promotes tumor cell proliferation.²³ Comprehensive circRNA analysis shows that Hsa_circ_0005075 is a new circRNA biomarker that is involved in the development of hepatocellular carcinoma.²⁴ Hsa_circ_102682 in the placental tissue has potential diagnostic value for preeclampsia.¹⁴ Because preeclampsia is related to hypertension and proteinuria and DN is mainly manifested by hypertension and proteinuria, we hypothesized that Hsa_circRNA_102682 is related to DN.

Our current results showed that Hsa_circRNA_102682 was highly expressed in diabetic patients with normal Hcy, and its expression was low in diabetic patients with high Hcy. Further, there was a correlation between Hcy and Hsa_circRNA_102682. As Hcy is associated with hypertension and an independent risk factor for DN,^{25,26} Hsa_circRNA_102682 might be a potential biomarker for DN. Comparison of the clinical data of the two groups of patients showed that the creatinine value was significantly different between the two groups. Further analysis showed a negative correlation between Hsa_circRNA_102682 and creatinine value. Because the creatinine value is a diagnostic indicator of renal disease, our results suggest an association between Hsa_circRNA_102682 and DN.

DN is a pathological change in kidney tissues caused by long-term hyperglycemia.²⁷ It is the most important cause of renal failure in end-stage renal disease (ESRD), and it is also one of the main causes of death in patients with diabetes.²⁸ Major changes in the kidneys of patients with DN include ultrafiltration of the glomeruli, glomerular hypertrophy, thickening of the glomerular basement membrane, deposition of mesangial matrix, and increased urine protein excretion, which eventually develop into glomerulosclerosis.^{29,30} Fibrosis is a terminal pathological change in most chronic diseases. Renal fibrosis gradually impairs kidney function as fibrosis worsens.³¹⁻³³ In recent years, the prevalence

of DN has been increasing rapidly. The onset of DN is insidious. Once a patient with DN enters the stage of severe proteinuria, the disease progresses to ESRD about 14 times faster than in other kidney diseases.³⁴ Therefore, early diagnosis, prevention, delay of the development, and progression of DN are of great significance to improve the survival and quality of life of diabetic patients. In the current study, the levels of fibrotic factors TGF- β and CTGF were high in patients with diabetic hyperhomocysteinemia and were negatively correlated with Hsa_circRNA_102682. These data suggest that Hsa_circRNA_102682 may be involved in DN pathogenesis. Serum Hcy is a metabolite of methionine and is related to coronary heart disease, diabetes, cerebral infarction, atherosclerosis, hypertension, and stroke. Hyperhomocysteinemia is increasingly reported as an independent risk factor of DN.^{35,36} A number of studies have shown that there is a certain relationship between hyperhomocysteinemia and DN. Hyperhomocysteinemia can increase end-glycosylation metabolites in diabetes, exacerbate vascular endothelial damage and cause renal microcirculation disorders.³⁷ Moreover, elevated plasma Hcy levels promote the generation of oxygen free radicals, causing damage to endothelial cells, aggravating albuminuria, and then further increasing blood Hcy, thereby forming a vicious circle. Hyperhomocysteinemia is an independent risk factor for the development of DN, and it is a reliable indicator for evaluating renal function damage in DN.³⁸ In our results, serum TGF- β and CTGF levels were positively correlated with Hcy and creatinine levels. Serum creatinine is an important clinical indicator for renal insufficiency. Our ROC analysis data indicated that the AUC of Hsa_circRNA_102682 was 0.9735, which was higher than that of creatinine (0.8460), and approximately the same as the AUC of their combination (0.9730). This result suggests that Hsa_circRNA_102682 might be more sensitive than creatinine for testing renal function in DN. These data suggest that Hsa_circRNA_102682 is associated with DN inflammation, which results in Hcy increasing and finally renal dysfunction. Furthermore, the diagnostic accuracy of Hsa_circRNA_102682 is higher than that of creatinine in diabetic nephropathy.

In summary, in view of the diagnostic roles of Hcy and creatinine in DN and the correlations of Hsa_circRNA_102682 to TGF- β and CTGF, as well as Hcy and creatinine, Hsa_circRNA_102682 might be a potential diagnostic indicator of DN.

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CONFLICTS OF INTEREST

No potential conflicts of interest were reported by the authors.

DATA AVAILABILITY STATEMENT

Data are available on request to the authors.

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