

Diagnostic Roles of circXPNPEP3 as Biomarker for Diabetic Nephropathy

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Background: Diabetic nephropathy (DN) is a common chronic microvascular complication of diabetes mellitus (DM). Circular RNAs (circRNAs) have emerged as ideal biomarkers for various diseases. Recent studies have shown that circXPNPEP3 is upregulated in high glucose-induced human umbilical vein endothelial cells. In this study, we aimed to examine the expression levels of circXPNPEP3 in the serum of patients with DN and to evaluate its diagnostic potential for this condition.

Methods: The expression levels of circXPNPEP3 in DN tissues and serum were detected using quantitative real-time polymerase chain reaction (qRT-PCR). DN was confirmed by biopsy or clinically defined as a urine albumin/creatinine ratio (uACR) > 30 mg/g or an estimated glomerular filtration rate (eGFR) < 60 mL/min per 1.73 m². The association between circXPNPEP3 expression levels and the clinical features of patients with DN was investigated. Using healthy individuals as controls, the diagnostic value of circXPNPEP3 for DN was evaluated by receiver operating characteristic (ROC) curve analysis. Finally, a potential RNA-interaction-network involving circXPNPEP3 was constructed using bioinformatics approaches.

Results: The expression levels of circXPNPEP3 were significantly upregulated in both DN tissues and serum compared to those in non-DN tissues and serum from healthy controls. The dysregulation of circXPNPEP3 was significantly correlated with albuminuria categories and glomerular filtration rate (GFR) categories. Serum expression of circXPNPEP3 distinguished patients with DN from controls, yielding an area under the ROC curve (AUC) of 0.8389, with a sensitivity of 70.59% and a specificity of 86.27%. Finally, hsa-miR-135b-5p, hsa-miR-135a-3p, and hsa-miR-1237-3p were predicted to be potential targets of circXPNPEP3 in DN, and a competing endogenous RNA (ceRNA) network involving circXPNPEP3 was constructed based on bioinformatic predictions.

Conclusion: CircXPNPEP3 is upregulated in DN tissues and serum. Its elevated expression in serum shows promise as a non-invasive diagnostic biomarker for DN.

Keywords: diabetic nephropathy, circular RNA, biomarker, serum

Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by persistent hyperglycemia resulting from impaired insulin secretion or action. In 2021, the global prevalence of diabetes reached 537 million affected adults, with projections indicating a rise to nearly 783 million by 2045. This disease poses a substantial global health burden, contributing to 6.7 million deaths annually and incurring healthcare costs exceeding USD 966 billion, with a disproportionately high impact on low- and middle-income countries.^{1,2} Diabetic nephropathy (DN) is a prevalent chronic microvascular complication associated with DM and a leading cause of chronic kidney disease and end-stage renal disease (ESRD), which necessitates dialysis and even renal transplantation.^{3,4} Moreover, a considerable number of patients with DN face a significant risk of cardiovascular disease, and cardiovascular-related mortality remains high even among those who have not reached ESRD.⁵ Furthermore, in a 10-year prospective study, the all-cause mortality rate for diabetes patients with DN was approximately 30%, contrasted with only about 8% in those without DN, contributing to a substantial economic and social burden.⁶ Despite the rising incidence of DN, current treatment strategies remain largely focused on symptom management, with a notable absence of effective interventions capable of halting or reversing disease progression.⁵ The early stages of DN are often asymptomatic or present with non-specific clinical

features, particularly in patients who do not show the classic pattern of DN. This frequently leads to delayed diagnosis and treatment, further aggravating the progression to ESRD.⁷

The conventional diagnosis of DN relies on the detection of a reduced glomerular filtration rate (GFR) or an elevated urinary albumin excretion rate.⁸ However, these parameters lack specificity for DN and demonstrate limited sensitivity and specificity for identifying early-stage diabetic kidney injury. Renal impairment may already be present even in the absence of detectable microalbuminuria; by the time albuminuria is evident, significant structural damage may have already occurred.⁹ Although renal biopsy is regarded as the gold standard for differentiating DN from non-diabetic kidney diseases, it is an invasive procedure associated with technical limitations and potential complications, thereby restricting its broad clinical utility.¹⁰ Despite the exploration of various laboratory and omics-based biomarkers in recent years, their clinical translation has been hindered by poor cost-effectiveness and insufficient data analytical capabilities.^{9,11–14} Consequently, there is an urgent need to identify and validate novel, specific biomarkers for diagnosis and monitoring of DN, as well as serving as potential therapeutic targets.

In recent years, circular RNAs (circRNAs) have emerged as a novel class of non-coding RNAs characterized by their unique structural and functional properties. Unlike linear RNAs, circRNAs form a covalently closed loop structure, which confers high stability, evolutionary conservation, and tissue-specific expression patterns.^{15,16} Owing to these distinctive features, circRNAs have been recognized as promising biomarkers for various diseases, particularly in cancers including renal cancer.^{10,17} For instance, Zheng et al reported that circPPAP2B promotes metastasis in clear cell renal cell carcinoma (ccRCC) by regulating the miR-182-5p/CYP1B1 axis and may serve as a biomarker for distinguishing patients with and without distant metastasis.¹⁸ Similarly, Wang et al suggested that circASAP1 modulates ferroptosis and tumor development in ccRCC, indicating its potential as a prognostic biomarker.¹⁹ However, research on the role of circRNAs as biomarkers in DN remains limited. Growing evidence has revealed that aberrant expression of circRNAs is closely associated with the development and progression of DN.²⁰ For example, hsa_circ_000953 was shown to regulate podocyte injury and autophagy via the miR-665-3p/Atg4b pathway in DN.²¹ Peng et al found that circRNA_010383 could target miR-135a to regulate TRPC1 expression, thereby modulating renal fibrosis in DN.²² Given the stability of circRNAs in peripheral blood and their functional involvement in DN pathogenesis, serum circRNAs represent promising non-invasive diagnostic biomarkers for early detection of DN.

Recently, circXPNPEP3 (hsa_circ_0008360) was demonstrated to be upregulated in the high glucose (HG)-induced human umbilical vein endothelial cells (HUVECs).²³ Additionally, Wen et al reported that circXPNPEP3 expression is dysregulated in the peripheral blood of patients with rheumatoid arthritis and may serve as a potential diagnostic biomarker for this condition.²⁴ CircRNAs commonly function as miRNA sponges that sequester specific miRNAs and attenuate their repression of target mRNAs, thereby forming competitive endogenous RNA (ceRNA) networks.²⁵ However, whether circXPNPEP3 can act as a biomarker for DN and its potential functional mechanisms remain unexplored. In this study, we first examined the expression levels of circXPNPEP3 in DN and non-DN tissues. We then determined its expression levels in serum from patients with DN and assessed its value as a diagnostic biomarker. Finally, using bioinformatic analysis, we predicted a ceRNA network centered on circXPNPEP3 to identify potentially interacting miRNAs and their downstream target mRNAs.

Methods

Patients and Samples

A total of 51 patients with DN admitted to the Department of Nephrology, Shanghai University of Medicine & Health Sciences Affiliated Zhoupu Hospital between 2022 and 2024 were enrolled in this study. Fifty-one age- and gender-matched individuals who underwent routine health examinations were selected as the control group. Blood samples were collected from all 51 patients with DN and 51 controls, and plasma was separated within 2 hours after collection. DN was diagnosed according to clinical guidelines,^{26,27} either confirmed by biopsy or clinically defined by an eGFR below 60 mL/min per 1.73 m² or a urine albumin/creatinine ratio (uACR) greater than 30 mg/g.⁸ All samples were stored at –80°C until RNA extraction. This study was approved by the Institutional Ethics Committee of the Shanghai University of Medicine & Health Sciences Affiliated Zhoupu Hospital, Shanghai Pudong New District Zhoupu Hospital. Written

informed consent was obtained from all participants prior to sample collection. The sample size was justified by using Power Analysis for ROC Curves module in MedCalc Statistical Software (Version 23.3.7)²⁸ based on the expected area under the ROC curve (AUC). Assuming an AUC of 0.75 for the biomarker under investigation versus a null hypothesis value of 0.5, with a power of 90% and a two-sided alpha of 0.05, a minimum of 50 subjects (25 cases and 25 controls) was required. The study was performed in accordance with the Declaration of Helsinki.

RNA Extraction and qRT-PCR Analyses

According to the manufacturers' instructions, total RNA was extracted from 500 μ L of serum using TRIzol reagent (Invitrogen, Carlsbad, California, USA), of which quality and quantity were examined with Nanodrop ND-2000 (Thermo Fisher Scientific, Inc). The A260/A280 ratios for all samples ranged from 1.9 to 2.1, indicating high-purity RNA with minimal protein contamination. PrimeScript™ RT Master Mix (TaKaRa, Dalian, China) and SYBR Green Premix Ex Taq™ II (TaKaRa, Dalian, China) were used to perform reverse transcription and qRT-PCR according to the manufacturer's instructions, respectively. GAPDH was selected as the internal standard control, and relative expression of genes were calculated using the $2^{-\Delta\Delta C_t}$ method. The following primers were used: forward primer of 5'-TCGAGAAGTTTGGGATGGTC-3' and reverse primer of 5'-TTTGTGTCTGCGAAGTGCAT-3' for circXPNPEP3;²⁹ and forward primer of 5'-CAATGACCCCTTCATTGACC-3' and reverse primer of 5'-TTGATTTTGGAGGGATCTCG-3' for GAPDH.³⁰

Prediction of circXPNPEP3-miRNA-mRNA ceRNA Network

The sequence and origin of circXPNPEP3 were acquired from Circular RNA Interactome (<https://circinteractome.nia.nih.gov/>). The candidate miRNAs that could be sponged by circXPNPEP3 were predicted miRanda v3.3a with a minimum alignment score of 140 and maximum free energy of -10 kcal/mol, which are widely applied thresholds to reduce false-positive predictions. The differentially expressed miRNAs (DE-miRNAs) in GSE51674 dataset³¹ between kidney specimens from patients with DN and non-diabetic renal disease were extracted via limma package (v3.60.6) ($adj.p < 0.05$ and $|\log_2FC| > 1$). The differentially expressed mRNAs (DE-mRNAs) were identified in the transcriptomic dataset GSE166239 using DESeq2 (v1.44.0) with stringent thresholds of $adj.p < 0.05$ and $|\log_2FC| > 1$. Besides, the single-cell RNA sequencing (scRNA-seq) dataset GSE131882³² was also explored for the DE-mRNAs. Then, the multiMiR package (v1.26.0) was employed to predict target genes of the DE-miRNAs. Finally, the circRNA-miRNA-mRNA network was constructed based on the "miRNA sponge" theory and visualized via the Cytoscape.

Statistical Analysis

GraphPad Prism 6.02 (GraphPad Software, La Jolla, CA, USA) and SPSS version 22.0 (SPSS Inc, Chicago, IL, USA) were employed to conduct the statistical analyses. The results of qRT-PCR were presented as mean \pm standard deviation (SD). Non-paired t-tests were used to compare the relative expression of circXPNPEP3 in plasma from DN patients and control patients. Furthermore, to explore the relationships between circXPNPEP3 expression levels and clinic characteristics, non-paired t-tests and one-way analysis of variance (ANOVA) were used. Pearson correlation analysis was used to explore the correlation between expression levels of circXPNPEP3 in kidney tissues and plasma of patients with DN. The area under the receiver operating characteristic (ROC) curve was used to evaluate the value of circXPNPEP3 as biomarker for DN. *P* values < 0.05 were considered as statistically significant.

Results

The Expression Levels of circXPNPEP3 Were Upregulated in DN Tissues

CircXPNPEP3 is derived from the XPNPEP3 gene and consists of exon 3, with a spliced sequence length of 408 bp (Figure 1). Divergent primers were used to quantify the expression levels of circXPNPEP3 in DN tissues and non-DN tissues. The results showed that circXPNPEP3 was significantly upregulated in DN tissues compared to non-DN tissues (Figure 2A).



Figure 1 CircXPNPEP3 (hsa_circ_0008360) is generated from chr22:41277774–41278181|+ through back-splicing (indicated by the blue arrows) of exon 3 (indicated by the blue lines) of XPNPEP3.

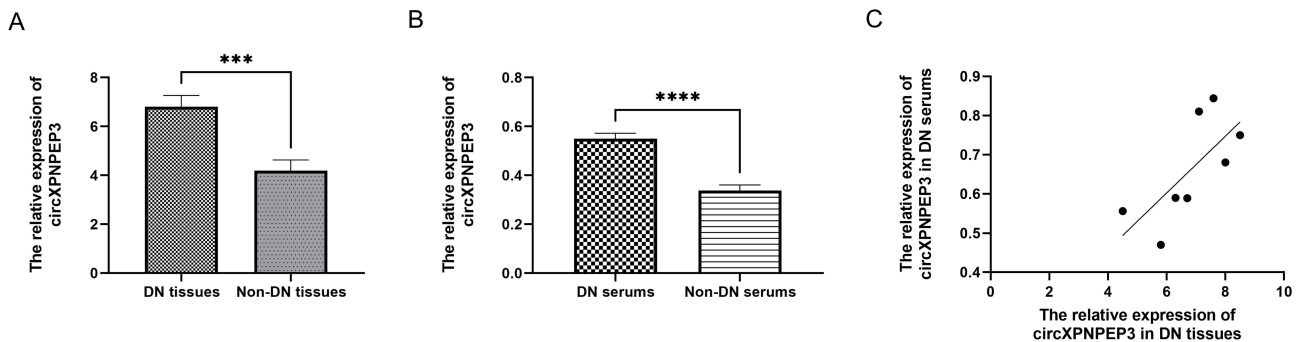


Figure 2 The expression levels of circXPNPEP3 in DN tissues and serums. **(A)** CircXPNPEP3 was upregulated in DN tissues compared to non-DN tissues. ($n = 8$; $***P < 0.001$). **(B)** CircXPNPEP3 was upregulated in DN serums compared to non-DN serums. ($n = 51$; $****P < 0.0001$). **(C)** The expression levels of circXPNPEP3 in DN serums were positively correlated with the expression levels of circXPNPEP3 in DN tissues. ($n = 8$; $r = 0.7239$).

The Expression Levels of circXPNPEP3 Were Upregulated in the Serums of DN Patients

The expression levels of circXPNPEP3 in serum samples from 51 patients with DN and 51 controls were measured using qRT-PCR. The baseline demographics and clinical parameters of the participants were summarized in [Table 1](#). The results demonstrated that circXPNPEP3 was significantly upregulated in the serum of DN patients compared to that of the controls ([Figure 2B](#)). Additionally, Pearson correlation analysis was performed to evaluate the relationship between circXPNPEP3 expression levels in serum and renal tissues from DN patients. A significant positive correlation was observed ($r = 0.7239$, $P < 0.05$) ([Figure 2C](#)).

The Stability of circXPNPEP3

The stability of circXPNPEP3 is a critical factor for its feasibility as a clinical biomarker, as the utility of circulating biomarkers heavily depends on their stability under typical handling and storage conditions.³³ We therefore investigated the stability of circXPNPEP3 in serum. For the freeze-thaw stability test, one set of samples was subjected to 2 and 4 complete freeze-thaw cycles. For the bench-top stability test, another set of samples was kept at room temperature (25°C) for 0, 2, and 4 hours. As shown in [Figure 3A](#), circXPNPEP3 levels showed no significant degradation after being subjected to up to four freeze-thaw cycles. Similarly, circXPNPEP3 levels remained stable in serum stored at room temperature for up to 4 hours ([Figure 3B](#)). These findings demonstrated that circXPNPEP3 is highly resistant to degradation induced by repeated freeze-thaw cycles or extended exposure to room temperature, underscoring its strong potential for clinical application as a robust biomarker.

Table 1 The Baseline Demographics and Clinical Parameters of the Participants

	Diabetic Nephropathy (DN)	Healthy Controls	P value
Age (years)	59.00 ± 9.22	57.67 ± 12.61	0.6235
Male (%)	34 (66.7)	36 (70.6)	0.8313
Body mass index (kg/m ²)	26.31 ± 3.53	26.22 ± 5.48	0.9513
Albumin (g/L)	35.98 ± 7.022	39.22 ± 3.592	0.1913
Systolic blood pressure (mmHg)	141.5 ± 10.42	137.0 ± 13.12	0.1405
Diastolic blood pressure (mmHg)	78.91 ± 15.93	70.25 ± 10.21	0.1527
Fasting glucose (mmol/L)	8.305 ± 3.585	5.861 ± 1.121	0.0048
HbA1c (%)	8.045 ± 2.254	5.704 ± 0.8331	0.0002
TC (mmol/L)	4.840 ± 2.245	4.712 ± 0.8649	0.7994
TG (mmol/L)	1.558 ± 0.567	1.258 ± 0.609	0.0679
UA (umol/L)	411.9 ± 115.3	327.5 ± 61.14	0.0027
Crea (umol/L)	173.2 ± 108.2	62.00 ± 9.481	< 0.0001
eGFR (mL/min/1.73 m ²)	52.98 ± 28.28	106.4 ± 14.06	< 0.0001
uACR (mg/g)	830.9 ± 794.7	17.91 ± 7.990	< 0.0001
Insulin use, n (%)	30 (58.8)	0 (0)	< 0.0001

Relationship Between Expression Levels of circXPNPEP3 and Clinic Factors and Its Diagnostic Significance for DN

Subsequently, the association between serum circXPNPEP3 expression levels and the clinical characteristics of patients with DN was evaluated. As summarized in Table 2, circXPNPEP3 expression was significantly higher in patients belonging to higher GFR categories compared to those in lower GFR categories ($P = 0.0004$). Similarly, serum circXPNPEP3 levels were significantly elevated in patients with higher albuminuria categories relative to those with lower categories ($P = 0.0303$). However, no significant correlation was observed between circXPNPEP3 expression and age or gender.

ROC curve analysis was performed to assess the diagnostic value of serum circXPNPEP3 for DN. The AUC was 0.8389 (95% confidence interval [CI]: 0.7599–0.9179; $P < 0.0001$; Figure 4). At the optimal cutoff value of 0.4145, determined by Youden's index, circXPNPEP3 exhibited a sensitivity of 70.59% (95% CI: 57.00–81.29%) and a specificity of 86.27% (95% CI: 74.28–93.19%), with a positive predictive value (PPV) of 55.7%, and a negative predictive value (NPV) of 92.3%.

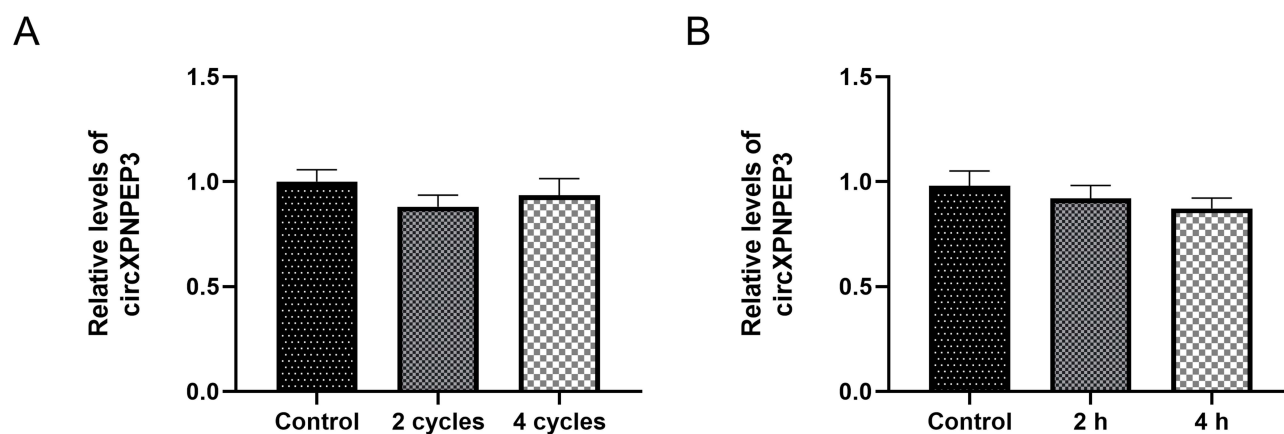


Figure 3 Stability of circXPNPEP3. (A) Stability after repeated freeze-thaw cycles (2 to 4 cycles). (B) Stability after storage at room temperature (25°C) for various durations (2 to 4 hours).

Table 2 Correlation Between circXPNPEP3 Expression Levels in Serums and Clinic Features of Patients with DN

Clinic Features	Number of Patients	Mean \pm SD	P value
Age			
<60	14	0.5493 \pm 0.1814	0.9853
\geq 60	37	0.5503 \pm 0.0978	
Gender			
Male	34	0.5367 \pm 0.1616	0.4273
Female	17	0.5753 \pm 0.1641	
GFR categories			
G1-G2	16	0.4377 \pm 0.1145	0.0004
G3a-G3b	22	0.5669 \pm 0.1293	
G4-G5	13	0.6580 \pm 0.1829	
Albuminuria categories			
A1-A2	22	0.4940 \pm 0.1574	0.0303
A3	29	0.5917 \pm 0.1546	

The ceRNA Network Involving circXPNPEP3 in DN

First, the sequence of circXPNPEP3 was obtained from Circular RNA Interactome and sequences of all miRNAs were downloaded at miRbase (<https://www.mirbase.org>). Then, miRanda v3.3a was used to predict the candidate miRNAs that could be sponged by circXPNPEP3. A total of 86 circRNA-miRNA pairs were identified. On the other hand, the DE-miRNAs between kidney tissues from patients with DN and kidney tissues from patients with non-diabetic renal disease were extracted via limma package (v3.60.6) from GSE51674 dataset. Differentially expressed miRNAs (DE-miRNAs) were defined as those with adjusted p-value < 0.05 (Benjamini-Hochberg method) and $|\log_2FC| > 1$, resulting in 126 DE-miRNAs. To resolve miRNA nomenclature discrepancies, miRBase-based identifier conversion was performed via the miEAA 2.1 platform (https://ccb-compute2.cs.uni-saarland.de/mieaa/mirna_version_converter/),³⁴ resulting in 117 annotated DE-miRNAs. Subsequently, an intersection analysis between these DE-miRNAs and computationally predicted

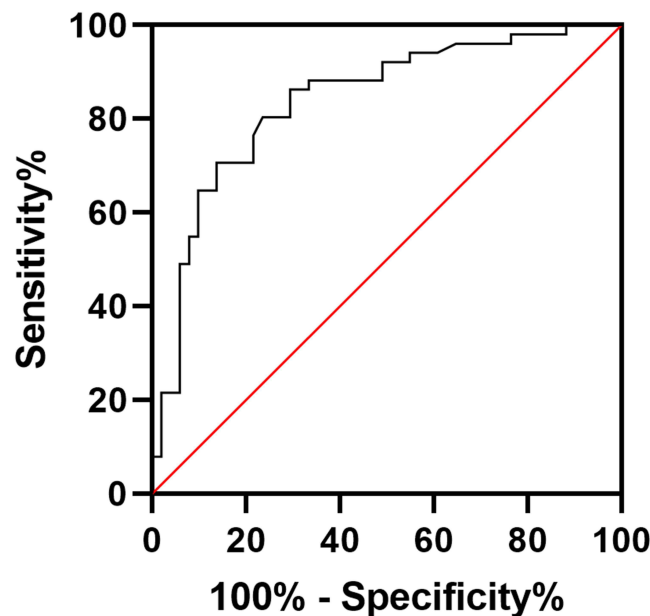


Figure 4 The diagnostic significance of circXPNPEP3 in serums for DN. The AUC was 0.8389 (95% CI: 0.7599–0.9179) with sensitivity of 70.59% (95% CI: 57.00–81.29%) and specificity of 86.27% (95% CI: 74.28–93.19%).

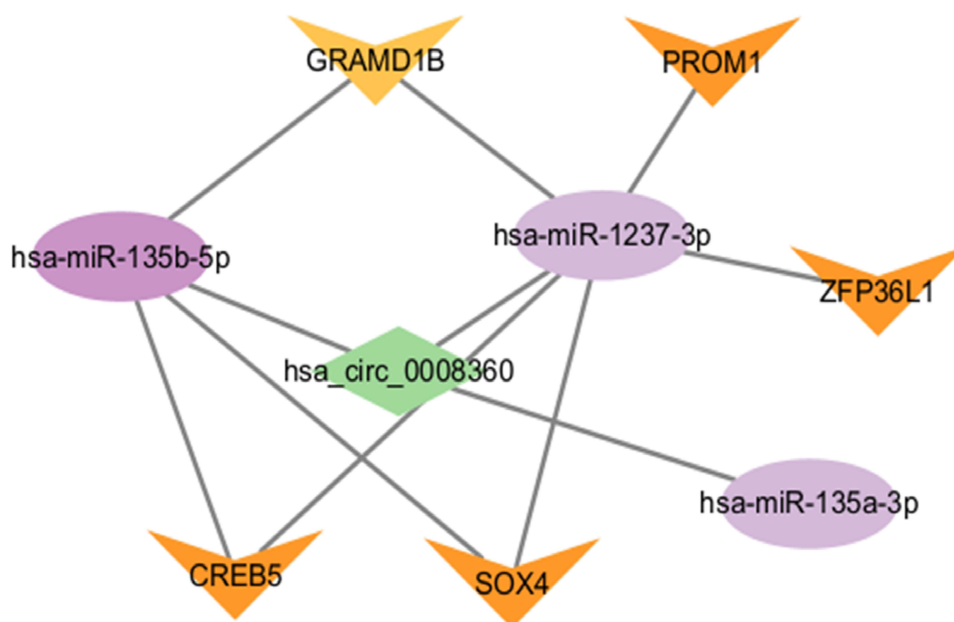


Figure 5 The predicted circXPNPEP3-miRNA-mRNA ceRNA network in DN.

miRNA targets of circXPNPEP3 revealed three overlapping candidates: hsa-miR-135b-5p, hsa-miR-135a-3p, and hsa-miR-1237-3p. The use of intersection rather than union criteria was intended to increase robustness by prioritizing candidates supported by both computational prediction and expression evidence.

Then, the multiMiR package (v1.26.0), a consolidated platform for aggregating miRNA-target interactions across multiple databases, was employed to predict target genes of the DE-miRNAs, which employed validated miRNA-target interactions retrieved from curated databases (miRecords, miRTarBase and TarBase) and in silico predictions derived from algorithmic databases (DIANA-microT-CDS, EIMMo, MicroCosm, miRanda, miRDB, PicTar, PITA and TargetScan). The result suggested 5579 unique target genes were identified with this analysis. By analyzing the transcriptomic dataset GSE166239 using DESeq2 (v1.44.0), 1208 significant DE-mRNAs had been identified. Furthermore, 125 DE-mRNAs had been screened from the scRNA-seq dataset GSE131882. Intersection analysis of the bulk and single-cell results identified 21 overlapping DE-mRNAs, suggesting consistent dysregulation across transcriptomic resolutions. Later, the predict target genes of the DE-miRNAs and the DE-mRNAs identified from GSE databases were intersected, resulting in 10 shared DE-mRNAs.

Finally, based on the anti-correlation principle—which reflects canonical miRNA-mediated mRNA suppression—negatively correlated miRNA-mRNA pairs were prioritized by filtering out positively correlated differential relationships. This analysis identified GRAMD1B, PROM1, ZFP36L1, SOX4, and CREB5 as potential downstream mRNAs regulated by circXPNPEP3. Functionally relevant miRNA-mRNA interactions were subsequently visualized using Cytoscape to construct a regulatory network (Figure 5).

Discussion

Chronic hyperglycemia, a hallmark of DM, can contribute to the development of severe complications, including microvascular pathologies such as retinopathy, nephropathy, and neuropathy, as well as macrovascular sequelae like cardiovascular disease and peripheral artery disease.³⁵ In DN, circRNAs have been implicated in the regulation of renal cell damage through influences on cell cycle progression, differentiation, apoptosis, and the secretion of inflammatory cytokines and extracellular matrix proteins.²⁰ For instance, circTAOK1 has been shown to play a critical role in the pathogenesis of DN by modulating the miR-142-3p/SOX6 axis under HG conditions, thereby exacerbating renal injury by promoting inflammatory responses, oxidative stress, extracellular matrix (ECM) accumulation, and apoptosis in renal cells.³⁶ Lin et al found that circUBXN7 was significantly upregulated in the plasma of patients with DN and correlated

with renal functions. It was transcriptionally activated by SP1 and, in turn, enhanced SP1 mRNA stability and activation via interaction with IGF2BP2.³⁷ These findings highlight the potential of circRNAs as therapeutic targets in DN.²¹ However, few studies have focused on the clinical utility of circRNAs as non-invasive biomarkers for the diagnosis and prognosis of DN.

In this study, we first showed that circXPNPEP3 was upregulated in DN tissues compared to non-DN tissues. Subsequently, circXPNPEP3 was also found to be upregulated in the serum of DN patients relative to non-DN controls. Notably, a positive correlation was observed between the expression levels of circXPNPEP3 in renal tissues and serum samples from DN patients. Furthermore, based on the association between serum circXPNPEP3 levels and clinical features of DN, ROC curve analysis suggested its possible role as a diagnostic biomarker for DN.

A well-established function of circRNAs is to act as miRNA sponges, thereby regulating the expression of genes targeted by these miRNAs.^{17,38} To evaluate the functions of circXPNPEP3 in DN progression, the potential cross-talk RNA network involving circXPNPEP3 was investigated by bioinformatic analysis. The *in silico* analysis predicted that circXPNPEP3 may potentially function in DN through regulating different mRNAs via sponging diverse miRNAs, including hsa-miR-135b-5p, hsa-miR-135a-3p, and hsa-miR-1237-3p.

Liu et al reported that hsa-miR-135b-5p inhibits the expression of VHL and upregulates HIF1 α , thereby promoting endothelial cell proliferation and angiogenesis in a mouse model of diabetic retinopathy.³⁹ Additionally, miR-135b-5p expression was observed to decrease in retinal cells with prolonged HG exposure, yet it was found to be up-regulated in diabetic retinas during the progression of diabetic retinopathy.⁴⁰ These findings suggest that the precise role of hsa-miR-135b-5p in the microvascular complications of DM requires further investigation. Icli et al demonstrated that hsa-miR-135a-3p acts as an anti-angiogenic miRNA by modulating angiogenesis and tissue repair through the regulation of p38 signaling pathway in endothelial cells.⁴¹ The expression levels of hsa-miR-1237-3p have been associated with concentrations of specific fatty acids, indicating a potential role in the regulation of metabolic abnormalities.⁴² Furthermore, Yu et al found that downregulated hsa-miR-1237-3p participates in the LINC00960/MMP-2 interaction pair, a key ceRNA network implicated in the progression of DN.⁴³

Upregulated expression of SOX4 has been reported to inhibit insulin secretion,⁴⁴ and the role of SOX4 in microvascular complications of DM has been extensively studied. In diabetic retinopathy, Wei et al found that silencing the upregulated expression of SOX4 enhanced the viability of human retinal endothelial cells and suppressed their inflammation, migration, and angiogenesis under HG conditions.⁴⁵ Additionally, Yang et al demonstrated that SOX4 is involved in epithelial-mesenchymal transition during the progression of diabetic retinopathy.⁴⁶ In DN, SOX4 has been shown to modulate renal injury and renal interstitial fibrosis under HG stimulation.⁴⁷ Shi et al demonstrated the involvement of CREB5 in regulating apoptosis and epithelial-mesenchymal transition in proximal convoluted tubule epithelial cells during early DN.⁴⁸ The RNA-binding protein ZFP36L1, a member of the ZFP36 family, plays important roles in diverse physiological and pathological processes through post-transcriptional regulation. Tarling et al identified ZFP36L1 as a key regulator of *Cyp7a1* mRNA and bile acid composition; its suppression reduced adiposity, impaired lipid absorption, and elicited antisteatotic effects in ZFP36L1-deficient mice.⁴⁹ Similarly, GRAMD1 is widely recognized as a master regulator of accessible cholesterol transport and the maintenance of cholesterol homeostasis.⁵⁰ Given the established roles of bile acid regulation in insulin resistance and lipid homeostasis in DN,^{51,52} ZFP36L1-dependent bile acid metabolism and GRAMD1-mediated cholesterol homeostasis may contribute significantly to the progression of DM and DN. Furthermore, Almasry et al reported that PROM1 is upregulated under hyperglycemic conditions and is associated with diabetes-induced structural changes in the retinas of diabetic rats, suggesting its potential role in the progression of diabetic retinopathy.⁵³ In conclusion, our findings indicate that circXPNPEP3 functions as a miRNA sponge, sequestering hsa-miR-135b-5p, hsa-miR-135a-3p, and hsa-miR-1237-3p. This interaction may lead to dysregulation of downstream target genes—including SOX4, CREB5, ZFP36L1, GRAMD1B, and PROM1—thereby contributing to extracellular matrix accumulation, impaired angiogenesis, and disrupted cholesterol homeostasis, which are key hallmarks of DN progression.^{41,43,46,50}

With the advancement of the high-throughput sequencing technologies and bioinformatics, circRNAs have emerged as a promising class of biomarkers due to their unique characteristics and extremely stability. Moreover, their stability, coupled with tissue-specific expression patterns and abundance in body fluids such as serum, plasma, and urine, makes

circRNAs ideal candidates for non-invasive liquid biopsies.⁵⁴ Despite the considerable potential of circRNAs as diagnostic biomarkers, several challenges must be addressed to facilitate their translation into clinical practice. First, the lack of standardized detection protocols remains a major obstacle. Current methods for circRNA detection, including qRT-PCR, rolling circle amplification, and droplet digital PCR (ddPCR), require further optimization and validation to ensure reproducibility and accuracy across different laboratories.^{54–56} Second, establishing appropriate cutoff values to differentiate healthy individuals from those with DN is fundamental to the application of circRNAs as diagnostic biomarkers. This necessitates large-scale validation studies to confirm the sensitivity and specificity of candidate circRNAs for DN. Third, the dynamic range and stability of circRNAs across various bodily fluids, including serum, as used in this study, must be thoroughly investigated to ensure their reliability. Standardization of RNA extraction and profiling procedures from these fluids is also essential.^{54,55,57} Fourth, the functional mechanisms through which differentially expressed circRNAs contribute to DN pathogenesis need to be elucidated to better understand their regulatory roles, which is critical for interpreting the biological significance of circRNA expression changes in different disease contexts.³⁸ Fifth, the integration of artificial intelligence (AI) with circRNA transcriptome analysis may offer significant opportunities for a more comprehensive understanding of human biological systems, thereby accelerating the development of diagnostics and therapeutics for DN and other pathological conditions.⁵⁷

Although the clinical diagnostic values and potential regulatory ceRNA network of circXPNPEP3 were preliminary analyzed, there were several limitations to our study. Firstly, the case-control, single-center design was adopted for initial biomarker screening in this tentative study, future studies with cross-sectional, multi-center design will be necessary for clinical validation. Secondly, the modest sample size in this discovery-phase study limits the generalizability of our findings. Future work with expanded cohorts is underway to validate these circRNA biomarkers. Thirdly, it is important to note that our study design, which defined DN cases mainly based on uACR thresholds, precludes a direct ROC curve-based comparison of diagnostic accuracy between circXPNPEP3 and uACR, as the latter was used as the grouping variable. Future studies in a prospectively recruited, unselected cohort are essential and are the next step to formally compare the diagnostic performance of circXPNPEP3 against and in combination with uACR. Fourth, this study is limited by its retrospective design and the lack of longitudinal data. Therefore, we cannot yet assess the dynamic changes of circXPNPEP3 in response to disease progression or therapeutic intervention. Last, experimental studies are needed to validate our results regarding the ceRNA network of circXPNPEP3 in the DN pathogenesis.

Conclusion

CircXPNPEP3 is upregulated in both tissues and serum of patients with DN, and its expression level is positively correlated with clinical features of DN. It shows promise as a non-invasive diagnostic biomarker for DN and may participate in disease progression by sponging hsa-miR-135b-5p, hsa-miR-135a-3p, and hsa-miR-1237-3p.

However, several challenges must be overcome before circRNAs can be translated into clinical use. Detection methods require standardization, and larger multi-center prospective studies are needed to establish optimized detection protocols and clinically relevant cutoff values. In addition, longitudinal studies are essential to evaluate the potential of circXPNPEP3 as a dynamic biomarker for monitoring treatment response. Furthermore, the functional role of circXPNPEP3, currently supported only by bioinformatic predictions, needs to be validated through mechanistic experiments.

Ethics Approval

This research was approved by the Institutional Ethics Committee of the Shanghai Pudong New District Zhoupu Hospital, Shanghai University of Medicine & Health Sciences Affiliated Zhoupu Hospital (Ref No.: ZPYLL-2018-02). Informed consent was obtained from all participants prior to the study and for the publication of this research.

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Disclosure

The authors report no conflicts of interest in this work.

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