Overexpression of LncRNA-PVT1 promotes proliferation migration, invasion and fibrosis in diabetic nephropathy via suppressing miR-93-5p

CURRENT STATUS: POSTED

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DOI: 10.21203/rs.2.14072/v1

SUBJECT AREAS
Urology & Nephrology

KEYWORDS
diabetic nephropathy; LncRNA-PVT1; miR-93-5p; PTEN; PI3K/Akt/mTOR pathway; mouse mesangial cells
Abstract

Background: This research aimed to explore the molecular mechanism of LncRNA-plasmacytoma variant translocation 1 (PVT1) in diabetic nephropathy (DN). Methods: Mouse mesangial cells (MMCs) were obtained from diabetic model mice. The real-time fluorescence quantitative polymerase chain reaction (RT-qPCR), Western blot, luciferase reporter gene assay, EdU assay, flow cytometry analysis, AnnexinV-PI double staining, scratch assay, Transwell assay, enzyme-linked immunosorbent assay (ELISA) and luciferase reporter gene assay were performed to reveal the effect of PVT1 on the proliferation, migration, invasion and fibrosis of MMCs cultured in high glucose. Results: PVT1 was overexpressed in both mice kidney tissue and high glucose cultured MMCs. Meanwhile, PVT1 could not only promoted proliferation, migration, invasion and fibrosis of high glucose cultured MMCs, but also regulated the cell cycle from G0/G1 to S phase. Moreover, PVT1 negatively regulated the expression of miR-93-5p in MMCs. Furthermore, the PVT1-miR-93-5p regulatory relation activated the PI3K/Akt/mTOR pathway, which in turn regulated cell proliferation and insulin signaling in high glucose cultured MMCs. Conclusions: PVT1 might promoted proliferation, migration, invasion and fibrosis in high glucose cultured MMCs, which further affected the development of DN. Furthermore, PVT1-miR-93-5p regulatory relation might take part in DN progression via activating the PI3K/Akt/mTOR pathway.

Background

Diabetic nephropathy (DN) is a chronic disease characterized by proteinuria, glomerular hypertrophy, decreased glomerular filtration and renal fibrosis with loss of renal function [1]. As a microvascular complication, DN affects up to 40% of diabetic patients and can lead to end-stage kidney disease, accounting for millions of deaths worldwide [2]. The
detail reason for the development of DN is unclear. Until now, various factors including hyperglycemia, advanced glycosylation products and activation of cytokines have been proved to be related with DN [3]. Although many effects have been made on the clinical thearp of DN [4, 5], the modern medical treatment is still not able to completely prevent the development and deterioration of DN due to the insufficient understanding of DN pathological mechanism [6]. Long noncoding RNAs (lncRNAs) are important class of pervasive genes play important regulatory roles in disease pathways [7-10]. The dysregulation of lncRNAs have been widely reported in various diseases including DN [11, 12]. As a member of lncRNAs, Plasmacytoma variant translocation 1 (PVT1) is proved to be associated with the progression of diabetes[13]. In animal model, autophagy ameliorates cognitive impairment through activation of PVT1 and apoptosis in diabetes mice [14]. The association between variants in the PVT1 and end-stage renal disease (ESRD) attribute to both type 1 and type 2 diabetes, which further indicating the function of PVT1 expression in a variety of renal cell types including DN [15]. A previous study shows that PVT1 take part in the development and progression of DN [16]. Actually, the biological function of PTV1 during disease process is commonly realized by targeting some genes [17]. The mediation effect of PTV1 during extracellular matrix accumulation in kidney cells of DN is realized by certain target microRNAs (miRNAs) [18]. A previous study show that serum miR-93 is low-expressed in diabetic nephropathy patients, indicating an important role of miR-93 in the development and progression of DN [19]. Although sporadic researches have mentioned the biological function of PTV1 and miR-93 in DN, the detail molecular mechanism of PTV1 during the progression of DN is still unclear.

Objectives: In this study, the mechanism of lncRNA-PTV1 in DN was explored based on the cells obtained from the mouse mesangial cells (MMCs). The real-time fluorescence
quantitative polymerase chain reaction (RT-qPCR), Western blot, luciferase reporter gene assay, EdU assay, flow cytometry analysis, AnnexinV-PI double staining, scratch assay, Transwell assay, enzyme-linked immunosorbent assay (ELISA) and luciferase reporter gene assay were performed to reveal the morphology, proliferation and apoptosis in MMCs. The study might provide a new theoretical basis for DN treatment and prevention.

Methods

Animal

A total of 20 SPF diabetic model mice (4 weeks, Strain: BKS.Cg-+Leprdb/Leprdb/J, db/db, 20-30 g) and normal mice (4 weeks, db/m, 20-30g) were obtained from Model Animal Research Center of Nanjing University. Mice were fed in a standalone environment at 22°C and 50% relative humidity under the alternating day and night of 12 h/12 h. After 4 weeks of feeding, the anesthesia with sodium pentobarbital (50mg/kg) was performed on mice by intraperitoneal injection. Then, all mice were sacrificed by using cervical dislocation, followed by the kidneys stripped and frozen in liquid nitrogen. This study was approved by the ethics committee of our hospital, and all experiments were in accordance with the guide for the care and use of laboratory animals established by United States National Institutes of Health (Bethesda, MD, USA).

Cell culture

Mouse mesangial cells (MMCs) were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. MMCs were cultured in DMEM complete medium containing 10% FBS (Gibco) and penicillin (Gibco). All cells were cultured in a humidified humidity incubator (MCO-15AC, SANYO) with 5% CO₂ at 37 °C. Cells were digested with 0.25% trypsin when the cells reached 80% confluency, followed by subculture every other day.

Cell transfection and grouping
The PVT1 gene was silenced by RNA interference technology, and primer sequences such as PVT1 siRNA (si-PVT1) (synthesized by Shanghai Shenggong Bioengineering Co., Ltd.) were shown in Table 1. Transient co-transfection of MMCs was performed using the liposome Lipofectamine RNAiMAX kit (Invitrogen). Transfected cells were divided into three groups including blank control group (Mock), negative control group (si-NC), and si-PVT1 experimental group (siPVT1). After transfection, the cells were replaced with 10% FBS no-anti-DMEM medium for 4 hours, and then incubated for 12 hours in serum-free DMEM medium to synchronize the cells in G0 phase. Cells cultured in DMEM full-medium containing 5.55 mmol/L D-Glucose simulated normal physiological environment were named as control group, while cells cultured in DMEM full-medium containing 30 mmol/L D-Glucose simulated diabetic physiological environment were named as high-sugar group. Thus, there were finally 4 groups in current study including control group (NG), high glucose group (HG), high glucose negative control group (HG + siNC) and high glucose siPVT1 group (HG + siPVT1). After 48 hours of high glucose culture, the cells and the supernatant of each group were collected and stored at -80 °C. The qRT-PCR was used to detect RNA interference efficiency.

MiR-93-5p mimics, mim-93-5p inhibitor, mim-NC and Inhibitor NC (Shanghai Jima Pharmaceutical Technology Co., Ltd.) were added to MMCs (80% confluency) respectively, followed by cultivation in DMEM medium containing 30 mmol/L D-Glucose. The transfected cells were divided into five groups: Mock, mim-NC, mimics, INC and inhibitor. Moreover, siNC, siPVT1, miR-93-5p inhibitor and inhibitor NC were co-transfected with MMCs (60% confluency), and named as siNC+INC, siNC+ miR-93-5p inhibitor, siPVT1+ INC and siPVT1+ miR-93-5p inhibitor group respectively.

The qRT-PCR assay

The expression level of genes including miR-93-5p was detected of by qRT-PCR. Briefly,
cells cultured for 48 h were collected to extract total RNA by using TRIZOL reagent (Invitrogen), and reverse-transcribed by PCR amplification instrument. The qRT-PCR was performed on A ABI7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The PCR program included 40 cycles of 95°C for 10 min, 95°C for 10 s, 60°C for 20 s and 72°C for 34 s. The data was analyzed by the \(2^{\Delta\Delta Ct}\) method [20], and all oligonucleotide primers were designed and synthesized by Biotechnology Bioengineering (Shanghai) Co., Ltd. The experiment was repeated for 3 times. All primer sequences for samples and internal references were listed in Table 1.

*Western blot analysis*

Proteins expression was measured by Western blot. Briefly, a total of 20 μg total protein was extracted by lysis buffer, followed by quantifying using a BCA kit (ThermoFisher Co., Ltd.). Then, samples were subjected to 10 % SDS-PAGE, and transferred onto PVDF membrane. Afterwards, the membrane was blocked with 5 % skim milk in TBST solution. Subsequently, membrane was sequentially incubated with primary antibodies including E-cadherin (1:1000, 3195, CST), N-cadherin (1:1000,13116, CST), Vimentin (1:1000, 5741, CST), GAPDH (1:1000, 5174, CST), Col. IV(1:1000, ab6586, Abcam), FN (1:10000, ab2413, Abcam), TGF-β1 (1:10000, ab92486, Abcam), PAI-1 (1:10000, ab66705, Abcam), Bcl-2 (1:1000, ab196495, Abcam), Bax (1:1000, ab199677, Abcam), cleaved caspase-3 (1:1000, ab49822, Abcam), cleaved PARP (1:1000, ab32064, Abcam), CyclinD1 (1:5000, ab226977, Abcam), CDK4 (1:3000, ab137675, Abcam), PI3K (1:1000, 4292, CST), p-PI3K (1:1000, 4228, CST), Akt (1:1000, 9272, CST), p-Akt (1:1000, 4060, CST), mTOR (1:1000,2972 CST), p-mTOR(1:1000, 2971, CST) and secondary antibodies (goat anti-rabbit IgG, 1:10000, Sigma). GAPDH was used as the internal reference. At last, blots were visualized by enhanced chemiluminescence Plus, and integrate optical density was measured by software Lab Works 4.5.
**EdU and colony formation assay**

Cells in each group were cultured for 48 hours after transfection, and EdU cell proliferation assay and colony formation assay were performed. For EdU assay, cells in all groups were performed with EdU labeling, fixation, Apollo staining and DNA staining based on EdU cell proliferation detection kit (Guangzhou Ruibo Biotechnology Co., Ltd.). All images were acquired by laser confocal microscope (NIKON A1). For colony formation assay, cells were washed with PBS and digested with 1% trypsin. All the cells were primarily added into 6-well plates (300 cells/well) with 2.5 mL medium in each well. Two weeks later, cells were fixed with 4% paraformaldehyde solution and then stained with Swiss-Gimsa for 15 min. Then, photographs were taken using an inverted phase contrast microscope (Olympus Ckx53), and the number of clones was automatically counted using ImageJ (1.48 V) software. Cell colony formation rate was calculated as (number of colonies/total number of cells seeded) ×100%.

**AnnexinV-PI assay**

Cells of each group were stained with Annexin V-PI kit (Invitrogen), and the apoptosis of each group was detected by MUSETM cytometer (EMD Millipore, USA). Briefly, after 48 hours of transfection, cells were centrifuged, washed twice with PBS, and collected (1-5×10^5 cells). A total of 500μl Binding Buffer, 5μl Annexin V-EGFP and 500μl Propidium Iodide were added to the cell samples (37°C, dark environment, 5-15 min). Finally, results were detected by flow cytometry (Ex = 488 nm, Em = 530 nm), followed by the apoptotic ratio calculation.

**Cell cycle analysis**

The flow cytometry was used to detect the cell cycle. Simply, at 48 hours after transfection, cells were mixed with 1 ml trypsin and 1-2 ml DMEM medium containing FBS, followed by centrifugation and fix with 70% ethanol. Subsequently, MuseTM Cell Cycle
Reagent was added to the cell precipitation and incubated at room temperature for 30 min. Finally, the cell cycle was measured by MUSE™ cytometer (Merck Milliber, USA). Cell Quest software was used for data analysis (results were expressed as cell percentage).

**Scratch assay**

Cell scratch assay was used to detect cell migration in current study. Simply, after adjusting the cell density of each group, the cells were inoculated into the 6-well plate. After drawed a line across the surface of culture medium, washed by PBS and added fresh culture medium, cells were continuous culture for 48 h. Then, these cells were observed and photographed under inverted microscope (Olympus Ckx53) to calculate the cell migration rate. Cell mobility was calculated as $(1 - \text{scratch width at measurement / initial scratch width}) \times 100\%$.

**Transwell assay**

After transaction for 48 h, cells suspension was added in transwell chamber (Corning Corporation, Midland, MI, USA) and matrigel (BD Biosciences) - coated transwell chamber, respectively. Then, the medium with 10% serum was added to the lower compartment of transwell chamber, followed by incubation with 5% CO$_2$ at 37 °C for 48 h. Then, the upper compartment of transwell cell was washed by PBS, fixed by 4% paraformaldehyde and stained with 0.25% Coomassie blue. Finally, five visual fields (´ 200) were randomly selected for observation under an inverted microscope (Olympus Ckx53).

**ELISA for inflammatory factors**

MMC$s$ cells were cultured for 48 hours after transfection, and the supernatants of each group were collected. The concentration of Col. IV (Sigma), PAI-1 (CST company) FN protein (Assay pro) and TGF-β1 (CST) in cells of each group were detected by ELISA kit (BioSource International, Camarillo, CA, USA). All the operations were carried out
according to the kit instructions.

*Luciferase reporter gene assay*

Prediction of PVT1 interacting miRNAs was performed based on StarBase 3.0 database. Then, the wild-type or mutant for miR-93-5p and PVT1 were designed respectively according to the predicted results. The PVT1 mutant sequence and the wild sequence fragment were cloned to the pmirGLO luciferase control reporting vector (Promega, USA). Then, HEK-293T cells were co-transfected with the mutant sequence in combination with miR-93-5p mimics or miR-93-5p negative control (Shanghai Jima Pharmaceutical Technology Co., Ltd.), which named as MT + mimics group and MT + NC group respectively. In addition, the negative control of wild-type sequence combined with miR-93-5p mimics (WT + mimics group) or miR-93-5p (WT + NC group) were co-transfected with HEK-293T. After transfection for 48 h, luciferase assay was determined by dual luciferase reporter assay kit (Promega).

*Statistical analysis*

All statistical analyses were performed using SPSS (version 20.0) and GraphPad.Prism (version: 5.01) statistical software. The results were presented in the form of mean ± standard deviation (SD). The data between two groups or among groups were analyzed by the Student t test or One-Way ANOVA respectively. P < 0.05 was considered to be statistically significant.

*Results*

*PVT1 was up-regulated in mice kidney tissue and high glucose cultured MMCs*

The result of qRT-PCR showed that PVT1 was significantly up-regulated (P<0.05) in the kidney tissue of db/db DN mice than that in db/m mice (Figure 1A). Furthermore, the expression analysis of PVT1 in glomerular MC under simulated diabetes in vitro showed that compared with NG group, PVT1 was significantly up-regulated (P<0.05) in HG group
PVT1 promoted proliferation of MMCs cultured in high glucose

RNA interference showed that the expression of PVT1 in HG + siPVT1 group was significantly lower than that in HG group (P < 0.05), and there was no significant difference between HG group and HG + siNC group (P > 0.05), indicating that siPVT1 could effectively interfere with the expression of PVT1 (Figure 2A). Moreover, EdU assay (Figure 2B) showed that compared with the NG group, the fluorescence intensity and proportion of positive cells in the HG group were both significantly increased (all P < 0.05). However, the fluorescence intensity and proportion of positive cells in the HG + siPVT1 group were lower than those in the HG + siNC and HG groups (all P < 0.05). Furthermore, colony formation assay (Figure 2C) showed that compared with the NG group, the clone formation ability of HG group cells was significantly improved (P < 0.05). Moreover, the proliferation of cell clones in HG group was significantly higher than that in HG+siPVT1 group (P < 0.05). However, there was no significant difference in the proliferation ability between HG group and HG + siNC cell clones (P > 0.05). In addition, flow cytometry showed that the apoptosis trend was opposite to that of the proliferation (Figure 2D). Moreover, Western blot showed that (Figure 2E) the expression of Bax, caspase-3, and PARP in the HG group were significantly lower than those in the NG group. Meanwhile, the expression of Bax, caspase-3, and PARP in HG + siPVT1 group were significantly higher than those in HG group (P < 0.05). The expression of anti-apoptotic factor Bcl-2 was opposite to that of pro-apoptotic factors Bax, caspase-3, and PARP. These results indicated that PVT1 might promote the proliferation and inhibited apoptosis of MGCs cells cultured in high glucose.

Effect of LncRNA-PVT1 on cell cycle of MMCs cultured in high glucose

Cell cycle of each group were detected by flow cytometry (Figure 3A-B). The result showed that compared with NG group, the proportion of cells in S phase and G0/G1 phase was
significantly increased and decreased respectively in HG group (all $P < 0.05$), suggesting that high glucose might promote MMCs into S phase. Meanwhile, compared with HG group, the percentage of cells in G0/G1 phase was significantly increased in HG + siPVT1 group ($P < 0.05$), but decreased in S phase and G2/M phase ($P < 0.05$), suggesting that silencing LncRNA-PVT1 might block the transition from G0/G1 phase to S phase in MMCs. Furthermore, the results of qRT-PCR and Western blot assay were used to reveal the expression of cyclin D1 and CDK4 (Figure 3C-E). The result showed that CyclinD1 and CDK4 in the HG + siNC group was significantly overexpressed than those in the NG group (all $P < 0.05$). Meanwhile, the expression of CyclinD1 and CDK4 in the HG + siPVT1 group was not significantly different from those in the HG group ($P > 0.05$). The relative expression of CyclinD1 and CDK4 in the HG + siPVT1 group was significantly lower than those in the HG group ($P < 0.05$).

**PVT1 Promoted migration and invasion of MMCs cultured in high glucose**

The results of scratch and Transwell assay (Figure 4A-B) showed that compared with the NG group, the cell migration and invasion ability were enhanced in HG group ($P < 0.05$). Meanwhile, compared with the, the difference in cell migration and invasion ability between HG group and HG + siNC group was not significant (all $P > 0.05$). Moreover, compared with HG+siPVT1 group, the cell migration and invasion ability were significantly lower than that in HG group ($P<0.05$). Furthermore, the expression of EMT-related protein was detected by Western blot (Figure 4C). The result showed that compared with NG group, the expression of E-cadherin was decreased, the expression of N-cadherin and vimentin were up-regulated ($P<0.05$). Compared with HG group, the expression of E-cadherin was significantly higher than that of HG + siPVT1 group ($P < 0.05$). Meanwhile, compared with NG group, the expression of N-cadherin and vimentin were significantly higher than those of HG group (all $P < 0.05$).
**PVT1 promoted fibrosis of MMCs cultured in high glucose**

The expression of fibrosis factors Col. IV, FN, TGF-β1 and PAI-1 were detected by ELISA (Figure 5 A-D). The result showed that the contents of Col. IV, FN, TGF-β1 and PAI-1 in the supernatant of HG group were significantly higher than those in NG group (P<0.05). Compared with HG + siNC group, there was no significant difference of these content between HG group and HG + siNC group (all P > 0.05). Meanwhile, the contents of Col. IV, FN, TGF-β1 and PAI-1 in HG+siPVT1 group were significantly lower than those in HG group (P<0.05). Furthermore, Western blot analysis showed that the expression trends of Col. IV, FN, TGF-β1 and PAI-1 were consistent with the results of ELISA (Figure 5 E).

**MiR-93-5p was the target gene of PVT1**

Since PVT1 was highly expressed in the HG group, the targeting relationship between miR-93-5p and PVT1 was predicted by StarBase3.0 software using bioinformatics technology (Figure 6 A-B). The result showed that the luciferase activity in the WT + mimics group was significantly lower than that in the WT + NC group (P < 0.05). However, there was no significant difference in the luciferase activity between the MT + mimics group and the MT + NC group (P > 0.05). Moreover, qRT-PCR was used to detect the expression of PVT1 in miR-93-5p mimics group and miR-93-5p inhibitor group (Figure 6C). The result showed that the expression of PVT1 in the inhibitor group was significantly higher than that in the inhibitor group (INC group). The expression of PVT1 in mimics group was significantly lower than that in mim-NC group (P < 0.05). However, the expression levels of PVT1 among mim-NC group, INC group and Mock group were not significant (all P > 0.05). In addition, qRT-PCR was used to detect the expression level of miR-93-5p in each group (Figure 6D). The result showed that the relative expression of miR-93-5p in HG group was significantly lower than that in NG group (P < 0.05). Meanwhile, the relative expression of miR-93-5p in HG + siPVT1 group was significantly higher than that in HG + siNC group (P
< 0.05). These results indicated that PVT1 might negatively regulate the expression of miR-93-5p in MMCs.

**MiR-93-5p eliminated the role of PVT1 in high glucose cultured MMCs**

Inhibition efficiency analysis of miR-93-5p inhibitor showed that the expression of miR-93-5p in the inhibitor group was significantly lower than that in the Mock group and the INC group (all P < 0.05), indicating that the miR-93-5p inhibitor might effectively inhibit the expression of miR-93-5p (Figure 7A). The interaction analysis between miR-93-5p and PVT1 showed that compared with the siNC + INC group, the cell proliferation, migration and invasion ability in siNC + miR-93-5p inhibitor group were significantly increased (all P < 0.05), and the cell proliferation, migration and invasion ability in siPVT1 + INC group were significantly decreased (all P < 0.05) (Figure 7B-E). This result indicated that the effect of PVT1 overexpression induced by miR-93-5p inhibitor was removed by siPVT1.

**PI3K/Akt/mTOR pathway was activated by PVT1-miR-93-5p under high glucose**

The expression of PI3K/Akt/mTOR pathway related protein and protein phosphorylation were detected by Western blot (Figure 8). The result showed that the relative expression of p-PI3K, p-Akt, p-mTOR in siNC + miR-93-5p inhibitor group was significantly higher than those in siNC + INC group (all P < 0.05). The relative expression levels of p-PI3K, p-Akt and p-mTOR in siPVT1 + miR-93-5p inhibitor group were not significantly different from those in siNC + INC group (all P > 0.05). Meanwhile, the relative expression levels of p-PI3K, p-Akt and p-mTOR in siPVT1 + INC group were significantly lower than those in siNC + INC group (all P < 0.05). This result indicated that the miR-93-5p inhibitor counteracted the effect of siPVT1 on phosphorylation of the PI3K/Akt/mTOR pathway related protein.

**Discussion**

DN has become the single most common condition found in patients with renal disease. Although genes such as IncRNA-PVT1 has been proved to associated with DN, the detail
molecular mechanism of PVT1 in DN progression is still unclear. The results of current analysis showed that PVT1 was overexpressed in both mice kidney tissue and high glucose cultured MMCs. Meanwhile, PVT1 could not only promoted proliferation, migration, invasion and fibrosis of high glucose cultured MMCs, but also regulated the cell cycle from G0/G1 to S phase. Moreover, PVT1 negatively regulated the expression of miR-93-5p in MMCs. Furthermore, the PVT1-miR-93-5p regulatory relation activated the PI3K/Akt/mTOR pathway, which in turn regulated cell proliferation and insulin signaling in high glucose cultured MMCs.

MCs play a central role in the development of DN because they regulate glomerular filtration rate through their contractility [21]. Over-proliferation of MCs is believed to take part in the development of NC [22]. During this process, the overexpression of lncRNAs are proved to be vital for diabetic kidneys and high glucose-treated MCs [23]. Li et al. showed that lncRNAs such as lncRNA-1700020I14Rik contributed to the fibrosis of MMCs cultured with high glucose medium [24]. A previous study based on cell counting kit-8, Western blot, and qRT-PCR showed that proliferation and fibrosis indexes are reversed in MCs with IncRNA-ENSMUST00000147869 overexpression, which further indicating a potential role of IncRNAs in proliferation and fibrosis of MCs [25]. This effect of IncRNAs in the regulation of proliferation and fibrosis in DN has been proved by Wang et al. [26]. Actually, as a member of IncRNAs, PVT1 also participates in proliferation, migration, invasion and fibrosis of various disease [27, 28]. A previous study shows that PVT1 overexpression is a prognostic biomarker and regulates migration and invasion in small cell lung cancer [29]. Zhao et al. indicated that PVT1 promoted the proliferation and migration in pancreatic cancer cells via acting as a molecular targeting miR-448 [30]. Lucrecia Alvarez et al. showed that PVT1 expression levels increased up to 5-fold in response to hyperglycemic conditions, showing an effect of glucose on PVT1 regulation in
DN [31]. In the current study, the EdU assay, colony formation assay, Western blot, flow cytometry analysis, ELISA, scratch assay and Transwell assay showed that high expression of IncRNA-PVT1 significantly increased the proliferation, migration, invasion ability as well as cell fibrosis in MMCS cells. Thus, we speculated that PVT1 might promoted proliferation, migration, invasion and fibrosis in high glucose cultured MMCs, which further affected the development of DN.

Some miRNAs (such as miR-1204 and miR-1205) mapping to the PVT1 locus play important roles in disease progression [32]. A previous study shows that IncRNA-miR-93 regulatory relation can inactivate PI3K/AKT pathway, which further take part in the process of tumor cell growth [33]. MiR-93 is a critical metabolic/epigenetic switch in the diabetic milieu linking the metabolic state to chromatin remodeling [34]. Zou et al. indicated that plasma level of miR-93 was associated with higher risk of diabetes related diseases [35]. Furthermore, some regulatory pathways such as PI3k/Akt/mTOR play an vital role in biological process of diabetic kidneys [36]. The phosphorylation of AKT and mTOR are landmark reaction during DN [37]. A previous study shows that Akt/mTOR signaling pathway is a potential drug therapy target to prevent renal fibrosis in DN [38]. Actually, miRNAs such as miR-196b and miR-451 can regulate cancer cell proliferation and invasion via PI3K/AKT/mTOR signaling pathway [39, 40]. In the current study, the Western blot show that miR-93-5p inhibitor counteracted the effect of siPVT1 on phosphorylation of PI3K/Akt/mTOR pathway proteins. Thus, we speculated that the PVT1-miR-93-5p regulatory relation activated the PI3K/Akt/mTOR pathway, which in turn regulated cell proliferation and insulin signaling in high glucose cultured MMCs.

Conclusions

PVT1 might promoted proliferation, migration, invasion and fibrosis in high glucose cultured MMCs, which further affected the development of DN. Furthermore, PVT1-miR-93-
5p regulatory relation activated the PI3K/Akt/mTOR pathway, which in turn regulated cell proliferation and insulin signaling in high glucose cultured MMCs.

Abbreviations

LncRNA- plasmacytoma variant translocation 1 (PVT1)

Mouse mesangial cells (MMCs)

diabetic nephropathy (DN)

real-time fluorescence quantitative polymerase chain reaction (RT-qPCR)

enzyme-linked immunosorbent assay (ELISA)

end-stage renal disease (ESRD)

microRNAs (miRNAs)

PVT1 siRNA (si-PVT1)

negative control group (si-NC)

blank control group (Mock)

si-PVT1 experimental group (siPVT1)

control group (NG)

high glucose group (HG)

high glucose negative control group (HG + siNC)

high glucose siPVT1 group (HG + siPVT1)

Declarations

Ethics approval and consent to participate

This study was approved by the ethics committee of Chongqing Medical University, and all experiments were in accordance with the guide for the care and use of laboratory animals established by United States National Institutes of Health.
Consent to publish

All contributing authors have agreed to submit this manuscript and all authors approved to publish this study.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding: None.

Authors’ Contributions

JZL, QZ and XHJ were involved in the conception and design of the study, and development of the manuscript. YHL was participated in the conduct of the experiment and collection of data. JS participated in the drafting or critical revision of the manuscript. All authors read and approved the final manuscript.

Acknowledgments: None.

References

1. Ritz E: Diabetic nephropathy. Saudi J Kidney Dis Transpl 2006, 17(4):481-490.

2. Yan D, Choi ME: Autophagy in diabetic nephropathy. J Endocrinol 2015, 224(1):R15.

3. Su Y: Recent advances in understanding the biochemical and molecular mechanism of diabetic nephropathy. Biochem Biophys Res Commun 2013, 433(4):359-361.

4. Huang Z, Mou Y, Xu X, Zhao D, Lai Y, Xu Y, Chen C, Li P, Peng S, Tian J: A Novel Derivative of Bardoxolone Methyl Improves Safety for Treatment of Diabetic Nephropathy. J Med Chem 2017, 60(21):acs.jmedchem.7b00971.
5. Egido J, Rojasrivera J, Mas S, Ruizortega M, Sanz AB, Gonzalez PE, Gomezguerrero C: Atrasentan for the treatment of diabetic nephropathy. *Expert Opin Investig Drugs* 2017, **26**(6).

6. Correa-Rotter R, Gonz̲á I-ML: Early detection and prevention of diabetic nephropathy: a challenge calling for mandatory action for Mexico and the developing world. *Kidney Int* 2005, **68**(98):S69.

7. UA Ø, Derrien T, Beringer M, Gumireddy K, Gardini A, Bussotti G, Lai F, Zytnicki M, Notredame C, Huang Q: Long noncoding RNAs with enhancer-like function in human cells. *Med Sci (Paris)* 2010, **143**(1):46-58.

8. Maass PG, Rump A, Schulz H, Stricker S, Schulze L, Platzer K, Aydin A, Tinschert S, Goldring MB, Luft FC: A misplaced IncRNA causes brachydactyly in humans. *J Clin Invest* 2012, **122**(11):3990-4002.

9. Kotake Y, Nakagawa T, Kitagawa K, Suzuki S, Liu N, Kitagawa M, Xiong Y: Long non-coding RNA ANRIL is required for the PRC2 recruitment to and silencing of p15(INK4B) tumor suppressor gene. *Oncogene* 2011, **30**(16):1956.

10. Khaitan D, Dinger ME, Mazar J, Crawford J, Smith MA, Mattick JS, Perera RJ: The melanoma-upregulated long noncoding RNA SPRY4-IT1 modulates apoptosis and invasion. *Cancer Research* 2011, **71**(11):3852.

11. Hu M, Wang R, Li X, Fan M, Lin J, Zhen J, Chen L, Lv Z: LncRNA MALAT1 is dysregulated in diabetic nephropathy and involved in high glucose-induced podocyte injury via its interplay with β-catenin. *J Cell Mol Med* 2017, **21**(11):2732-2747.

12. The efficiency of serum IncRNA GAS5/miR-21 as biomarkers in patients with diabetes and diabetic nephropathy. *China Medical Abstracts (Internal Medicine)* 2018, **v.35**(1):55-56.
13. Hanson RL, Craig DW, Millis MP, Yeatts KA, Kobes S, Pearson JV, Lee AM, Knowler WC, Nelson RG, Wolford JK: Identification of PVT1 as a candidate gene for end-stage renal disease in type 2 diabetes using a pooling-based genome-wide single nucleotide polymorphism association study. Diabetes 2007, 56(4):975.

14. Li Z, Hao S, Yin H, Gao J, Yang Z: Autophagy ameliorates cognitive impairment through activation of PVT1 and apoptosis in diabetes mice. Behav Brain Res 2016, 305:265-277.

15. M Lucrecia A, Distefano JK: Functional characterization of the plasmacytoma variant translocation 1 gene (PVT1) in diabetic nephropathy. Plos One 2011, 6(4):e18671.

16. Allison SJ: Diabetic nephropathy: A IncRNA and miRNA megacluster in diabetic nephropathy. Nat Rev Nephrol 2016, 12(12):713-713.

17. He RQ, Qin MJ, Lin P, Luo YH, Ma J, Yang H, Hu XH, Chen G: Prognostic Significance of LncRNA PVT1 and Its Potential Target Gene Network in Human Cancers: a Comprehensive Inquiry Based Upon 21 Cancer Types and 9972 Cases. Cell Physiol Biochem 2018, 46(2):591.

18. Alvarez ML, Khosroheidari M, Eddy E, Kiefer J: Role of microRNA 1207-5P and its host gene, the long non-coding RNA Pvt1, as mediators of extracellular matrix accumulation in the kidney: implications for diabetic nephropathy. Plos One 2013, 8(10):e77468.

19. Yang Y, Zhang Y, Xie A: Expression and significance of serum miRNA-93 in diabetic nephropathy patients. Experimental & Laboratory Medicine 2012.

20. Livak KJ ST: Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2(-Delta Delta C(T))Method. METHODS 2001, Dec;25(4):402-408.
21. Li W, Ding Y, Smedley C, Wang Y, Chaudhari S, Birnbaumer L, Ma R: Increased glomerular filtration rate and impaired contractile function of mesangial cells in TRPC6 knockout mice. Sci Rep 2017, 7(1):4145.

22. Ding T, Chen W, Li J, Ding J, Mei X, Hu H: High Glucose Induces Mouse Mesangial Cell Overproliferation via Inhibition of Hydrogen Sulfide Synthesis in a TLR-4-Dependent Manner. Cell Physiol Biochem 2017, 41(3):1035.

23. Yan G, Chen ZY, Yan W, Yan L, Ma JX, Li YK: Long non-coding RNA ASncmtRNA-2 is upregulated in diabetic kidneys and high glucose-treated mesangial cells. Experimental & Therapeutic Medicine 2017, 13(2):581-587.

24. Ai-Ling LI, Peng R, Sun Y, Peng HM, Hong YI, Zhang Z: Effect of IncRNA-170002014Rik on the fibrosis in mouse mesangial cells in high glucose concentration. Basic & Clinical Medicine 2017.

25. Wang M, Yao D, Wang S, Yan Q, Lu W: Long non-coding RNA ENSMUST00000147869 protects mesangial cells from proliferation and fibrosis induced by diabetic nephropathy. Endocrine 2016, 54(1):1-12.

26. Wang M, Wang S, Yao D, Yan Q, Lu W: A novel long non-coding RNA CYP4B1-PS1-001 regulates proliferation and fibrosis in diabetic nephropathy. Molecular & Cellular Endocrinology 2016, 426(C):136-145.

27. Yang YR, Zang SZ, Zhong CL, Li YX, Zhao SS, Feng XJ: Increased expression of the IncRNA PVT1 promotes tumorigenesis in non-small cell lung cancer. Int J Clin Exp Pathol 2014, 7(10):6929.

28. Zhao L, Kong H, Sun H, Chen Z, Chen B, Zhou M: LncRNA-PVT1 promotes pancreatic cancer cells proliferation and migration through acting as a molecular sponge to regulate miR-448. J Cell Physiol 2018, 233(5).

29. Huang C, Liu S, Wang H, Zhang Z, Yang Q, Gao F: LncRNA PVT1 overexpression is
a poor prognostic biomarker and regulates migration and invasion in small cell lung cancer. *Am J Transl Res* 2016, **8**(11):5025.

30. Zhao L, Kong H, Sun H, Chen Z, Chen B, Zhou M: **LncRNA-PVT1 promotes pancreatic cancer cells proliferation and migration through acting as a molecular sponge to regulate miR-448. J Cell Physiol** 2018, **233**(5):4044-4055.

31. Alvarez ML, Distefano JK: **Functional Characterization of the Plasmacytoma Variant Translocation 1 Gene (PVT1) in Diabetic Nephropathy. PLOS ONE** 2011, **6**(4).

32. Huppi K, Volfovsky N, Runfola T, Jones TL, Mackiewicz M, Martin SE, Mushinski JF, Stephens R, Caplen NJ: **The identification of microRNAs in a genomically unstable region of human chromosome 8q24. Mol Cancer Res** 2008, **6**(2):212.

33. Zhang L, Liang X, Li Y: **Long non-coding RNA MEG3 inhibits cell growth of gliomas by targeting miR-93 and inactivating PI3K/AKT pathway. Oncol Rep** 2017, **38**(4):2408.

34. Badal SS, Yin W, Long J, Corcoran DL, Chang BH, Luan DT, Kanwar YS, Overbeek PA, Danesh FR: **miR-93 regulates Msk2-mediated chromatin remodelling in diabetic nephropathy. Nat Commun** 2016, **7**:12076.

35. Zou HL, Wang Y, Gang Q, Zhang Y, Sun Y: **Plasma level of miR-93 is associated with higher risk to develop type 2 diabetic retinopathy. Graefes Arch Clin Exp Ophthalmol** 2017, **255**(6):1-8.

36. Huang C, Lin MZ, Cheng D, Filip B, Pollock CA, Chen XM: **KCa3.1 mediates dysfunction of tubular autophagy in diabetic kidneys via PI3k/Akt/mTOR signaling pathways. Sci Rep** 2016, **6**:23884.

37. Mavroeidi V, Petrakis I, Stylianou K, Katsarou T, Giannakakis K, Perakis K, Vardaki E, Stratigos S, Ganotakis E, Papavasiliou S: **Losartan affects glomerular AKT and **
mTOR phosphorylation in an experimental model of type 1 diabetic nephropathy. J Histochem Cytochem 2013, 61(6):433-443.

38. Lu Q, Zuo WZ, Ji XJ, Zhou YX, Liu YQ, Yao XQ, Zhou XY, Liu YW, Yin XX: Ethanol Ginkgo biloba leaf extract prevents renal fibrosis through Akt/mTOR signaling in diabetic nephropathy. Phytomedicine 2015, 22(12):1071-1078.

39. Du J, Liu S, He J, Liu X, Qu Y, Yan W, Fan J, Li R, Xi H, Fu W: MicroRNA-451 regulates stemness of side population cells via PI3K/Akt/mTOR signaling pathway in multiple myeloma. Oncotarget 2015, 6(17):14993-15007.

40. Li NA, Wang W, Xu B, Gong H: miR-196b regulates gastric cancer cell proliferation and invasion via PI3K/AKT/mTOR signaling pathway. Oncol Lett 2016, 11(3):1745-1749.

Tables

Table 1 The siRNAs and amplified sequences of genes and their primers
| Primer            | Sequence                                      |
|------------------|-----------------------------------------------|
| **siPVT1**       | Forward: 5′-GCUUGGAGGCUGAGGAGUUTT-3′          |
|                  | Reverse: 5′-AACUCCUCAGCCUCCAAGCTT-3′          |
| **siNC**         | Forward: 5′-UUCUCCGAACGUGUCACGUTT-3′          |
|                  | Reverse: 5′-ACGUGACACGUUCGGAGAATT-3′          |
| **LncRNA-PVT1**  | Forward: 5′-ATTGAGATGTGAAGCGTTGA-3′           |
|                  | Reverse: 5′-AGGCACCTTTCCAGTT-3′               |
| **CyclinD1**     | Forward: 5′-AGCTCCTGTGCTGGAAGTGGA-3′          |
|                  | Reverse: 5′-AGTGTTCAATGAAATCGTGGG-3′          |
| **CDK4**         | Forward: 5′-AGTAATGGGACCGTCAAGC-3′            |
|                  | Reverse: 5′-CACCAAGACTGGGAAAGG-3′             |
| **miR-93-5P**    | Forward: 5′-AGGCCCAAAGTGCTGTTCGT-3′           |
|                  | Reverse: 5′-GTGCAGGGTCCGAGG-3′                |
| **U6**           | Forward: 5′-CTCGCTTCGGCAGCACA-3′              |
|                  | Reverse: 5′-AACGCTTCAGAATTGCCT-3′             |
| **GAPDH**        | Forward: 5′-TGACGTGCGCTGGAGAAGAC-3′           |
|                  | Reverse: 5′-CCGGCATCGGAAGTGGAAGAG-3′          |

**Figures**
Expression of LncRNA-PVT1 in renal tissues and mesangial cells. A, qRT-PCR was used to detect the expression of LncRNA-PVT1 in kidney tissues of db/db DN mice; *, P<0.05 compared with db/m mice group. B, detection of LncRNA-PVT1 in qRT-PCR Expression in membrane cells; *, P < 0.05 compared to the NG group.
Figure 2

Effect of IncRNA-PVT1 on proliferation of MMCs cells under high glucose conditions. A, RNA interference efficiency test; *, P<0.05 compared with HG group. B, EdU test detected the proliferation ability of each group (×200). C, clonal formation assay detected the colony forming ability of each group. D, flow cytometry was used to detect the apoptosis results of each group. E, Western blot was used to detect the expression of apoptotic proteins in each group. One-Way ANOVA one-way analysis of variance was used among the groups; Tukey's multiple comparisons test was used for two groups comparison after AVOVA analysis. *, P < 0.05 when compared with NG group; #, P < 0.05 when compared with HG group.
Figure 3

Effect of IncRNA-PVT1 on cell cycle of MMCs under high glucose conditions. A, the results of cell cycle distribution detected by flow cytometry. B, the proportion of cell cycle distribution in each group. C, the relative expression of CyclinD1 in
each group. D, the relative expression of CDK4 in each group. E, the relative expression of CyclinD1 and CDK4 in each group. One-Way ANOVA one-way analysis of variance was used among the groups; Tukey's multiple comparisons test was used for two groups comparison after AVOVA analysis. *, P < 0.05 when compared with NG group; #, P < 0.05 when compared with HG group.
Effects of IncRNA-PVT1 on migration and invasion of MMCs cells under high glucose conditions. A, cell scratch test was used to detect the migration ability of each group (×200). B, Transwell chamber assay was used to detect the invasive ability of each group (×200). C, Western blot was used to detect the expression of EMT protein in each group. One-Way ANOVA one-way analysis of variance was used among the groups; Tukey's multiple comparisons test was used for two groups comparison after AVOVA analysis. *, P < 0.05 when compared with NG group; #, P < 0.05 when compared with HG group.
Figure 5

Effect of lncRNA-PVT1 on fibrosis of MMCs cells under high glucose conditions. A-D, ELISA was used to detect the content of Col. IV, FN, TGF-β1, and PAI-1 in each group. E, the relative expression levels of fibrosis marker proteins Col. IV, FN, TGF-β1 and PAI-1 in each group detected by Western blot. One-Way ANOVA one-way analysis of variance was used among the groups; Tukey’s multiple comparisons test was used for two groups comparison after AVOVA analysis. *, P < 0.05 when compared with NG group; #, P < 0.05 when compared with HG group.
MiR-93-5p was the target gene of LncRNA-PVT1. A, StarBase3.0 software predicted the targeting binding sites of miR-93-5p to LncRNA-PVT1. B, the result of dual luciferase reporter gene activity detection; *, *P<0.05 when compared with WT+NC group. C, the overexpression and interference of PVT1 expression in the miR-93-5p group; *, *P<0.05 when compared with the mim-NC group; #, *P<0.05 when compared with the INC group. D, the relative expression of miR-93-5p in the group; *, *P<0.05 when compared with NG group; #, *P<0.05 when compared with the HG group. One-Way ANOVA one-way analysis of variance was used among the groups; Tukey's multiple comparisons test was used for two groups comparison after AVOVA analysis. *, *P<0.05 when compared with NG group.

Data were expressed as mean ± standard deviation.
Figure 7

MiR-93-5p eliminated the role of LncRNA-PVT1 in high glucose cultured MMCs. A, miR-93-5p inhibitor inhibition rate; *, P < 0.05 when compared with the Mock group. B, Edu detection of miR-93-5p and LncRNA-PVT1 interaction in MMCs cell proliferation. C, scratch test was used to detect the effect of miR-93-5p interaction with LncRNA-PVT1 on cell migration ability in MMCs cells. D, Transwell assay was used to detect the miR-93-5p interaction with LncRNA-PVT1 in MMCs cells. E, Western blot analysis revealed the effect of miR-93-5p and LncRNA-PVT1 interaction on MMCs fibrosis. *, P < 0.05 when compared with siNC + INC; #, P < 0.05 when compared with siNC + miR-93-5p inhibitor; &, P < 0.05 when compared with siPVT1+INC group.
LncRNA-PVT1-miR-93-5p activated the PI3K/Akt/mTOR pathway. Western blot was used to detect the relative expression of PI3K/Akt/mTOR protein in each group.

One-Way ANOVA one-way analysis of variance was used among the groups; Tukey's multiple comparisons test was used for two groups comparison after AVOVA analysis. *, P < 0.05 when compared with siNC + INC; #, P < 0.05 when compared with siNC + miR-93-5p inhibitor; &, P < 0.05 when compared with siPVT1 + INC group.

Supplementary Files

This is a list of supplementary files associated with the primary manuscript. Click to download.

NC3Rs ARRIVE Guidelines Checklist (fillable).pdf