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PVT1 protects diabetic peripheral neuropathy via PI3K/AKT pathway

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Abstract. – OBJECTIVE: To investigate whether Plasmacytoma Variant Translocation 1 (PVT1) could regulate the occurrence and progression of diabetic peripheral neuropathy (DPN) via activating the PI3K/AKT pathway.

MATERIALS AND METHODS: Diabetes model in rats was constructed by streptozotocin (STZ) injection. PVT1 expression in diabetic rats and control rats was detected by quantitative real time-polymerase chain reaction (qRT-PCR). Rats were injected with PVT1 overexpression lentivirus or vector, respectively, followed by determination of mechanical withdrawal threshold (MWT), thermal withdrawal latency (TWL) and sensory nerve conduction velocity (SNCV). Cell apoptosis of dorsal root ganglia (DRG) was accessed by TUNEL. Western blot was performed to detect the expressions of neurodegeneration-related genes and neurogenesis-related genes. The regulatory effect of PVT1 on the PI3K/AKT pathway was detected by Western blot.

RESULTS: PVT1 was downregulated in diabetic rats compared with that of controls. Diabetic rats presented higher MWT, TWL and SNCV. Cell apoptosis of DRG was pronounced in diabetic rats. The amount of inflammation-related glial cells increased in diabetic rats. PVT1 overexpression remarkably decreased MWT and TWL. PVT1 downregulated expressions of neurodegeneration-related genes and upregulated neurogenesis-related genes. Western blot results suggested that PI3K/AKT pathway in diabetic rats was blocked, which was reversed by PVT1 overexpression.

CONCLUSIONS: PVT1 is lowly expressed in diabetic rats, leading to decreased mechanical withdrawal threshold, thermal withdrawal latency and sensory nerve conduction velocity. PVT1 protects diabetic peripheral neuropathy via PI3K/AKT pathway.

Key Words:
PVT1, PI3K/AKT Pathway, Diabetic Peripheral Neuropathy, Dorsal Root Ganglia.

Introduction

Peripheral neuropathy is one of the most common neurological disorders caused by multiple etiologies. It can affect the sensory, motor, and autonomic functions of affected patients. Diabetic peripheral neuropathy (DPN) is the most frequent complication of diabetes mellitus (DM). In DM patients with over 10-year disease course, more than 50% present varying degrees of peripheral neuropathy[1]. DPN is manifested with impaired small nerve fibers and autonomic nerves, which is the main reason for the loss of working ability of DM patients[2]. In recent years, the incidences of DM and DPN have risen. It is reported that DPN costs 10.9 billion of medical expense in the U.S. every year[3,4]. Currently, there is no effective treatment for DPN. Symptomatic treatments are the preferred methods for DPN patients, such as neurotrophy and circulation improvement. Due to the high incidence and serious impacts on DPN, it has achieved great concern in clinical researches.

LncRNA (long non-coding RNA) is a type of non-coding RNA located in the nucleus or cytoplasm with more than 200 nt in length. It has a relatively long nucleotide chain with a specific and complex secondary spatial structure, which provides multiple positions to combine with target proteins. LncRNA is involved in a complex and precise regulatory network alongside with DNA and RNA, thereafter regulating downstream gene function[5,6]. LncRNA exerts tissue specificity, cell specificity, developmental stage specificity, space-time specificity and disease specificity, which is widely involved in cell differentiation, metabolism and proliferation[7,8].

Plasmacytoma Variant Translocation 1 (PVT1) is located in the 8q24.21 region containing the well-known MYC proto-oncogene. Studies have

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shown that MYC overexpression promotes PVT1 accumulation in primary tumors9. In addition, in vitro experiments showed that PVT1 overexpression can inhibit the apoptosis of colon cancer cells, suggesting a poor prognosis of colon cancer patients. PVT1 promotes the proliferation of hepatocellular carcinoma by stabilizing nuclear NOP210, 11. However, the biological function of PVT1 in DPN and its potential mechanism are not fully elucidated.

Materials and Methods

Diabetes Rat Model Construction

Male Sprague Dawley rats weighing 280-310 g were selected. After 12 h fasting, 1% streptozotocin (STZ) diluted in citrate buffer was intraperitoneally injected in rats at a dose of 55 mg/kg. Rats in the control group received an intraperitoneal injection of isodose citrate buffer. 48 h later, a blood sample of each rat was collected from the tail vein for detecting fasting blood-glucose level. The diabetes rat model was considered to be successfully established when the fasting blood-glucose level in rats was detected to be higher than 16.67 mmol/L. Diabetic rats were further injected with PVT1 overexpression lentivirus or vector at the 6th week. Rats were sacrificed one week after lentivirus injection for extracting DRG. Lentiviruses were obtained from GenePharma (Shanghai, China). This investigation was approved by the Xiangyang Central Hospital Ethics Committee.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA in treated cells was extracted using TRIzol method (Invitrogen, Carlsbad, CA, USA) for reverse transcription according to the instructions of PrimeScript RT reagent Kit (TaKaRa, Otsu, Shiga, Japan). The RNA concentration was detected using the spectrometer. QRT-PCR was then performed based on the instructions of SYBR Premix Ex Taq TM (TaKaRa, Otsu, Shiga, Japan). The relative gene expression was calculated using the 2-ΔCt method. Primers used in the study were as follows: PVT1, F: 5'-GGGGTACCCTCCGGGCAGAGCGCGTGTG-3', R: 5'-CGGGATCCTAGACACGAGGCCGGCCACGC-3'; GFAP, F: 5'-CTGCGGCTCGATCAACTCA-3', R: 5'-TCCAGCGACTCAATCTTCCTC-3'; TNF-α, F: 5'-AGGTCCATGTGGAGCTTGAC-3', R: 5'-GCCATTGCCTCATACTGCGT-3'; P2X7, F: 5'-CAACGGGTCAGACGGGAAG-3', R: 5'-GAATTTCCACTCACCTACCACC-3'; SOD1, F: 5'-GGTGGGCCAAAGGATGAAGAG-3', R: 5'-CCACAAGCCAAACGACTTCC-3'; Drd2, F: 5'-CTCTTCGGACTCAATAACGCAG-3', R: 5'-GACGATGGAGGAGTAGACCAC-3'; Uch-L1, F: 5'-CCCCGCCAAACCAGAGAAG-3', R: 5'-TTTTGCCATTGGGCATGGTCT-3'; S100B, F: 5'-TGGCCCTCATCGACGTTTTC-3', R: 5'-ATGTTCAAAGAACTCGTGGCA-3'; Notch1, F: 5'-GAGGCGTGGCAGACTATGC-3', R: 5'-CTTGTACTCCGTCAGCGTGA-3'; GAPDH, F: 5'-ACCCACTCCTCCACCTTTGA-3', R: 5'-CTGTTGCTGTAGCCAAATTCGT-3'.

Western Blot

Cells were lysed with phenylmethylsulfonyl fluoride (PMSF) (Beyotime, Shanghai, China), followed by centrifugation at 12 000 rpm/min for 20 min. The supernatant was collected for detecting the total protein concentration using the BCA (bicinchoninic acid) method (Pierce, Rockford, IL, USA). Protein samples were separated by gel electrophoresis and transferred to PVDF (polyvinylidene difluoride) membranes (Roche, Basel, Switzerland). After incubation with primary and secondary antibodies, protein bands were determined using Image J Software.

Determination of Mechanical Withdrawal Threshold (MWT)

Rats were previously placed on a mental chamber for 15-min habituation period. Mechanical stimulation was triggered at the mid-plantar for 6-8 s to observe the withdrawal responses. A positive response was considered an immediate withdrawal during the stimulation period or at the time when removing the von Frey filaments (BME-403, Tianjin, China). Withdrawal responses induced by body activity were not recorded as positive responses. 50% MWT was finally calculated.

Determination of Thermal Withdrawal Latency (TWL)

Rats were previously placed on a transparent acrylic box for 15-min habituation period. Noxious heat stimulation was performed using the Thermal paw Stimulation System (BME-410C, Tianjin, China) at the rat mid-planter. The paw withdrawal latency was recorded from the beginning of heat stimulation to the elevation of the foot. Each hind paw was tested three times with 15 min intervals.

Determination of Sensory Nerve Conduction Velocity (SNCV)

Rat sciatic nerve trunks at sciatic tubercle and talocalcaneal joint were exposed. The proximal and distal stimulus electrode were located in the ischiatic notch and inner ankle, respectively. The recording electrode was 10 cm proximal from the stimulus site. The reference electrode was placed between the stimulus electrode and the recording electrode. Rats were stimulated with 3 V of monopulse. The latency of the action potential from the proximal to the distal sciatic nerve and the distance between recording electrode and stimulus electrodes were recorded.

TUNEL (Terminal Dexynucleotidyl Transferase (TdT)-Mediated dUTP Nick end Labeling)

Paraffin-embedded slices were dehydrated and incubated with Protease K at 37°C for 60 min. After washing with phosphate-buffered saline (PBS) three times, slices were blocked at room temperature for 10 min, followed by reaction with 100 μL of TdT solution in the dark for 60 min. Diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, MO, USA) was added and slices were sealed by neutral gum for detection of apoptotic rate.

Isolation of DRG Non-Neurons

After rat anesthesia with urethane, DRGs were isolated and cultured in DMEM (Dulbecco's modified Eagle's medium) (Gibco, Rockville, MD, USA). The attached nerves and surrounding connective tissues were removed, and the DRGs were minced with dissecting spring scissors. DRGs were incubated in 5 mL of DMEM supplemented with trypsin (0.5 mg/mL; type III, Sigma-Aldrich, St. Louis, MO, USA), collagenase (1.0 mg/mL; type IA, Sigma-Aldrich, St. Louis, MO, USA) and DNase (0.1 mg/mL; type IV, Sigma-Aldrich, St. Louis, MO, USA) at 35°C in a shaking bath for 35-40 min. Enzymatic digestion was terminated by soybean trypsin inhibitor (1.25 mg/mL; type II-S, Sigma-Aldrich, St. Louis, MO, USA). The isolated non-neuronal cells (satellite glial cells) were transferred into a 35 mm culture dish.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM Corp., Armonk, NY, USA) was used for data processing and analysis. The data were expressed as Mean±SD. The t-test was used to compare the mean values of two independent samples. $p<0.05$ was considered statistically significant.

Results

PVT1 Was Highly Expressed in DRG of Diabetic Rats

First, we constructed a diabetes rat model by STZ injection. Diabetic rats presented remarkably higher blood glucose level than that of control rats (Figure 1A). QRT-PCR indicated that PVT1 expression in rat DRG gradually decreased in a time-dependent manner (Figure 1B). Transfection of PVT1 overexpression lentivirus in diabetic rats effectively upregulated PVT1 expression (Figure 1C). These results suggested that PVT1 expression may be associated with abnormal function of DRG.

Effect of PVT1 on MWT, TWL, and SNCV in Diabetic Rats

PVT1 overexpression remarkably elevated MWT and TWL in diabetic rats, which were still

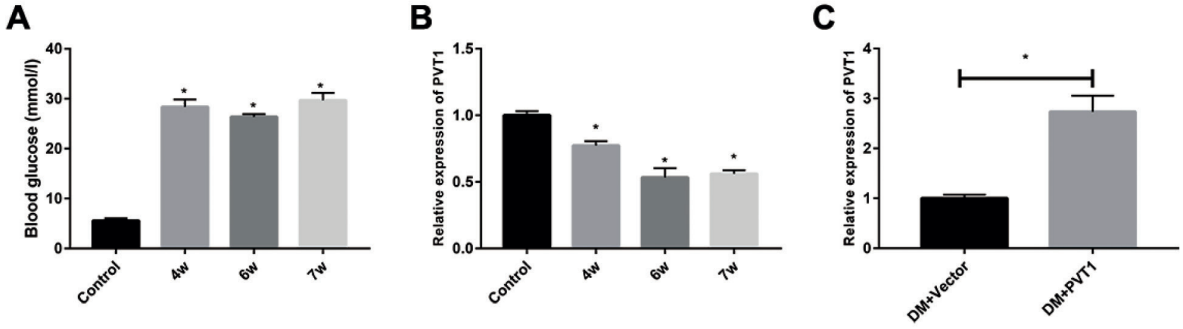

Figure 1. PVT1 was highly expressed in DRG of diabetic rats. ***A,*** Diabetic rats presented remarkably higher blood glucose level than that of control rats. ***B,*** PVT1 expression in rat DRG gradually decreased in a time-dependent manner. ***C,*** Transfection of PVT1 overexpression lentivirus in diabetic rats effectively improved PVT1 expression.

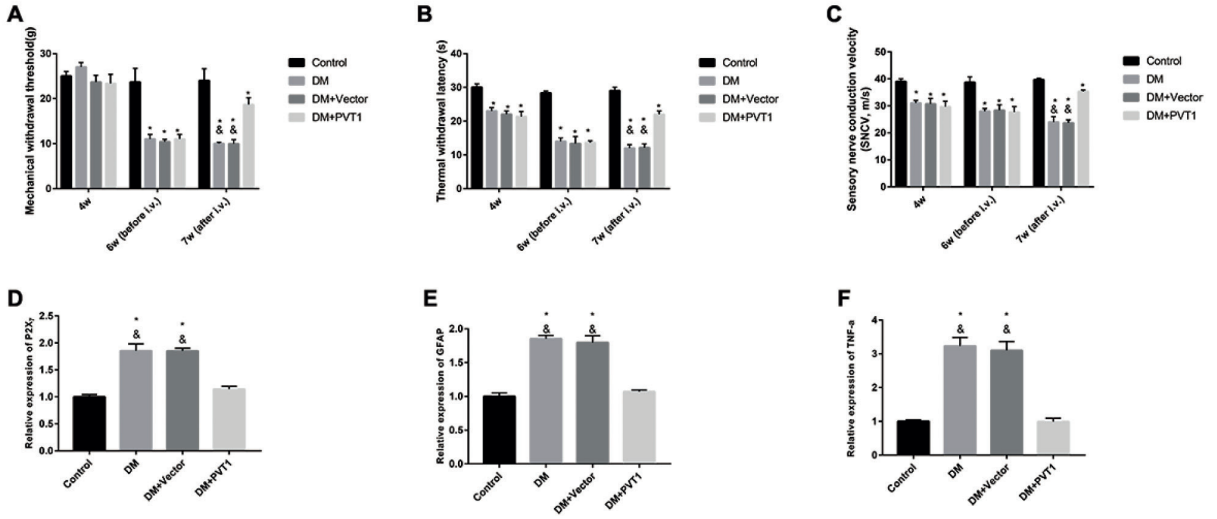

Figure 2. Effect of PVT1 on MWT, TWL and SNCV in diabetic rats. ***A-B,*** PVT1 overexpression remarkably elevated MWT ***(A)*** and TWL ***(B)*** in diabetic rats. However, they were still lower than those of control rats. ***C,*** SNCV in diabetic rats began to decrease since the fourth week, which was partially reversed by PVT1 overexpression. ***D,*** P2X7 expression remarkably increased in diabetic rats, which was reversed by PVT1 overexpression. ***E,*** GFAP level was upregulated, which was reversed by PVT1 overexpression. F, The mRNA level of TNF-α was reversed by PVT1 overexpression.

lower than those of control rats (Figure 2A and 2B). SNCV in diabetic rats began to decrease in the fourth week, which was partially reversed by PVT1 overexpression (Figure 2C). Previous studies have suggested that P2X7 receptors are associated with diabetic neuropathic pain12. Therefore, we hypothesized that PVT1 can regulate P2X7 expression. Our results showed that P2X7 expression remarkably increased in diabetic rats, which was reversed by PVT1 overexpression (Figure 2D). An increased amount of satellite glial cells (SGCs) is also associated with neuropathic pain13. QRT-PCR results indicated that GFAP level, the biomarker for SGCs, was upregulated, which was reversed by PVT1 overexpression as well (Figure 2E). Elevated mRNA level of TNF-α in DRG was also reversed by PVT1 overexpression, indicating the anti-inflammation effect of PVT1 (Figure 2F).

Effect of PVT1 on Apoptosis of DRG

TUNEL results demonstrated obvious cell apoptosis of DRG after PVT1 overexpression (Figure 3A). Subsequently, we detected expressions of neurodegeneration-related genes (Uchl1, Sod1) and neurogenesis-related genes (Drd2, Notch1 and S100b). We found that PVT1 overexpression remarkably inhibited expressions of Uchl1 and Sod1 (Figure 3B). On the contrary, expressions of Drd2, Notch1 and S100b were upregulated by overexpression of PVT1 (Figure 3C).

Effect of PVT1 on PI3K/AKT Pathway

The PI3K/AKT pathway is a classical pathway in the regulation of cell apoptosis. We hypothesized whether PVT1 regulates DRG function through the PI3K/AKT pathway. Western blot results showed that the PI3K/AKT pathway was remarkably inhibited in DRG of diabetic rats (Figure 4A). Overexpression of PVT1 inhibited PI3K/AKT pathway as well (Figure 4B). The above results suggested that PVT1 participates in DPN through PI3K/AKT pathway.

Discussion

Recent studies have suggested that DPN is a consequence of multiple factors and peripheral neuropathy is believed to be the leading cause. Both myelinated and unmyelinated afferent fibers are involved in regulating diabetic neuropathic pain14. It is reported that long-term severe hyperglycemia, metabolic disorders, microcirculatory abnormalities, neurotrophic factor deficiency, increased oxidative stress-free radicals, and autoimmune disorders may contribute to DPN[15,16].

This study explored the effect of PVT1 on neurological function in diabetic rats. Compared with normal mice, PVT1 expression in DRG of diabetic rats remarkably decreased. Diabetic rats presented impaired nerve conduction, manifesting elevated MWT and TWL. Meanwhile, cell apop-

tosis of DRG and expressions of inflammation-related glial cells increased in diabetic rats. Further experiments showed that PVT1 downregulates neurodegeneration-related genes and upregulates neurogenesis-related genes. To investigate the mechanism of PVT1 in regulating DPN development, we examined the effect of PVT1 on the PI3K/AKT pathway. The results indicated that the overexpression of PVT1 can significantly activate the PI3K/AKT pathway in DRG.

Accumulating studies have shown that cell apoptosis is an important mechanism of DPN. Sustained hyperglycemia can induce apoptosis of cervical ganglion cells in diabetic rats, leading to neurite growth inhibition, diameter reduction, string-of-beads and growth cone contraction. Axon growth and neuronal apoptosis are greatly stimulated by the injection of nerve growth factors. Hence, sustained hyperglycemia-induced apoptotic response is considered an important factor in neurodegeneration[17]. In the STZ-induced diabetic rat model, the expression levels of Bcl-2, Bax and Caspase-3 in sciatic nerve remarkably increased. The antioxidant treatment remarkably alleviates cell apoptosis and improves SNCV18. In the early stage of DPN, the thicknesses of the inner plexus and inner layer of the retina were reduced, so as the amount of residual ganglion cells. TUNEL results indicated nerve cell loss in early-stage DPN[19]. In this work, we found that PVT1 can significantly inhibit the apoptosis of DRG cells in diabetic rats. With the in-depth researches on cell apoptosis in DPN, it is possible to develop novel treatments for preventing DM-induced complications via targeting apoptosis.

There were some limitations in this research. STZ-induced diabetes model in rats used in this study was greatly similar to symptoms of DM patients, including chronic peripheral pain[20], vascular permeability increase[21], nerve ischemia damage[22], increased sensory nerve activity, and decreased nerve conduction velocity[23]. Diabetic rats in the study presented a significant increase in blood glucose and water intake, accompanied by decreased body weight and motor dysfunction[24]. However, rats presented the extremely unhealthy state of diabetic rats and large individual difference in body weight during the animal procedures, which may markedly influence the nociceptive threshold[25,26]. Most studies believed that diabetic

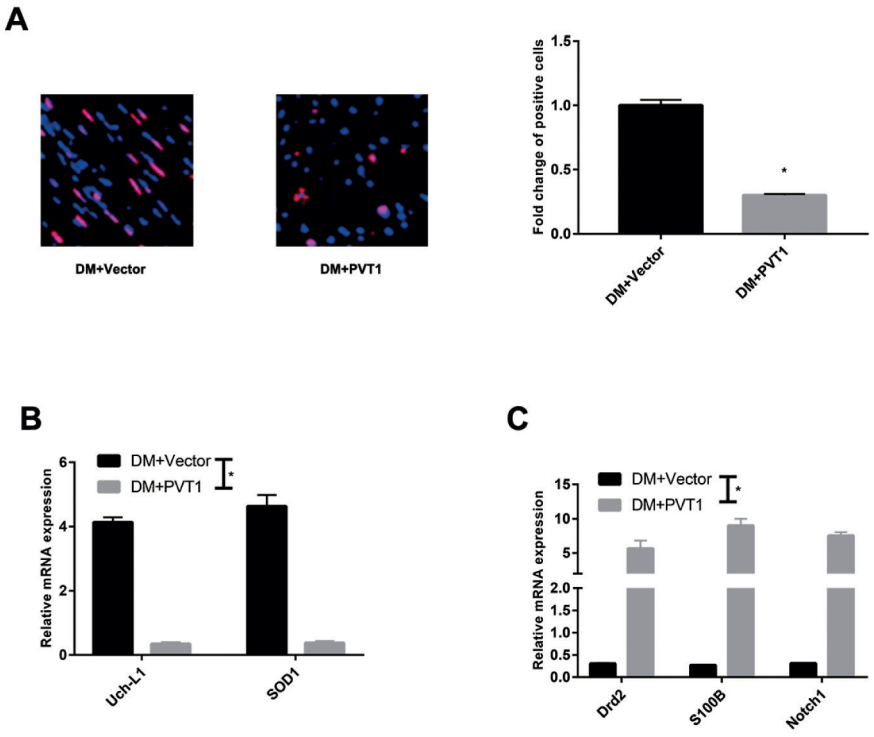

Figure 3. Effect of PVT1 on apoptosis of DRG. ***A,*** TUNEL results demonstrated significant cell apoptosis of DRG after PVT1 overexpression. ***B,*** PVT1 overexpression remarkably inhibited expressions of Uchl1 and Sod1. ***C,*** Expressions of Drd2, Notch1 and S100b were upregulated by overexpression of PVT1.

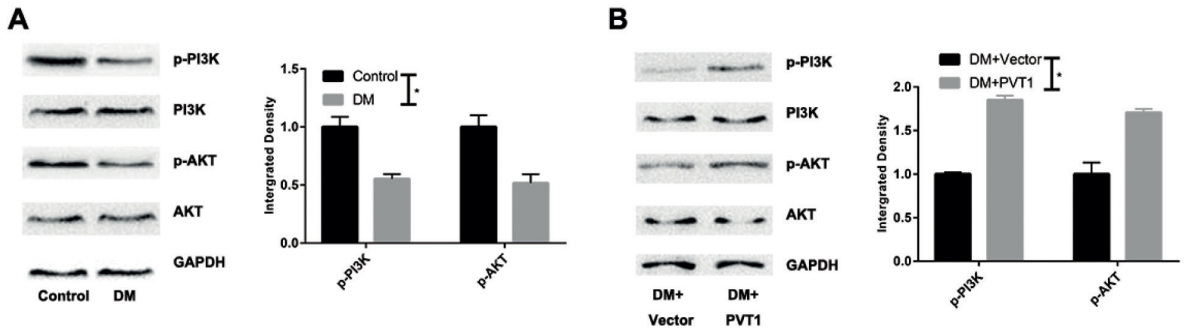

Figure 4. Effect of PVT1 on the PI3K/AKT pathway. ***A,*** Western blot results showed that PI3K/AKT pathway was remarkably inhibited in DRG of diabetic rats. ***B,*** The overexpression of PVT1 inhibited PI3K/AKT pathway in DRG of diabetic rats.

rats exert decreased MWT and SNCV, increased action potential and alterations in the second messenger system (PKA and PKC). On the contrary, relative studies showed controversial results on TWL in diabetic rats. The different correlation between TWL and DM is presumably related to rat species, age, experimental environment and blood glucose level[27-30]. Further investigations should be conducted by using different species of experimental animals, including GK rat, KK mouse, ob/ob mouse, db/db mouse, etc.

Conclusions

We found that PVT1 was lowly expressed in diabetic rats, resulting in decreased mechanical withdrawal threshold, thermal withdrawal latency and sensory nerve conduction velocity. PVT1 protects diabetic peripheral neuropathy via inhibiting the PI3K/AKT pathway.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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