miR-199a-3p is involved in the pathogenesis and progression of diabetic neuropathy through downregulation of SerpinE2

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Abstract. The present study aimed to investigate the expression status of miRNA-199a-3p in patients with diabetic neuropathy (DN) and the mechanism by which this miRNA is involved in the genesis of DN. The expression of miRNA-199a-3p in plasma of peripheral blood was compared between patients with diabetes and a family history of diabetes and control volunteers by reverse transcription-quantitative polymerase chain reaction (RT-qPCR); in 60 diabetes patients, 45 (75%) demosntrated upregulated miR-199a-3p expression compared with control volunteer plasma. RT-qPCR was also used to detect miRNA-199a-3p expression in paired lower limb skin tissues from 30 patients with DN and 20 control volunteers; miR-199a-3p expression in patients with DN was significantly higher than in the control group. Next miR-199a-3p expression levels were evaluated with respect to the clinic-pathological parameters of diabetes; increased expression of miR-199a-3p was significantly associated with increased disease duration (P=0.041), glycated hemoglobin (HbA1C) levels (P=0.033), and fibrinogen levels (P=0.003). Finally, the effects on downstream mRNA expression levels were investigated as a result of manipulating miR-199a-3p levels. miR-199a-3p overexpression inhibited the expression of the extracellular serine protease inhibitor E2 (SerpinE2). Therefore, it may be hypothesized that miR-199a-3p can induce DN via promoting coagulation in skin peripheral circulation, through the downregulation of SerpinE2. The present findings suggested that miR-199a-3p may have potential as a novel therapeutic target for the treatment of patients with DN.

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Introduction

The prevalence of diabetes is increasing rapidly, and type 2 diabetes now accounts for 20-50% of cases of new-onset diabetes in young people (1,2). Diabetic peripheral neuropathy (DN) is the most common complication in both type 1 and type 2 diabetes (3). To explore a more effective treatment for DN, efforts have been focused on the molecular mechanisms underlying the etiology of DN. Serine protease inhibitors, termed serpins, are key regulators of many biological events (4). In blood, they circulate as inactive proforms or zymogens, and once activated, are quickly and irreversibly inhibited by circulating inhibitors, in particular by serine protease inhibitors, termed serpins. The extracellular serine protease inhibitor E2 (SerpinE2), also called Protease Nexin-1 (PN-1) belongs to the Serpin gene superfamily (5). Santoro *et al* (6) demonstrated that SerpinE2 prevents cartilage catabolism by inhibiting the expression of matrix metalloproteinase 13, one of the most relevant collagenases, involved in cartilage breakdown in OA. Another study using PN-1-deficient mice revealed that SerpinE2 confers antithrombotic and antifibrinolytic properties that had previously not been recognized (4). However, the effect of this factor upon diabetes neuropathy remains unknown.

MicroRNAs (miRNAs or miRs) are a family of non-coding RNAs that inhibit gene expression by interacting directly with the 3'-untranslated regions (3'-UTRs) of target mRNAs (7,8). Through these interactions, they can inhibit translation of the targeted mRNAs or degrade them (9). miRNAs have multiple functions, affecting many aspects of all kinds of diseases. Although there is much evidence linking altered miRNA expression with various cancers (10,11), the role of miRNAs in diabetes has not been sufficiently examined. Through suppressing key genes involved in disease development and progression, miRNAs can affect many disease-related signaling pathways via local or systemic regulation (12). The association between changes in miRNA expression and the development of diabetes neuropathy presents us with a new angle to explore pathogenesis and progression of diabetes: In diabetes, several miRNAs including miR-590-3p, miR-155 and

miR-323b-5p have been demonstrated to be associated with genesis and prognosis of diabetes patients (13-15). Limited studies have been performed on miRNA expression profiles in patients with DN, especially in DN-induced pain, and miRNA expression has not previously been linked to SerpinE2 (16).

In the present study, miRNA-199a-3p expression was detected in the plasma of patients with diabetes and healthy controls in order to study the pathogenesis of diabetes. In addition, miRNA-199a-3p expression was examined in lower limb tissue samples isolated from patients with DN and from healthy volunteers. Finally, the molecular mechanisms underlying the effects of miRNA-199a-3p in DN were examined using endothelial cell lines.

Materials and methods

Patients and samples. Plasma was collected from 60 patients who have a family history of type II diabetes, at the Department of Pain Management, The Central Hospital of Wuhan (Wuhan, China) between May 2014 and March 2015. The 60 diabetes patients were aged 35-65 and included 32 female and 28 male patients. All diabetes patients had at least one parent or sibling who also suffered from diabetes. Plasma samples isolated from 5 healthy volunteers were used as controls. Lower limb skin samples from 30 DN patients and 20 volunteer samples were collected between July 2011 and October 2015. All samples were cut into small pieces and stored at -80˚C. Written consent was obtained from all patients and prior approval for the study was obtained from the Institutional Research Ethics Committee of Wuhan Central Hospital.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted from plasma obtained from patients and control volunteers using the *mir*Vana PARIS kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA was then reverse transcribed using the PrimeScript™ RT-PCR kit (Takara Bio, Inc., Otsu, Japan) and qPCR performed using Real Time PCR Master Mix SYBR-Green PCR (Toyobo Co., Ltd., Osaka, Japan). Thermocycling conditions were as follows: A total of 45 cycles of denaturation at 95˚C for 15 sec, annealing at 55˚C for 30 sec, and extension at 72˚C for 30 sec. U6 was used as the internal control. The primers used were as follows: miR-199a-3p, forward 5'-GCGGCGGACAGTAGTCTGCAC-3', reverse 5'-ATCCAGTGCAGGGTCCGAGG-3'; U6, forward 5'-CTC GCTTCGGCAGCACA-3', reverse 5'-AACGCTTCACGA ATTTGGT-3'. Relative miRNA expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (17). All assays were carried out in triplicate.

Total RNA from DN lower limb skins and control group tissues was obtained using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA (1 μg) was reverse transcribed and subjected to qPCR using the same method as that used for plasma microRNA.

miRNA-199a-3p expression was measured using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The process was repeated in the CRL-4025 cell line to measure the transfection efficiency of miR-199a-3p mimic and inhibitor.

Cell lines, cell culture and transient transfection. CRL-4025, a human dermal microvascular endothelium cell line, was purchased from the Typical Training Content Preservation Committee Cell Bank, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Vascular Cell Basal Medium (cat. no. PCS-100-030; American Type Culture Collection, Manassas, VA, USA), supplemented with Microvascular Endothelial Cell Growth Kit-VEGF (cat. no. PCS-110-041; American Type Culture Collection) and 12.5 μg/ml blasticidine and penicillin/streptomycin at 37˚C in a humidified atmosphere with 5% CO_2. To manipulate miR-199a-3p expression, miRNA mimics (5'-UACCCC UCC CCCCAUCCCGCCUGCCCACCCCCCCCCCCCCCC CGUGUUCAGACUACCUGUUCAGGAAGUAGUGGU UGUACAGUAGUCUGCACAUUGGUUAGGCUGGUU AGGGAAGUGCG-3') and inhibitor (5'-AAGAACCUGCUC CGUCGCCCCAGUGUUCAGACUACCUGUUCAGGACAA UGCUGUUGUACAGUAGUCUGCACAUUGGUUAGA CUGGGCAUGGGACAG-3'; Thermo Fisher Scientific, Inc.) were used to transiently transfect CRL-4025 cell lines. Small interfering RNA (siRNA) targeting different coding regions of human SerpinE2 and its scrambled siRNA sequence (NC) were synthesized by Thermo Fisher Scientific Inc. The siRNA sequences were as follows: siRNA1, sense 5'-AAGACATTG TGACAGTGGCTA-3', antisense 3'-TTCTGTAACACTGTC ACCGAT-5'; siRNA2, sense 5'-AAGACCATAGACAGCTGG ATG-3', antisense 3'-TTCTGGTATCTGTCGACCTAC-5'; and scramble siRNA, sense 5'-AAGACCAACTGACAGTGG CTA-3' and antisense 3'-TTCTGTAATTAGGTCACCGAT-5'. Transfections were performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Cells were plated at a density of $6x10^4$ per well in a 6-well plate. The expression of miR-199a-3p was measured 48 h after reaching confluence; protein expression was measured 72 h after reaching confluence.

Luc-UTR vectors and tumor protein p53 (P53)-expressing vector. The full-length SerpinE2 3'-UTR was cloned into the *Sac*1 and *Mlu*1 sites of the pMIR-REPORT luciferase vector (Ambion; Thermo Fisher Scientific, Inc.). A mutated vector with the first 5 nucleotides complementary to the miR-199a-3p seed-region mutated was constructed as a control.

Full-length P53 cDNA entirely lacking the 3'-UTR was purchased from Gene Chem (Shanghai, CHINA) and subcloned into the pcDNA3.1(+) vector (Invitrogen; Thermo Fisher Scientific, Inc.). The blank pcDNA3.1(+) vector was applied as a negative control.

Western blot analysis. Cells were lysed using radioimmunoprecipitation assay lysis buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) for 30 min on ice, sonicated for 5-10 sec and then centrifuged at 12,000 x g for 20 min at 4˚C. Protein concentrations were determined using a DC™ Protein Assay (Bio-Rad Laboratories, Inc.). Equal amounts (40 μg) of extracted protein samples were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with 2% milk in PBS containing 0.1% Tween-20 at room temperature for 2 h, and subsequently probed with the following primary antibodies overnight at 4˚C: Anti-SerpinE2 (cat. no. ab154591; 1:1,000;

Table I. miR-199a-3p expression in type 2 diabetes patients clinical and pathological properties.

Parameters	High miR-199a-3p expression, N=36 (%)	Low miR-199a-3p expression, N=24 (%)	χ^2-test
Sex			P=0.853
Male	(16) (44.4)	(10) (41.7)	
Female	(20) (55.6)	(14) (58.3)	
Age (years)			P=0.203
≥45	(22) (61.1)	(11) (45.8)	
<45	(14) (38.9)	(13) (54.2)	
Disease duration (years)			P=0.041[a]
≥10	(16) (44.4)	(3) (12.5)	
<10	(20) (55.6)	(21) (87.5)	
HbA1C (%)			P=0.033[a]
≥7	(22) (61.1)	(9) (37.5)	
<7	(14) (38.9)	(15) (62.5)	
BMI			P=0.971
≥28	(25) (69.4)	(17) (70.8)	
<28	(11) (30.6)	(7) (29.2)	
APTT			P=0.179
≥25	(11) (30.6)	(11) (45.8)	
<25	(25) (69.4)	(13) (54.2)	
PT			P=0.076
≥11	(10) (27.8)	(12) (50.0)	
<11	(26) (72.2)	(12) (50.0)	
FIB			P=0.003[a]
≥4	(27) (75.0)	(5) (20.9)	
<4	(9) (25.0)	(19) (79.1)	

[a]P<0.05. HbA1C, glycated hemoglobin; BMI, body mass index; APTT, activated partial thromboplastin time; PT, prothrombin time; FIB, fibrinogen; miR, microRNA.

Abcam, Cambridge, MA, USA), anti-tissue plasminogen activator (tPA; cat. no. ab157469; 1:1,000; Abcam), anti-β-actin (cat. no. sc-130300; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and anti-GADPH (cat. no. ab37168; 1:1,000; Abcam). Membranes were then incubated with the following horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature: Anti-rabbit (cat. no. A0545; 1:5,000; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and anti-mouse (cat. no. A9044; 1:5,000; Sigma-Aldrich; Merck KGaA). The protein bands were detected using an enhanced chemiluminescence detection system (Pierce; Thermo Fisher Scientific, Inc.).

Immunohistochemistry. Immunohistochemical staining was performed on 4-μm thick paraffin-embedded skin tissue sections with an anti-SerpinE2 primary antibody (cat. no. ab154591; 1:200; Abcam). Briefly, sections were deparaffinized in xylene and hydrated with graded ethanol. Antigen retrieval was performed using 0.01 mM citrate buffer (pH 6.0) in a pressure cooker at 95˚C for 20 min, and endogenous peroxidase activity was blocked with incubation with 3% hydrogen peroxide for 10 min at room temperature. Sections were incubated with the primary antibody in a moist chamber overnight at 4˚C, washed 3 times in PBS and then incubated with a horseradish peroxidase-conjugated secondary antibody (cat. no. 31460; 1:5,000; Thermo Fisher Scientific, Inc.) for 1 h at 37˚C. Anibody-antigen complexes were visualized using a 3,3'-diaminobenzidine detection kit (Dako; Agilent Technologies, Inc., Santa Clara, CA, USA). Samples were counterstained with 100% hematoxylin for 10 sec at room temperature. The mean percentage of SerpinE2-positive tumor cells was determined under an inverted microscope. At least 5 random fields under x400 magnification were assessed from each section.

Luciferase activity of different promoter constructs and chromatin immunoprecipitation (ChIP). Transcriptional factor binding sites in the human miR-199a-3p promoter region were predicted using the JASPAR 2016 server (http://jaspar.genereg.net) and the ECR browser software (https://ecrbrowser.dcode.org/). Putative P53 binding site: 5'-GGGCTTT-3'; -1747 bp to -1741 bp. Mutant P53 binding site: 5'-AAATGGG-3'. ChIP assays were performed on CRL-4025 cells transfected with P53 or empty vector using the Magna ChIP Assay Kit (EMD Millipore, Billerica, MA, USA). Protein-DNA complexes were precipitated with normal

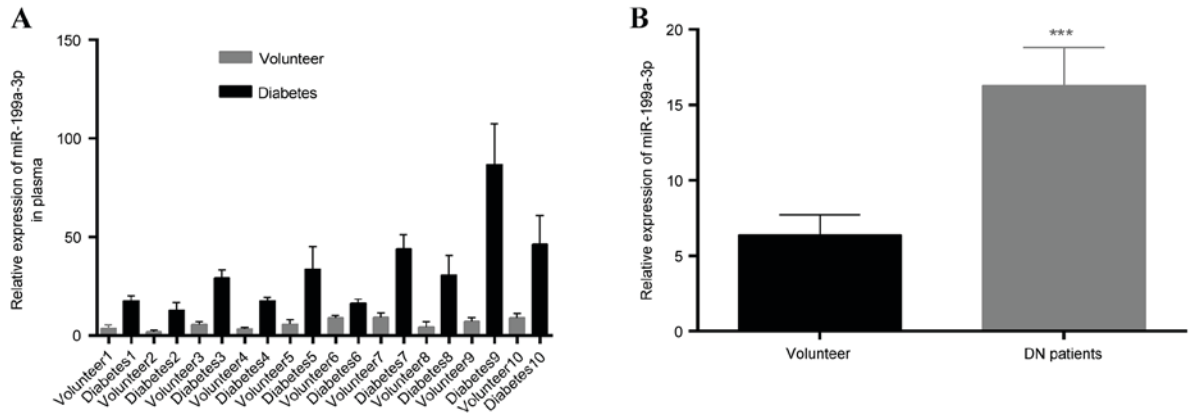

Figure 1. miR-199a-3p is upregulated in diabetes plasma and tissues. (A) RT-qPCR analysis of the expression levels of miR-199a-3p in the plasma of 10 pairs of diabetes patients of the same sex with family history of diabetes and healthy volunteers. miR-199a-3p expression in plasma samples from diabetes patients appeared to be upregulated compared with in plasma from volunteers. (B) RT-qPCR analysis of miR-199a-3p expression levels in 30 pairs of tissues from patients with DN and healthy volunteers. ***P<0.001 vs. volunteers. miR-199a-3p expression was significantly higher in DN tissues than in volunteers. miR, microRNA; RT-qPCR, reverse transcription- quantitative polymerase chain reaction; DN, diabetic neuropathy.

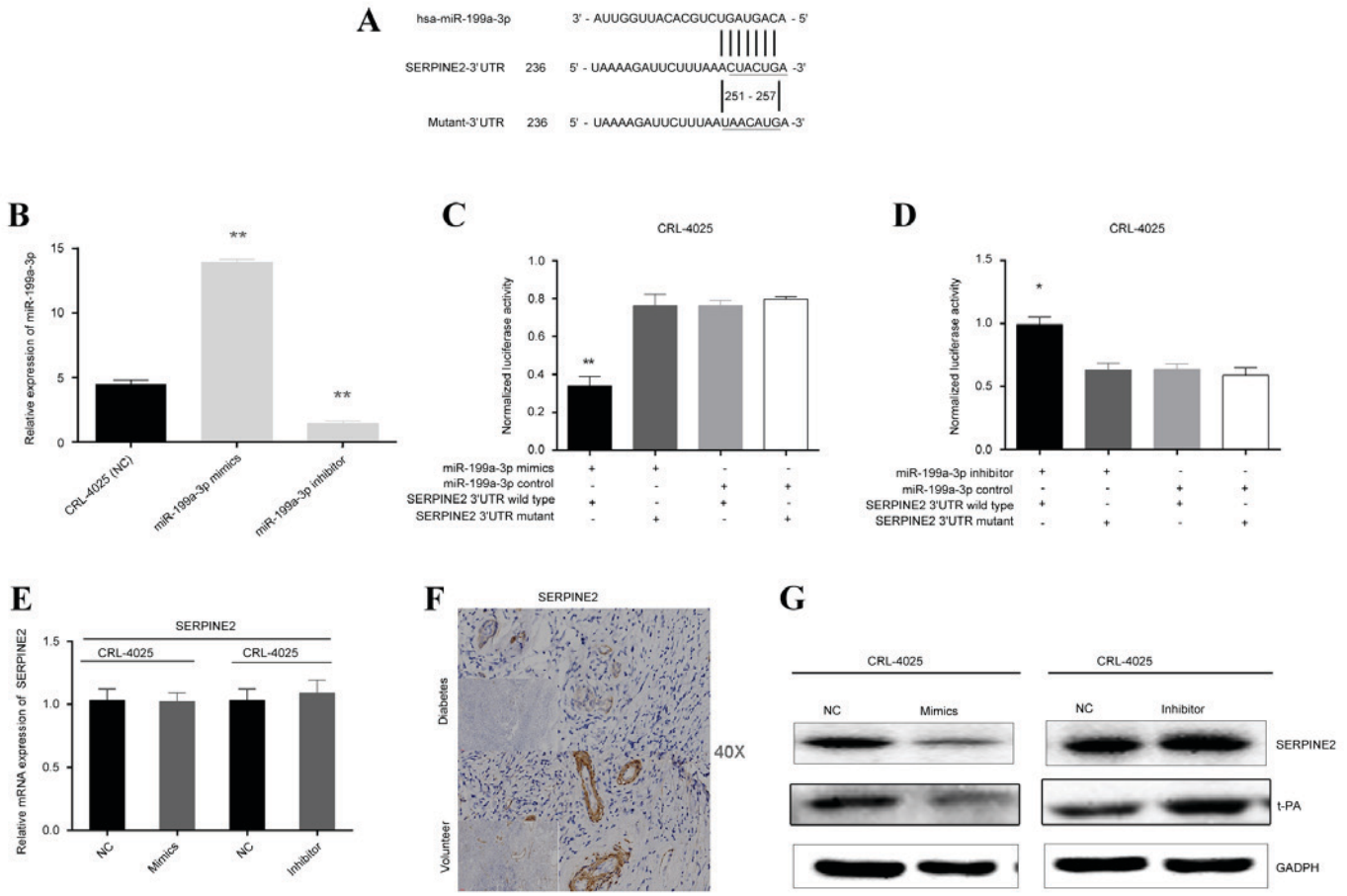

Figure 2. SERPINE2 is a direct target of miR-199a-3p. (A) Sequence alignment of miR-199a-3p with the SERPINE2 3'-UTR. The seed-recognizing sites (underlined) in the SERPINE2 sequence matched with the seed regions of miR-199a-3p. (B) Following transfection of CRL-4025 cells with a miR-199a mimic, the expression of miR-199a was upregulated, whereas transfection with an inhibitor effectively suppressed the expression of miR-199a-3p. **P<0.01 vs. NC. (C) Luciferase assay in CRL-4025 cells demonstrated that miR-199a-3p mimics significantly suppressed luciferase activity in wild type reporter constructs, compared with NC. (D) miR-199a-3p inhibitor significantly promoted luciferase activity in wild type constructs, compared with NC. *P<0.05 vs. NC. (E) Reverse transcription-quantitative polymerase chain reaction demonstrated that the level of SERPINE2 mRNA in CRL-4025 cells was not affected by transfection with miR-199a-3p mimics or miR-199a-3p inhibitor. (F) Immunohistochemistry revealed that SERPINE2 was downregulated in DN microvasculature compared with volunteers. (G) Transfection of CRL-4025 cells with miR-199a-3p mimics or miR-199a-3p inhibitor affects SERPINE2 and tPA protein expression levels. Protein expression of SERPINE2 was negatively associated with the expression of miR-199a-3p. All data are presented as the mean ± standard deviation. SERPINE2, serine protease inhibitor E2; miR, microRNA; UTR, untranslated region; NC, negative control; DN, diabetic neuropathy; tPa, tissue plasminogen activator.

IgG (cat. no. I5006; 1:1,000; Sigma-Aldrich; Merck KGaA) and anti-P53 (cat. no. P5813; 1:200; Sigma-Aldrich) at 4˚C overnight with rotation.

Statistical analysis. All data are expressed as the mean ± standard deviation. Comparisons of clinical-pathologies were performed by the log-rank test and χ^2 test. P<0.05 was considered to indicate a statistically significant difference. Statistical analysis was performed using SPSS statistical software (version 15.0; SPSS, Inc., Chicago, IL, USA).

Results

miR-199a-3p is upregulated in the plasma of patients with a family history of type II diabetes. RT-qPCR was employed to detect miR-199a-3p expression in the plasma of the 60 patients with diabetes and a family history of diabetes and healthy volunteers. miR-199a-3p expression appeared to be increased in patients with diabetes compared with the controls (Fig. 1A). In addition, the expression of miR-199a-3p was measured in 30 DN lower limb skin samples and 20 volunteer tissues. We found that miR-199a-3p expression was significantly increased in skin tissues compared with volunteer tissues (P<0.05; Fig. 1B).

miR-199a-3p expression is positively associated with clinical features of type 2 diabetes patients, especially peripheral blood coagulation. To examine the relationships between miR-199a-3p expression and biological characters of diabetes, the 60 patients were divided into two groups according to miR-199a-3p expression (Table I). High disease duration, glycated hemoglobin (HbA1C) and fibrinogen (FIB) were demonstrated to be associated with high miR-199a-3p expression (P<0.05; Table I). This suggests that high miR-199a-3p expression in diabetes patients may be considered as indicative of DN progression.

miR-199a-3p promotes coagulation by targeting SerpinE2 in dermal microvascular endothelium. To study the mechanism by which miR-199a-3p may act, several computational algorithms, such as TargetScan version 7.0 (18), and miRBase (19-23), were used to predict potential miR-199a-3p target sites. The seed sequences of mature miR-199a-3p and the 3'-UTR of SERPINE2 mRNA were matched (Fig. 2A). Following transfection with a miR-199a-3p mimic, the expression of miR-199a-3p was significantly upregulated, whereas transfection with an inhibitor effectively suppressed the expression of miR-199a-3p (Fig. 2B).

To confirm SERPINE2 as the target of miR-199a-3p, the full-length SERPINE2 3'-UTR was subcloned into a luciferase reporter vector (pMIR-REPORT β-galactosidase control vector). Following transfection of SERPINE2 3'-UTR pMIR-REPORT into CRL-4025 cells, luciferase activity associated with SERPINE2 expression was demonstrated to be inhibited by co-transfection with miR-199a-3p mimics compared with co-transfection with miR-199a-3p control (P<0.01; Fig. 2C). This reduction in luciferase activity was also demonstrated to be dependent on the presence of a wild type, rather than mutated, SERPINE2 3'-UTR; this inhibition was abolished when the predicted miR-199a-3p target sequences in the SERPINE2 3'-UTR were mutated (Fig. 2A and C). Furthermore, inhibition of endogenous miR-199a-3p by the inhibitor in CRL-4025 cells was able to increase luciferase expression compared with control (P<0.05; Fig. 2D). The changes in luciferase activity all occurred in the absence of any changes in the mRNA expression level of SERPINE2 (Fig. 2E). Immunohistochemical analysis of skin tissues also demonstrated the difference in SERPINE2 expression in microvascular endothelia cells between diabetic patients and volunteers (Fig. 2F). To directly assess the effect of miR-199a-3p on SERPINE2 protein expression, miR-199a-3p was transfected into CRL-4025 cells the protein expression levels of SERPINE2 detected by western blotting; overexpression of miR-199a-3p was demonstrated to reduce SERPINE2 protein levels compared with NC (Fig. 2G). Conversely, transfection with miR-199a-3p inhibitor increased SERPINE2 protein expression levels compared with NC (Fig. 2G).

miR-199a-3p promotes coagulation by targeting SerpinE2, thus inhibiting the tPA pathway. Since SERPINE2 lies upstream of the tPA signaling pathway, the effect of miR-199a-3p dysregulation on this pathway was examined in CRL-4025 cells. Increased miR-199a-3p in cells was associated with decreased expression of tPA compared with NC, whereas, inhibition of miR-199a-3p resulted in increased expression of tPA compared with NC (Fig. 2G). miR-199a-3p was, therefore, posited to affect downstream signaling by targeting SERPINE2.

P53 activation of the promoter of miR-199a-3p and downregulates SERPINE2. P53 was reported to regulate gene expression through its interaction with transcriptional factors and is downregulated in diabetes (13). Activation of β cell glucokinase, initially triggers replication, then results in apoptosis associated with DNA double-strand breaks and activation of the tumor suppressor P53 (24). P53 downregulates the expression of DNA methyltransferase 1 in NT2 cells, while overexpression of P53 restores the expression of miR-199-3p/5p and miR-214 (25). To determine how P53 regulates the expression of miR-199a-3p, the effects of P53 on the expression of miR-199a-3p and SERPINE2 were examined. Compared with control, overexpression of P53 in CRL-4025 cells decreased expression of SERPINE2 (Fig. 3A). By contrast, knockdown of P53 increased that of SERPINE2 compared with control (Fig. 3B). These results suggest that P53 is responsible for the regulation of miR-199a-3p and SERPINE2 expression. To determine whether P53 directly transcribes miR-199a-3p, a putative P53-binding site upstream of the transcriptional start site of miR-199a-3p was characterized: P53 stimulated the activity of the luciferase reporter containing the putative P53-binding site but not the reporter with the mutated binding site or without the putative P53-binding site (Fig. 3C). ChIP assays demonstrated the miR-199a-3p promoter occupancy of P53 (Fig. 3D). Taken together, these data strongly suggest that P53 activates miR-199a-3p transcription through direct binding to its promoter.

Discussion

Since the discovery of miRNAs, many miRNAs have been found to have different expression patterns in different

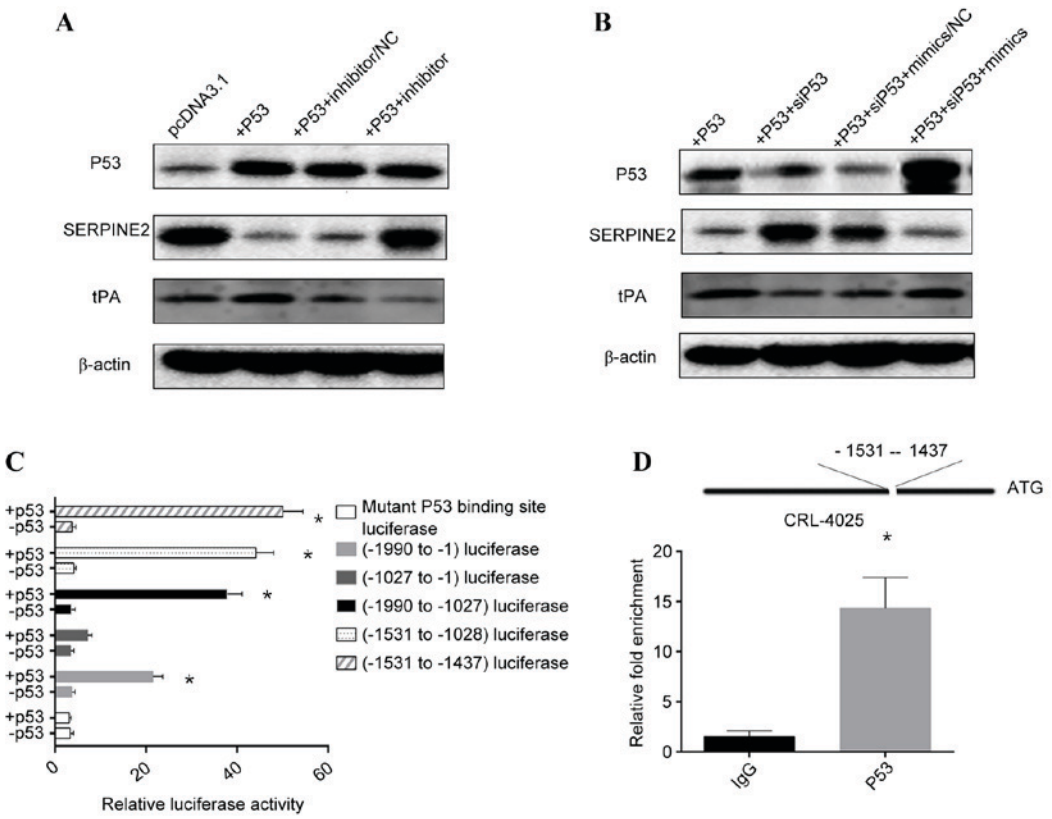

Figure 3. P53 regulates miR-199a-3p expression and then regulates SERPINE2 expression. (A) When miR-199a-3p expression is downregulated (lane 4), the P53 downregulating effects on SERPINE2 expression and the subsequent pathways are offset, compared with NC. (B) When miR-199a-3p expression is upregulated (lane 4), the siP53 upregulating effects on SERPINE2 expression and the subsequent pathways are offset compared with NC. (C) Dual-luciferase assay of the putative miR-199a-3p promoter in CRL-4025 cells transfected with P53 and empty vector controls. (D) Chromatin immunoprecipitation analysis demonstrated that P53 occupies the putative miR-199a-3p promoter in CRL-4025 cells. All data are presented as the mean ± standard deviation. *P<0.05, as indicated. P53, tumor protein p53; miR, microRNA; SERPINE2, serine protease inhibitor E2; NC, negative control.

diseases, that are regulated by mechanisms as varied as the presence of deletions, amplifications or mutations involving miRNA loci, epigenetic silencing or the dysregulation of transcription factors that target specific miRNAs (26,27).

There have been a number of miRNA-screening studies in the plasma or serum of diabetes patients (28). Inducible transgenic overexpression of miR-802 in mice causes impaired glucose tolerance and attenuates insulin sensitivity, whereas reduction of miR-802 expression improves glucose tolerance and insulin action (29). Another study suggested that a defect in the NF-κB-miR-146a negative feedback loop may be involved in the pathogenesis of DN through regulating TNF-α, interleukin 6 (IL-6), and interleukin 1β (IL-1β) in the sciatic nerve of diabetic rats (30). The present study examined miRNAs that are involved in DN occurrence from the coagulation status angle. A previous study identified miR-1908, miR-199a-5p and miR-199a-3p as endogenous promoters of metastatic invasion, angiogenesis and colonization in melanoma by convergently targeting apolipoprotein E and the heat shock factor DnaJ heat shock protein family (Hsp40) member A4 (31). Furthermore, miR-199a-3p dysregulation has been noted in liver cancer (32) and in leukemia (33). miR-199a-3p has been associated with many kinds of cancer, but, to the best of our knowledge, its role in diabetes has not previously been explored specifically. In the present study, the expression of miR-199a-3p was upregulated in the plasma of patients with diabetes and tissues of patients with DN compared with volunteers. In addition, high expression was significantly associated with high disease duration, HbA1C and FIB.

Through manipulation of its expression, miR-199a-3p was found to inhibit tPA *in vitro* through the regulation of the expression of SERPINE2. SERPINE2 upregulates tPA, which is associated with hypoxia-ischemia and excitotoxicity causes of DN injuries and participates in the processes through proteolytic and receptor-mediated pathways (34). The present study observed downregulation of tPA protein expression levels in endothelial cells and DN tissues, and tPA protein levels were inversely associated with miR-199a-3p expression. Luciferase activity assays demonstrated that miR-199a-3p bound to the 3'-UTR of SERPINE2 mRNA. The effect of miR-199a-3p on SERPINE2 mRNA levels was also investigated, and it was found that miR-199a-3p did not modulate SERPINE2 mRNA expression levels, which indicated that miR-199a-3p targets SERPINE2 through translation inhibition rather than mRNA degradation. Furthermore, the miR-199a-3p-associated inhibition of tPA expression could be offset by upregulating SERPINE2 expression. Conversely, the increase in tPA expression caused by downregulation of miR-199a-3p could be offset by inhibition of SERPINE2.

miRNA studies have demonstrated that P53 expression affects the expression of many miRNAs, however, the mechanisms through which miRNAs are regulated remain poorly understood (35). P53 accumulation may be responsible for impaired wound healing in patients with diabetes (36). The present study demonstrated a close association between miR-199a-3p expression and P53: Overexpression of P53 enhanced miR-199a-3p expression and downregulated SERPINE2 expression. miR-199a-3p was, therefore, identified as a downstream target of P53. These results demonstrated that P53 regulated miR-199a-3p expression through interacting with the promoter section as a transcriptional factor. The fact that P53 regulated miR-199a-3p expression suggests that miR-199a-3p may serve a role in DN.

In summary, the present study suggests that miR-199a-3p, induced by P53, functions as a pro-coagulating factor in DN by targeting SERPINE2, and subsequently suppresses expression of downstream tPA. miR-199a-3p, as a fascinating molecule involved in the pathogenesis and progression of DN, may serve as both a biomarker in early diagnosis of DN and a directing factor in treatment of DN.

References

1. Shi TJ, Zhang MD, Zeberg H, Nilsson J, Grünler J, Liu SX, Xiang Q, Persson J, Fried KJ, Catrina SB, *et al*: Coenzyme Q10 prevents peripheral neuropathy and attenuates neuron loss in the db-/db- mouse, a type 2 diabetes model. Proc Natl Acad Sci USA 110: 690-695, 2013.
2. Haw JS, Tantry S, Vellanki P and Pasquel FJ: National strategies to decrease the burden of diabetes and its complications. Curr Diab Rep 15: 65, 2015.
3. Palmer BF and Clegg DJ: Electrolyte and acid-base disturbances in patients with diabetes mellitus. N Engl J Med 373: 548-559, 2015.
4. Bouton MC, Boulaftali Y, Richard B, Arocas V, Michel JB and Jandrot-Perrus M: Emerging role of serpinE2/protease nexin-1 in hemostasis and vascular biology. Blood 119: 2452-2457, 2012.
5. Valiente M, Obenauf AC, Jin X, Chen Q, Zhang XH, Lee DJ, Chaft JE, Kris MG, Huse JT, Brogi E and Massagué J: Serpins promote cancer cell survival and vascular co-option in brain metastasis. Cell 156: 1002-1016, 2014.
6. Santoro A, Conde J, Scotece M, Abella V, Lois A, Lopez V, Pino J, Gomez R, Gomez-Reino JJ and Gualillo O: SERPINE2 inhibits IL-1α-induced MMP-13 expression in human chondrocytes: Involvement of ERK/NF-κB/AP-1 pathways. PLoS One 10: e0135979, 2015.
7. Alsaweed M, Lai CT, Hartmann PE, Geddes DT and Kakulas F: Human milk miRNAs primarily originate from the mammary gland resulting in unique miRNA profiles of fractionated milk. Sci Rep 6: 20680, 2016.
8. Yuan Y, Kang R, Yu Y, Liu J, Zhang Y, Shen C, Wang J, Wu P, Shen C and Wang Z: Crosstalk between miRNAs and their regulated genes network in stroke. Sci Rep 6: 20429, 2016.
9. Inui M, Martello G and Piccolo S: MicroRNA control of signal transduction. Nat Rev Mol Cell Biol 11: 252-263, 2010.
10. Hur K, Toiyama Y, Okugawa Y, Ide S, Imaoka H, Boland CR and Goel A: Circulating microRNA-203 predicts prognosis and metastasis in human colorectal cancer. Gut 66: 654-665, 2017.
11. Liu FY, Zhou SJ, Deng YL, Zhang ZY, Zhang EL, Wu ZB, Huang ZY and Chen XP: MiR-216b is involved in pathogenesis and progression of hepatocellular carcinoma through HBx-miR-216b-IGF2BP2 signaling pathway. Cell Death Dis 6: e1670, 2015.
12. Liu FY, Deng YL, Li Y, Zeng D, Zhou ZZ, Tian DA and Liu M: Down-regulated KLF17 expression is associated with tumor invasion and poor prognosis in hepatocellular carcinoma. Med Oncol 30: 425, 2013.
13. Wang X, Li W, Ma L, Gao J, Liu J, Ping F and Nie M: Association study of the miRNA-binding site polymorphisms of CDKN2A/B genes with gestational diabetes mellitus susceptibility. Acta Diabetol 52: 951-958, 2015.
14. Chen Y, Wang X and Shao X: A combination of human embryonic stem cell-derived pancreatic endoderm transplant with LDHA-repressing miRNA can attenuate high-fat diet induced type II diabetes in mice. J Diabetes Res 2015: 796912, 2015.
15. Song C, Ji Y, Zou G and Wan C: Tetrandrine down-regulates expression of miRNA-155 to inhibit signal-induced NF-κB activation in a rat model of diabetes mellitus. Int J Clin Exp Med 8: 4024-4030, 2015.
16. Chattopadhyay M, Zhou Z, Hao S, Mata M and Fink DJ: Reduction of voltage gated sodium channel protein in DRG by vector mediated miRNA reduces pain in rats with painful diabetic neuropathy. Mol Pain 8: 17, 2012.
17. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2-(Delta Delta C(T)) method. Methods 25: 402-408, 2001.
18. Nam JW, Rissland OS, Koppstein D, Abreu-Goodger C, Jan CH, Agarwal V, Yildirim MA, Rodriguez A and Bartel DP: Global analyses of the effect of different cellular contexts on microRNA targeting. Mol Cell 53: 1031-1043, 2014.
19. Kozomara A and Griffiths-Jones S: miRBase: Annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res 42 (Database Issue): D68-D73, 2014.
20. Kozomara A and Griffiths-Jones S: miRBase: Integrating microRNA annotation and deep-sequencing data. Nucleic Acids Res 39 (Database Issue): D152-D157, 2011.
21. Griffiths-Jones S, Saini HK, van Dongen S and Enright AJ: miRBase: Tools for microRNA genomics. Nucleic Acids Res 36 (Database Issue): D154-D158, 2008.
22. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A and Enright AJ: miRBase: microRNA sequences, targets and gene nomenclature. Nucleic Acids Res 34 (Database issue): D140-D144, 2006.
23. Griffiths-Jones S: The microRNA registry: Nucleic Acids Res 32 (Database issue): D109-D111, 2004.
24. Tornovsky-Babeay S, Dadon D, Ziv O, Tzipilevich E, Kadosh T, Schyr-Ben Haroush R, Hija A, Stolovich-Rain M, Furth-Lavi J, Granot Z, *et al*: Type 2 diabetes and congenital hyperinsulinism cause DNA double-strand breaks and p53 activity in beta cells. Cell Metab 19: 109-121, 2014.
25. Chen BF, Suen YK, Gu S, Li L and Chan WY: A miR-199a/miR-214 self-regulatory network via PSMD10, TP53 and DNMT1 in testicular germ cell tumor. Sci Rep 4: 6413, 2014.
26. Seviour EG, Sehgal V, Lu Y, Luo Z, Moss T, Zhang F, Hill SM, Liu W, Maiti SN, Cooper L, *et al*: Functional proteomics identifies miRNAs to target a p27/Myc/phospho-Rb signature in breast and ovarian cancer. Oncogene 35: 801, 2016.
27. Thomas H: Diabetes: Enterovirus dysregulates islet miRNAs. Nat Rev Endocrinol 12: 2, 2016.
28. Jansen F, Wang H, Przybilla D, Franklin BS, Dolf A, Pfeifer P, Schmitz T, Flender A, Endl E, Nickenig G and Werner N: Vascular endothelial microparticles-incorporated microRNAs are altered in patients with diabetes mellitus. Cardiovasc Diabetol 15: 49, 2016.
29. Kornfeld JW, Baitzel C, Könner AC, Nicholls HT, Vogt MC, Herrmanns K, Scheja L, Haumaitre C, Wolf AM and Knippschild U: Obesity-induced overexpression of miR-802 impairs glucose metabolism through silencing of Hnf1b. Nature 494: 111-115, 2013.
30. Yousefzadeh N, Alipour MR and Soufi FG: Deregulation of NF-κB-miR-146a negative feedback loop may be involved in the pathogenesis of diabetic neuropathy. J Physiol Biochem 71: 51-58, 2015.
31. Pencheva N, Tran H, Buss C, Huh D, Drobnjak M, Busam K and Tavazoie SF: Convergent multi-miRNA targeting of ApoE drives LRP1/LRP8-dependent melanoma metastasis and angiogenesis. Cell 151: 1068-1082, 2012.
32. Lee CG, Kim YW, Kim EH, Meng Z, Huang W, Hwang SJ and Kim SG: Farnesoid X receptor protects hepatocytes from injury by repressing miR-199a-3p, which increases levels of LKB1. Gastroenterology 142: 1206-17.e7, 2012.
33. Alemdehy MF, Haanstra JR, de Looper HW, van Strien PM, Verhagen-Oldenampsen J, Caljouw Y, Sanders MA, Hoogenboezem R, de Ru AH, Janssen GM, *et al*: ICL-induced miR139-3p and miR199a-3p have opposite roles in hematopoietic cell expansion and leukemic transformation. Blood 125: 3937-3948, 2015.
34. Omouendze PL, Henry VJ, Porte B, Dupré N, Carmeliet P, Gonzalez BJ, Marret S and Leroux P: Hypoxia-ischemia or excitotoxin-induced tissue plasminogen activator- dependent gelatinase activation in mice neonate brain microvessels. PLoS One 8: e71263, 2013.

35. Garibaldi F, Falcone E, Trisciuoglio D, Colombo T, Lisek K, Walerych D, Del Sal G, Paci P, Bossi G, Piaggio G and Gurtner A: Mutant p53 inhibits miRNA biogenesis by interfering with the microprocessor complex. Oncogene 35: 3760-3770, 2016.

36. Morimoto Y, Bando YK, Shigeta T, Monji A and Murohara T: Atorvastatin prevents ischemic limb loss in type 2 diabetes: Role of p53. J Atheroscler Thromb 18: 200-208, 2011.