Original Article
MicroRNA-27a adjusts diabetic nephropathy patients and inhibits TGF-β/Smad signaling pathway

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Abstract: The aim of this study was to determine the function and mechanism of microRNA-27a in diabetic nephropathy. Firstly, microRNA-27a expression of diabetic nephropathy patients was up-regulated. Up-regulation of microRNA-27a expression promoted apoptosis, caspase-3, FN, Col-IV, and α-SMA protein expression, suppressed E-cadherin protein expression, and induced TGFβ1 and p-Smad2 protein expression in diabetic nephropathy model vitro. Down-regulation of microRNA-27a expression suppressed apoptosis, caspase-3, FN, Col-IV, and α-SMA protein expression, induced E-cadherin protein expression, and reduced TGFβ1 and p-Smad2 protein expression in diabetic nephropathy model vitro. The results showed that microRNA-27a adjusts diabetic nephropathy patients and inhibits renal TGF-β/Smad signaling pathway, provide new insights into the progression of diabetic nephropathy.

Keywords: microRNA-27a, diabetic nephropathy, EMT, TGF-β/Smad

Introduction

Diabetic nephropathy (DN) is the most common complication induced by diabetes, which is also one of the major causes responsible for renal failure [1]. The morbidity of DN shows an annually increasing trend, rendering it a global public health problem severely impairing human health [2]. According to the statistics of WTO, 0.37 billion people will be affected by diabetes in the world by 2025, among which 30% will developed into DN [3]. The major pathological features of DN include glomerular hypertrophy, hyperplasia, basement membrane thickening and increased extracellular matrix, which gradually develop into glomerulosclerosis, interstitial fibrosis as well as loss of function. Eventually, they will lead to chronic renal failure, reduced quality of life and even being life-threatening [2]. DN can be caused by a variety of reasons, and its pathogenesis is complicated, which remains incompletely illuminated yet [4].

Epithelial-mesenchymal transition (EMT) indicates a phenomenon that epithelial cells transform into mesenchymal cells under certain physiological or pathological conditions [5]. Such process is accompanied with disappearance of multiple epithelial cell markers and the appearance of mesenchymal cell markers, as well as reduced cell adhesion, disappeared polarity, changed cell activities and enhanced invasion ability [6]. It is currently considered that EMT plays an important role in physiological and pathological processes such as embryogenesis and development, tissue fibrosis, as well as tumor invasion and metastasis [7].

Transforming growth factor TGF-β/Smad signal pathway has been extensively studies, which has a large family, together with numerous functions and complicated mechanisms, and it is closely related to the genesis and development of DN [8]. It can induce the hyperplasia and hypertrophy of glomerular cells and increased extracellular matrix, and results in glomerulosclerosis and interstitial fibrosis, thereby giving rise to irreversible progression of DN [9]. A series of post-receptor signal molecules are required for TGF-β signal transduction, while Smad protein is the only intracellular kinase substrate of TGF-β receptor known so far, which mediates TGF-β signal transduction [10].

As is discovered in a number of studies, miRNA is involved in the genesis and development of
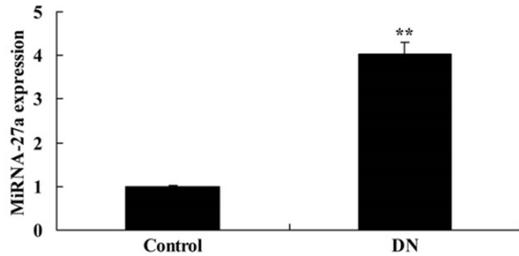
Figure 1. MicroRNA-27a expression of diabetic nephropathy patients.

DN, which forms an extremely complicated network. The discovery of miRNA has provided a new thought for investigating the pathogenesis of DN [11]. A large number of miRNAs have been discovered and studied; in addition, increasing importance has been attached to their functions in vivo [12]. miRNAs regulate about 1/3 of human genes, which are almost involved in a series of physiological and biochemical processes, such as cell growth, differentiation and apoptosis, carbohydrate metabolism, fat metabolism, insulin secretion, brain formation, cardiogenesis and stem cell differentiation [12]. Furthermore, they are closely related to the genesis of numerous diseases, including tumor [13]. Different from the modes of action of other genes, they have independent modes of transcription, and their products are not translated into proteins [13]. They only identify and bind with specific targets, thereby regulating target gene expression at post-transcription level [14].

Renal fibrosis is the pathological characteristic indicating progression of DN into end-stage renal failure. α-SMA is a characteristic protein expressed by myofibroblast, which is only expressed in medial vascular wall in mature kidney [15]. Research in recent years has verified that under certain pathological conditions, epithelial cell, endothelial cell and mesangial cell in parietal layer of glomerulus, as well as tubulointerstitial cell can trans-differentiate into myofibroblast (Myo-FB), thus expressing α-SMA [16]. Expression of α-SMA in renal tissue can reflect severity of renal fibrosis in an indirect way [17]. FN is an important component of extracellular matrix (ECM), which is produced by mesangial cell under normal condition. Phenotype trans-differentiation of mesangial cell into Myo-FB can be seen under pathological condition, where ECM components such as FN can be massively expressed

[16]. Thus, this study aimed to investigate whether the function and mechanism of microRNA-27a in diabetic nephropathy.

Materials and methods

Patient samples and quantitative real time (qRT)-PCR

Peripheral blood of diabetic nephropathy patients and normal control healthy volunteers were collected and centrifuged at 2000 g for 10 min. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA) from serum and cell by transfection. cDNA was synthesized using Takara RNA PCR kit (Baoshengwu, Dalian, China). mRNA expression was measured by CFX 96TM Connect Real-Time system (BioRad, USA) using SYBR Select Master Mix (BioRad, USA). The primer sequences of microRNA-27a: 5'-TGCGCAAGGATGACACGCA-3'; the primer sequences of U6: 5'-ATTGGAACGATACAGAGAAG-ATT-3' and 5'-GGAACGCTTCACGAATTTG-3'. Relative expression of miR-27a was analysed using the 2-ΔΔCT method.

Cell culture and transfection

NRK-52E cells was purchased from the Cell Culture Centre of the Institute of Biomedicine and Health (Guangzhou, PRC) and grown in DMEM (Gibco, China) containing 4.5 g/l glucose and 10% FBS (Hyclone, USA) at 37°C and 5% CO_2. MicroRNA-27a mimics, anti-microRNA-27a mimics and negative control mimics were purchased from Sangon Biotech (Shanghai) Co., Ltd. NRK-52E cells was transfected by Lipofectamine 2000 according to the instructions specified by the manufacturer (Invitrogen).

Cell apoptosis assay

After transfection, NRK-52E cells (1×106 cell) was stained with FITCA nnexin V and PI (BD Pharmingen, San Diego, CA, USA) at darkness for 15 min. Cell apoptosis were assayed by a FACScan flow cytometer (BD Biosciences, Mountain View, CA, USA) and analyzed using CellQuest software (BD Biosciences, Mountain View, CA, USA).

Western blot analysis and caspase-3 activity

Total proteins were extracted from cells in RIPA buffer (Kaiji, Shanghai, China) and protein con-
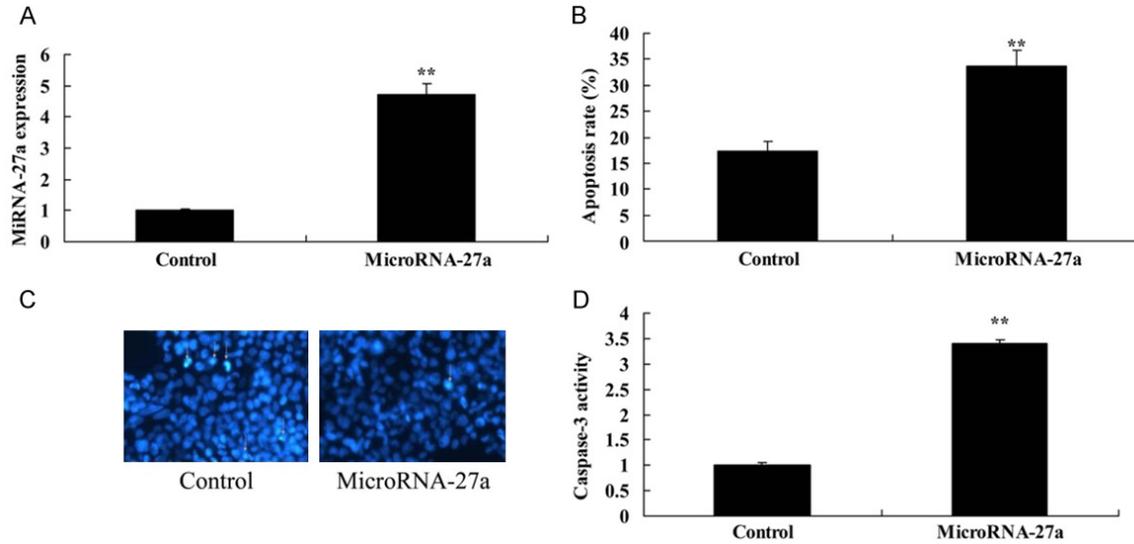
Figure 2. Up-regulation of microRNA-27a expression promoted apoptosis and caspase-3. MicroRNA-27a expression (A), apoptosis rate (B), cell apoptotic nueleolus (C) and caspase-3 activity (D). Control, control group; MicroRNA-27a, over-expression microRNA-27a group. **p<0.05 compared with control group.

centration was determined using BCA assay (Kaiji, Shanghai, China). Equal amounts of protein (50 µg) were electrophoresed on 8-12% SDS-PAGE gels, transferred to PVDF (Millipore, Billerica, USA). Membranes were blocked in TBS-T buffer containing 5% non-fat dry milk and exposed to E-cadherin (1:500, Santa Cruz, USA), TGFβ1 (1:200, Santa Cruz, USA), p-Smad2 (1:200, Santa Cruz, USA) and GAPDH (1:500, Santa Cruz, USA) at 4°C overnight. Blots were washed with TBS-T buffer and then incubated with the anti-rabbit HRP-conjugated secondary antibody (1:2000, Santa Cruz, USA) at 37 °C for 1 h and visualized with the Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, USA). The band of protein were quantified using Bio-Rad gel imaging system (BioRad, USA) and Quantity one 4.6 software (BioRad, USA).

5 µg of protein was used to analyze caspase-3 activity using caspase-3 activity Kit (Kaiji, Shanghai, China) and the optical density of each well was read at 450 nm.

Statistical analysis

All results are presented as the mean ± SEM. One-way ANOVA and Student's Newman-Keuls test for comparisons were used to determine differences between control and experimental groups. p<0.05 was considered statistically significant.

Results

MicroRNA-27a expression of diabetic nephropathy patients

First, microRNA-27a expression was analyzed by employing qRT-PCR. Compared with control normal group, microRNA-27a expression of diabetic nephropathy patients was up-regulated (**Figure 1**).

Up-regulation of microRNA-27a expression promoted apoptosis and caspase-3

To evaluate the effect of microRNA-27a affects on cell apoptosis in diabetic nephropathy, we used microRNA-27a mimics to increase microRNA-27a expression in diabetic nephropathy vitro. As showed in **Figure 2**, microRNA-27a mimics effectively increased microRNA-27a expression, also promoted apoptosis rate, and induced cell apoptotic nueleolus and caspase-3 activity in diabetic nephropathy vitro.

Up-regulation of microRNA-27a expression promoted FN, Col-IV, and α-SMA mRNA expression

We determined whether microRNA-27a affects on FN, Col-IV, and α-SMA mRNA expression using qRT-PCR. Up-regulation of microRNA-27a expression significantly promoted FN, Col-IV, and α-SMA mRNA expressionin diabetic nephropathy vitro (**Figure 3**).
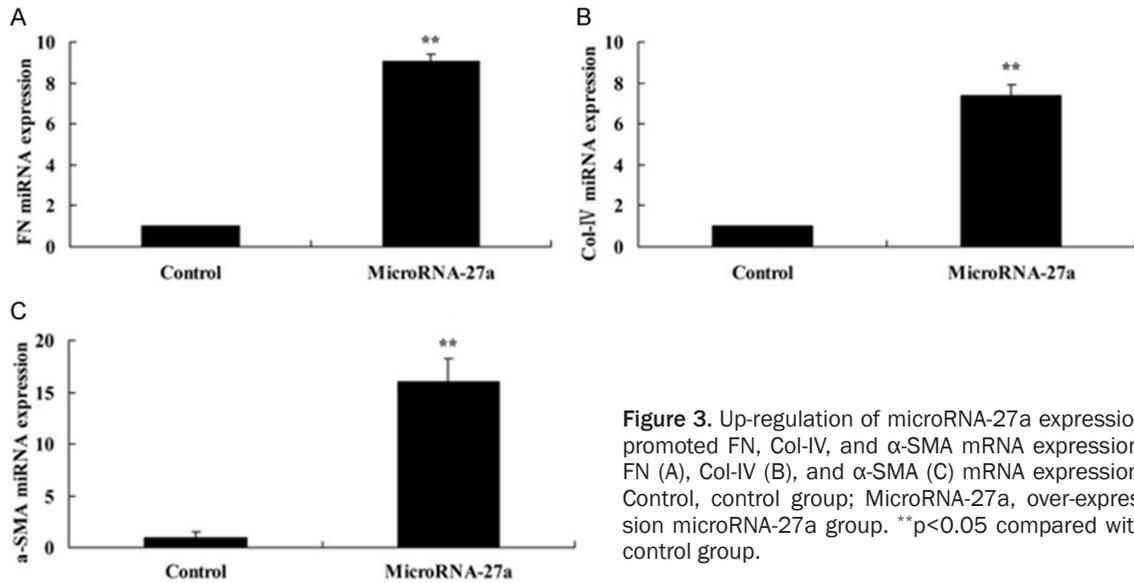
Figure 3. Up-regulation of microRNA-27a expression promoted FN, Col-IV, and α-SMA mRNA expression. FN (A), Col-IV (B), and α-SMA (C) mRNA expression. Control, control group; MicroRNA-27a, over-expression microRNA-27a group. **p<0.05 compared with control group.
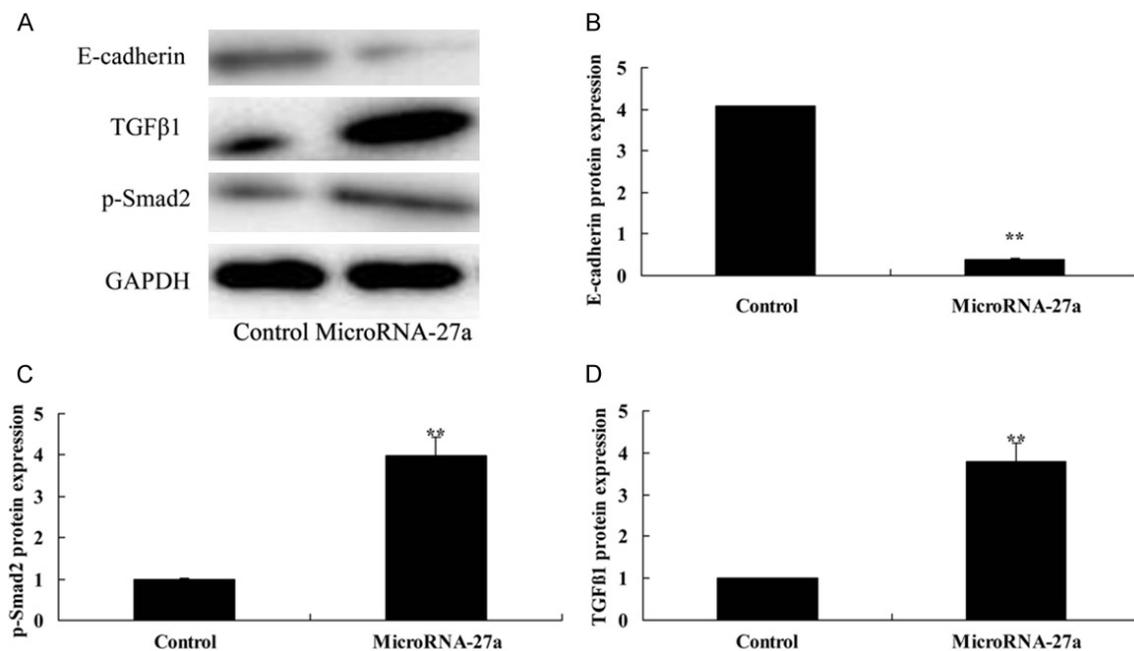

Figure 4. Up-regulation of microRNA-27a expression suppressed E-cadherin protein expression, and induced TGFβ1 and p-Smad2 protein expression. E-cadherin, TGFβ1 and p-Smad2 protein expression (A) and statistical analysis of E-cadherin, TGFβ1 and p-Smad2 protein expression (B-D). Control, control group; MicroRNA-27a, over-expression microRNA-27a group. **p<0.05 compared with control group.

Up-regulation of microRNA-27a expression suppressed E-cadherin protein expression, and induced TGFβ1 and p-Smad2 protein expression

To determine whether microRNA-27a affects E-cadherin and GF-β1/smad2 signaling pathway, we used western blot analysis. Western blotting showed that the up-regulation of micro-RNA-27a expression significantly suppressed E-cadherin protein expression, and induced TGFβ1 and p-Smad2 protein expression in diabetic nephropathy vitro (**Figure 4**).

Down-regulation of microRNA-27a suppressed apoptosis and caspase-3

We then sought to identify microRNA-27a down-regulation on apoptosis and caspase-3 in dia-
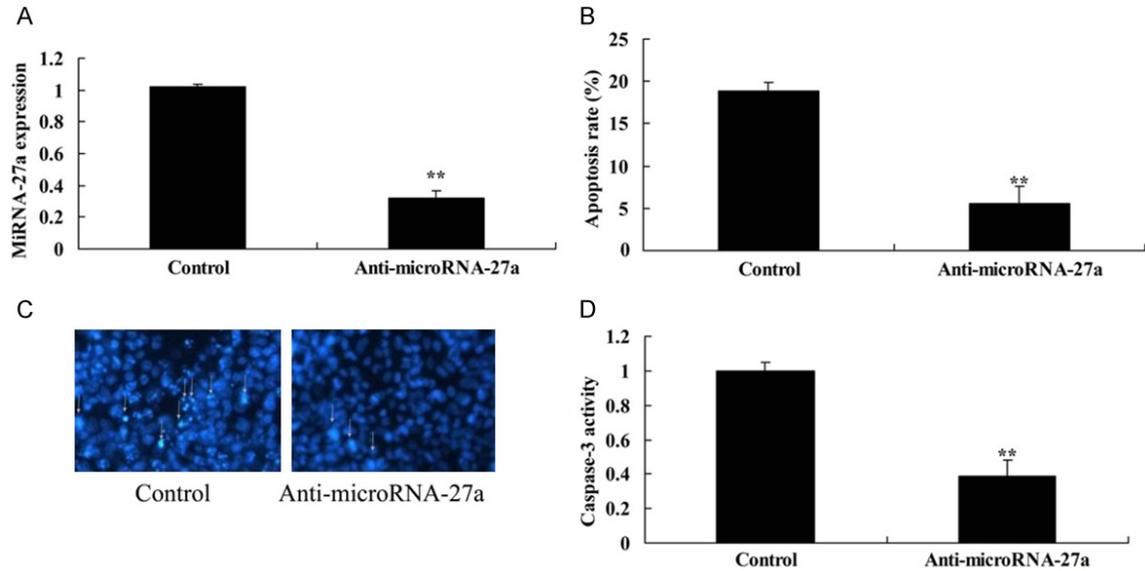
Figure 5. Down-regulation of microRNA-27a suppressed apoptosis and caspase-3. MicroRNA-27a expression (A), apoptosis rate (B), cell apoptotic nueleolus (C) and caspase-3 activity (D). Control, control group; Anti-microRNA-27a, down-expression microRNA-27a group. **$p < 0.05$ compared with control group.
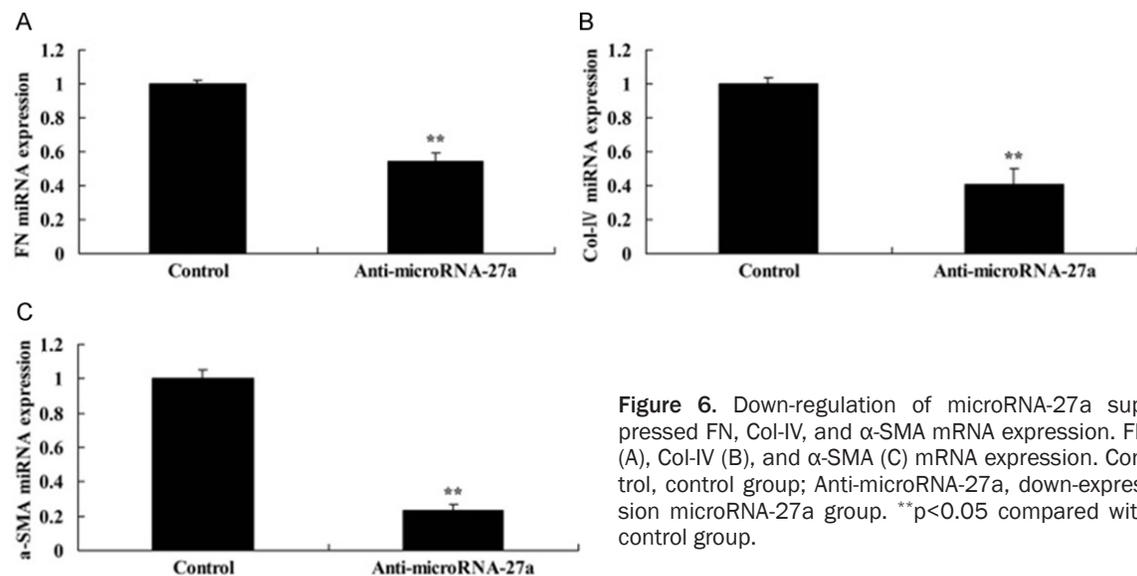

Figure 6. Down-regulation of microRNA-27a suppressed FN, Col-IV, and α-SMA mRNA expression. FN (A), Col-IV (B), and α-SMA (C) mRNA expression. Control, control group; Anti-microRNA-27a, down-expression microRNA-27a group. **$p < 0.05$ compared with control group.

betic nephropathy. Our study revealed that microRNA-27a expression was suppressed by anti-microRNA-27a mimics (**Figure 5A**). Furthermore, the down-regulation of microRNA-27a suppressed apoptosis rate, and reduced cell apoptotic nueleolus and caspase-3 activity in diabetic nephropathy vitro (**Figure 5B-D**).

Down-regulation of microRNA-27a suppressed FN, Col-IV, and α-SMA mRNA expression

In addition, we explored whether microRNA-27a down-regulation regulate FN, Col-IV, and α-SMA miRNA expression in diabetic nephropathy vitro. However, microRNA-27a down-regulation suppressed FN, Col-IV, and α-SMA mRNA expression in diabetic nephropathy vitro (**Figure 6**).

Down-regulation of microRNA-27a induced E-cadherin protein expression, and reduced TGFβ1 and p-Smad2 protein expression

We investigated the down-regulation of microRNA-27a on E-cadherin and GF-β1/smad2 signaling pathway, we used western blot analysis
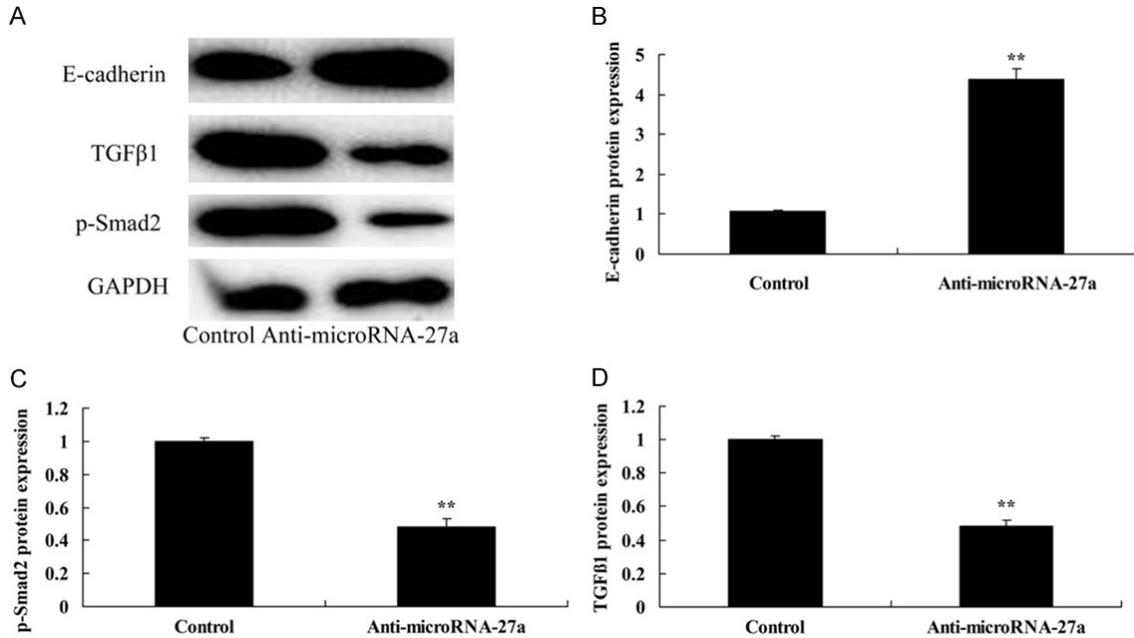
Figure 7. Down-regulation of microRNA-27a induced E-cadherin protein expression, and reduced TGFβ1 and p-Smad2 protein expression. E-cadherin, TGFβ1 and p-Smad2 protein expression (A) and statistical analysis of E-cadherin, TGFβ1 and p-Smad2 protein expression (B-D). Control, control group; Anti-microRNA-27a, down-expression microRNA-27a group. **p<0.05 compared with control group.

to analyze E-cadherin, TGFβ1 and p-Smad2 protein expression. Notably, Down-regulation of microRNA-27a induced E-cadherin protein expression, and reduced TGFβ1 and p-Smad2 protein expression in diabetic nephropathy vitro (**Figure 7**).

Discussion

DN is the one of the most severe complications of diabetes, which is also one of the major causes leading to end-stage uremia [18]. Hyperglycemia is a major factor resulting in severe glomerular and renal tubular injury, among which, renal tubulointerstitial fibrosis plays an extremely important role in the progression of renal failure in diabetics [19]. Previous studies have verified that EMT of renal tubular epithelial cell is an important reason for renal interstitial fibrosis [3, 4]. Our study showed microRNA-27a expression of diabetic nephropathy patients was up-regulated. Moreover, these results suggested that microRNA-27a mimics promoted apoptosis and caspase-3 activity in diabetic nephropathy vitro.

TGF-β, a kind of multi-functional polypeptide cytokine, can be expressed in human tissues such as connective tissue, liver, kidney, lung, brain, skin and muscle [20]. TGF-β is composed of secretory peptide TGF-β, bone morphogenetic proteins (BMPs), activins, inhibins and mullerian inhibitor substance. TGF-β receptors are demonstrated to exist in almost all cells, and they have extensive biological activities [9]. A plenty of studies have indicated that TGF-β signal pathway controls a series of cellular responses, including cell proliferation and differentiation, extracellular matrix formation, body immunity, embryogenesis and injury repair [9]. Abnormality in its signal transduction is closely associated with multiple diseases, like oncogenesis, tissue fibrosis, autoimmune disease, as well as cardiovascular and cerebrovascular diseases [21]. Our results also indicated that up-regulation of microRNA-27a expressioninduced TGFβ1 and p-Smad2 protein expression,promoted FN, Col-IV, and α-SMA mRNA expression in diabetic nephropathy vitro. Min et al. showed that TGF-β-associated miR-27a inhibits dendritic cell-mediated differentiation in Th1 and Th17 cells [22].

TGF-β/Smad signal pathway is a huge family that is extensively distributed in a variety of tissues in multiple organisms ranging from fruit fly

to human being [23]. It has been studied for years, and is shown to regulate cell proliferation, differentiation and apoptosis through complicated receptor signal transduction pathways on cell surface in the manners of autocrine and paracrine [24]. Furthermore, it plays a crucial role in embryogenesis, extracellular matrix formation, wound healing, immune function, bone formation and reconstruction [24]. Smad7 is a negative feedback inhibitor in the TGF-β/Smad pathway, which can inhibit its transduction through interfering the phosphorylation of Smad2 and Smad3 [21]. Importantly, we showed thatdown-regulation of microRNA-27a suppressed apoptosis and caspase-3 activity,reduced TGFβ1 and p-Smad2 protein expression in diabetic nephropathy. Ma et al. showed that miR-27a adjusts differentiation of embryonic stem cells through Smads [25].

Type IV collagen, an important indicator for the genesis and development of DN, is closely related to blood glucose control and course of disease [26]. Determination of urinary albumin can be affected by random factors like diet, urine volume and exercise; thus, the combined determination of type IV collagen and urinary albumin can be served as a monitoring indicator of DN [27]. In our study, microRNA-27a down-regulation suppressed FN, Col-IV, and α-SMA mRNA expression in diabetic nephropathy vitro; up-regulation of microRNA-27a expression significantly promoted FN, Col-IV, and α-SMA mRNA expression in diabetic nephropathy vitro. Tanaka et al. indicated that miR-27 is associated with chemoresistance in esophageal cancer through α-SMA expression [28].

E-cadherin is a calcium-dependent adhesion molecule, which is an important component constituting the tight junction among renal tubular epithelial cells. It plays a crucial role in maintaining cell integrity and polarity [29]. DN is accompanied by changes in E-cadherin expression [30]. It can be speculated that decreased E-cadherin expression can be seen in early renal injury induced by ureteral obstruction, which thus results in re-arrangement of Na+K+ATPase on renal tubular epithelial cell membrane surface (namely, to change from restricted distribution on lateral and basal surface of cell membrane to uniform dispersion on the entire cell membrane), leading to structural changes in renal tubular epithelial cells [31]. These data indicate that the down-regulation

of microRNA-27a induced E-cadherin protein expression, the up-regulation of microRNA-27a expression suppressed E-cadherin protein expression in diabetic nephropathy. Zhao at el. suggested miR-27a-3p suppresses tumor metastasis through E-cadherin in hepatocellular carcinoma [32].

In summary, we presented that microRNA-27a adjusts diabetic nephropathy patients and promoted FN, Col-IV, and α-SMA mRNA expression through TGF-β/Smad signaling pathway. Taken together, these results showed that microRNA-27a provide new insights into the progression of diabetic nephropathy.

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