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Original Article

Tannins from pomegranate seeds ameliorate renal injury in streptozotocin-induced diabetic rat through the activation of microRNA-495 via regulating SMAD7

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Abstract: The present study was to investigate the effects of tannins from pomegranate seeds (TA-PS) on renal injury in streptozotocin (STZ)-induced experimentally type 1 diabetic mice. By treating STZ-induced diabetic mice with TA-PS, we found that the upregulation of albuminuria and urine calcium induced by hyperglycemia was reversed by TA-PS in experimentally type 1 diabetic mice. However, TA-PS treatment had no obvious effects on fasting blood glucose and body weight. Additionally, the regulating effect of TA-PS on renal fibrogenesis was investigated, and the down-regulation of E-cadherin and the up-regulation of α-SMA in kidney might be a better explanation for renal fibrogenesis in type 1 diabetic mice. However, the levels of E-cadherin and α-SMA were reversed by TA-PS treatment in diabetic nephropathy mice. Importantly, bioinformatics analysis suggested that miR-495 was a regulator of SMAD7. The levels of miR-495 and SMAD7 were simultaneously suppressed; however, tannins treatment could reverse the downregulation of miR-495 and SMAD7 in the kidney of diabetic rat. In conclusion, our work demonstrated that tannins from pomegranate seed could significantly ameliorate the pathological changes in the renal fibrosis induced by hyperglycemia in rat, and the underlying mechanism was mediated, at least partially, through the activation of microRNA-495 via enhancing SMAD7.

Keywords: Diabetic nephropathy, tannins, pomegranate seeds, SMAD7 microRNA-495

Introduction

Diabetic nephropathy (DN), as one of the major complications of diabetes, is now the most common cause of chronic kidney failure and end-stage renal disease in the worldwide [1]. From a clinical perspective, albuminuria, glomerular hypertrophy and excellular matrix protein accumulation are the major clinical associations which contribute to the development of final renal failure [2]. Reducing proteinuria is very important and is the main target for the treatment of diabetic nephropathy. Although DN has been regarded as an irreversible and rapidly progressing disease, progression to kidney failure may be slowed by the use of the pomegranate seed extracts [3-5]. Pomegranate [Punica granatum L. (Punicaceae)], as one of nature's powerful fruits, has markedly high antioxidant capacity [6], anti-inflammatory activity [7], anti-atherosclerotic [8] and cardioprotective [9], which have been attributed to its high polyphenol content. Actually, tannins as the major active ingredients of pomegranate seed extracts possess the pharmacological actions in a various of tissue damages [10, 11]. However, the roles of tannins from pomegranate seeds in hyperglycemia-induced albuminuria and renal injury have not been clearly delineated.

MicroRNAs (miRs) are endogenous non-coding RNAs and single-stranded RNA molecules of ~22 nucleotides in length that serve as important post-transcriptional gene regulators. The key features of miRs control cell proliferation and differentiation of various cell types. A growing number of studies have demonstrated that the pathogenic change in various tissues has been linked to miRs [12, 13]. A recent study indicates that urinary miRs profiling predict the development of microalbuminuria in patients

with type 1 diabetes [14]. Moreover, miR-346, miR-26a, miR-130b and miR-495 are involved in the pathological mechanism of DN [14-17]. MiR-495 is initially reported to be present in brain tissues and can regulate the proliferation and apoptosis of cancer cells [18]. In human umbilical vein endothelial cells, miR-495 regulates the proliferation and apoptosis by targeting chemokine CCL2 [19]. Recent studies have shown that miR-495 via suppressing monocarboxylate transporter 1 (MCT1) attenuates type 2 diabetes in mice [20]. However, the role of miR-495 in hyperglycemia-induced type 1 diabetic rat has not been explored until recently.

In this study, bioinformatics analysis suggested that miR-495 was a regulator of drosophila mothers against decapentaplegic 7 (SMAD7). There was a possibility that miR-495 might be involved in hyperglycemia-induced renal injury and was utilized as a therapeutic target for tannins from pomegranate seeds in experimentally type 1 diabetic rat.

Materials and methods

Animal treatment and cell culture

Six-month-old male Sprague-Dawley rats (Slac Laboratory Animal, Shanghai, China) were allowed to acclimate to the environment for 1 week. All experimental procedures were carried out in accordance with the guidelines of the First Affiliated Hospital of Soochow University. All chemicals and reagents were purchased from Sigma (Oakville, Ontario, Canada), except where noted. The rats were randomly divided into three groups: (1) Vehicle group (n = 8); (2) Streptozotocin (STZ)-induced diabetic nephropathy rats (DN, n = 8); (3) STZ with tannins-treated group received tannins orally at a dose of 1000 mg/kg/day (TA-PS, n = 8). The rats were induced hyperglycemia by intraperitoneal injection of STZ, dissolved in citrate buffer (0.1 M at pH 4.2), at 45 mg/kg body weight for singly injection. The rats in vehicle group were injected with solvent. All rats were sacrificed 12 weeks after STZ injection. The fasting blood glucose (FBG) levels were measured with a blood glucose monitoring system (Roche). Body weight and FBG were recorded every three weeks during experimental period.

The rat NRK-52E cells were obtained from the Chinese Academy of Sciences (Institute of Shanghai Cell Biology and Chinese Type Culture Collection, China), and maintained in DMEM (Dulbecco's modified Eagle's medium; Invitrogen), supplemented with 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 100 units/ml penicillin, and 100 mg/ml streptomycin (Invitrogen) at 37°C in a humidified, 5% CO_2, 95% air atmosphere. The medium was replenished every day.

Chemistries in urine

At the each point, mice selected randomly in each group were placed in metabolic cages to collect 24 h urine and record the amount of urine volume, which was used for detecting urinary total protein and the concentration. The concentrations of calcium (Ca) from urine were measured by standard colorimetric methods using a micro-plate reader (Bio-Tek, USA). The level of urine Ca was corrected by the concentration of urine Cr.

Histomorphology and immunohistochemistry

Formalin-fixed and paraffin-embedded kidney tissues were cut into about 4 μm-thick sections, which were stained with periodic acid-Schiff (PAS) staining, and visualized under a microscope (Leica DM 2500).

The kidney was evaluated immunohistochemically using anti-rat FN and SMAD7. Paraffin embedded tissues were cut into about 4 μm sections, mounted on glass slides and stained using indirect immunoperoxidase. The paraffin sections were baked in oven at 65°C for 24 h, then dewaxing to water, rinsed with PBS three times (5 min per time). Well washed sections were placed in the EDTA buffer for microwave antigen retrieval, the fire to boil, then low heat to boil after an interval of 10 min. After natural cooling, the sections were washed with PBS 3 times. The sections were put into 3% hydrogen peroxide solution and incubated at room temperature for 10 min, which was to block endogenous peroxidase, then washed with PBS 3 times, closed with 5% bovine serum albumin (BSA) for 20 min after drying (close charge). After removal of BSA liquid, each section was added with 50 μl diluted primary antibody overnight at 4°C, then washed with PBS 3 times. After the removal of PBS liquid, each slice which added with 50-100 μl secondary antibody was incubated at 4°C for 50 min, then

washed with PBS 3 times, each slice was added with 50-100 μl freshly prepared DAB solution with the help of microscope for controlling color. After being washed, sections were counterstained with hematoxylin, rinsed with tap water, dehydrated and mounted, and visualized under a microscope (Leica DM 2500).

Luciferase reporter gene activity assay

The 3'UTR of SMAD7 gene containing the predicated target sites for miR-495 was obtained by PCR amplification. The fragment was inserted into the multiple cloning sites in the pMIR-REPORT luciferase microRNA expression reporter vector (Ambion, Austin, USA). HEK-293 cells were co-transfected with 0.1 μg of luciferase reporters containing SMAD7 3'UTR and miR-495 mimics by Lipofectamine 2000 (Invitrogen, Carlsbad, USA). We harvested the cell lysates after 48 h transfection and measured the luciferase activity with a dual luciferase reporter assay kit according to manufacturer's instruction.

Reverse transcription-polymerase chain reaction (RT-PCR)

The RNA extraction was performed according to the TRIzol manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). RNA integrity was verified by agarose gel electrophoresis. Synthesis of cDNAs was performed by reverse transcription reactions with 2 μg of total RNA using moloney murine leukemia virus reverse transcriptase (Invitrogen) with oligo dT (15) primers (Fermentas) as described by the manufacturer. The first strand cDNAs served as the template for the regular polymerase chain reaction (PCR) performed using a DNA Engine (ABI 7300). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control was used to normalize the data to determine the relative expression of the target genes. The reaction conditions were set according to the kit instructions. The PCR primers used in this study were shown as following: SMAD7, Forward 5'-TTTTGAGGTGTGGTGGG-3' and Reverse 5'-TAGGCAGATCGGTGCAAGTGA-3'; FN, Forward 5'-GAGTGGAGGCGATTGAAGTGCCGT-3' and Reverse 5'-CTGGTGGACGATTGACGAAGTAGAA-3'; collagen I, Forward 5'-ACACGCAGAATGACGTACGCGATT-3' and Reverse 5'-TTTCGTGCTACGATCGGCAGTGTA-3'; OPN, Forward 5'-TGAGACTGGCAGTGGTTTGC-3' and Reverse 5'-CCACTTTCACCGGGAGACA-3'; NGAL, Forward 5'-GACTCAACTCAGAACTTGATCCCT-3' and Reverse 5'-AGCTCTGTATCTGAGGGTAGCTGT-3'; PAI-1, Forward 5'-GAGCCAGATTCATCATCAACG-3' and Reverse 5'-CTGCAATGAACATGCTGAGG-3'; miR-495, Forward 5'-GCGCGTGAGCAGGCTGGAGAAATT-3' and Reverse 5'-AAACAAACATGGTGCA-3'; GAPDH, Forward 5'-GGATTTGGTCGTATTGGG-3' and Reverse 5'-GGAAGATGGTGATGGGATT-3'.

Western blotting

The kidney was homogenized and extracted in NP-40 buffer, followed by 5-10 min boiling and centrifugation to obtain the supernatant. Samples containing 50 μg of protein were separated on 10% SDS-PAGE gel, transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). After saturation with 5% (w/v) non-fat dry milk in TBS and 0.1% (w/v) Tween 20 (TBST), the membranes were incubated with the following antibodies, α-SMA, E-cadherin and SMAD7 (Santa Cruz Biotechnology, USA), collagen I and collagen IV (Bellinzona, Switzerland), at dilutions ranging from 1:500 to 1:2,000 at 4°C over-night. After three washes with TBST, membranes were incubated with secondary immunoglobulins (Igs) conjugated to IRDye 800 CW Infrared Dye (LI-COR), including donkey anti-goat IgG and donkey anti-mouse IgG at a dilution of 1:10,000-1:20,000. After 1 hour incubation at 37°C, membranes were washed three times with TBST. Blots were visualized by the Odyssey Infrared Imaging System (LI-COR Biotechnology). Signals were densitometrically assessed (Odyssey Application Software version 3.0) and normalized to the β-actin signals to correct for unequal loading using the mouse monoclonal anti-β-actin antibody (Bioworld Technology, USA).

Statistical analysis

All data were expressed as the mean ± standard deviation (SD). The statistical analyses were performed using the SPSS 13.0 statistical software package (SPSS Inc., USA). One-way analysis of variance (ANOVA) was used to perform comparisons among the different groups, and $P < 0.05$ was considered to be statistically significant.

Results

Body weight and fasting blood glucose

The body weight of non-diabetic control rat steadily increased during the experimental

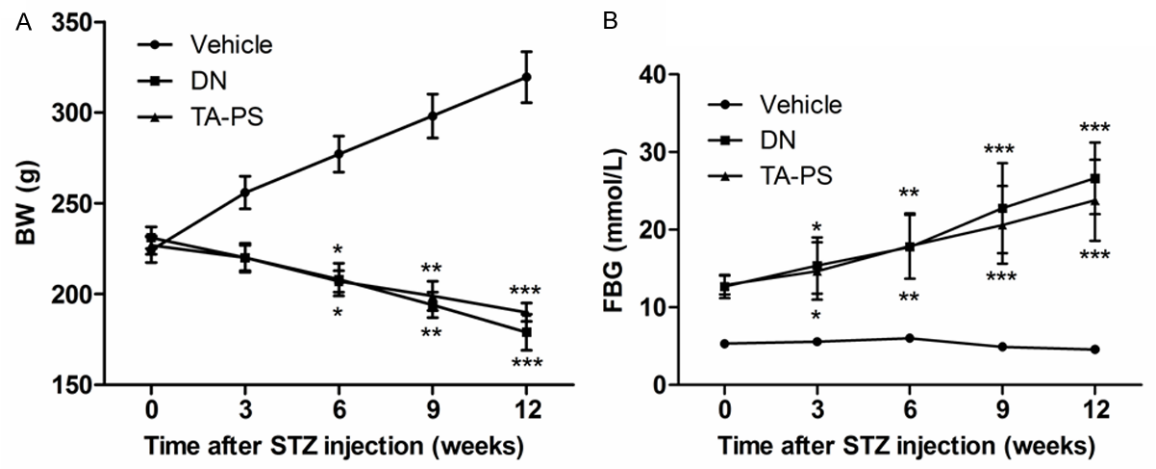

Figure 1. Body weight (A) and fasting blood glucose (B) were recorded every three weeks during experimental period. Values are expressed as mean ± SD, n = 10 in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, versus vehicle group.

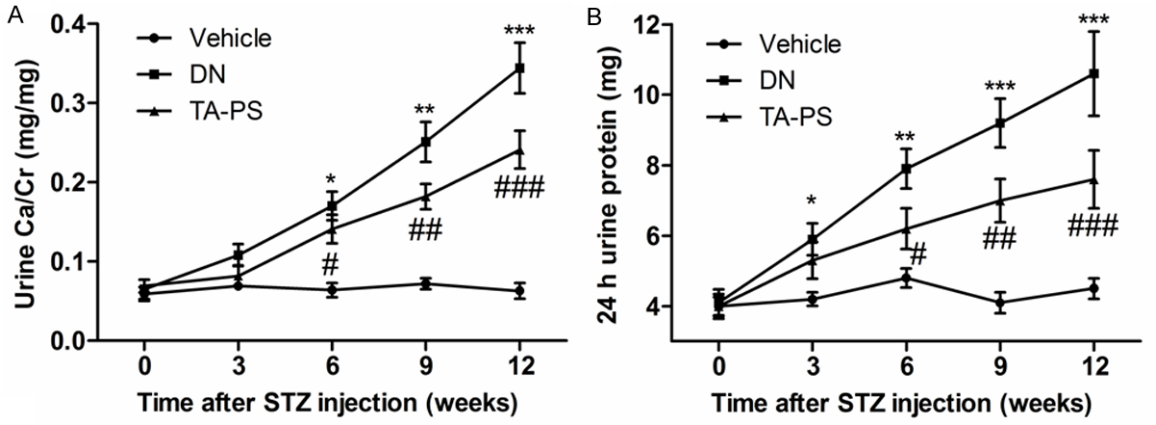

Figure 2. Urine calcium (A) and 24 h urine protein (B) were recorded every four weeks during experimental period. Values are expressed as mean ± SD, n = 10 in each group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, versus vehicle group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, versus DN group.

period (**Figure 1A**). However, after 3 weeks STZ injection, the body weight of rat in DN group and TA-PS treatment group was significantly decreased as compared to those of the control group (**Figure 1A**). One week after the STZ injection, fasting blood glucose (FBG) was measured, and whole blood was collected from the orbital vein to confirm diabetes at levels greater than 11.1 mmol/L. The FBG level was maintained within the normal range of 3.9-6.1 mmol/L in the control group during the experimental period (**Figure 1B**). The FBG in both the DN and TA-PS treatment group was significantly increased as compared to that of the control group. The FBG value of rat in DN group and TA-PS treatment group rose from 12.7 mmol/L and 12.9 mmol/L at week 0 to 26.6 mmol/L and 23.8 mmol/L at week 12 respectively (**Figure 1B**).

Tannins improve urine calcium and albuminuria in diabetic rat

Acute toxicity study revealed the LD_{50} of TA-PS was 7.82 g/kg (7.82 ± 1.65 g/kg) body weight. As shown in **Figure 2**, at each time point (except week 0), the levels of urine calcium (**Figure 2A**) and 24 h total urinary protein (**Figure 2B**) in the DN group were significantly increased compared with the non-diabetic control group during the experimental period. Three weeks after STZ injection, the levels of urine calcium and 24 h total urinary protein in the DN group were 0.108 ± 0.012 mg/mg and 5.9 ± 0.51 mg, sig-

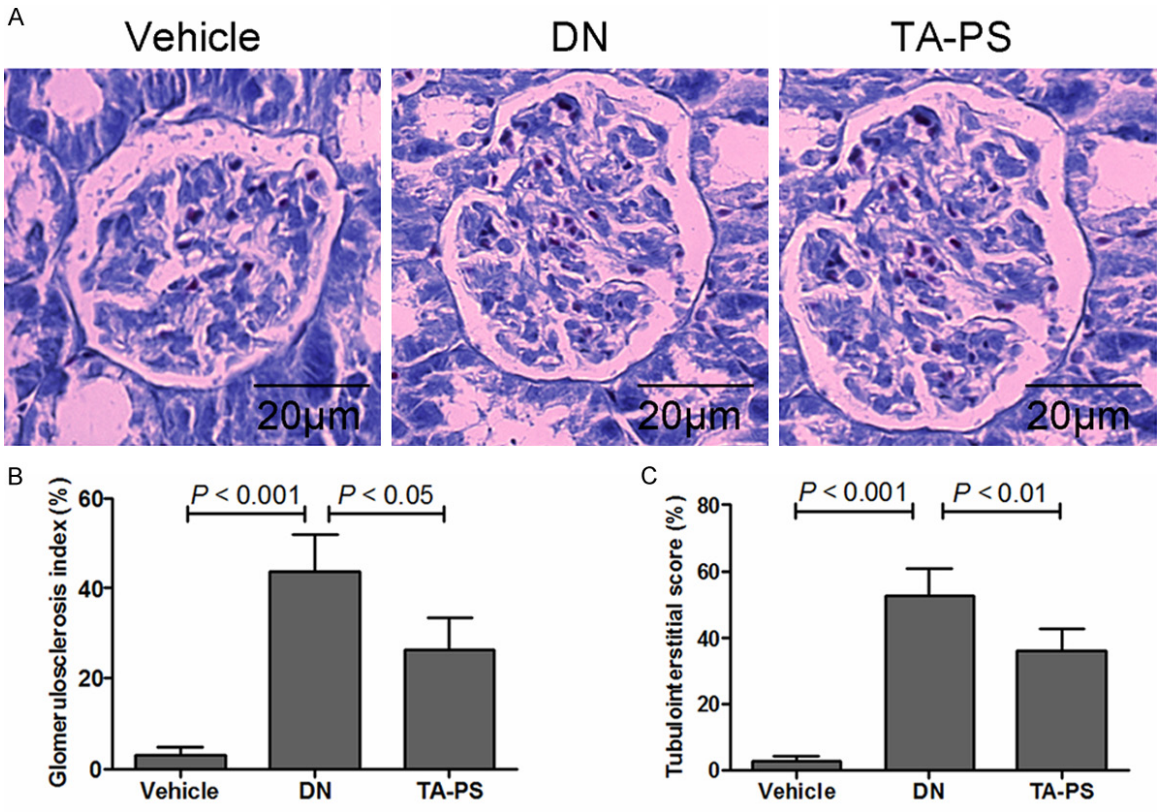

Figure 3. Renal pathology PAS staining (× 400) were performed after 12 weeks by tannins treatment in experimentally type 1 diabetic mice (A). GSI, glomerulosclerosis index (B); TIS, tubulointerstitial score (C).

nificantly higher than 0.069 ± 0.007 mg/mg and 4.2 ± 0.23 mg in the non-diabetic control group ($P < 0.05$). However, the levels of urine calcium in TA-PS group were 0.141 ± 0.015 mg/mg at week 6, 0.182 ± 0.013 mg/mg at week 9 and 0.241 ± 0.027 mg/mg at week 12, significantly lower than 0.170 ± 0.018 mg/mg at week 6 ($P < 0.05$), 0.251 ± 0.022 mg/mg at week 9 ($P < 0.01$) and 0.344 ± 0.035 mg/mg at week 12 ($P < 0.001$) in the DN group (**Figure 2A**). Meanwhile, the levels of 24 h total urinary protein in TA-PS group were 6.2 ± 0.54 mg at week 6, 7.0 ± 0.65 mg at week 9 and 7.6 ± 0.77 mg at week 12, significantly lower than 7.9 ± 0.68 mg at week 6 ($P < 0.05$), 9.2 ± 0.77 mg at week 9 ($P < 0.01$) and 11.6 ± 1.06 mg at week 12 ($P < 0.001$) in the DN group (**Figure 2B**).

Tannins ameliorate renal injury in diabetic rat

To determine the role of tannins in hyperglycemia-induced renal fibrogenesis in diabetic rat, histological examination by periodic acid-schiff (PAS) was implemented. The DN group showed significant glomerular and tubulointerstitial damage, including glomerular sclerosis, mesangial expansion, tubular dilatation, atrophy and interstitial fibrosis. Tannins administration could markedly ameliorate renal interstitial collagen accumulation in diabetic rat (**Figure 3A**). Moreover, these data were supported by analysis of renal pathology by means of GSI (**Figure 3B**) and TIS (**Figure 3C**). These results show that tannins from pomegranate seeds significantly improve renal physiological structure and function in diabetic nephropathy rat. Effects of tannins treatment on gene expression of renal injury markers were measured. When compared with the non-diabetic rats, diabetic rats expressed elevated levels of the renal injury markers OPN, NGAL and PAI-1. This was in sharp contrast with DN group, genes expression levels demonstrated a decrease in TA-PS treatment group (**Figure 4A**). Next, we examined the expression of extracellular matrix protein FN and collagen I, which were the biomarkers of renal fibrosis. Real-time PCR showed a marked increase in FN and collagen I in the diabetic rat as compared to the control group, however, the increase in diabetic rat was significantly inhibited by TA-PS administration (**Figure 4B**). Consistent with the mRNA levels, the west-

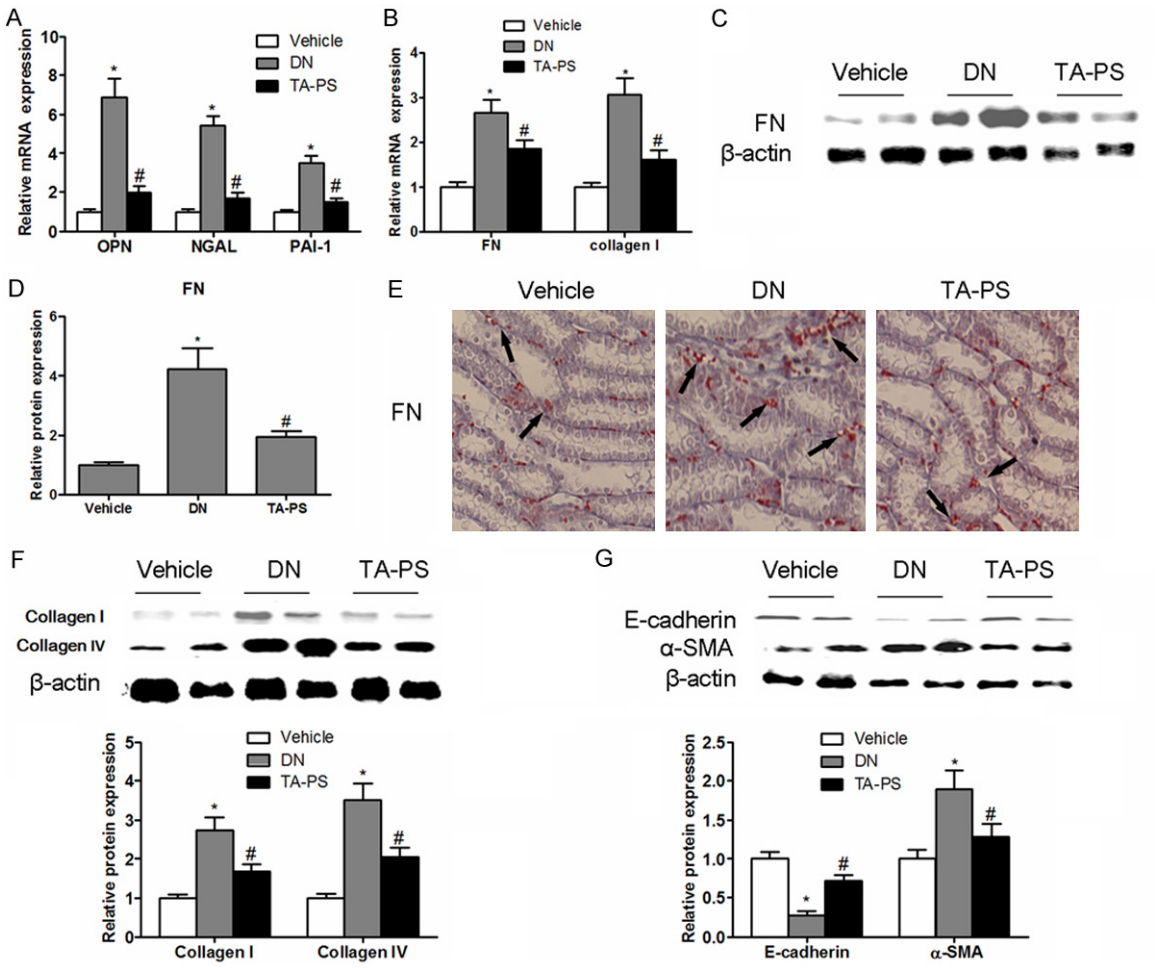

Figure 4. Gene expression of the renal injury markers OPN, NGAL and PAI-1 was measured by real-time PCR (A). Real-time PCR quantification of FN and collagen I expression in the kidney (B). Western blotting analysis of FN levels (C) and the relative statistical values of FN (D). Immunostaining of kidney sections with anti-FN antibody (E). Western blot analysis of collagen I and collagen IV (F). Western blot analysis of α-SMA and E-cadherin (G). Values are expressed as mean ± SD, n = 10 in each group. $^{*}P < 0.05$ versus vehicle group, $^{\#}P < 0.05$ versus DN group.

ern blotting (**Figure 4C** and **4D**) and immunohistochemical staining (**Figure 4E**) showed increased levels of FN in total kidney lysates from diabetic rat. However, the upregulated level of FN was reversed by TA-PS treatment in diabetic rat. Furthermore, in tannins-treated rat, there was marked induction of E-cadherin and marked reduction of α-SMA, collagen I and collagen IV in the TA-PS group compared with the control group (**Figure 4F** and **4G**). These data indicate that tannins from pomegranate can blunt the enhanced renal fibrosis seen in STZ-induced diabetic rat.

miR-495/SMAD7 signaling involves in hyperglycemia-induced renal injury

Renal fibrosis is a hallmark of DN and has been shown to be inhibited by SMAD7 [21]. We thus examined if the blockade of diabetic kidney injury by tannins is associated with miR-495/SMAD7 signaling. As shown in **Figure 5A**, miR-495 regulated SMAD7 through the predicted binding sites in its 3'-UTR. Next, we designed luciferase construct by incorporating either wild-type or mutant 3'-UTR of SMAD7, which constitutively expressed luciferase unless repressed by the incorporated 3'-UTR. The results indicated that PLemiR-495 resulted in a lower luciferase activity than control group, leading to a nearly 80% decline in the luciferase activity compared with control group ($P < 0.05$). However, mutant SMAD7 3'-UTR and PLemiR-495 did not show much difference as compared to control group (**Figure 5B**). These results demonstrate that miR-495 is a regulator of SMAD7. Intriguingly, when the expression of miR-495 in NRK-52E cell inhibited, the pro-

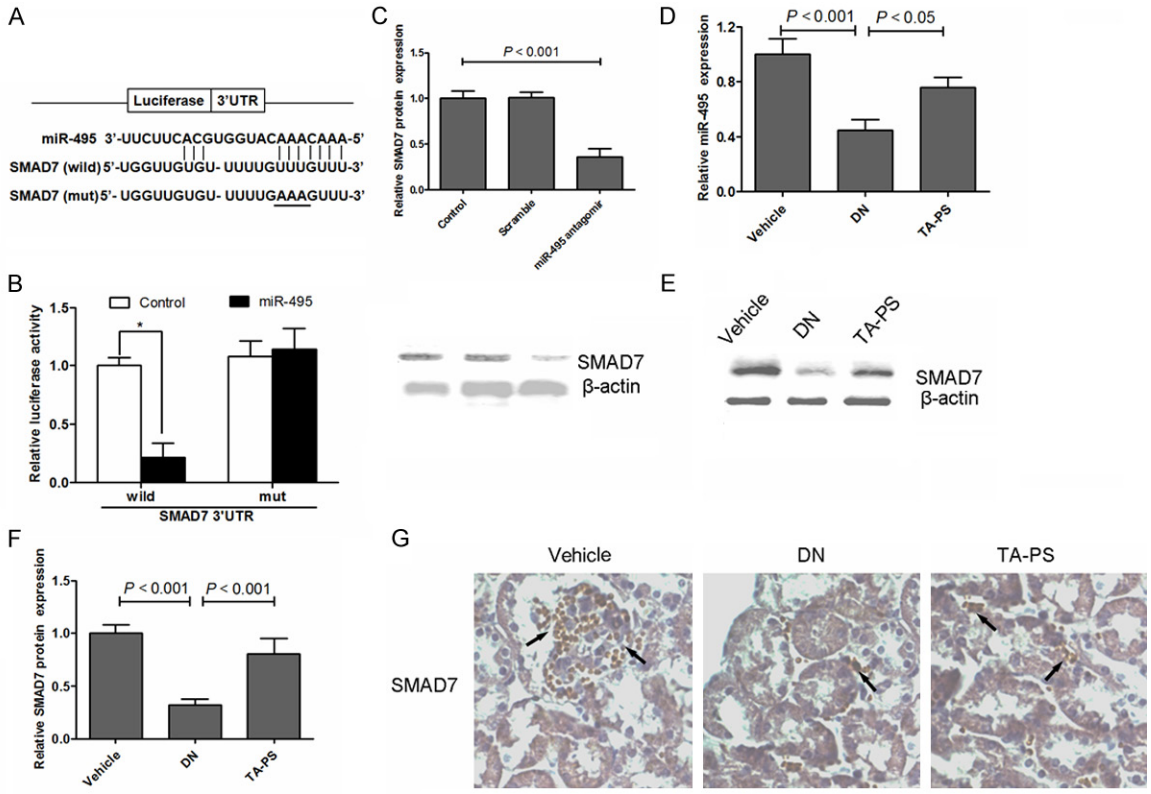

Figure 5. Schematic representation of the putative miR-495 binding site in the SMAD7 3'UTR as in Targetscan (A). Luciferase activity assay (B). The protein expression of SMAD7 was measured by western blotting in the present of miR-495 antagomir in NRK-52E cells (C). The expression of miR-495 in the kidney of rat (D). The protein expression (E) and relative statistical values (F) of SMAD7 in the kidney of rat. The protein expression of SMAD7 in the kidney of rat was measured by immunofluorescent staining (G).

tein expression of SMAD7 was significantly decreased as a response for inhibiting miR-495 (**Figure 5C**). In diabetic rat, the levels of miR-495 and SMAD7 were simultaneously suppressed, however, tannins treatment could reverse the downregulation of miR-495 and SMAD7 in the kidney of diabetic rat (**Figure 5D-G**).

Discussion

Renal fibrosis is a common downstream event leading to renal failure, thus, understanding the development of renal fibrosis has important implications for therapeutic intervention of kidney disease [22]. Moreover, microalbuminuria is also a risk factor for renal fibrosis. Although the inhibitors of the renin-angiotensin system are thought to confer some advantage in delaying the onset of diabetic renal complications in those with microalbuminuria, toxic side and the resistance effects after long-term application remain the main obstacle for clinical doctors [23]. Moreover, the scientific efforts and significant progress in understanding the DN, but the precise mechanisms underlying DN are still unknown. Therefore, it is extremely urgent to explore the novel therapeutic targets and strategies for DN clinic treatment.

There is growing evidence that the extracts of pomegranate seed significantly decrease the levels of blood glucose in diabetic rat [24]. Pomegranate seed oil can decrease weight gain and reduce the risk for type 2 diabetes in wild type CD-1 mice by improving insulin sensitivity [5, 8, 25]. Previous study has found that tannin from green tea is found to lighten the kidney under such oxidative stress and suppress the progression of the renal failure [26]. However, the pharmacological function of tannin from pomegranate seed in hyperglycemia-induced renal injury has not seen the relevant report at present. In the present study, these observations document that the upregulation of albuminuria and urine calcium induced by

hyperglycemia in experimentally type 1 diabetic mice, however, tannin from pomegranate seed could reverse STZ-induced albuminuria and disequilibrium of calcium homeostasis. In PAS assay, diabetic rat showed significant glomerular and tubulointerstitial damage which reflected renal tissue had damaged. Interestingly, the administration of tannins from pomegranate seed improved pathological changes caused by renal fibrosis.

In our study, we firstly examined miR-495 and SMAD7 expressions in the kidney of diabetic rat. Results indicated miR-495 and SMAD7 were significantly down-regulated in the kidney of diabetic rat compared with non-diabetic controls. Next, we attempted to confirm whether there was a regulatory relationship between miR-495 and SMAD7. We provided the first evidence that miR-495 could directly regulate SMAD7 expression by binding with its 3'UTR, which was supported by the following reasons: miR-495 gain-of-function significantly decreased the luciferase activity in HEK293FT cells. Moreover, in NRK-52E cell, miR-495 inhibitor decreased endogenous SMAD7 expressions at protein levels. SMAD7 is a downstream signaling molecule of TGF-β1 and can repress epithelial-to-mesenchymal transition (EMT), which plays an important role in renal interstitial fibrosis with DN [27]. Meantime, the siRNA experiment shows that SMAD7 can directly regulate α-SMA and E-cadherin expression. SMAD7 siRNA knockout endogenous SMAD7 gene in vitro, α-SMA expression is increased and E-cadherin expression is decreased, suggesting that smad7 can directly control the gene expression of EMT [28]. SMAD7 gene transfer inhibits renal TGF-β/ SMAD3 and nuclear factor κB (NF-κB) signaling pathways, and improves diabetic nephropathy in mice [29]. In our experiment, tannins could reverse the decreased E-cadherin and increased α-SMA in the kidney of diabetic rat. Meantime, the mRNA and protein expression of SMAD7 were significantly upregulated. Therefore, we have absolute proof that tannins from pomegranate seeds play an important role in renoprotective effect via enhancing the levels of SMAD7.

In conclusion, our work demonstrated that tannins from pomegranate seed could significantly ameliorate the pathological changes in the renal fibrosis induced by hyperglycemia in rats, and the underlying mechanism was mediated, at least partially, through the activation of microRNA-495 via enhancing SMAD7.

Disclosure of conflict of interest

None.

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