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Original Article

The role of TGF-β1, P53 and microRNA 192 in the pathogenesis of diabetic nephropathy in diabetic rats

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Abstract: Objective: To investigate the role of TGF-β1, P53 and microRNA 192 in the pathogenesis of diabetic nephropathy. Methods: 40 SD rats were randomly divided into control group (10) and experiment group (30). First of all, the rat model of diabetic nephropathy was established. Then, HE staining of rats' renal tissues was used to examine the model effect; subsequently, immunohistochemical staining, Western Blot and real-time quantitative PCR were performed to compare the expression of TGF-β1 and P53 protein, as well as the expression levels of TGF-β1 mRNA, P53 mRNA and microRNA 192 in two groups. Results: the model success rate in experiment group was 86%. The expression levels of TGF-β1 mRNA, P53 mRNA and microRNA 192 in the experiment group were significantly higher than those in the control group ($P<0.05$). The expressions of TGF-β1 and P53 protein in the experiment group were significantly higher than those in the control group ($p<0.05$). Conclusion: The expression levels of TGF-β, P53 protein and microRNA 192 were significantly increased in renal tissues of diabetic nephropathy rats, which might promote the development of diabetic nephropathy.

Keywords: TGF-β, P53, microRNA 192, diabetic nephropathy

Introduction

Diabetic nephropathy (DN) is one of the complications caused by diabetes that did not get timely and effective treatment [1], eventually, progress to ESRD (end-stage renal disease, ESRD), which is one of the most important factors that lead to organ damage and even death in diabetic patients [2]. Diabetic nephropathy is the most common cause of renal failure without any obvious clinical manifestations in the early stage. At the early stage of renal disease, if the patient did not receive timely and effective intervention, more than half of the patients will progress to ESRD 10 years later, and the disease is irreversible which seriously affects the quality of their daily life, and even threaten their life [3]. The pathogenesis of diabetic nephropathy is not clear that there is no effective clinical treatment; at present, we try to reduce kidney damage mainly by lowering blood glucose [4]. Thus, research on the pathogenesis of diabetic nephropathy helps to provide new ideas for clinical treatment of the disease.

TGF-β can promote cell proliferation and hypertrophy, as well as the accumulation of extracellular matrix. More and more evidences show that tumor suppressor P53 plays an important role in severe kidney disease, but it is not clear in the pathogenesis of diabetic nephropathy. In tumor cells, P53 can combine with the miRNA 192 promoter sequences and promote its expression. Previous studies have indicated that miRNA plays an important role in diabetic complications, especially in the occurrence and development of diabetic nephropathy. MiR-192 is one of the important downstream factors of TGF-β to promote the expression of collagen gene. Therefore, this study used male SD rats as the research object to investigate the role of TGF-β1, P53 and microRNA 192 in diabetic nephropathy in animal model.

Materials and methods

General information

40 male SD rats were selected, clean grade, 250~300 g, 8 weeks old, and the animals were

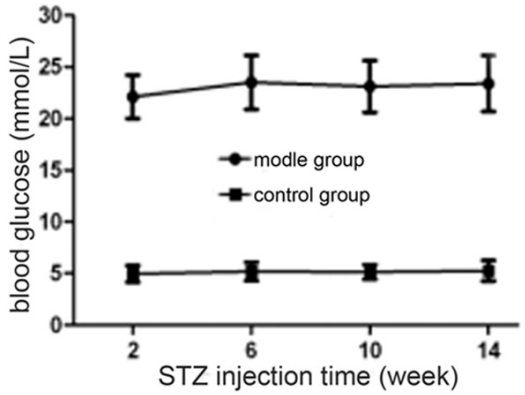

Figure 1. The change of blood glucose in two groups.

provided by department of laboratory animal science, Peking University Health Science Center (animal license number: SCXK (Jing) 2011-0012). Before the experiment, the rats were fed in a relatively quiet enviroment with good ventilation facilities, fresh air, suitable temperature and appropriate light; drinking and eating ad libtum. All the procedures in the whole experiment were in accordance with the relevant provisions of the management of animal experiments [5]. All rats were reared under the same environment for 7 d and then randomly divided into control group (C, n = 10) and experiment group (H, n = 30).

Research method

Animal model construction: After adaptive feeding for a week, the rats were fasted for 12 hours. 2% solution was made by mixing streptozotocin (STZ) with 0.1 mol/L of aseptic citrate buffer uniformly. Rats in the experiment group were injected with STZ at 40 mg/kg dose in caudal vein, and control group was injected with equal volume of citrate buffer. All rats were continuously fed with standard diet for 2 weeks; detect blood glucose; eliminate the rats with blood glucose ≤ 16.7 mmol/L during the process of model constructing. The rats in control group were not screened. After model constructed, continuously feed for 12 weeks, and detect the blood glucose level every 4 weeks. Rats were sacrificed and the kidneys were taken for the following experiments 1d before the end of the experiment.

Hematoxylin-eosin staining

The kidney tissues were trimmed and embedded in paraffin, and 4 μm thick slices were prepared for H-E staining. The specific steps are as follows: firstly the sections were baked in oven at 60°C for 20 minutes, then removed from oven and soaked in xylene immediately for 20 minutes, after that, dehydrated by gradient alcohol, and finally rinsed by clean water for H-E staining. We first used hematoxylin to stain nucleus for 2 minutes, and then used 1% hydrochloric acid alcohol to carry on colour separation for 2 seconds after water rinse, later placed the slices at room temperature about 10 minutes to show blue; after showing blue, we then used eosin to stain the cytoplasm, and later used gradient alcohol again to dehydrate gradually, and then used xylene for vitrification, finally we used neutral balata to seal. Nuclei were observed in blue, while cell cytoplasm or collagenous fibers were in red at different degrees under microscope.

Immunohistochemical staining

After dewaxing and dehydration, paraffin slices were incubated in 3% hydrogen peroxide solution at room temperature for 10 min to seal the activity of endogenous peroxidase. After washing with PBS, primary antibodies (Rabbit anti rat TGF-β1 antibody and Rabbit anti mouse p53 antibody, purchased from America Santa Cruz company) were added in and the slices were incubated at 4°C overnight, and then rewarmed at 37°C for 20 min; after washing with PBS. secondary antibodies (Goat anti rabbit IgG antibody, purchased from America Santa Cruz company) were added in and the slices were incubated at 37°C for 20 min; after washing with PBS, DAB was dropped in, and finally the slices were counterstained with hematoxylin, differentiated and showed blue, dehydrated, transparented and sealed. 8 visual fields (×400) were randomly selected from each slice under the same conditions, and 200 cells were counted in each field. The immunohistochemical results were analyzed by NIS-ElementsBR 3.1 image analysis software. We counted all the positive cells and calculated positive rate.

Expression of P53 protein and TGF-β1 in renal tissues of model rats with diabetes mellitus by Western Blot

The total protein of the two groups was extracted on the ice, and the protein was quantified by the BCA method. SDS-PAGE gel electrophoresis was performed with equal amounts of

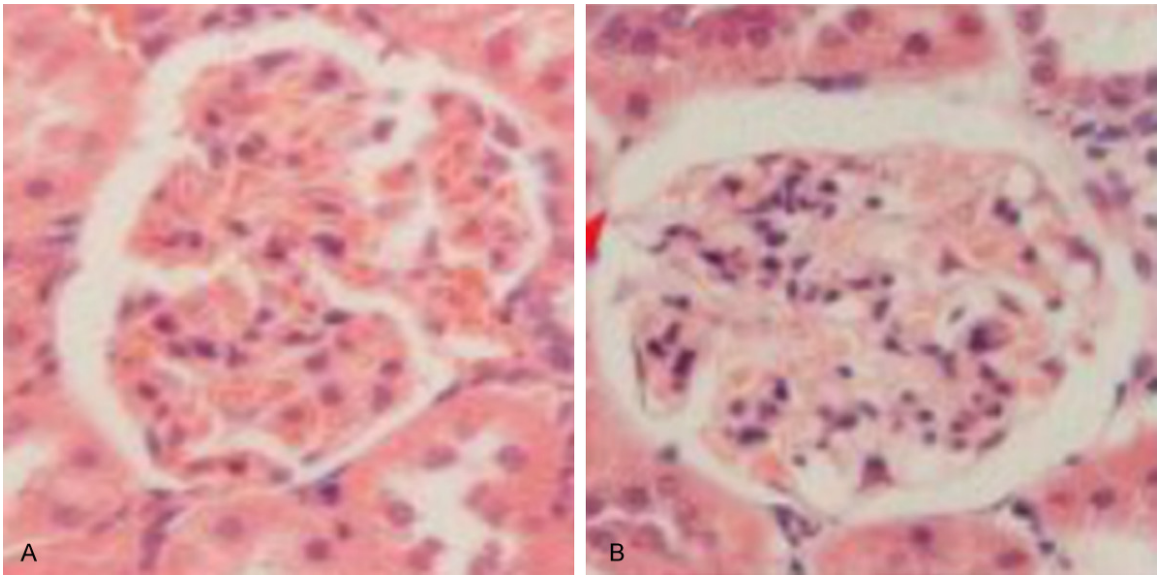

Figure 2. HE staining results of diabetic nephropathy. A: Control group; B: Experiment group.

protein; the protein was transferred to PVDF film by Bio-rad at 20 V for 20 min. 5% BSA was applied to seal for 1 h, TGF-β1 and P53 were incubated with β-actin at 4°C overnight respectively; after washed the membrane, Goat anti rabbit IgG (purchased from Wuhan Dr biological Co., Limited company) labeled by HRP were used to incubate at room temperature for 2 h. After a second wash of the membrane, ECL reagent was added, and Odyssey image acquisition system was used to scan image. The Lab Works 4.5 software (American UVP Company) was used to carry out quantitative analysis of the bands, and the optical density ratio of the target band and the β-actin band was used as the final result.

Expression of TGF-β1 mRNA, P53 mRNA, microRNA 192 in renal tissues of model rats with diabetes mellitus by real-time quantitative PCR detection

The preserved kidney tissues were taken out and about 40 mg kidney tissues were selected after thawing, then TRIzol 1 ml was added in for grinding. After fully grinded, the tissues were removed out and transferred into a clean centrifuge tube and gently mixed. After 5 min, 0.2 ml chloroform was added in, and then put aside in the room temperature for another 5 min after force shaking. And then, the mixture was centrifuged under conditions of 4°C and 12000 rmp/min; 10 min later, supernatant was absorbed and gently mixed with same volume of isopropanol, placed for 20 minutes and centrifuged again for 10 min, the supernatant was discarded and precipitation was washed to obtain RNA; then, the obtained RNA was dissolved by 1% DEPC and put in the new PCR tube. Take 1 μl to detect the concentration of nucleic acids by ultraviolet spectrophotometry.

PCR reaction system 20 μl, its components are as follows: 12.5 μl ddH_2O, 2.5 ul 10×Taq Buffer with KCL, 2.0 μl dNTP (2.5 mmol/L), 1.0 μl primers, 0.15 μl TaqE (5 U/ul), 2.0 ul DNA template. miR-192 primers: 5'-CTGACCTATGAATTGACAGCC-3'; p53 primers: forward, 5'-CGAAAGAAGACAGGCAGACTTTTCG-3'; p53 reverse, 5'-GAAGGTAAGGATAGGTCGGCGGTTC-3'; TGF-β1 primers: forward, 5'-CGGCAGCTGTACATTGACTT-3', reverse, 5'-TCAGCTGCACTTGCAGGAGC-3'. Real-time PCR was carried out by using Light Cycler 480 system, the reaction conditions are as follows: 95°C for 7 min; 95°C for 10 s, and 60°C for 15 s, after 40 cycles, the temperature was increased to 95°C for melting curve, data were analyzed by $2^{-\Delta\Delta Ct}$ method.

Statistical method

All data collected in this study were analyzed by SPSS18.0 software, the measurement data were expressed by means ± SD, count data was expressed by frequency, data were compared by T test and chi square test, $P < 0.05$

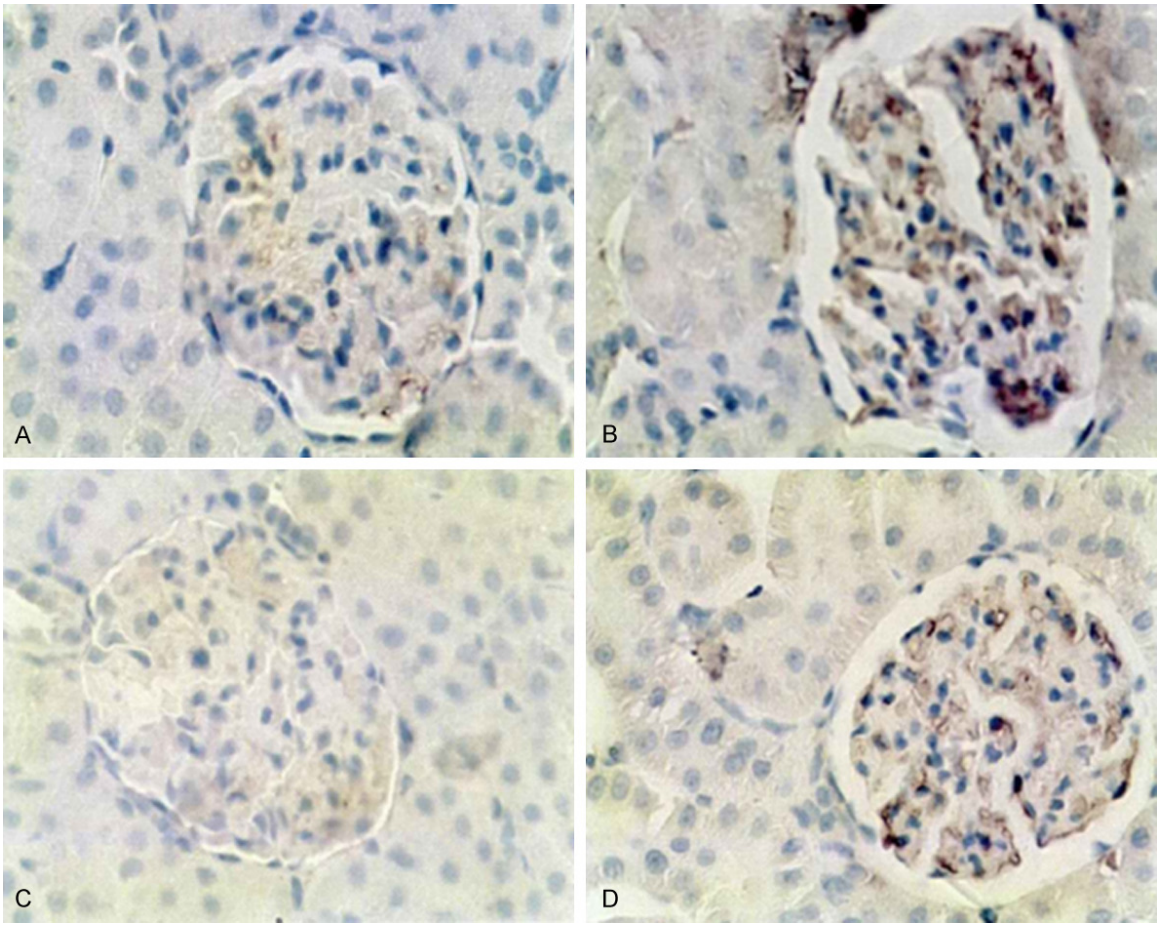

Figure 3. Immunohistochemistry results of the expression of TGF-β1 and P53 protein in two groups. (A, C) control group. (B, D) experimental group. Note: (A and B) are the expression of TGF-β1; (C and D) are the expression of P53 protein.

Table 1. The expression level comparison of TGF-β1 and P53 protein in renel tissues of two groups (mean ± standard deviation)

Groups	TGF-β1	P53
Control group (n = 10)	0.213±0.004	0.125±0.005
Experiment group (n = 30)	0.415±0.007	0.386±0.006
T value	0.584	0.469
P value	0.021	0.019

indicated that the difference has statistical significance.

Result

The general situation and the change of blood glucose in the model rats

The success rate of the experimental group was 86%. Mortality after model established was 6.1%. Rats in the experimental group began to show symptoms like: eat and drink more, wasting, urine volume increased significantly, slouch, slow move and response, arched body, dry and withered hair; the mean blood glucose was 22.1-23.5 mmol/L^2 weeks after STZ injection; rats in control group were in moderate shape, with flat and shiny hair, good spirit, free movement and quick response, the mean blood glucose in control group was 4.96-5.27 mmol/L, the results of blood glucose in the two groups were shown in **Figure 1**. From **Figure 2**, we can see that compared with control group, glomerular hypertrophy is significant, mesangial region is diffuse broadened, and glomerular basement membrane is thickened in experiment group.

Positive rate comparison between TGF-β1 and P53

The positive rate of TGF-β1 in experiment group and control group was 87.8% and 5% respec-

Table 2. Comparison of each indicator in kidney tissues of two groups (mean ± standard deviation)

Groups	TGF-β1 mRNA	P53 mRNA	MicroRNA 192
Control group (n = 10)	0.195±0.01	0.109±0.02	0.08±0.005
Experiment group (n = 30)	0.539±0.02	0.586±0.01	0.32±1.01
T value	0.326	0.521	0.624
P value	0.013	0.025	0.031

tively, there was a statistically significant difference between the two groups (P<0.05); the positive rate of P53 in experiment group and control group was 85.2% and 7.1% respectively, there was a significant difference between the two groups (P<0.05), see **Figure 3**.

Expression level contrast of TGF-β1 and P53 protein

After model constructed, the expression levels of TGF-β1 and P53 protein in experiment group were significantly higher than those in the control group (p<0.05), and the difference was statistically significant, see **Table 1**.

The detection results of the expression of TGF-β1 mRNA, P53 mRNA and microRNA 192

After model constructed, expression levels of TGF-β1 mRNA, P53 mRNA and microRNA 192 in the experimental group were significantly higher than those of control group, and the difference was statistically significant (P<0.05), see **Table 2**.

Discussion

With the improvement of social economy and the change in eating habits, the number of diabetic patients in China and even in the world is growing at a rapid rate every year, and the diabetic nephropathy patients are increasing too. Diabetic nephropathy is a great threat to the life and health of the patient to a great extent. So the exploration of an effective treatment can significantly improve patient's life quality and reduce the mortality rate. At present, the mechanism of clinical treatment is mostly to reduce blood glucose by oral hypoglycemic drugs, thereby to reduce the damage of kidney [8], this method can be used to restore the renal function of patients at a certain level, but it is not very ideal. In recent years, studies have shown that combined with drugs that beneficial to kidney can achieve a better result [9], however, there has not been attempted in clinical practice.

The results of this study showed that, after model constructed, the expression level of TGF-β1 mRNA, P53 mRNA and microRNA 192 of the experiment group were significantly higher than those of control group, which fully proved that the expression levels of TGF-β1, P53 and microRNA 192 were significantly increased after the onset of diabetic nephropathy. There are researches showed that TGF-β1 could be used as a predictive factor for diabetic nephropathy, and it is helpful for the diagnosis of diabetic nephropathy [10, 11] when TGF-β1 found to be significantly increased, which is in accordance with our results.

Abnormal glucose metabolism in diabetic nephropathy is the main factor that causes the increase of TGF-β1 expression, and another important factor for the increase is hemodynamic change in the early stage of diabetes mellitus, such as the increase of trans-membrane pressure and high filtration of glomerular capillary. In addition, the local rennin-angiotensin system excitement caused over secretion of angiotensin II is also a factor of TGF-β1 increase under high glucose condition. In addition to high glucose, glycosylated products also can stimulate the expression of TGF-β1, causing kidney damage. It is found that the TGF-β1 increased in mesangial cells cultured by high glucose, and TGF-β1 can directly stimulate the excessive accumulation of extracellular matrix, resulting in the thickening of the capillary basement membrane and the proliferation of mesangial cells.

In addition, the results of this study also showed that, after model constructed, the expression of P53 protein and miRNA 192 of experiment group were significantly higher than those of control group, suggesting the expression of P53 protein and miRNA 192 increased during the development of diabetic nephropathy, and promoted the progress of diabetic nephropathy. Studies have shown that, P53 can promote the expression of miRNA 192 in tumor cells, and conversely miRNA 192 can regulate the expression of P53 [12-14]. Some studies indicate a significant miRNA 192 expression increases in mesangial cells induced by high

glucose, and miRNA 192 has an important role in the pathogenesis of kidney disease [15, 16]. Another study reported that miR-192 was highly expressed in type I or type II diabetic nephropathy induced by streptozotocin, and was capable of acting with SIP1 target sequence to encode mRNA and further regulate SIP1, and the down regulation of SIP1 further decreased E-box, which subsequently caused an increase in collagen I [17]. The decrease or loss of miR-192 would promote the generation of fibrosis in DN [18]. MiRNA screening results of renal biopsy in DN patients with different stages showed the biggest change was miRNA-192, in particular, decreased significantly in stage III renal disease, moreover, the low expression of miR-192 was related with the low glomerular filtration rate and interstitial fibrosis of renal tubular. Wang et al. [19] found TGF-β1 could significantly reduce the expression level of miR-192 in renal tubular epithelium cells, podocytes and mesangial cells.

It can be inferred, there is a certain link between the expression of TGF-β1, P53 and miRNA 192, and they can possibly promote the development and progression of diabetic nephropathy through coordination and interaction. However, at present, there is no study could further confirm the relationship, and further studies are required to clarify.

In conclusion, the high level expression of TGF-β1, P53 and miRNA 192 in DN rats could co-promote the occurrence and progression of DN; our research provided experimental foundation for the deep research on the pathogenesis of DN and suggested a new approach and new ideal for clinical treatment of DN.

Disclosure of conflict of interest

None.

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