MiR-92b-3p is induced by advanced glycation end products and involved in the pathogenesis of diabetic nephropathy

Wang Liping Corresp., [1] , **Geng Jia Nan** [2] , **Sun Bo** [3] , **Sun Chengbo** [4] , **Wang Siyuan** [5] , **Shi Yan** [5] , **Yu Xiayan** [5]

[1] Department of Biobank, Clinical Medical School of Yangzhou University, Northern Jiangsu People's Hospital, Yangzhou, China
[2] Department of Experimental Pharmacology and Toxicology, Jilin University, Changchun, China
[3] Department of Experimental Pharmacology and Toxicology, jilin University, Changchun, China
[4] Department of Experimental Pharmacology and Toxicology,, Jilin University, Changchun, China
[5] Department of Experimental Pharmacology and Toxicology, Jilin University, Changchun, China

Corresponding Author: Wang Liping
Email address: 446296987@qq.com

Background. The current study aims to explore the effects of advanced glycation end products (AGEs) on the microRNA (miRNA) expression profile in kidney tissues in a murine diabetic nephropathy (DN) model, and to investigate the possible underlying mechanisms.

Methods. Wister rats were randomly divided into three equal experiment groups, the AGE group, the RSA group and the control group. The rats in the AGE group and the RSA group were administered with AGEs and rat serum albumin (RSA) via the tail vein, respectively, whereas those in the control group received no injection. Following the extraction of total RNA from the murine kidney tissues, miRNA profiling was performed using a miRNA microarray to determine the global differences in miRNA expression between the three experiment groups. The identified panel of aberrantly expressed miRNA candidates were subsequently validated by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Bioinformatics analysis was performed to identify the target genes and enriched signaling pathway for the validated miRNAs.

Results. Compared with RSA group, miR-7d-3p, miR-92b-3p, miR-181b-5p and miR-196c-5p were found to be significantly up-regulated in the AGE group, whereas miR-7a-1-3p, miR-186-5p, miR-192-5P, miR-196b-5p and miR-345-5p were shown to be down-regulated ($p<0.05$). Among the nine miRNA candidates, miR-92b-3p was validated by qRT-PCR analysis. Subsequent bioinformatic study indicated that SMAD7 could be a potential downstream gene target of miR-92b-3p. Both immunohistochemical staining and western blotting showed that SMAD7 expression was significantly suppressed in the kidney tissues harvested from the AGE group compared to those from the control and the RSA group.

Discussion. The results of the current study revealed a correlation between increased renal level of miR-92b-3p and elevated serum level of AGEs in a murine model of DN.

MiR-92b-3p is induced by advanced glycation end products and involved in the pathogenesis of diabetic nephropathy

Wang Liping [1,2], Geng Jianan [2], Sun Bo [2], Sun Chengbo [2], Wang Siyuan [2], Shi Yan [2] and Yu Xiaoyan [2#]

1 Department of Biobank, Clinical Medical School of Yangzhou University, Northern Jiangsu People's Hospital, Yangzhou 225001, China;
2 Department of Experimental Pharmacology and Toxicology, School of Pharmacy, Jilin University, Changchun 130021, China

Xiaoyan Yu, yuxy@jlu.edu.cn

Abstract

Backgroud

The current study aims to explore the effects of advanced glycation end products (AGEs) on the microRNA (miRNA) expression profile in kidney tissues in a murine diabetic nephropathy (DN) model, and to investigate the possible underlying mechanisms.

Methods

Wister rats were randomly divided into three equal experiment groups, the AGE group, the RSA group and the control group. The rats in the AGE group and the RSA group were administered with AGEs and rat serum albumin (RSA) via the tail vein, respectively, whereas those in the control group received no injection. Following the extraction of total RNA from the murine kidney tissues, miRNA profiling was performed using a miRNA microarray to determine the global differences in miRNA expression between the three experiment groups. The identified panel of aberrantly expressed miRNA candidates were subsequently validated by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Bioinformatics analysis was performed to identify the target genes and enriched signaling pathway for the validated miRNAs.

Results

Compared with RSA group, miR-7d-3p, miR-92b-3p, miR-181b-5p and miR-196c-5p were found to be significantly up-regulated in the AGE group, whereas miR-7a-1-3p, miR-186-5p, miR-192-5P, miR-196b-5p and miR-345-5p were shown to be down-regulated ($p<0.05$). Among the nine miRNA candidates, miR-92b-3p was validated by qRT-PCR analysis. Subsequent bioinformatic study indicated that SMAD7 could be a potential downstream gene target of miR-92b-3p. Both immunohistochemical staining and western blotting showed that SMAD7 expression was significantly suppressed in the kidney tissues harvested from the AGE group compared to those from the control and the RSA group.

Conclusions

The results of the current study revealed a correlation between increased renal level of miR-92b-3p and elevated serum level of AGEs in a murine model of DN.

Keywords

Diabetic nephropathy (DN). Advanced glycation end products (AGEs). Smad7 protein (Smad7). MiRNA-92b-3p (MiR-92b-3p)

Introduction

Diabetic nephropathy (diabetic nephropathy, DN) is one of the most serious complications and the leading cause of death in diabetic patients, as well as a major contributing factor to end-stage renal disease (Kato et al. 2012).Common pathological features of DN include extracellular matrix (ECM) protein aggregation, mesangial cell proliferation and hypertrophy, and glomerular podocyte dysfunction (Qian et al. 2008). Patients with advanced DN can also develop glomerular lesions and proteinuria. In recent years, the pathogenesis and treatment of DN have become a main focus of clinical research on diabetes and renal diseases. It is generally accepted that mutual reinforcement of metabolic dysregulation and hemodynamic abnormalities

contributes to a vicious cycle of deteriorating renal pathologies in patients with chronic hyperglycemia.(Forbes et al. 2007; Schena & Gesualdo 2005). Despite this, the exact mechanism for DN pathogenesis is extremely complex and remains poorly understood, which has hampered the development of effective diagnostic tools and therapies.

AGEs are a structurally diverse group of glycated natural polymers generated by irreversible non-enzymatic reactions between the aldehyde groups of various reducing sugars, such as glucose and fructose, and the amino groups present in proteins and lipids. A wide range of studies have shown that the interaction of AGEs and the receptors for advanced glycation end products (RAGE) can trigger the activation of inflammatory response pathways and the accumulation of oxidative stress signals, often through affecting the levels of various cytokines, hormones and free radical species (Wautier et al. 2001). AGEs are naturally present in healthy individuals at very low levels which generally increase with aging. However, diabetes patients often exhibit abnormally high concentrations of AGEs due to sustained hyperglycemia, which increases the availability of circulating sugar substrates that can modify proteins, lipids and even DNA molecules, altering their structural integrity and biological functions. AGE-induced crosslinking on protein substrates can cause degradation and functional disorders. Research has also suggested a link between DNA glycation and increased frequency of mutagenesis. The irreversible nature of AGE-promoted chemical modifications contributes to the so-called hyperglycemic memory effect, in which the glycated products cannot be eliminated by the restoration of normal blood sugar levels. Studies have shown significantly higher AGEs levels in the kidneys of diabetic animals and human patients (Kalousova et al. 2002; Kanauchi et al. 2001; Turk et al. 2003; Vlassara et al. 1994). It has also been shown that intravenous injection of AGEs can induce pathophysiological changes similar to those caused by DN in rats (Zhou et al. 2004).

MicroRNAs (miRNAs) are a class of non-coding single-stranded RNA molecules with a typical length of approximately 22 nucleotides. Since the discovery of the first miRNA in Caenorhabditis elegans in 1993 (Lee et al. 1993). The structural features and functional roles of these oligonucleotides have been elucidated in details in a plethora of literatures. MiRNAs are now seen to be closely involved in the regulation of a wide range of cellular activities, including but not limited to cell development, replication, aging and apoptosis, through their ability to complement with the 3'untranslated region (3'-UTR) of the target mRNAs (Bronze-da-Rocha 2014; Mathieu & Ruohola-Baker 2013). It is estimated that approximately 30% of all protein-encoding genes in human genome are regulated by miRNAs (Hammond 2006). Recently, studies have suggested that miRNAs might also be mechanistically implicated in the early development of DN. For instance, Zhang et al. reported that overexpression of miR-451 could cause the down-regulation of MAP kinase kinase 3 (MKK3) and p38 mitogen-activated protein kinase (p38MAPK). Furthermore, elevated miRNA-451 level was found to inhibit glomerular mesangial cell proliferation both in vitro and in vivo (Zhang et al. 2012). In another study, miRNA profiling of a murine DN model based on db/db mice demonstrated that the expression levels of miR-196a, miR-98 and miR-29c significantly increased, while those of miR-21, miR-451, miR-709 and miR-187 declined. In particular, ectopic expression of miR-21 showed inhibitory effect on mesangial cell proliferation and urine albumin excretion in the diabetic mice, suggesting its potential therapeutic utility in treating early DN (Zhang et al. 2009). Wang et al found that laboratory-cultured human and mouse mesangial cells stimulated by high concentrations of glucose and transforming growth factor-β exhibited an abnormally high level of miR-377 in a manner similar to what was shown in the murine DN model. Overexpression of miR-377 was subsequently associated with diminished cellular levels of superoxide dismutase and p21-activated kinase, resulting in up-regulation of fibronectin production. Based on these findings, the authors argued that miR-377 could contribute to DN pathogenesis by altering ECM architecture and stability (Wang et al. 2008a). Other miRNAs, including miR-21(Zhang et al. 2009), miR-192 (Kato et al. 2007), miR-377(Wang et al. 2008b) have also been reported in association with DN-related pathologies.

Based on the above findings, the current study aims to investigate whether AGEs could affect the miRNA expression profile in murine kidney. In addition, the study also seeks to probe the mechanistic roles of miRNAs in DN pathogenesis.

Materials and methods

Preparation of glycated serum

The preparation of glycated serum was performed according to a previously described protocol with minor modifications (Vlassara et al. 1992). Briefly, the glycated serum consisted of 5 mg/mL of rat serum albumin, 0.5 mol/L of glucose, 100 U/mL of penicillin and 100 U/mL of streptomycin in phosphate buffered saline (PBS, pH 7.2). The serum was filter-sterilized and incubated at 37 °C for two months in the dark. Following the incubation, excess glucose was removed by extensive dialysis against (PBS pH 7.2) and the resulting liquid was concentrated to a final protein level of 25 mg/mL. As a control, nonglycated serum was prepared following the same method, except that no glucose was added. Finally, the serum samples were aliquoted and frozen at -20 °C until use.

Animal experiments

Ethical approval was obtained from the Animal Care and Welfare Committee of Jilin University prior to the animal experiments. A total of ten healthy adult male Wistar rats, weighted between 220 to 250 g, were obtained from the Experimental Animal Center of Jilin University. The rats were fed a standard nutritional granular diet for one week and then randomly divided into three equal experiment groups, the control group, the RSA group and the AGE group. The rats in the RSA group and in the AGE group received daily intravenous injection of nonglycated and glycated sera, respectively, at a dose of 100 mg/kg. In contrast, the control group underwent no treatment. All rats were maintained under otherwise identical experimental conditions and weighed once a week throughout the entire period of the experiment. Six weeks after the first injection, murine urine samples were collected twice with a 24h interval in between for analysis of urinary albumin excretion (UAE), followed by the withdrawal of blood from abdomen main veins. The rats were then euthanized immediately, and kidneys were harvested and stored at -80 °C until use.

Preparation of total RNA from murine kidney tissues and miRNA microarray analysis

The murine kidney tissues were stored in RNAlater RNA Stabilization Solution (Thermo Fisher Scientific, PA, USA) at -80 °C. Total RNA was extracted using the Trizol method. The quality of the extracted RNA was verified by measuring the A260/A280 ratio on a spectrophotometer and by agarose-formaldehyde gel electrophoresis. Approximately 5 μg of RNA was size-fractionated using a YM-100 microcon centrifugal filter (Millipore, MA, USA) to isolate fragments shorter than 300 nucleotides, which were then 3'-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail to allow subsequent fluorescent dye staining. Overnight hybridization was conducted using a micro-circulation pump (Atactic Technologies, TX, USA) on a μParaflo microfluidic chip (LC Sciences, TX, USA) (Zhu et al. 2007). Following RNA hybridization, detection was performed by circulating tag-specific Cy3 dye through the microfluidic chip. Images were collected using a GenePix 4000B Microarray Scanner (LC Sciences, TX, ,USA) and digitized using the Array-Pro Analyzer Software (Media Cybernetics, MD, USA). The results were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (LC Sciences, TX, USA) (Bolstad et al. 2003). $P < 0.05$ was considered statistically significant.

Quantitative real-time polymerase chain reaction (qRT-PCR)

MiRNAs and their putative target mRNAs identified by sequencing and bioinformatic analysis were individually reverse-transcribed using specific primers (Table 1) on the ABI PRISM 7900 Sequence Detection System (Applied Biosystems, CA, USA). U6 small-nuclear RNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene were used as internal standards. Ct values were converted to fold changes. All experiments were performed in triplicate. $P<0.05$ was considered statistically significant.

Bioinformatic analysis

The putative miRNA-mRNA interactions were evaluated by using the algorithm of Targetscan version 6.2 (http://www.targetscan.org/), miRanda version 3.3a (http://www.microrna.org/microrna/home.do) and PITA version 6 (https://genie.weizmann.ac.il/pubs/mir07/mir07_data.html). The miRNA binding–site prediction in targets was based on their correspondent completed 3' UTR sequences through Ensembl BioMart web services. High-confidence miRNA-mRNA pairs resulted from consideration of a Targetscan context+ score percentile >50, miRanda max energy <-20 kcal/mol and PITA max energy <-10 kcal/mol.

Western blotting

Western blotting was performed according to a previously described procedure (Towbin et al. 1979). Kidney tissues were suspended in Radio Immunoprecipitation Assay(Beyotime Biotechnology, Shanghai,China)cracking liquid and homogenized by sonication. The resultant mixture was centrifuged at 3000 rpm for 10 min at 4 ºC, and proteins in the supernatant were separated on a 12% dodecyl sulfate-polyacrylamide gel, followed by transfer onto a polyvinylidene difluoride membrane. The membrane was then blocked with 5% skimmed milk, stained with 1:1000 Rabbit anti-SMAD7 antibody (Abcam, UK) overnight at 4 ºC, and then with 1:5000 Goat anti-HRP antibody at 37 ºC for 1 h (ZSGB, Beijing,China). The membrane was developed using the BeyoECL Plus kit (Beyotime Biotechnology, Shanghai, China).

Immunohistochemistry

Immunohistochemistry was performed based on a previously described protocol (Zhu et al. 2010). (Polyclonal rabbit anti-SMAD7 antibody (Abcam, UK) was used as the primary antibody at a dilution level of 1:150. Polyclonal goat anti-HRP antibody(ZSGB, Beijing, China) was used as the second antibody at Ready-to-use.

Results

Examination of urinary secretion and renal pathological changes

We began our study by first measuring the UAE of each rat in the three experiment groups. UAE serves as a critical diagnostic indicator of the severity of DN and can be used for the staging of the disease. No obvious difference was observed in the average volume of the collected urine among the three groups (Table 2). In addition, the average 24-h UAE of the AGE group was found to be higher than those of the control and the RSA groups (Table 2). However, the increase was found not to be statistically significant. Based on the experiment data, we concluded that the mice injected with glycated sera displayed some early signs of renal injury but could still be considered normoalbuminuric.

We next performed histopathological examination on the harvested murine renal tissues using H&E and PAS staining. As illustrated in HE(Fig. 1), the rats in the AGE group showed significant signs of glomerular hypertrophy, characterized by mesangial cell hyperplasia and ECM expansion. Furthermore, tissue staining also revealed collapse of the glomerular capillary loops in the AGE group. In comparison, neither the control group nor the RSA group displayed any of the abovementioned pathological changes PAS(Fig. 2). The rats in these groups exhibited no capillary leakage, adhesion of glomerular tufts to Bowman's capsules, or mesangial expansion.

Identification of aberrantly expressed miRNAs and qRT-PCR validation

Based on the results obtained from UAE rate measurement and histochemical examinations, we selected six rats from the AGE group and the RSA group for a comparative analysis of miRNA expression profiles in their kidney tissues. A total of 121 miRNA candidates were detected. Based on the cut-off criteria that we applied, we identified a panel of nine miRNAs with aberrant expression levels in the AGE group compared to the RSA group ($p<0.05$ and signal >500), among which four, including miR-7d-3p, miR-92b-3p, miR-181b-5p and miR-196c-5p, were found to be up-regulated and the other five, namely miR-7a-1-3p, miR-186-5p, miR-192-5P, miR-196b-5p and miR-345-5p, were down-regulated (Fig. 3). Noticeably, the expression level of miR-92b-3p showed a difference between the two experiment groups with a statistical significance of $p<0.01$. Among these miRNA candidates, seven that showed at least a two-fold difference in the expression levels between the two groups (signal > 500), consisting of miR-21-5p, miR-92b-3p, miR-140-3p, miR-196a-5p, miR-181b-5p, miR-186-5p and miR-192-5p were selected for qRT-PCR validation. As summarized, the results confirmed that the expression of miR-92b-3p was indeed significantly increased in rats that were administered with injections of glycated sera.

Differences miRNAs predicted target genes

The potential functions of miR-92b-3p were analyzed by three miRNA prediction algorithms, including TargetScan, PITar and miRanda. A total of 265 potential target genes of miR-92b-3p were identified by all three algorithms. The predicted miRNA candidates were shown by GO analysis to be involved in a diverse range of biological functions, including cellular metabolic processes, phosphorylation, cell proliferation and differentiation, cell apoptosis, as well as signal transduction. Based on these results, we selected four miRNAs with established pathological roles in DN, including transcription factor 21 (TCF-21), transforming growth interacting factor 1 (TGIF1), mothers against decapentaplegic homolog 7 (SMAD7) and TNF receptor associated factor 3 (TRAF3). We speculated that miRNA-92-3p could be implicated in the pathogenesis of DN through targeting SMAD7 (Table 3). Therefore, we subsequently focused on investigating the mechanistic role of SMAD7 in DN pathogenesis.

Evaluation of SMAD7 expression in murine kidney tissues

We next verified the expression levels of SMAD7 in kidney tissues collected from different experiment groups. On the one hand, immunohistochemical results showed a marked drop in the level of SMAD7 in the kidneys of the AGE group compared to both the control group and the RSA group, as indicated by the lighter staining of the cell nuclei (Fig. 4). Consistently, Western blotting also confirmed that the expression level of SMAD7 in the AGE group was significantly lower than those in the other two groups (Fig. 5). These results supported the existence of a correlation between down-regulated SMAD7 expression and increased level of serum AGEs.

Discussion

In the current study, we sought to investigate the regulatory effects of AGEs on miRNA expression in a murine model. Compared to the controls, the mice that received intravenous injections of glycated sera showed various DN-associated symptoms, such as the development of albuminuria as evidenced by the elevated UAE, and glomerular hypertrophy based on histological examinations. We then compared the miRNA expression profiles of the AGE group and RSA group using a

combination of RNA sequencing and bioinformatic analysis, which identified a panel of 451 up-regulated and 320 down-regulated miRNA candidates. Subsequent qRT-PCR validation confirmed the significant increase of miR-92b-3p expression in the AGE group. Results retrieved from different miRNA databases suggested that miR-92b-3p could be involved in a variety of biological functions, including cellular metabolic process, phosphorylation, cell proliferation and differentiation, cell apoptosis and signal transduction. In addition, several DN-related genes, including, TCF-21, TGIF1, SMAD7 and TRAF3, were shown to be the potential downstream regulatory targets of miR-92b-3p. Indeed, elevated level of SMAD7 was observed in renal tissues via both immunohistological staining and Western blotting. Taken together, these results
Implied that miR-92b-3p could be mechanistically implicated in mediating the renopathological effects of AGEs.

It is well accepted that AGEs contribute to DN pathogenesis by altering the chemical structures of various macromolecules with important biological functions. Very recently, there is emerging evidence that suggests the involvement of miRNAs in AGE-mediated pathological changes in diabetic kidneys. In a study published by Li and colleagues, the miRNA expression profile in monocyte/macrophage-lineage THP-1 cells treated with AGEs was probed by microarray analysis and compared to that of cells cultured in regular medium (Li et al. 2011). The investigation found the level of miR-214 to be significantly elevated in both the AGE-treated cells and in patients with chronic renal failure (Li et al. 2011). In addition, the authors' experimental data suggested that up-regulation of miR-214 was likely associated to activation of receptors for AGEs, which in turn led to inhibition of PTEN and cell apoptosis (Li et al. 2011). In another study, comparative microarray analysis of miRNA profiles linked AGE induction with suppression of miR-200b and miR-200c (Wu et al. 2014). This was shown to cause dysregulation of the downstream target genes, including RhoA and ROCK, and treatment with miR-200b mimics could alleviate AGE-promoted endothelial cell injury (Wu et al. 2014). Consistent with these studies, our current finding that miR-92b-3p expression was significantly increased in AGE-injected mice further corroborated the hypothesis that miRNAs could serve as a mechanistic "bridge" between hyperglycemia-related metabolic changes and impairment of crucial signaling pathways implicated in DN pathologies.

A series of recent studies have considerably furthered our understanding of the mechanistic implication of SMAD7 in the pathogenesis of DN (Chen et al. 2011; Meng et al. 2015;Braga et al.2014). In this regard, the transforming growth factor-β (TGF-β)-Smad signaling pathway has been shown to play a critical role in the regulation of ECM homeostasis, a process closely associated with the development of the disease. Smad7 negatively modulates the TGF-β pathway by competing for receptor binding against Smad2 and Smad3, both of which function as activators (Hayashi et al. 1997). Furthermore, SMAD7has also been indicated to exert its inhibitory effect by inducing the ubiquitin-dependent proteasomal degradation of the TGF-β receptor (Kavsak et al. 2000). Based on these mechanistic findings, it is not surprising that overexpression of Smad7 was found to result in marked improvement of renal fibrosis in a murine unilateral ureteral obstruction model (Lan et al. 2003). Conversely, diabetic mice deficient in Smad7 exhibited more severe renal fibrosis and albuminuria, as well as elevated levels of inflammation factors compared to the ones with a normal genotype (Chen et al. 2011). These findings were consistent with our observation of down-regulated SMAD7 expression in murine kidney tissues exposed to a high level of AGEs.

SMAD7 has been shown to be a downstream target of several microRNAs in connection with a variety of diseases including DN. MiR-21 was shown to aggravate diabetes-induced renal fibrosis by suppressing the expression of SMAD7 and PTEN (McClelland et al. 2015). This was echoed in another study conducted by Chung et al., in which several miRNA mimics, including those of anti-miR-29b, miR-21 and miR-192, were found to abolish the protective effect of SMAD7 against renal fibrosis (Chung et al. 2013). Interestingly, the authors reported that disruption of SMAD7 could stimulate the expression of miR-21 and miR-192, while repressing that of miR-29b, suggesting a complex regulatory relationship between miR-21 and SMAD7 with pathogenic implications for DN (Chung et al. 2013). Yu et al. revealed that SMAD7 was also a downstream target of miRNA-192, which could promote epithelial-to-mesangial transition in tumor cells (Yu et al. 2016). Similarly, miR-92a was demonstrated to act through the inhibition of SMAD7 expression to aggravate hypoxia/reoxygenation-induced myocardial injury and cell apotosis (Zhang et al. 2014). Our current results identified miR-92b-3p as a new potential modulator of SMAD7 and lent further support to the increasingly accepted notion that miRNAs could play important roles in mediating hyperglycemia-stimulated dysregulation of genes that contributes to pathological changes in renal tissues.

It is worth noting that we did not observe a significant difference between the average 24-h UAE of the AGE group and those of the other two groups. This implied that the alteration in miR-92b-3p expression occurred when the mice were still at the normoalbuminuric or microalbuminuric stage. Changes in miRNA expression profiles have been shown to be an early molecular event in the development of DN and have been suggested as a potential diagnostic biomarker. For example, in Jia et al.'s study, the level of miR-192 in renal tissues was found to be correlated with albuminuria and TGF β 1 expression even in normoalbuminuric DN patients, indicating its diagnostic value for detecting early forms of the disease（Jia et al, 2016）. The

current study hinted at the possibility of using miR-92b-3p as a potential early indicator of DN development. It would be interesting to see whether a similar trend could also be observed in DN patients, and whether a predictive model consisting of a panel of aberrantly expressed miRNAs could be constructed to identify early-stage DN in individuals that do not exhibit overt pathophysiological signs.

Acknowledgements

This work was supported by grants from the Technology Development planning of Jilin Province (no. 20160101206JC) and the Project from the Northern Jiangsu People's Hospital fund projects (no. yzucms201509)

Conclusions

The results of the current study revealed a correlation between increased renal level of miR-92b-3p and elevated serum level of AGEs in a murine model of DN.

Reference:

Bolstad BM, Irizarry RA, Astrand M, and Speed TP. 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 19:185-193.

Bronze-da-Rocha E. 2014. MicroRNAs expression profiles in cardiovascular diseases. *Biomed Res Int* 2014:985408.

Braga Gomes K, Fontana Rodrigues K, Fernandes A P. The role of transforming growth factor-beta in diabetic nephropathy[J]. International Journal of Medical Genetics, 2014, 2014.

Chen HY, Huang XR, Wang W, Li JH, Heuchel RL, Chung AC, and Lan HY. 2011. The protective role of Smad7 in diabetic kidney disease: mechanism and therapeutic potential. *Diabetes* 60:590-601.

Chung AC, Dong Y, Yang W, Zhong X, Li R, and Lan HY. 2013. Smad7 suppresses renal fibrosis via altering expression of TGF-beta/Smad3-regulated microRNAs. *Mol Ther* 21:388-398.

Forbes JM, Fukami K, and Cooper ME. 2007. Diabetic nephropathy: where hemodynamics meets metabolism. *Exp Clin Endocrinol Diabetes* 115:69-84.

Hammond SM. 2006. microRNA detection comes of age. *Nat Methods* 3:12-13.

Hayashi H, Abdollah S, Qiu Y, Cai J, Xu YY, Grinnell BW, Richardson MA, Topper JN, Gimbrone MA, Jr., Wrana JL et al. . 1997. The MAD-related protein Smad7 associates with the TGFbeta receptor and functions as an antagonist of TGFbeta signaling. *Cell* 89:1165-1173.

Jia Y, Guan M, Zheng Z, et al. miRNAs in urine extracellular vesicles as predictors of early-stage diabetic nephropathy[J]. Journal of diabetes research, 2016, 2016.

Kalousova M, Skrha J, and Zima T. 2002. Advanced glycation end-products and advanced oxidation protein products in patients with diabetes mellitus. *Physiol Res* 51:597-604.

Kanauchi M, Nishioka H, and Dohi K. 2001. Serum levels of advanced glycosylation end products in diabetic nephropathy. *Nephron* 89:228-230.

Kato M, Park JT, and Natarajan R. 2012. MicroRNAs and the glomerulus. *Exp Cell Res* 318:993-1000.

Kato M, Zhang J, Wang M, Lanting L, Yuan H, Rossi JJ, and Natarajan R. 2007. MicroRNA-192 in diabetic kidney glomeruli and its function in TGF-beta-induced collagen expression via inhibition of E-box repressors. *Proc Natl Acad Sci U S A* 104:3432-3437.

Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, and Wrana JL. 2000. Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. *Mol Cell* 6:1365-1375.

310 Lan HY, Mu W, Tomita N, Huang XR, Li JH, Zhu HJ, Morishita R, and Johnson RJ. 2003. Inhibition of renal
311 fibrosis by gene transfer of inducible Smad7 using ultrasound-microbubble system in rat UUO model. *J Am*
312 *Soc Nephrol* 14:1535-1548.
313 Lee RC, Feinbaum RL, and Ambros V. 1993. The C. elegans heterochronic gene lin-4 encodes small RNAs with
314 antisense complementarity to lin-14. *Cell* 75:843-854.
315 Li LM, Hou DX, Guo YL, Yang JW, Liu Y, Zhang CY, and Zen K. 2011. Role of microRNA-214-targeting
316 phosphatase and tensin homolog in advanced glycation end product-induced apoptosis delay in monocytes.
317 *J Immunol* 186:2552-2560.
318 Mathieu J, and Ruohola-Baker H. 2013. Regulation of stem cell populations by microRNAs. *Adv Exp Med Biol*
319 786:329-351.
320 McClelland AD, Herman-Edelstein M, Komers R, Jha JC, Winbanks CE, Hagiwara S, Gregorevic P, Kantharidis P,
321 and Cooper ME. 2015. miR-21 promotes renal fibrosis in diabetic nephropathy by targeting PTEN and
322 SMAD7. *Clin Sci (Lond)* 129:1237-1249.
323 Meng XM, Tang PM, Li J, and Lan HY. 2015. TGF-beta/Smad signaling in renal fibrosis. *Front Physiol* 6:82.
324 Qian Y, Feldman E, Pennathur S, Kretzler M, and Brosius FC, 3rd. 2008. From fibrosis to sclerosis: mechanisms of
325 glomerulosclerosis in diabetic nephropathy. *Diabetes* 57:1439-1445.
326 Schena FP, and Gesualdo L. 2005. Pathogenetic mechanisms of diabetic nephropathy. *J Am Soc Nephrol* 16 Suppl
327 1:S30-33.
328 Towbin H, Staehelin T, and Gordon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to
329 nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci U S A* 76:4350-4354.
330 Turk Z, Sesto M, Skodlar J, Ferencak G, Pokupec R, Turk N, and Stavljenic-Rukavina A. 2003. Products of
331 advanced glycation in patients with type 2 diabetes and vascular disease. *Ann Clin Biochem* 40:552-559.
332 Vlassara H, Bucala R, and Striker L. 1994. Pathogenic effects of advanced glycosylation: biochemical, biologic, and
333 clinical implications for diabetes and aging. *Lab Invest* 70:138-151.
334 Vlassara H, Fuh H, Makita Z, Krungkrai S, Cerami A, and Bucala R. 1992. Exogenous advanced glycosylation end
335 products induce complex vascular dysfunction in normal animals: a model for diabetic and aging
336 complications. *Proc Natl Acad Sci U S A* 89:12043-12047.
337 Wang ET, Sandberg R, Luo S, Khrebtukova I, Zhang L, Mayr C, Kingsmore SF, Schroth GP, and Burge CB. 2008a.
338 Alternative isoform regulation in human tissue transcriptomes. *Nature* 456:470-476.
339 Wang Q, Wang Y, Minto AW, Wang J, Shi Q, Li X, and Quigg RJ. 2008b. MicroRNA-377 is up-regulated and can
340 lead to increased fibronectin production in diabetic nephropathy. *FASEB J* 22:4126-4135.
341 Wautier MP, Chappey O, Corda S, Stern DM, Schmidt AM, and Wautier JL. 2001. Activation of NADPH oxidase
342 by AGE links oxidant stress to altered gene expression via RAGE. *Am J Physiol Endocrinol Metab*
343 280:E685-694.
344 Wu XD, Liu WL, Zeng K, Lei HY, Zhang QG, Zhou SQ, and Xu SY. 2014. Advanced glycation end products
345 activate the miRNA/RhoA/ROCK2 pathway in endothelial cells. *Microcirculation* 21:178-186.
346 Yu J, Lei R, Zhuang X, Li X, Li G, Lev S, Segura MF, Zhang X, and Hu G. 2016. MicroRNA-182 targets SMAD7
347 to potentiate TGFbeta-induced epithelial-mesenchymal transition and metastasis of cancer cells. *Nat*
348 *Commun* 7:13884.
349 Zhang B, Zhou M, Li C, Zhou J, Li H, Zhu D, Wang Z, Chen A, and Zhao Q. 2014. MicroRNA-92a inhibition
350 attenuates hypoxia/reoxygenation-induced myocardiocyte apoptosis by targeting Smad7. *PLoS One*

9:e100298.

Zhang Z, Luo X, Ding S, Chen J, Chen T, Chen X, Zha H, Yao L, He X, and Peng H. 2012. MicroRNA-451 regulates p38 MAPK signaling by targeting of Ywhaz and suppresses the mesangial hypertrophy in early diabetic nephropathy. *FEBS Lett* 586:20-26.

Zhang Z, Peng H, Chen J, Chen X, Han F, Xu X, He X, and Yan N. 2009. MicroRNA-21 protects from mesangial cell proliferation induced by diabetic nephropathy in db/db mice. *FEBS Lett* 583:2009-2014.

Zhou G, Li C, and Cai L. 2004. Advanced glycation end-products induce connective tissue growth factor-mediated renal fibrosis predominantly through transforming growth factor beta-independent pathway. *Am J Pathol* 165:2033-2043.

Zhu C, Liu J, and Wang X. 2010. [Detection of EGFR and COX-2 expression by immunohistochemical method on a tissue microarray section in lung cancer and biological significance]. *Zhongguo Fei Ai Za Zhi* 13:107-111.

Zhu Q, Hong A, Sheng N, Zhang X, Matejko A, Jun KY, Srivannavit O, Gulari E, Gao X, and Zhou X. 2007. microParaflo biochip for nucleic acid and protein analysis. *Methods Mol Biol* 382:287-312.