Dysregulation of lncRNAs GM5524 and GM15645 involved in high-glucose-induced podocyte apoptosis and autophagy in diabetic nephropathy

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Abstract. Diabetic nephropathy (DN) is an important microvascular complication of diabetes, and one of the leading causes of end-stage kidney disease. However, the mechanism of the DN pathogenic process remains unclear. Recently, long non-coding (lnc)RNA dysregulation has been regarded to cause the occurrence and development of various human diseases, although the functions of lncRNAs in human DN are poorly understood. The authors' previous study using microarray analysis identified hundreds of dysregulated lncRNAs in DN, although the functions of these lncRNAs were not demonstrated. Out of those dysregulated lncRNAs, Gm5524 was significantly upregulated in response to DN, while Gm15645 was significantly downregulated in response to DN. In the present study, this result was further validated by reverse transcription-quantitative polymerase chain reaction assays, and downregulating or overexpressing Gm5524 and Gm15645 in mouse podocytes. Notably, knockdown of Gm5524 and overexpression of Gm15645 induced mouse podocyte apoptosis and decreased cell autophagy in high-glucose culture conditions. In conclusion, the results of the present study revealed the roles of lncRNAs Gm5524 and Gm15645 in high-glucose induced podocyte apoptosis and autophagy during DN, which may further the understanding of the involvement of lncRNAs in DN, and provide a potential novel therapeutic target for this disease.

Introduction

Diabetes mellitus is one of the most common chronic diseases worldwide, and there were 422 million adults diagnosed with diabetes globally in 2014, according to the World Health Organization (1,2). As the number of novel diagnoses is increasing, this disease is attracting increased attention. Diabetic nephropathy (DN) is one of the principal microvascular complications of diabetes, and it is highly prevalent in 30-40% of hospitalized patients with diabetes (3,4). DN is additionally one of the leading causes of end-stage kidney disease, which only has an ~20% 5-year survival rate (5). DN has been characterized by a series of abnormal pathological alterations, including glomerular hypertrophy, mesangial proliferation, thickening of the glomerular basement membrane, and accumulation of the extracellular matrix (6,7). However, the dysregulated molecules and the mechanisms involved in this manifestation of disease remain poorly understood. Therefore, a better understanding of the pathogenesis and the identification of novel factors in DN may promote the development of novel therapeutics to address this complex disease.

Over the past decades, the rapid improvement of high-throughput sequencing techniques and bioinformatics methods has led to the advent of whole human genome sequencing. Annotation of sequencing results has revealed that <2% of the whole human genome is protein coding genes; whereas, the majority of the rest are non-coding genes, which yield numerous non-coding transcripts, including microRNAs and long non-coding RNAs (lncRNAs) (8-10). lncRNAs, novel examples of non-coding RNAs, are >200 nucleotides in length and lack any protein coding ability (11). Previously, studies have revealed that lncRNAs are widely expressed in almost all human tissues, and are involved in a number of important biological process, including X chromatin imprinting, stem cell differentiation, immune responses, cell fate decision, proliferation, and transcriptional and post-transcriptional regulation (12-14). Furthermore, dysregulation of lncRNAs has been demonstrated to contribute to the development of diverse human diseases, including types of cancer, neurological and cardiovascular diseases, and diabetes (15-17). For example, knockdown of lncRNA uc.48+ improved diabetic...
sympathetic neuropathy in type 2 diabetic rats through regulation of purinergic receptor P2X 7 expression and extracellularsignal-regulated kinase signaling (18).

In the case of DN, a number of lncRNAs have been demonstrated to be dysregulated by microarray analysis. Out of these lncRNAs, the function and underlying pathways associated with certain ones have been characterized and reported (19). For example, IncRNA taurine upregulated 1 alleviates extracellular matrix accumulation by acting as an endogenous sponge for microRNA (miR)-377 and thereby relieving the inhibition of peroxisome proliferator-activated receptor γ in DN (20). In addition, Wang et al (21) reported that IncRNA CYP4B1-PS1-001 expression was significantly downregulated in response to early DN, and CYP4B1-PS1-001 overexpression inhibited mesangial cell proliferation and fibrosis. Furthermore, IncRNA metastasis associated lung adenocarcinoma transcript 1 (MALAT1) expression is downregulated in kidney cortices from streptozotocin-induced DN cases, and decreased MALAT1 is involved in high glucose-induced podocyte injury via interacting with β-catenin (22). In the authors’ previous study, IncRNA expression patterns between a DN model and db/m control mouse kidney tissues were analyzed using microarray analysis (23). It was demonstrated that hundreds of lncRNAs are dysregulated in DN, and these lncRNAs may contribute to the pathogenesis of DN by modulating multiple molecular pathways (23). However, the functions of these lncRNAs in DN remain unclear. In the present study, the microarray results were further validated, and the function of two of these lncRNAs (Gm5524 and Gm15645) in podocytes was analyzed by loss- and gain-of-function assays.

Materials and methods

Animal model and tissue specimen preparation. A total of 12 8-week-old male mice, including six C57BL/KsJ db/db mice (experimental group; average weight 46.53±1.96 g) and six C57BL/KsJ m/db mice (control group; average weight 36.96±1.86 g) and 12 8-week-old male mice, including six C57BL/KsJ db/db mice and six C57BL/KsJ db/db mice, were purchased from Genscript (Nanjing, China). A total of 48 h following transfection, the cells were selected using puromycin for 48 h and harvested for further RT-qPCR or western blot analysis. The sequence of scrambled (Scr) shRNA sequences were synthesized and subsequently inserted into the pCDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. Gm5524 and Gm15645 overexpression vectors and the shRNA lentivirus vectors were purchased from Genscript (Nanjing, China). A total of 48 h following transfection, the cells were selected using puromycin for 48 h and harvested for further RT-qPCR or western blot analysis. The sequence of scrambled (Scr) shRNA (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). All the vectors were prepared using Midiprep kits (Qiagen GmbH, Hilden, Germany), and 2 µg plasmid were transfected into mouse podocytes with ~70% density in six-well plates using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer’s protocol. Gm5524, Gm15645 overexpression vectors and the shRNA lentivirus vectors were purchased from Genscript (Nanjing, China). A total of 48 h following transfection, the cells were selected using puromycin for 48 h and harvested for further RT-qPCR or western blot analysis. The sequence of scrambled (Scr) shRNA (Sigma-Aldrich; Merck KGaA; 50 ng/µl) is 5'-CCT AAG GTT AGG GGC CAT CCA CAG TCT TC-3'.

Flow cytometry apoptosis assay. Podocytes transfected with sh-Gm5524, sh-Gm15645, sh-NC vector, Gm5524 vector and Gm15645 vector were harvested 48 h following transfection using trypsin. The cell suspension was incubated with fluorescine isothiocyanate-Annexin V and propidium iodide for 15 min at room temperature in the dark and analyzed using a flow cytometer (FACSscan®; BD Biosciences, Franklin Lakes, NJ, USA) equipped with CellQuest software version 5.1 (BD Biosciences).

Evaluation of autophagy by transmission electron microscopy. Podocytes were washed and fixed in glutaraldehyde (2.5% in 0.1 mol/l phosphate buffer; pH 7.4) at 4°C overnight, post-fixed in 1% osmium tetroxide for 3 h at 4°C and dehydrated. Subsequently, the samples were embedded using a Poly/Bed 812 kit at 60°C for 24-48 h (Polysciences, Inc., Warrington, PA, USA). Samples were sliced into 70-nm ultra-thin sections.
and stained with uranyl acetate for 3 min at room temperature for transmission electron microscopic analysis (JEOL-1010; JEOL, Ltd., Tokyo, Japan).

Western blot analysis. The total proteins from mouse podocytes were extracted using radioimmunoprecipitation assay reagent (Beyotime Institute of Biotechnology, Haimen, China) supplemented with a protease inhibitor cocktail (Roche Molecular Diagnostics, Pleasanton, CA, USA). Subsequently, 40 µg extracted protein was separated by 8-15% SDS-PAGE, and transferred to 0.22-µm polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% milk in TBS with Tween-20 at room temperature for 2 h and subsequently incubated overnight at 4˚C with microtubule-associated proteins 1A/1B light chain 3B (LC3)I, LC3II (cat. no. 4108; 1:1,000), autophagy protein 5 (Atg5; cat. no. 12994; 1:1,000), ubiquitin-like modifier-activating enzyme ATG7 (Atg7; cat. no. 8558; 1:1,000), caspase 3 (cat. no. 9662; 1:1,000), cellular tumor antigen p53 (p53; cat. no. 2524; 1:1,000), apoptosis regulator BAX (Bax; cat. no. 2772; 1:1,000), apoptosis regulator Bcl-2 (Bcl2; cat. no. 3498; 1:1,000) and GAPDH (cat. no. 5174; 1:1,000) antibodies (all from Cell Signaling Technology, Inc., Danvers, MA, USA). The membranes were blocked with 5% milk in TBS with Tween-20 at room temperature for 2 h and subsequently incubated overnight at 4˚C with microtubule-associated proteins 1A/1B light chain 3B (LC3)I, LC3II (cat. no. 4108; 1:1,000), autophagy protein 5 (Atg5; cat. no. 12994; 1:1,000), ubiquitin-like modifier-activating enzyme ATG7 (Atg7; cat. no. 8558; 1:1,000), caspase 3 (cat. no. 9662; 1:1,000), cellular tumor antigen p53 (p53; cat. no. 2524; 1:1,000), apoptosis regulator BAX (Bax; cat. no. 2772; 1:1,000), apoptosis regulator Bcl-2 (Bcl2; cat. no. 3498; 1:1,000) and GAPDH (cat. no. 5174; 1:1,000) antibodies (all from Cell Signaling Technology, Inc., Danvers, MA, USA). Membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibodies (cat. nos. 7077 and 7076; 1:5,000; Cell Signaling Technology, Inc.) for 2 h at room temperature. Protein bands were visualized using enhanced chemiluminescence (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and a Western Blotting Detection and Imaging system (Bio-Rad Laboratories, Inc.). Enhanced chemiluminescence chromogenic substrate was quantified by densitometry (Quantity One 4.6 software; Bio-Rad Laboratories, Inc.).

Statistical analysis. All the statistical analyses were conducted using SPSS Statistics 18.0 software (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance followed by the Least Significant Difference test and Student’s t-test (two-tailed) were used to analyze the in vitro assay data. The data are presented as the mean ± standard error of the mean of at least three independent assays. P<0.05 was considered to indicate a statically significant difference.

Results

Gm5524 is upregulated and Gm15645 is downregulated in mouse DN. In the authors’ previous study, IncRNA microarray analysis of an IncRNA profile in mouse DN kidney tissues and control tissues was performed (23). It was demonstrated that hundreds of IncRNAs were differentially expressed in DN tissues compared with control kidney tissues. Among these altered IncRNAs, Gm5524 was significantly upregulated and Gm15645 was significantly downregulated in DN tissues. RT-qPCR was used to further validate this in DN and control tissues, and the results demonstrated that Gm5524 was significantly upregulated and Gm15645 was significantly downregulated in DN tissues, as was the case with the microarray data (P<0.01; Fig. 1A and B). Furthermore, podocytes were treated with high glucose to imitate the in vivo DN conditions, and the expression of Gm5524 and Gm15645 was examined. The results of the RT-qPCR demonstrated...
that Gm5524 was additionally significantly upregulated and Gm15645 was significantly downregulated in podocytes under high glucose conditions (P<0.01; Fig. 1C and D). Additionally, specific shRNAs were designed for the two lncRNAs and transfected into mouse podocytes to knock down their expression. Furthermore, Gm5524 and Gm15645 overexpression vectors were constructed and transfected into mouse podocytes to upregulate their expression. The results of the RT-qPCR revealed that Gm5524 and Gm15645 expression was significantly deceased or increased, respectively, following transfection with shRNAs or overexpression vectors compared with control cells (P<0.01; Fig. 2A and B).

**Gm5524 and Gm15645 affect the podocyte autophagy process.** Increasing evidence has demonstrated that cell autophagy serves an important role in human diseases. To investigate whether cell autophagy may be affected by Gm5524 and Gm15645 in DN, electron microscopy analysis was performed. Firstly, the podocytes were cultured under normal conditions or high glucose following transfection with Gm5524 and Gm15645 shRNAs or overexpression vector (Fig. 2A and B). The visualization of autophagosomes and quantification of mean autophagosome number/cell demonstrated that podocytes exhibit more electrodense inclusions and lipid granules following overexpression of Gm5524 or knockdown of Gm15645, which was more marked in high glucose-treated podocytes. In the control cells, autophagosomes were decreased or no autophagosomes were observed (Fig. 2C-F). These results suggested that the presence of autophagy may contribute to DN when Gm5524 expression is increased and Gm15645 expression is decreased.

**Effect of Gm5524 and Gm15645 on mouse podocyte apoptosis.** To determine whether Gm5524 and Gm15645...
affected podocyte apoptosis, flow cytometry apoptosis assays were performed. Flow cytometry analyses of these cells demonstrated that podocytes exhibited a significantly higher apoptotic percentage following knockdown of Gm5524 expression under normal conditions, while the apoptotic percentage was significantly higher in high glucose-treated podocytes (P<0.05). Conversely, Gm5524 overexpression significantly decreased the podocyte apoptotic percentage under the two conditions (P<0.05; Fig. 3A and B). Furthermore, podocytes exhibited a higher apoptotic percentage following the upregulation of Gm15645 expression under normal conditions, and the apoptotic percentage was significantly increased under high glucose conditions compared with the control; however, Gm15645 downregulation significantly decreased the podocyte apoptotic percentage under the two conditions (P<0.05; Fig. 3C and D).

In cancer, for example, lncRNAs may function as either oncogenes or tumor suppressors, and thereby regulate cancer cell growth, metastasis and drug resistance. Although lncRNAs have been associated with tumor pathogenesis, the study of lncRNAs in other human diseases requires further investigation, including in DN. Notably, certain previous studies have identified that lncRNAs are additionally involved in the development of DN.

Discussion

The sequencing of diverse tissue samples and different cell lines has identified lncRNAs as a novel class of non-coding RNAs. Subsequently, emerging evidence has revealed that lncRNAs serve critical roles in various biological processes via regulation of target gene expression by recruiting chromatin-modifying complexes to their promoters in the nucleus, or by affecting mRNA stability and translational efficiency (25,26). Recent studies have demonstrated that lncRNAs are also critical regulators of disease processes, in addition to their functional roles in normal physiological processes (27-29).
Given the importance of lncRNAs in DN, lncRNA microarray analyses were initially performed to identify the differential lncRNA expression profiles between the kidney cortices of db/db DN mice and controls, in a previous study (23). As a result, 311 lncRNAs were demonstrated to be differentially expressed in the db/db DN tissues, including 105 upregulated lncRNAs and 206 downregulated lncRNAs. In addition to the results of the authors' previous study (23), Wang et al (21) reported that 1,018 lncRNAs (221 upregulated and 797 downregulated) were differentially expressed in kidney tissue from db/db mice with DN. Although the expression of hundreds of lncRNAs may be dysregulated in DN, their underlying functional roles remain unclear. In the present study, the expression of lncRNAs was further validated in mice kidney tissues with or without DN. Out of these lncRNAs, Gm5524 was upregulated and Gm15645 was downregulated, in accordance with the lncRNA microarray data and RT-qPCR validation results. Furthermore, flow cytometry apoptosis assays suggested that downregulation of Gm5524 and overexpression of Gm15645 increased the apoptotic rate of podocytes, a feature of the early stages of DN. In addition, the electron microscopy analysis revealed that alterations in Gm5524 and Gm15645 expression were able to induce podocyte autophagy.

Cellular autophagy and apoptosis are basic cellular processes, and are essential for the maintenance of normal tissue homeostasis under physiological conditions. Disorder of these processes has been reported in diabetes and its complications (30-32). For example, IncRNA myocardial infarction associated transcript has been demonstrated to be involved in diabetes-induced microvascular dysfunction by acting as a competing endogenous RNA for miR-150-5p to antagonize its repression of vascular endothelial growth factor in retinal endothelial cells (33). In addition, plasmacytoma variant translocation 1 gene is able to increase extracellular matrix (ECM) synthesis by upregulating transforming growth factor β1, plasminogen activator inhibitor 1 and fibronectin 1, which are the principal regulators of ECM accumulation in DN (34).

Figure 4. Effect of Gm5524 and Gm15645 on apoptosis and autophagy-associated protein expression levels in podocytes. (A) Protein expression levels of LC3I, LC3II, Atg5, Atg7, cleaved caspase 3, p53, Bax, Bel2 and p53 were detected by western blotting in podocytes following transfection with the empty vector, sh-Gm5524 vector and Gm5524 overexpression vector under normal or HG medium conditions. (B) Densitometry data from the blots. *P<0.05 vs. control; #P<0.05 vs. control+HG; &P<0.05 vs. Scr; $P<0.05 vs. Scr+HG. (C) Protein expression levels of LC3I, LC3II Atg5, Atg7, cleaved caspase 3, p53, Bax, Bel2 and p53 was detected by western blotting in podocytes following transfection with the empty vector, sh-Gm15645 vector and Gm15645 overexpression vector under normal or HG medium conditions. (D) Densitometry data from the blots. *P<0.05 vs. Scr; #P<0.05 vs. Scr+HG; $P<0.05 vs. control; &P<0.05 vs. control+HG. LC3, microtubule-associated proteins 1A/1B light chain 3B; Atg5, autophagy protein 5; Atg7, ubiquitin-like modifier-activating enzyme ATG7; p53, cellular tumor antigen p53; Bax, apoptosis regulator BAX; Bcl-2, apoptosis regulator Bcl-2; HG, high glucose; sh, short hairpin; Scr, scrambled.
and autophagy-associated factors were examined. It was demonstrated that the Bcl2 protein expression was decreased and Bax expression was increased in Gm5524-knockdown and Gm15645-overexpressing podocytes. Bcl-2 is an important anti-apoptotic protein, while Bax is an important pro-apoptotic protein. The alteration in their expression levels was consistent with the functional results. Additionally, the ratio of LC3II/LC3I protein expression is a well-established biochemical assay to determine the activation of autophagy, and the results of the present study demonstrated that the altered expression of Gm5524 and Gm15645 also affected the LC3II/LC3I ratio. These data suggested that Gm5524 and Gm15645 may affect cellular apoptotic and autophagy processes in the diabetic mouse kidney, and thus contribute to the development and progression of DN. However, further investigations are required to further elucidate the molecular mechanisms underlying the roles of Gm5524 and Gm15645 in the pathogenesis of DN.

In conclusion, the present study revealed that IncRNA Gm5524 and Gm15645 may affect podocytes apoptosis and autophagy via regulation of the Bcl2/Bax, and LC3/ATG signaling pathway in DN. Therefore, it was hypothesized that Gm5524 and Gm15645-mediated regulation of autophagy and apoptotic signaling pathways may be potential, useful therapeutic targets for the development of alternative treatment strategies for patients with DN.

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Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

YF and YL conceived and designed the study. YF, SC, JX, QZ and XY performed the experiments. YF, DD and WY analyzed the data. YF and DD wrote the paper. WY and YL reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The protocol used in the present study was approved by the Committee on the Ethics of Animal Experiments of The Second Affiliated Hospital of Nanjing Medical University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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