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LncRNA TCF7 triggered endoplasmic reticulum stress through a sponge action with miR-200c in patients with diabetic nephropathy

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Abstract. – OBJECTIVE: To investigate the functions and mechanisms of long non-coding RNA TCF7 (LncTCF7) in patients with diabetic nephropathy (DN).

PATIENTS AND METHODS: LncTCF7 and miR-200c expressions in DN (+) were detected by Real Time-Polymerase Chain Reaction (RT-PCR). In the established high-glucose group (HG) model, RT-PCR and Western blot were used to detect the expressions of lncTCF7 and key apoptotic genes. The effect of podocyte endoplasmic reticulum (ER) stress was detected in high glucose conditions after lncTCF7 siRNA transfection. The miR-200c level was detected in lncTCF7 overexpressing cell model and the luciferase reporter gene assay was performed to verify the potential binding site of lncTCF7 with miR-200c. Furthermore, ER stress associated genes were detected in patients with DN. Finally, in the HG model, the levels of ER stress were detected by WB after transfection with miR-200c inhibitor and lncTCF7 siRNA with miR-200c mimics.

RESULTS: Results showed that the lncTCF7 expression was up-regulated, while miR-200c was significantly downregulated in patients with DN (+). In the HG model, the expression of lncTCF7 was significantly increased and some key ER stress-associated genes, such as CHOP, XBP1, and cleaved caspase3, were significantly increased, while the anti-apoptotic protein Bcl-2 was significantly decreased. However, after inhibiting the lncTCF7 expression, these gene levels were reversed. In lncTCF7 overexpressing cells, miR-200c expression was significantly down-regulated compared with the control ($p<0.05$) and the luciferase reporter gene assay results showed that lncTCF7 could directly bind to miR-200c. In the HG model, after inhibiting lncTCF7 expression, the miR-200c level was increased, while the ER stress-associated proteins CHOP, XBP1, and cleaved caspase3 were significantly repressed. However, these proteins were reversed after inhibiting miR-200c expression. In addition, the expressions of ER stress-associated protein and apoptotic protein in human DN patients were consistent with HG cell model.

CONCLUSIONS: LncRNA TCF7 triggered endoplasmic reticulum stress through a sponge action with miR-200c in patients with diabetic nephropathy.

Key Words:
LncRNA-TCF7, MiR-200c, Diabetic Nephropathy, ER-stress.

Introduction

About 30-40% of diabetic patients will develop into nephropathy in the world. With the increase of diabetes incidence, diabetic nephropathy has become the main cause of end-stage renal disease worldwide and the incidence rate in China is also increasing[1]. The causes of diabetes progression to diabetic nephropathy are extremely complicated; some researchers have reported that chronic diabetes is associated with metabolism and hemodynamic stress, which promotes the modification of DNA, proteins and lipids and induces cell dysfunction and damage. Then, it stimulates inflammation and fibrosis. As a result, it causes various types of renal damage[2,3]. The diabetic nephropathy has a serious metabolic disorder process and once it develops into the end-stage of renal disease, it is often more difficult to treat than other renal diseases. Therefore, it is of great significance to prevent the progression and to treat diabetic nephropathy. Although the pathogenesis of diabetic nephropathy has been deeply, the mechanisms of its pathogenesis are not fully clear.

Long non-coding RNAs (lncRNAs) are a sort of long RNAs, which are longer than 200 nucleotides but do not have the protein-coding capacity, but they participate in regulating gene expression at epigenetic, transcriptional, and post-transcriptional levels in some biological processes of various diseases[4-9], including DN. For instance,

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lncTUG1 alleviates extracellular matrix accumulation by regulating PPARγ/miR-377 axis in DN[10]; lncMALAT1 is specifically expressed in DN and participates in high glucose-induced podocyte injury by interacting with β-catenin[11]. The disorder of lncTCF7 is common in many human cancers, such as colorectal cancer, liver cancer, non-small cell lung cancer, and glioma and plays a key role in promoting cancer cell self-renewal, migration, invasion, etc.[12-14]. However, the role of lncTCF7 in DN is poorly understood.

MicroRNAs (miRNAs) are sorts of small non-coding RNA molecules, containing about 22 nucleotides, which function in RNA silencing and post-transcriptional regulation in some biological processes[15,16]. Some studies[17-19] showed that some miRNAs played some roles in DN. MiR-200c is a member of miR-200 family, which has been shown to be dysregulated in a variety of cancer diseases and plays an important role in the development and metastasis of cancer, such as breast cancer, ovarian cancer, prostate cancer, etc.[20,21]. It may be important for the diagnosis and treatment of cancer, but its function in DN is still unclear.

ER stress are a series of complicated processes induced by unfolded protein reaction (UPR). The upstream signal transduction pathway includes some important molecules, such as ATF6, IRE1α, XBP1, and CHOP[22]. At present, Pang et al[23] have reported the role of ER-stress in DN, which showed that ERp44 depletion could promote ER stress, thereby aggravating DN. In addition, it plays a key role in ER-stress-induced podocyte apoptosis in glomerulosclerosis, and CHOP is the key factor in the apoptosis of ER-stress cells[24]. However, there are few reports on the related research of lncTCF7 in promoting the development of DN by regulating ER-stress.

In this work, we detected the expressions of lncTCF7 and several other lncRNAs in patients with DN, and initially found that lncTCF7 was highly expressed in DN (+). To further investigate the role of lncTCF7 in the development of human DN, we constructed a cellular model (HG) of DN to study its function and mechanism. We also detected the expression of miR-200c and further explored whether it could interact with lncTCF7.

Patients and Methods

Patient Samples

Patient samples were collected from 14 DN patients and 14 non-diabetic nephropathy patients, who were admitted in our hospital from June 2016 to July 2018. All tissue samples were frozen in liquid nitrogen at the condition of –80°C until usage. This investigation was approved by the Faculty of Medicine's Ethics Committee of our hospital and all patients signed the informed consent.

Cell Culture and Cell Transfection

Construction of high glucose (HG) cell model[25] Dulbecco's Modified Eagle's Medium (DMEM)/F12 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 μg/ml streptomycin and 100 U/ml penicillin were added into human podocyte cell line (CIHP) at the condition of 37°C with 5% CO_2. When cells reached about 70%, 33.3 mM HG glucose was added into the cells and then cultured at 37°C and 5% CO_2 culture incubator. The siRNAs were constructed and cloned into pcDNA3, lncTCF7 siRNA primer: 5'-AGCCAACATTGTTGGTTAT-3', the control primer: 5'-UUCUCCGAACGUGUCACGUTT-3'. CHIP cells were seeded in 96-well plates (1×10^5/well) until reached 70-80%. Before transfection, the transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), serum-free DMEM, and lncTCF7 siRNA or miR-200 inhibitor were mixed and incubated for 30 min, which were then added into cells with complete medium containing 15% FBS. At indicated time point after transfection, cells were harvested for further study.

RNA Extraction and Quantitative Real-Time PCR

The total RNAs of patient samples and CIHP cells in each group were extracted after treatment by using RNAiso Plus (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's protocol. Real Time-Polymerase Chain Reaction (RT-PCR) was performed by using PrimeScript™ RT reagent Kit (TaKaRa, Otsu, Shiga, Japan) according to the manufacturer's protocol. PCR primers were synthesized by Gene Pharma (Shanghai Gene Pharma, Shanghai, China) and sequences were listed in Table I. The levels of mRNA expression were detected by SYBR Premix Ex Taq II (TaKaRa, Otsu, Shiga, Japan). The mRNA expressions of XBP1, CHOP, Caspase3, Lnc-TCF7, Lnc-p21, Lnc-MEG3, and Lnc-MALAT1 were normalized to GAPDH and miR-200c was normalized to U6 and the $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expressions.

Table I. RT-PCR primer sequences.

Gene names	Primer sequences
Lnc-TCF7	Forward: 5'-AGGAGTCCTTGGACCTGAGC-3' Reverse: 5'-AGTGGCTGGCATATAACCAACA-3'
Lnc-p21	Forward: 5'-CCTGTCCCACTCGCTTTC-3' Reverse: 5'-GGAACTGGAGACGGAATGTC-3'
Lnc-MEG3	Forward: 5'-CTCAGGCAGGATCTGGCATA-3' Reverse: 5'-CCTGGAGTGCTGTTGGAGAA-3'
Lnc-MALAT1	Forward: 5'-AGCGGAAGAACGAATGTAAC-3' Reverse: 5'-GAACAGAAGGAAGAGCCAAG-3'
XBP1	Forward: 5'-GTTAAGACAGCGCTTGGGGA-3' Reverse: 5'-TGCACGTAGTCTGAGTGCTG-3'
CHOP	Forward: 5'-CACCACTCTTGACCCTGCTTCTCT-3' Reverse: 5'-GTTTCCTGGTTCTCCCTTGGTCTT-3'
Caspase3	Forward: 5'- ATTTGGAACCAAAGATCATACATGG-3' Reverse: 5'-CTGAGGTTTGCTGCATCGAC-3'
U6	Forward: 5'-CTCGCTTCGGCAGCACA-3' Reverse: 5'-AACGCTTCACGAATTTGCGT-3'
MiR-200c	Forward: 5'-CGTAATACTGCCGGGTAATGAT-3' Reverse: 5'-GTGTCGTGGAGTCGGCAA-3'
GAPDH	Forward: 5'-GGAGTCCACTGGTGTCTTCA-3' Forward: 5'-GGGAACTGAGCAATTGGTGG-3'

Protein Extraction and Western Blot

The total proteins were extracted from patient samples and CIHP cells in each group by using a radioimmunoprecipitation assay (RIPA) lysis buffer (Biyuntian, Shanghai, China) and protease inhibitor cocktails (Roche Diagnostic, Basel, Switzerland) according to manufacturer's instruction. The protein concentration was measured by using the bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). 50 μg proteins were added to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Pharmacia, Shanghai, China), which were blocked at room temperature for 1 h. Next, membranes were incubated with primary antibodies overnight at 4°C. XBP1 (1:1000), CHOP (1:2000), Caspase3 (1:1000), and GAPDH (1:5000) were bought from Abcam (Cambridge, MA, USA). After washed with Tris-Buffered Saline and Tween-20 (TBST) for 4 times, it was then incubated with the secondary antibodies (1:20000; BD Biosciences, Franklin Lakes, NJ, USA) for 1 h at room temperature. Protein bands were detected by Pierce enhanced chemiluminescence (ECL) Western blot substrate (Thermo Fisher Scientific, Waltham, MA, USA) with ECL detection system (Thermo Fisher Scientific, Waltham, MA, USA).

Luciferase Reporter Gene Assay

A TCF7 wild type (WT) or mutant (mut) 3'-UTR recombinant luciferase reporter plasmid containing the potential miR-200c binding site was constructed and cloned into the pGL3 basic vector (Promega, Madison, WI, USA). HEK-293T cells were seeded in 48-well plates for 24 h. Then, miR-200c mimics and miR-NC were co-transfected into HEK-293T cells with pGL3-TCF7-WT or pGL3- TCF7-mut for another 24 h. Plasmids with 200 ng were mixed with Lipofectamine 2000 and DMEM medium for 20 min, then the mixtures were added into HEK-293T cells for 24 h. After 24 h, the cells were lysed and the activities of firefly luciferase and *Renilla* luciferase were measured by using dual-luciferase reporter assay (Promega, Madison, WI, USA). The ratio of this two revealed the relative activity of luciferase.

Statistical Analysis

All statistical analyses were performed by using SPSS 20.0 (IBM, Armonk, NY, USA) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Data was expressed as the mean±SD, the significance between groups was analyzed by Student's *t*-test and multiple comparison between the groups was performed by the SNK method. If $p<0.05$, it was considered statistically significant.

Results

LncTCF7 was Highly Expressed in DN Patients

To explore the roles of lncTCF7 in DN patients, for the first time, we detected the expressions of lncTCF7 and another three lncRNAs in DN patients and non-DN patients. Results showed that the expression of lncTCF7 in DN patients was significantly increased than non-DN patients (Figure 1A) ($p<0.05$), while no significantly differences had been found in other lncRNAs (Figure 1B-D) ($p>0.05$), which indicated that lncTCF7 was increased in DN patients and might play some roles in DN.

ER Stress was Increased in DN Patients

In patients with DN, we detected the ER stress associated gene expressions to observe whether ER stress was affected. CHOP and XBP1 were two kinds of important proteins that regulated the process of ER stress. Results showed that the mRNA and protein expressions of CHOP and

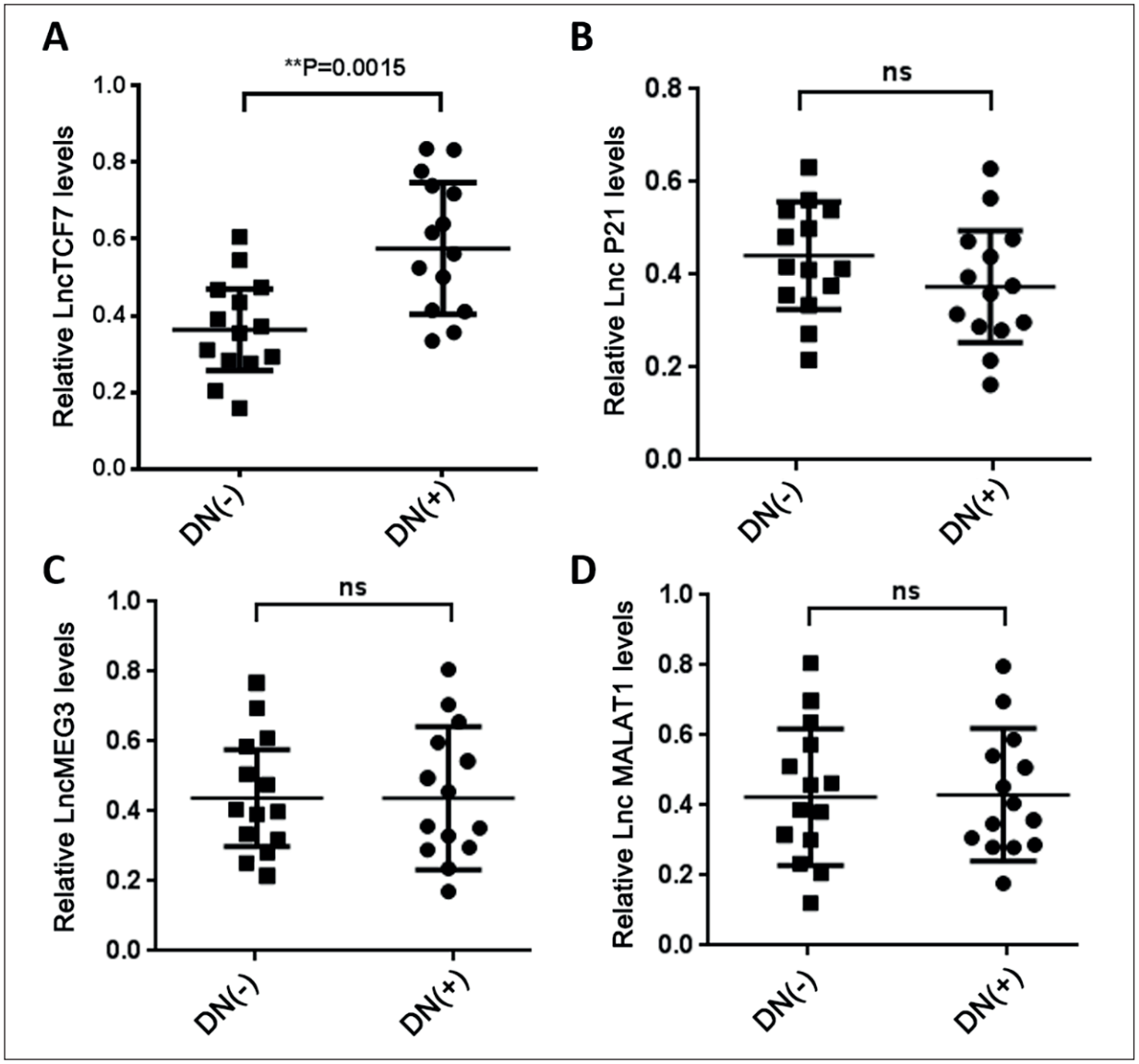

Figure 1. LncTCF7 was highly expressed in DN patients. **A,** MRNA levels of lncTCF7 in DN patients and non-DN patients were detected by RT-PCR. **B-D,** MRNA levels of lncRNA-p21, lnc-MEG3, and lnc-MALAT1 in DN patients and non-DN patients were also detected by RT-PCR. Data are shown as mean ± SD based on at least three independent experiments, $^{**}p<0.01$.

XBP1 were significantly increased in DN patients (Figure 2A-F) (p<0.05), indicating that ER stress was aggravated in the development of DN.

LncTCF7 Promoted ER Stress in HG Cell Model

To investigate the roles of lncTCF7 in DN, we constructed a HG cell model of human renal podocytes. The levels of lncTCF7 and ER stress associated molecules were detected by RT-PCR and WB. Results showed that lncTCF7 expression was significantly up-regulated in HG compared to NG group (Figure 3A) (p<0.01), which was consistent with the results of patients with DN. Furthermore, the expressions of CHOP, XBP1, and Caspase3 were significantly

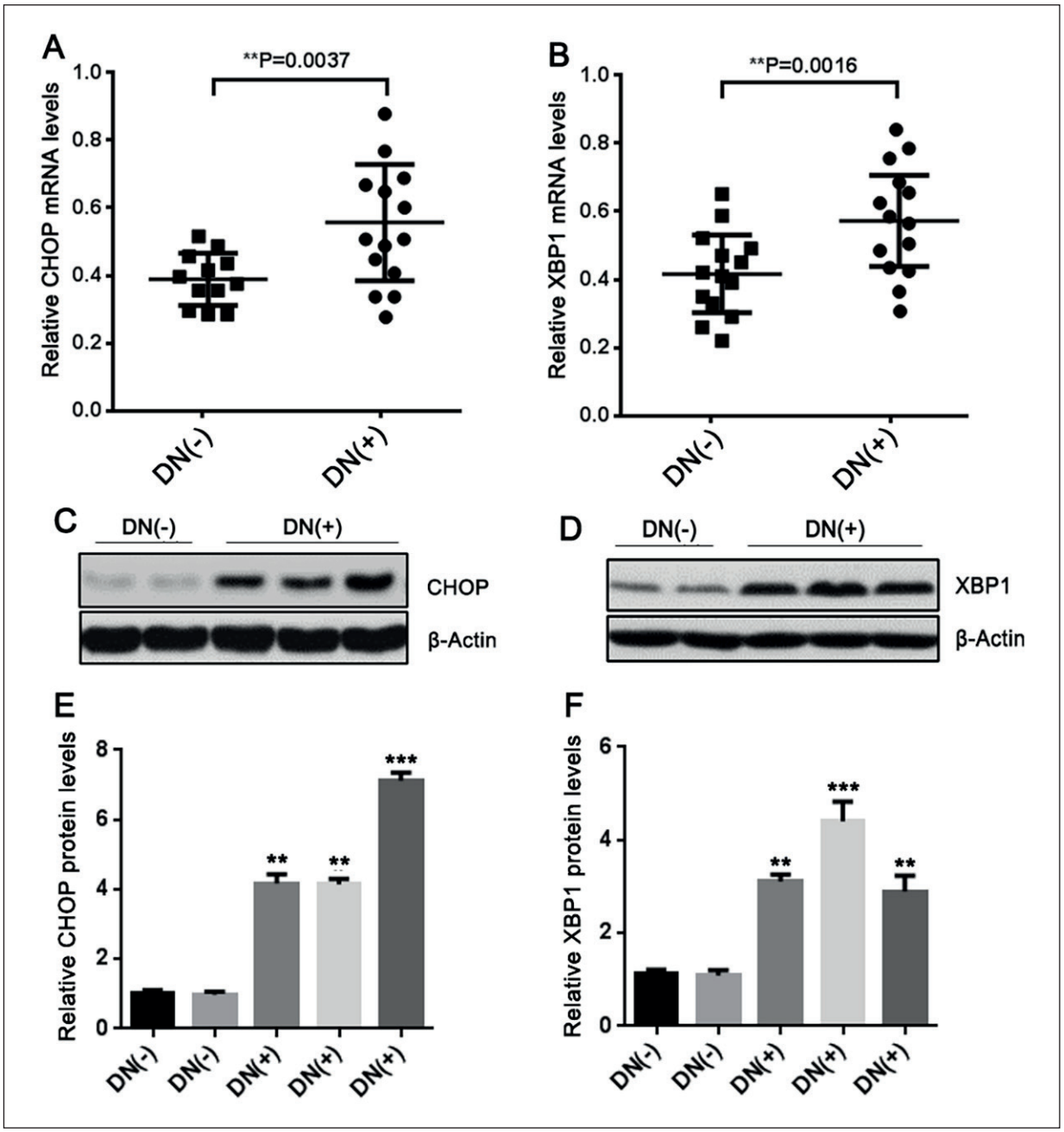

Figure 2. ER stress was increased in DN patients. **A-F,** MRNA levels and protein levels of CHOP and XBP1 were detected by RT-PCR and WB in DN patients and non-DN patients. The mRNA and protein levels of CHOP and XBP1 were significantly increased. Data are shown as mean ± SD based on at least three independent experiments, **p<0.01; ***p<0.001.

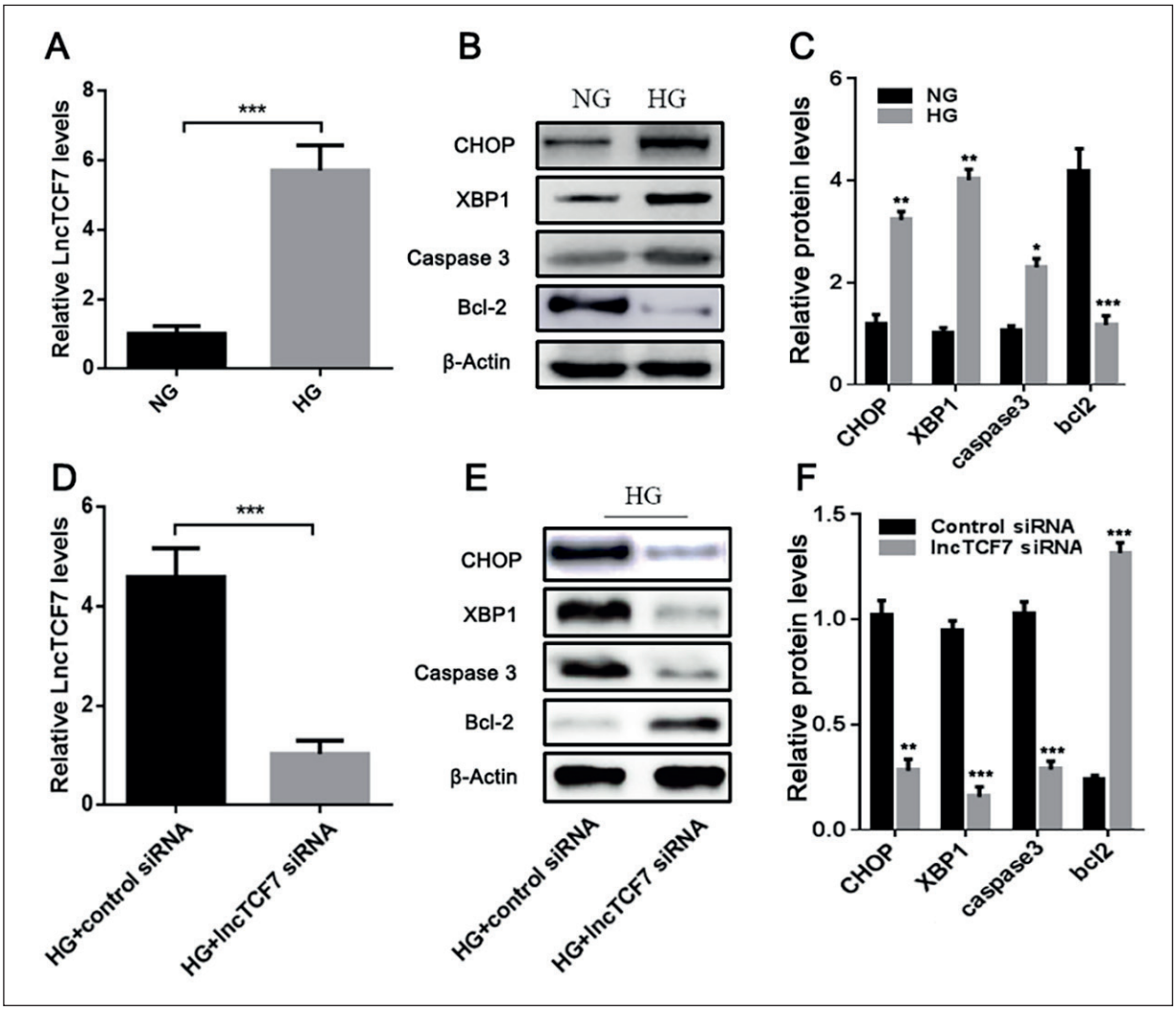

Figure 3. LncTCF7 promoted ER stress in HG cell model. **(A)** The lncTCF7 level in HG and NG cells was detected by RT-PCR and it was significantly increased. **B, C** The levels of CHOP, XBP1, Caspase3 and Bcl-2 were detected by WB, the levels of CHOP, XBP1, Caspase3 were significantly increased, while Bcl-2 was significantly repressed. **D,** LncTCF7 level was detected by RT-PCR after transfection with lncTCF7 siRNA and it was significantly repressed. **E, F** The levels of CHOP, XBP1, Caspase3 and Bcl-2 were detected by WB, and were reversed after lncTCF7 siRNA transfection. Data are shown as mean ± SD based on at least three independent experiments. *p<0.05; **p<0.01; ***p<0.001.

increased, while the anti-apoptotic protein Bcl-2 was significantly decreased (Figure 3B, C) (p<0.05). These results indicated that severe renal damage occurred in human renal foot cells in HG conditions, and initiated cell apoptosis. To further prove that lncTCF7 played a role in this process, lncTCF7 siRNA was constructed and transfected into HG podocytes to inhibit the lncTCF7 expression. Results showed that the level of lncTCF7 was repressed after transfection (Figure 3D) (p<0.01). In addition, WB analysis showed that the levels of CHOP, XBP1, Caspase3 and Bcl-2 were reversed (Figure 3E, F) (p<0.05), showing that lncTCF7 was involved in the process of ER stress damage in human renal foot cells under HG condition.

LncTCF7 Could Directly Bind with MiR-200c

To further investigate the downstream mechanisms that lncTCF7 played a role in this process, it was reported that lncTCF7 might directly bind to miR-200c[26], and miR-200c was reported to trigger ER stress, which played a protective role in Alzheimer's disease[27]. Therefore, we speculate that lncTCF7 promoted the ER stress process of

podocytes in DN by targeting with miR-200c. To verify this hypothesis, we first examined the expression of miR-200c in podocytes after lncTCF7 overexpression. Results showed that the expression of miR-200c was significantly decreased after overexpression of lncTCF7 (Figure 4A) ($p<0.05$). To further prove the potential binding site, the pGL3- TCF7-WT or pGL3- TCF7-mut were constructed. Luciferase reporter gene assay showed that the luciferase activity in co-transfection with miR-200c mimic and pGL3- TCF7-WT was significantly decreased, while it was increased in pGL3- TCF7-mut group in HETK-293T cells (Figure 4B) ($p<0.05$).

LncTCF7 Promoted ER Stress and Aggravated DN Through Regulating MiR-200c

To further validate the role of lncTCF7 that targeted with miR-200c and promoted ER stress in DN, we examined the expression of miR-200c in the patient samples and constructed podocyte in HG model. Results showed that miR-200c was significantly reduced in the DN patients compared with the control (Figure 5A) ($p<0.05$). And results showed that miR-200c was significantly down-regulated in HG cells compared with control NG, while it was significantly increased in HG cells after inhibition of lncTCF7 (Figure 5B) ($p<0.05$). Furthermore, we transfected the miR-200c inhibitor into the lncTCF7 siRNA transfected cells; results showed that the miR-200c level was significantly decreased (Figure 5C) ($p<0.05$). After that, the levels of CHOP, XBP1 and Caspase 3 were also detected. Data showed that the mRNA and protein levels of CHOP, XBP1 and Caspase 3 were up-regulated after inhibiting miR-200c expression in lncTCF7 siRNA cells. These findings indicated that lncTCF7 might promote ER stress and aggravate DN by regulating with miR-200c.

Discussion

Diabetic nephropathy (DN) is a kind of chronic loss of kidney function occurring in patients with diabetes mellitus, which is a slow-

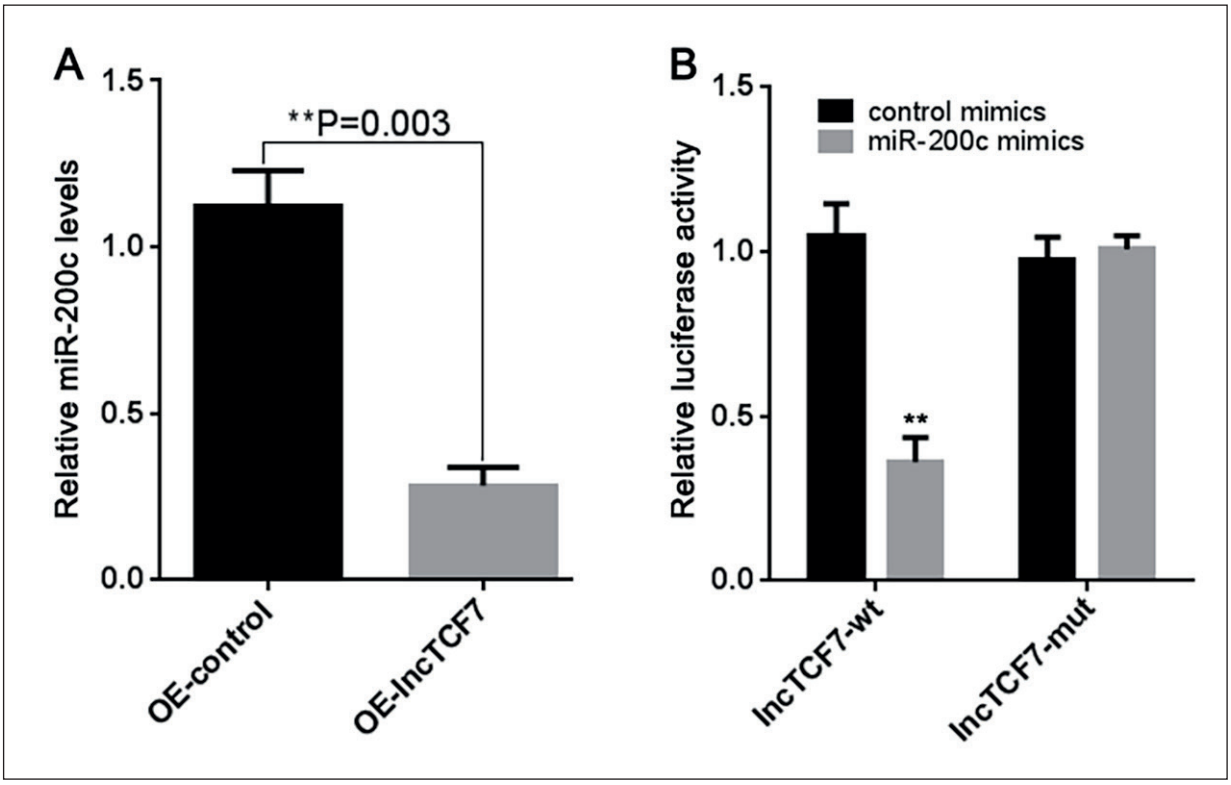

Figure 4. LncTCF7 could directly bind with miR-200c. **A,** MiR-200c level was detected after lncTCF7 transfection and it was significantly repressed. **B,** Luciferase reporter assay was performed to determine the binding site. It was significantly decreased in co-transfection with miR-200c mimic and pGL3- TCF7-WT, while it was reversed in other groups. Data are shown as mean ± SD based on at least three independent experiments, *$p<0.05$; **$p<0.01$.

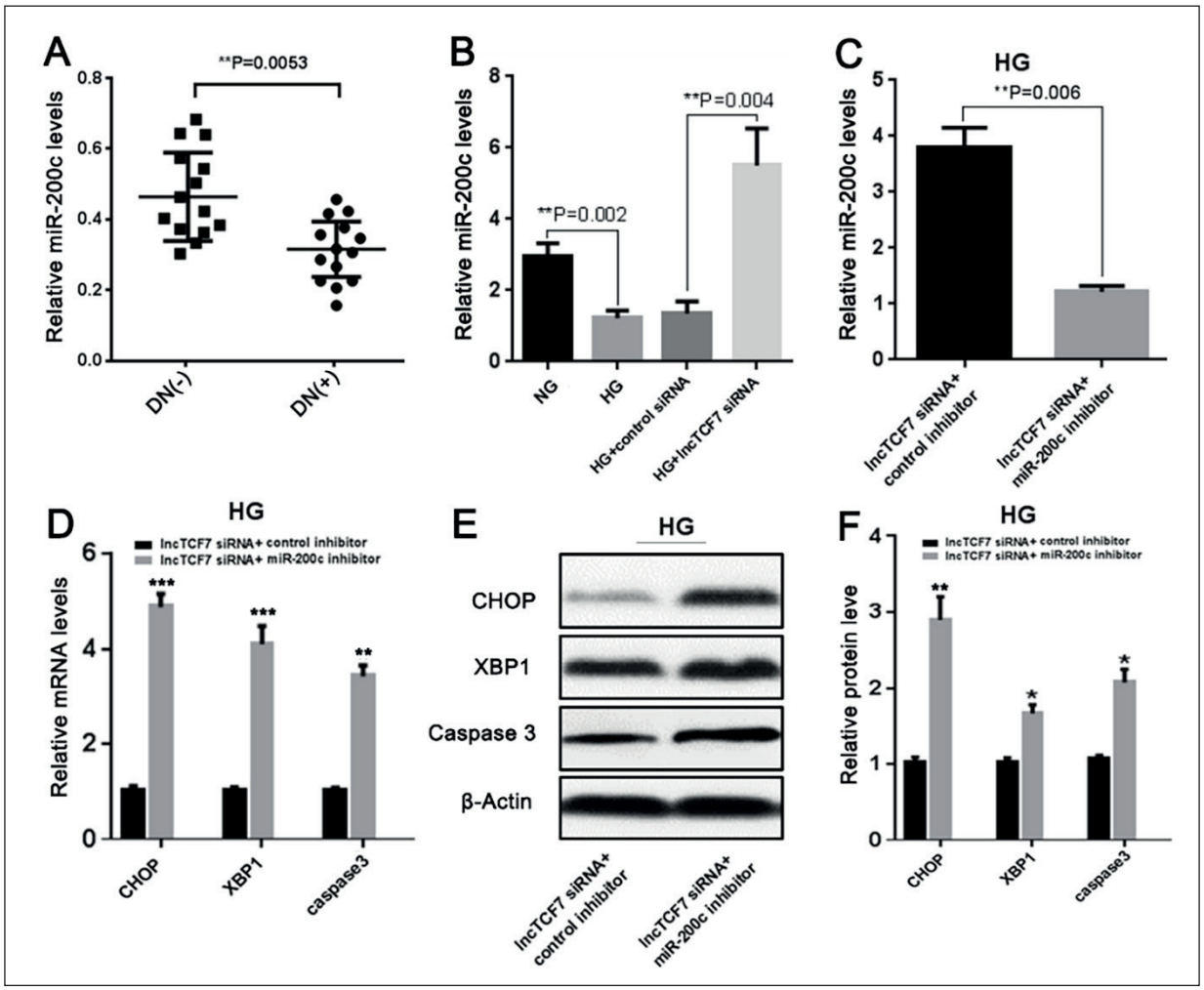

Figure 5. LncTCF7 promoted ER stress and aggravated DN through regulating miR-200c. **A,** Expression of miR-200c in DN patients was detected, which was significantly decreased in DN patients. **B,** MiR-200c levels were detected in HG, NG cells and HG cells after lncTCF7 siRNA transfection. (**C**, MiR-200c levels were detected in lncTCF7 siRNA transfected cells after transfected with miR-200c inhibitor, which was significantly decreased. **D-F,** MRNA and protein levels of CHOP, XBP1 and Caspase 3 were detected by RT-PCR and WB. Data are shown as mean ± SD based on at least three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

ly progressive disease for years. The incidence rate is still increasing and it is also associated with an increased risk of cardiovascular disease and death[28-32]. Studies have found that lncRNAs are involved in many kidney diseases including diabetic nephropathy. Huang et al[33] reported that the inhibition of lncNEAT1 could promote fibrosis by activating Akt/mTOR signaling pathway in DN. Long et al[34] showed that LncTUG1 could regulate mitochondrial biological function in DN. However, the roles and mechanisms of lncTCF7 in DN remained unclear. In our previous study, we found that lncTCF7 was increased in DN patients, which might play an important role in the processes of DN. However, the detailed mechanisms were not clear. It was reported that some lncRNAs contributed to the ER-stress process in DN[35,36]. To investigate the roles and mechanisms of lncTCF7 in the pathogenesis of human DN, we further detected the ER- stress in DN patients. Results showed that these associated genes, such as CHOP and XBP1, were significantly increased, indicating that ER stress was aggravated in the development of DN.

To detect whether lncTCF7 played some role in DN, a diabetic nephropathy cell model (HG) was established in this study, and the expression of lncTCF7 in this model was detected. Results showed that lncTCF7 was significantly

increased in the HG cell model, which was consistent with the results of DN patients. Furthermore, the levels of CHOP, XBP1 and Caspase3 were significantly increased, while Bcl-2 was significantly decreased, showing that severe renal damage occurred in HG cells and initiated cell apoptosis. To further demonstrate that lncTCF7 played a role in this process, lncTCF7 siRNA were constructed and transfected into HG podocytes to inhibit the lncTCF7 expression. Results showed that the levels of CHOP, XBP1, Caspase3, and Bcl-2 were reversed. These results suggested that lncTCF7 was involved in the process of ER stress damage in human renal foot cells under HG condition.

MiRNAs are involved in RNA silencing and post-transcriptional regulation in some biological processes[15,16]. MiR-200c has been shown to be dysregulated in a variety of cancer diseases and plays an important role in the development and metastasis of cancer, such as breast cancer, ovarian cancer, prostate cancer, etc.[20,21]. It is reported that lncTCF7 could promote the cell growth and self-renewal of glioma cells by targeting at miR-200c[37]. To further explore whether lncTCF7 could promote ER-stress damage through binding with miR-200c in DN, luciferase reporter gene assay was performed. Data showed that the luciferase activity in co-transfection with miR-200c mimic and pGL3- TCF7-WT was significantly decreased, while it was increased in pGL3- TCF7-mut group in HETK-293T cells, which indicated that lncTCF7 could promote ER-stress damage through binding with miR-200c in the process of DN.

To further validate the role of lncTCF7 that promoted ER stress by targeting with miR-200c in DN, the expressions of miR-200c in the patient samples and constructed podocyte in HG model were examined. Data showed that miR-200c was significantly reduced in DN patients. It was significantly down-regulated in HG cells compared with control NG, while it was significantly increased in HG cells after inhibition of lncTCF7. Furthermore, we transfected the miR-200c inhibitor into the lncTCF7 siRNA transfected cells. Results showed that the miR-200c level was significantly decreased. Finally, the mRNA and protein levels of CHOP, XBP1 and Caspase 3 were up-regulated after inhibiting miR-200c expression in lncTCF7 siRNA cells. These findings indicated that lncTCF7 might promote ER stress and aggravate DN through regulating with miR-200c.

Conclusions

We demonstrated that the lncTCF7 was up-regulated in patients with DN. Furthermore, it promoted the pathogenesis and ER stress by regulating with miR-200c, which might provide a potential biomarker for end-stage DN and might become a new therapeutic target for DN.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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