Downregulation of lncRNA NEAT1 inhibits mouse mesangial cell proliferation, fibrosis, and inflammation but promotes apoptosis in diabetic nephropathy

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Abstract: This study aimed to investigate the effect of long non-coding RNA nuclear enriched abundant transcript 1 (lnc-NEAT1) on mouse mesangial cells (MMCs) proliferation, apoptosis, fibrosis as well as inflammation in diabetic nephropathy (DN). MMCs (SV40 MES13 cells) were cultured under 30 mM glucose to construct DN cellular model (high glucose (HG) group); meanwhile, MMCs cultured under 5.6 mM glucose (normal glucose (NG) group) and 5.6 mM glucose plus 24.4 mM 3-O-methyl-D-glucose (osmotic control (OC) group) served as controls, and lnc-NEAT1 expression was determined by qPCR assay. Lnc-NEAT1 interference plasmids and control interference plasmids were transfected into DN cellular model as Sh-NEAT1 group and Sh-NC group. Cell proliferation, apoptosis, fibrosis, and inflammation were detected using Counting Kit-8, Annexin V/propidium iodide, western blot and quantitative polymerase chain reaction assays. Lnc-NEAT1 expression was elevated in HG group compared to NG group and OC group. Cell proliferation was decreased, and proliferative marker protein Cyclin D1 and proliferating cell nuclear antigen expressions also decreased in Sh-NEAT1 group compared to Sh-NC group. For cell apoptosis, apoptosis rate was increased, and apoptotic protein Cleaved Caspase3 expression enhanced but anti-apoptosis protein Bcl-2 expression decreased in Sh-NEAT1 group compared to Sh-NC group. For fibrosis markers (including fibronectin and collagen I) and inflammatory cytokines (including tumor necrosis factor-α, interleukin-1β and interleukin-6), their expressions were reduced in Sh-NEAT1 group compared to Sh-NC group. Lnc-NEAT1 is overexpressed, and its down-regulation inhibits cell proliferation, fibrosis, and inflammation but promotes cell apoptosis in HG-induced MMCs DN cellular model.

Keywords: Long non-coding RNA, enriched abundant transcript 1, diabetic nephropathy, proliferation, apoptosis, fibrosis, inflammatory cytokines

Introduction

Diabetic nephropathy (DN), with the functional derangement and structural remodeling of kidney triggered by hyperglycemic injury, is one of the most frequent microvascular complications of diabetes patients and the leading cause of end-stage renal disease worldwide, and is characterized by a series of pathologic abnormalities such as glomerular hypertrophy, mesangial proliferation, thickening of the glomerular basement membrane and accumulation of the extracellular matrix [1-6]. According to recent reports, around 30%-40% diabetes patients tend to develop DN, and about 50% of DN patients would further develop end-stage renal disease (ESRD), which has the requirement of dialysis or renal transplantation [7-9]. Prevention or counteraction of these disease progression is based on the well understanding of underlying mechanisms of DN [10]. Although several molecular events such as the amplification and perpetuation of inflammatory reactions, oxidative stress, activation of sorbitol-aldose reductase, elevated production of advanced glycation end-products, raised activity of intracellular protein kinase C and altered expression of cellular cyclin-dependent kinases have been revealed to participate in the physiopathology of DN, the mechanism in DN is still poorly understood due to its complicated pathogenesis [1, 7, 8, 11]. Thus, further exploration of underlying mechanism for DN progression is of great importance, which might provide novel strategies for preventing or treating DN.
Long non-coding RNA (IncRNA), a subgroup of RNAs with insufficient protein coding capability, is composed of more than 200 nucleotides [12, 13]. Increasing studies have shown that IncRNA plays crucial roles in multiple biological processes (including regulation of gene expression, recruitment of chromatin modification, X chromosome inactivation, chromosome recombination and protein folding). Also, some of them are identified as important regulators in the pathologic processes of several endocrine and metabolic diseases, such as diabetes, osteoporosis and fatty liver disease [14-16]. LncRNA nuclear enriched abundant transcript 1 (Inc-NEAT1), located on chromosome 11q13.1, works as an important structural component of paraspeckles [17]. Majority of previous studies focus on the role of Inc-NEAT1 in different carcinomas, and identify its ability to affect cell activities via targeting miRNAs or regulating signaling pathways in cancer progression [18-20]. Recently, some recent studies disclose Inc-NEAT1 is aberrantly expressed in diabetes mice, moreover, there are some studies show that Inc-NEAT1 has proinflammatory influence through affecting inflammatory pathways such as activation of mitogen-activated protein kinase (MAPK) pathways or Toll-like receptor 3 (TLR3)-p38 pathway in some inflammatory diseases [21, 22]. Considering inflammation is a key pathogenic mechanism in DN and Inc-NEAT1 is involved in inflammatory processes, we hypothesized that Inc-NEAT1 might participate in the pathology of DN, while the underlying mechanism of Inc-NEAT1 in DN was not deeply investigated [23, 24]. In this study, we constructed a DN cellular model with mouse mesangial cells (MMCs) under high glucose (HG) condition, and investigated the effect of Inc-NEAT1 on cell proliferation, apoptosis, fibrosis as well as inflammation in DN.

Materials and methods

Cells source

MMCs cell line (SV40 MES13 cells) was purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China).

Cells culture

SV40 MES13 cells were cultured in 90% Dulbecco’s modified eagle medium (DMEM) (Gibco, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) in a humidified incubator under 95% air and 5% CO2 at 37°C.

Measurement of Inc-NEAT1 expression in SV40 MES13 cells under HG condition

30 mM glucose (Sigma, USA), 5.6 mM glucose, 5.6 mM glucose plus 24.4 mM 3-O-methyl-D-glucose (Wako, Japan) were added to treat SV40 MES13 cells for 96 h, and these cells were divided into three groups accordingly as HG group, normal glucose group (NG group) and osmotic control group (OC group). Quantitative polymerase chain reaction (qPCR) was subsequently performed to measure Inc-NEAT1 expression in each group.

Construction and transfection of Inc-NEAT1 interference plasmids

Lnc-NEAT1 interference plasmids and control interference plasmids were constructed by Shanghai GenePharma Bio-Tech Company (Shanghai, China). These plasmids were transfected into SV40 MES13 cells under HG condition as Sh-NEAT1 and Sh-NC groups.

Measurement of cell proliferation

Cell proliferation ability was measured using Counting Kit-8 (CCK-8) (Sangon, China) at 0 h, 24 h, 48 h and 72 h after transfection according to the instructions of manufacturer. In addition, cell proliferative markers (Cyclin D1 and proliferating cell nuclear antigen (PCNA)) were also measured using qPCR and western blot at 48 h after transfection.

Measurement of apoptosis

Cell apoptosis rate was measured using Annexin V (AV) apoptosis detection kit with propidium iodide (PI) (BD, USA) at 24 h after transfection according to the instructions of manufacturer. In addition, apoptotic markers (Cleaved Caspase3 (C-Caspase3) and Bcl-2) were also measured using western blot at 24 h after transfection.

Measurement of cell fibrosis

To determine the effect of Inc-NEAT1 inhibition on MMCs, cell fibrosis markers (Fibronectin (FN) and collagen I) were measured using qPCR and western blot at 24 h after transfection.
Measurement of inflammatory cytokines

Inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-6 were measured using qPCR and western blot at 24 h after transfection.

Western blot

Total proteins were extracted from each group of cells with 1 mL RIPA (Sigma, USA) on ice and shocked every 5 mins during 30 mins for complete pyrolysis. Then, protein concentration was determined by Bicinchoninic Acid Kit (Sigma, USA), followed by thermal denaturation that performed at 98°C for 5 mins. Subsequently, 20 μg proteins were added to NuPAGE Bis-Tris protein gel (Invitrogen, USA), and after electrophoresis was finished, proteins were transferred to polyvinylidene fluoride membranes (Millipore, USA). 5% skim milk was used to block the membranes at 37°C for 1 h, and membranes were incubated with the corresponding primary antibodies overnight at 4°C. Then, membranes were further incubated with secondary antibodies at room temperature for 2 h. Lastly, the bands were visualized using EasyBlot ECL kit (Sangon, China). The antibodies applied in western blot assay are listed in Table 1.

qPCR

The qPCR process was as follows: (1) total RNA was extracted by PureZOL RNA isolation reagent (Bio-Rad, USA); (2) reverse transcription to cDNA was performed with 1 μg total RNA using iScript™ Reverse Transcription Supermix (Bio-Rad, USA); (3) TB Green™ Fast qPCR Mix (Takara, Japan) was applied to perform the qPCR, and qPCR amplification was conducted at 95°C for 3 mins, followed by 40 cycles of 95°C for 5 s, 61°C for 10 s, and then 72°C for 30 s. Finally, the qPCR results were calculated by 2-ΔΔCt formula, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal references. The primers used in qPCR assay were listed in Table 2.

Statistics

Statistics was conducted using SPSS 21.0 Software (IBM, USA) and graphs were drawn using GraphPad 6.01 Software (GraphPad Software, USA). Data were presented as mean ± standard deviation. Comparison among groups was detected using One-way ANOVA followed by Turkey’s multiple comparison test, and comparison between two groups was detected using t test. P value < 0.05 was considered as significant in this study.

Table 1. Antibodies applied in western blot

<table>
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<th>Antibody</th>
<th>Company</th>
<th>Dilution</th>
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</thead>
<tbody>
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</tr>
<tr>
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<td>GAPDH Rabbit mAb</td>
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<tr>
<td>Secondary Antibody</td>
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<td></td>
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<tr>
<td>Goat Anti-Rabbit IgG H&amp;L (HRP)</td>
<td>Cell Signaling Technology (USA)</td>
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Table 2. Primers applied in qPCR

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<th>Reverse Primer (5'-3')</th>
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<td>GGAGGCAAACAGGAGACAGA</td>
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<td>Cyclin D1</td>
<td>CCAAGCCCTGAGAGAACAGG</td>
<td>GCGCATCAGGAGAGGAAGG</td>
</tr>
<tr>
<td>PCNA</td>
<td>GCGGACCCCTACCCACATTG</td>
<td>ATGGTACGCTCCTTCTTTT</td>
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<td>FN</td>
<td>CAGTGACCCGAGTGCACAGG</td>
<td>CAGACACACACTACAGGAG</td>
</tr>
<tr>
<td>Collagen I</td>
<td>CAGGAGCTGGTCCGAGCA</td>
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<td>TNF-α</td>
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<td>TCGTACGAGAGGAGGAGGG</td>
</tr>
<tr>
<td>IL-1β</td>
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<td>TGTTACGCGAGCTTGATG</td>
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<td>IL-6</td>
<td>GTGTCGCCCTCGGGTCT</td>
<td>GTAATTGCTGCGACCTTGATG</td>
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<tr>
<td>GAPDH</td>
<td>GAGTCCACTGGCGCTTCCAC</td>
<td>ATCTTGAGGTTGTCATAACTC</td>
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lncRNA NEAT1 in diabetic nephropathy

Results

Comparison of Inc-NEAT1 expression among HG group, OC group and NG group

Inc-NEAT1 expression was remarkably elevated in HG group compared to NG group (P < 0.001), and it was also greatly higher in HG group than that in OC group (P < 0.001), while no difference of Inc-NEAT1 expression was found between OC group and NG group. These results indicated that Inc-NEAT1 was overexpressed in DN cellular model (Figure 1).

Comparison of Inc-NEAT1 expression after plasmids transfection in DN cellular model

In order to determine the effect of Inc-NEAT1 in DN, we transfected Inc-NEAT1 interference plasmids and control plasmids into DN cellular model. Inc-NEAT1 expression in Sh-NEAT1 group was dramatically reduced in Sh-NEAT1 group compared to Sh-NC group (P < 0.001) (Figure 2), suggesting the successful transfection of Inc-NEAT1 interference plasmids into DN cellular model.

Comparison of cell proliferation between Sh-NEAT1 group and Sh-NC group

In order to investigate the function of Inc-NEAT1 downregulation on cell proliferation in DN, we performed CCK-8 assay and found that cell proliferation was decreased in Sh-NEAT1 group at 48 h (P < 0.05) and 72 h (P < 0.05) compared to Sh-NC group (Figure 3A). Besides, western blot disclosed that expressions of both Cyclin D1 protein and PCNA protein were reduced in Sh-NEAT1 group compared to Sh-NC group (Figure 3B), and qPCR assay displayed that expressions of Cyclin D1 mRNA and PCNA mRNA were lower in Sh-NEAT1 group compared to Sh-NC group (P < 0.01) (Figure 3C, 3D). These results suggested that Inc-NEAT1 downregulation repressed cell proliferation in the DN cellular model.

Comparison of cell apoptosis between Sh-NEAT1 group and Sh-NC group

Cell apoptosis rate was increased in Sh-NEAT1 group than that in Sh-NC group (P < 0.001) (Figure 4A, 4B). Western blot revealed that expression of apoptotic protein C-Caspase3 was enhanced in Sh-NEAT1 group compared to Sh-NC group, while expression of anti-apoptosis protein Bcl 2 was decreased in Sh-NEAT1 group compared to Sh-NC group (Figure 4C). These results indicated that Inc-NEAT1 downregulation inhibited cell proliferation in a DN cellular model.

Comparison of cell fibrosis between Sh-NEAT1 group and Sh-NC group

Expression of cell fibrosis marker mRNAs were measured by qPCR assay, which displayed that
In this study, we found that: (1) lnc-NEAT1 was overexpressed and its downregulation repressed cell proliferation but enhanced apoptosis in a DN cellular model; (2) lnc-NEAT1 downregulation decreased fibrotic marker expression in a DN cellular model; (3) lnc-NEAT1 downregulation reduced inflammatory cytokine expressions in a DN cellular model.

LncRNA, which has been identified to have a vital role in a wide spectrum of diseases, typically acts as scaffolds for multiple proteins and forms complexes to regulate gene or protein expression [25-30]. Emerging studies have disclosed that the IncRNA dysregulation which responds to hyperglycemia may enhance the metabolic impairment and renal lesions in DN [9, 25, 31-33]. For instance, lnc-ASncmtRNA-2 is upregulated in DN mice and HG-treated MMCs, and it promotes glomerular fibrosis through upregulating expression of pro-fibrotic factor transforming growth factor β1 (TGFβ1) [25]. Besides, lnc-LINC00968 represses p21 in DN cellular model (MMCs under HG condition) via binding with zeste homolog 2 (EZH2), thereby facilitates cell proliferation and fibrosis [32]. Furthermore, lnc-Gm4419 knockdown inhibits cell proliferation, represses expressions of pro-inflammatory cytokines (including monocyte chemoattractant protein-1 (mcp-1), IL-1β and TNF-α), and decreases expressions of fibrosis biomarkers (including FN and collagen IV) through reducing activation of nuclear factor kappa light-chain enhancer of activated B cells (NF-κB)/NACHT, leucine-rich repeats (LRR) and (pyrin domain) PYD domain-containing protein 3 (NLRP3) inflammasome signaling pathway in DN cellular model (MMCs under HG condition) [33]. These previous studies reveal that IncRNA may be involved in the pathology of DN and they present potentials to serve as treatment targets.

**Discussion**

In this study, we found that: (1) Inc-NEAT1 was overexpressed and its downregulation repressed cell proliferation but enhanced apoptosis in a DN cellular model; (2) Inc-NEAT1 downregulation decreased fibrotic marker expression in a DN cellular model; (3) Inc-NEAT1 downregulation reduced inflammatory cytokine expressions in a DN cellular model.

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logic processes by affecting cell proliferation or apoptosis in several diseases such as diabetes, traumatic brain injury, as well as multiple cancers [23, 37-40]. Whereas, limited information of lnc-NEAT1 in diabetes was found. One previous study conducted microarray analyses on diabetic Akita mice heart and displayed that lnc-NEAT1 expression is downregulated [23]. However, another study disclosed that lnc-NEAT1 is overexpressed and it promotes cell apoptosis as well as autophagy in diabetic rats with myocardial ischemia reperfusion injury [41]. Hence, these previous data reveal that the influence of lnc-NEAT1 in diabetes or diabetic complications is still controversial, and the underlying mechanism of lnc-NEAT1 in DN is rarely disclosed. Since inflammation is the important pathogenetic mechanism in DN and lnc-NEAT1 has potential to serve as a pro-inflammatory factor; and lnc-NEAT1 expression

![Figure 4. Cell apoptosis in Sh-NEAT1 group and Sh-NC group. As to cell apoptosis, AV/PI assay revealed that cell apoptosis rate was elevated in Sh-NEAT1 group compared to Sh-NC group (A, B). Moreover, western blot displayed that apoptotic protein C-Caspase3 expression was increased but anti-apoptosis protein Bcl 2 expression was decreased in Sh-NEAT1 group compared to Sh-NC group (C). NEAT1, nuclear enriched abundant transcript 1; AV/PI, Annexin V propidium iodide. Comparison between two groups was determined by t test. P value < 0.05 was considered significant. ***P < 0.001.](image)

![Figure 5. Expression of cell fibrosis markers in Sh-NEAT1 group and Sh-NC group. The qPCR assay disclosed that miRNA expressions of cell fibrosis markers including FN and Collagen I were reduced in the Sh-NEAT1 group compared to the Sh-NC group (A, B), and western blot revealed that expressions of FN and Collagen I proteins were lower in the Sh-NEAT1 group compared to Sh-NC group (C). NEAT1, nuclear enriched abundant transcript 1; qPCR, quantitative polymerase chain reaction (qPCR); FN, fibronectin. Comparison between two groups was determined by t test. P value < 0.05 was considered significant. *P < 0.05; **P < 0.01.](image)
is dysregulated in diabetic rats in the aforementioned previous studies, we suspected that lnc-NEAT1 might act as a key component in the pathology of DN. In the present study, we measured lnc-NEAT1 expression in MMCs (SV40 MES13 cells) with different glucose conditions, and we found that lnc-NEAT1 expression was increased in the HG group compared to the OC group and NG group, indicating that lnc-NEAT1 was overexpressed in the DN cellular model. Moreover, we conducted CCK-8 assay, AV/PI assay and western blot (detection of apoptotic markers) to investigate the influence of lnc-NEAT1 on cell proliferation and apoptosis in DN, and we observed that lnc-NEAT1 downregulation inhibited cell proliferation but enhanced cell apoptosis in DN cellular model. Our data suggested that lnc-NEAT1 might serve as a potential target for preventing or treating the abnormally increase of MMCs in DN.

Some previous studies have proposed that inhibition of renal fibrosis is important to alleviate the functional deterioration in DN, while the effect of lnc-NEAT1 on fibrosis is seldom reported. One related investigation in liver fibrosis mice displays that silencing of lnc-NEAT1 reduces collagen I expression by targeting miR-122 and Kruppel-like factor 6 in liver fibrosis mice, suggesting that lnc-NEAT1 may play a pro-fibrogenic role in liver fibrosis [9, 42-44]. As to the influence of lnc-NEAT1 on fibrosis in DN, no previous studies have been found. In our present study, we found that lnc-NEAT1 downregulation lowered the fibrotic markers’ expressions including FN and collagen I in DN cellular model, which might provide evidence to explore novel preventative or therapeutic measures in inhibiting fibrosis of DN.

Inflammation is involved in the pathogenesis of diabetes, and elevated inflammatory cytokines are revealed to be associated with the development of microvascular diabetic complications, including DN [45, 46]. In previous studies, one study discloses that lnc-NEAT1 knockdown reduces several inflammatory cytokines’ expressions including IL-6 and IL-8 in osteoarthritis synoviocytes [47]. Also, a study observes that lnc-NEAT1 downregulation suppresses the inflammatory response through modulating the intestinal epithelial barrier and regulating exosome-mediated polarization of macrophages in inflammatory cell line models [48]. Another study reveals that silencing of lnc-NEAT1 reduces the expressions of inflammatory cytokines including IL-6 and C-X-C motif chemokine ligand 10 (CXCL10) in monocytes from patients with systemic lupus erythematosus [21]. Furthermore, an interesting study has identified the proinflammatory role of lnc-NEAT1 as a factor of the innate immune response in normal cells through activating the genes transcription by sequestration of transcriptional factor splicing factor proline/glutamine-rich (SFPQ) [49]. These previous data reveal the pro-inflammatory influence of lnc-NEAT1 in some inflammatory diseases, while evidence of lnc-NEAT1 affecting inflammatory
response in DN is limited. In this present study, we used qPCR and western blot to explore the influence of Inc-NEAT1 downregulation on inflammatory cytokines including TNF-α, IL-1β and IL-6. We found that Inc-NEAT1 downregulation reduced the expressions of TNF-α, IL-1β and IL-6 in a DN cellular model, indicating that Inc-NEAT1 aggravated the inflammatory response. This might shed light on the application of Inc-NEAT1 downregulation in prevention or treatment of DN.

In conclusion, Inc-NEAT1 is overexpressed, and its downregulation inhibits cell proliferation, fibrosis, and inflammation but promotes cell apoptosis in a HG-induced MMCs DN cellular model.

Acknowledgements

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Disclosure of conflict of interest

None.

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References

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