Long non-coding RNA ASncmtRNA-2 is upregulated in diabetic kidneys and high glucose-treated mesangial cells

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Abstract. Diabetic nephropathy (DN) is one of the most frequent complications associated with type I and II diabetes mellitus. Kidneys from patients with DN are characterized by mesangial matrix expansion and increased thickness of the glomerular basement membrane, which are induced by reactive oxygen species (ROS) production. Previous studies have been conducted to investigate this; however, the detailed mechanism of DN progression remains to be elucidated. The present study evaluated the expression of antisense mitochondrial non-coding RNA-2 (ASncmtRNA-2) in an experimental DN model and cultured human mesangial cells. When mice that exhibited genetic type II diabetes developed DN, ASncmtRNA-2 expression was significantly increased (P=0.017) and was positively correlated with pro-fibrotic factor transforming growth factor β1 (TGF-β1) expression and its downstream gene, fibronectin. Inhibition of ROS through administration of the nitric oxide synthase inhibitor, NG-nitro-L-Arginine methylester (L-NAME), significantly reduced (P=0.022) the upregulation of ASncmtRNA-2 in DN. In cultured human renal mesangial cells (HRMCs), ASncmtRNA-2 was upregulated by high glucose stimuli in a time-dependent manner. Glucose-induced upregulation of ASncmtRNA-2 was also reduced by co-incubation of HRMCs with L-NAME. Notably, specific short hairpin RNA against ASncmtRNA-2 significantly downregulated the expression of HRMCs with L-NAME. The present study suggests that ASncmtRNA-2 is upregulated by ROS and may promote glomerular fibrosis in DN via positively regulating the expression of pro-fibrotic factors. These findings may provide novel potential therapeutic and preventative treatments for DN.

Introduction

Diabetes mellitus (DM) is one of the most prevalent metabolic disorders worldwide, particularly in western countries. Over the past few decades, incidence rates of DM have markedly increased due to unhealthy diet, lack of exercise and a deteriorating environment (1,2). DM may lead to complications such as diabetic nephropathy (DN), which is the leading cause of end stage renal disease in the USA (3). Diabetic kidneys are characterized by the development of albuminuria, enhanced glomerular filtration and pressure inside the glomerulus (4,5). Of all patients with type I and II DM, ~1/3 are affected by DN in their daily life (4). As DN progresses, excessive reactive oxygen species (ROS) are generated, inducing irreversible renal damage due to continuous hyperglycemic conditions (6,7). Various enzymatic signaling pathways that generate ROS, such as nitric oxide synthase (NOS) and xanthine oxidase ( XO) are stimulated in DN (8). However, the specific mechanism by which ROS regulates DN remains unclear.

A large proportion of regulatory RNAs are long non-coding RNAs (lncRNAs), which typically contain >200 nucleotides and serve important roles in the cytoplasm and nucleus (9). LncRNAs have been studied extensively in recent years, leading to the discovery of their important roles in tumorigenesis (9,10), RNA transcription (11) and mRNA translation (12,13). Furthermore, it has been demonstrated that IncRNAs are associated with DM progression (14). For example, IncRNA H19 is reportedly associated with the gestational DM-induced transformation of islet structure and function (14) and expression of the colorectal neoplasia differentially expressed IncRNA is affected by insulin and insulin-like growth factor signaling pathways in DM (15,16).

Although it has recently been revealed that IncRNAs are associated with the progression of DM (17), no IncRNA of mitochondrial origin has been identified that is associated with DM or its complications. It is well-documented that mitochondria maintain ROS levels; high levels of ROS induce dysfunction of mitochondria, which in turn produce ROS at a higher rate (18). Due to the central roles of mitochondria in ROS production and ROS-mediated oxidative stress, it was hypothesized that lncRNAs transcribed from the mitochondrial genome may serve important roles in oxidative stress-mediated disease progression, such as DN.

Keywords: antisense mitochondrial noncoding RNA2, diabetic nephropathy, oxidative stress, NL-nitro-L-arginine methyl ester, high glucose

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Antisense mitochondrial non-coding RNA-2 (ASncmtRNA-2; 2,104 nt) is a mitochondrial lncRNA that is expressed in the mitochondria and exported to the nucleus (19-21). Previous studies have revealed that it is involved in the tumorigenesis and mitochondrial retrograde signaling pathways (19-21). Notably, it has recently been demonstrated that ASncmtRNA-2 is overexpressed during aging and replicative senescence in human endothelial cells (18). This novel study indicated that ASncmtRNA-2 potentially serves a role in physiological oxidative stress. However, it remains unclear whether ASncmtRNA-2 is associated with pathological oxidative stress, such as the conditions observed in DN.

The present study aimed to investigate the role of ASncmtRNA-2 in oxidative stress-mediated DN fibrosis. Expression of ASncmtRNA-2 under ROS stimuli was assessed in an experimental model of DN and in cultured mesangial cells, and the possible mechanism through which ASncmtRNA-2 functions in DN was investigated.

Materials and methods

Experimental DN mouse model. A total of 16 four-week-old male C57BLKS/J mice weighing 15-18 g were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China) and used as a control group. A total of 32 four-week-old male C57BLKS/J-Lepr+/− (db/db) mice (weights, 30-32 g, Model Animal Research Center of Nanjing University), which genetically develop DM shortly following birth, were randomly divided into two groups: A DN+NG-nitro-L-Arginine methyl ester (L-NAME) group (n=16 each). L-NAME, which is an NOS inhibitor that also inhibits ROS, was purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). All mice were housed at 22˚C, 45% humidity and a 12-h light/dark cycle. Mice had ad libitum access to water and food. The drinking water supply for the mice from the DN+L-NAME group contained L-NAME (90 mg/kg) throughout the experiment (22). In each group, RNA from the kidneys was harvested at weeks eight and 16 from three randomly selected mice. Remaining mice were sacrificed with 1.5% Pelltobarbitalum Natricum (0.1 ml/20 g; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at 37˚C with DNase I (Invitrogen; Thermo Fisher Scientific, Inc.) and harvested at 57˚C for 24 h and for subsequent analysis. Due to the lack of commercial primary antibody against ASncmtRNA-2, only mRNA levels were detected throughout the study.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA from mouse tissues and cultured cells were isolated using TRIzol reagent (Takara Biotechnology Co., Ltd., Dalian, China) and incubated at 37˚C with DNase I (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturers' protocols. A total of 1 µg RNA was reverse transcribed into cDNA with a volume of 20 µl (Takara Biotechnology Co., Ltd.) and qPCR was subsequently performed using an ABI PRISM 7900 system with a SYBR Premix Ex Taq Perfect Real-Time kit (Takara Biotechnology Co., Ltd.). Briefly, PCR was performed as follows: Initial denaturation at 95˚C for 2 min, 35 cycles at 95˚C for 30 sec and at 55˚C for 60 sec. Fluorescence was recorded after each cycle using the ABI7900 (Applied Biosystems; Thermo Fisher Scientific, Inc.). GAPDH was included as an internal reference gene. Primers for corresponding genes are listed in Table I. Each assay was performed in triplicate unless otherwise stated. The gene expression was normalized to GAPDH with the delta delta Ct method as explained in details in previous article (24).

Measurement of intracellular ROS. To detect the effects of L-NAME and high glucose on ROS production, the intracellular ROS level was evaluated using an non-fluorescent probe, 2',7'-dichlorofluorescein diacetate (DCFH-DH: Beyotime Institute of Biotechnology, Nanjing, China). Briefly, HRMCs were seeded in a 96-well plate (1x10^5 cells/well) and cultured with low (LG) or high glucose (HG), with each medium supplemented with or without L-NAME. Culture medium was removed and plates were washed with PBS three times at 24 h following culture. DCFH-DH was diluted with DCFH/F12 to a final concentration of 10 µM and co-incubated with HRMCs at 37˚C for 20 min. Fluorescence was measured using a Tecan fluorescence plate reader (Tecan Group, Ltd., Mannedorf, Switzerland) at 485 nm for excitation and 530 nm for emission. Each measurement was performed in triplicate and repeated at least three times.

Statistical analysis. Data were presented as the mean ± standard deviation and analyzed using a Student’s t-test. Statistical analyses were performed using Excel software (Microsoft, Inc., Richmond, WA, USA). Results were obtained from three independent experiments and P<0.05 was considered to indicate a statistically significant difference.

Results

Establishment of experimental DN model. To assess the expression of ASncmtRNA-2 in DN samples and ROS-reduced diabetic kidney samples, a mouse model of DN was established. L-NAME is an NOS inhibitor that is able to reduce the production of ROS in diabetic kidneys (25), hence administering...
diabetic mice with L-NAME is an effective method to evaluate the effects of reduced ROS on the expression of ASncmtRNA-2 in diabetic kidneys. At week 24, blood samples were harvested from each group to analyze physical and biochemical parameters (Table II), including glucose, glycated hemoglobin, total cholesterol, triglycerides, high-density lipoproteins and low-density lipoproteins. The differences observed in these parameters between groups demonstrated that mice from the DN group were suffering from a metabolic disorder whereas those in the control group were not and that administration of L-NAME was able to relieve these disorders. These findings suggest that mice with genetically developed DM developed DN at week 24, and L-NAME effectively ameliorated ROS-mediated renal damage in the experimental DN model. Mice were sacrificed by the end of week 24 and their kidneys were harvested. Half of each kidney was formalin-fixed and paraffin-embedded for histological analysis whereas the other half was frozen to allow for isolation of tissue RNA.

Urine samples were collected at weeks 8, 16 and 24 to assess renal function. As illustrated in Fig. 1A, there was no significant difference in UACR over time for control mice. A significant progressive increase in UACR was observed in DN mice over time compared with the control group (P<0.05). In the DN+L-NAME group, UACR was also significantly increased compared with the control group (P<0.05); however, the UCAR levels in this group were markedly lower than

Table I. Primers used in quantitative polymerase chain reaction.

| Genes    | Primer sequence                                      |
|----------|------------------------------------------------------|
| hASncmtRNA-2 | Forward: 5'-ACCGTGCAAGGTCATCAATACTCAG-3'           |
|          | Reverse: 5'-CCGTAATTCATCTACATCTCAACT-3'            |
| hTGFβ1   | Forward: 5'-TGACATTTGCTTGTCGTTGCT-3'              |
|          | Reverse: 5'-TGACATTTGCTTGTCGTTGCT-3'              |
| hFN      | Forward: 5'-CTGACCTACAGCCGACACC-3'                |
|          | Reverse: 5'-TGCTGACATTTGCTTGTCGTTGCT-3'          |
| hGAPDH   | Forward: 5'-GCTAAACGAGGTCACACTG-3'                |
|          | Reverse: 5'-CTAAACGAGGTCACACTG-3'                |
| mASncmtRNA-2 | Forward: 5'-ACGTTGTCACCTGTCGTTGCT-3'          |
|          | Reverse: 5'-ACTGCTCTACCTGTCGTTGCT-3'            |
| mTGFβ1   | Forward: 5'-ACGGTTCCATACCCGGCCAT-3'               |
|          | Reverse: 5'-TCATCCGCTGGCCATTTCCT-3'              |
| mFN      | Forward: 5'-AGGTCGTTGTAACCGATTTG-3'              |
|          | Reverse: 5'-TGTTGACATTTGTAACCGATTTG-3'           |
| h, human; m, mouse; ASncmtRNA-2, antisense mitochondrial non-coding RNA-2; TGFβ1, transforming growth factor β1; FN, fibronectin.

Table II. Physical and biochemical parameters after 24 weeks of study in control, DN and DN+L-NAME group mice.

| Parameters                          | Control     | DN         | DN + L-NAME |
|-------------------------------------|-------------|------------|-------------|
| Number                              | 10          | 9          | 10          |
| Body weight (g)                     | 22.09±2.05  | 41.93±1.32 | 47.97±0.87  |
| Kidney/body weight ratio (%)        | 0.45±0.12   | 0.39±0.28  | 0.31±0.31   |
| Glucose (mmol/l)                    | 6.72±0.01   | 23.80±0.01 | 14.41±0.009 |
| HbA1c (%)                           | 5.3±0.09    | 9.6±0.34   | 7.6±0.12    |
| HbA1c (mmol/mol)                    | 34.426±0.98 | 83.607±3.76| 59.563±1.31 |
| Total cholesterol (mmol/l)          | 4.9±0.17    | 11.8±1.01  | 9.4±0.97    |
| Triglycerides (mmol/l)              | 1.5±0.24    | 3.7±0.33   | 2.6±0.42    |
| HDL (mmol/l)                        | 1.3±0.32    | 2.7±0.11   | 1.7±0.21    |
| LDL (mmol/l)                        | 4.5±0.56    | 10.3±0.31  | 7.4±0.11    |

Data are presented as the mean ± standard deviation. *One mouse from this group succumbed at the 22nd week. DN, diabetic nephropathy; L-NAME, NG-nitro-L-Arginine methylester; HbA1c, glycated hemoglobin; HDL, high density lipoprotein; LDL, low density lipoprotein.
Expression of ASncmRNA-2 is upregulated in mice with DN and decreased by L-NAME. Three mice from each group were randomly chosen and sacrificed at weeks 8, 16 and 24 to assess the expression levels of ASncmRNA-2. Data from RT-qPCR revealed that the mRNA expression level of ASncmRNA-2 in the DN group was significantly increased (2.6-fold) compared with control mice at week 16, and 3.8-fold at week 24 (both P<0.05). Furthermore, the mRNA expression levels of ASncmRNA-2 in the DN+L-NAME group were significantly higher (P<0.05) than the control group and markedly lower than the DN group at weeks 16 and 24 (Fig. 2A). These results indicate that ASncmRNA-2 increased throughout the development of DN and that inhibition of ROS by L-NAME may reduce the upregulation of ASncmRNA-2 in DN. Transforming growth factor β1 (TGFβ1) and its downstream gene, fibronectin (FN), are well-known pro-fibrotic factors in DN (26). To investigate
whether the expression of ASncmtRNA-2 is associated with these pro-fibrotic factors, mRNA levels of TGFβ1 and FN were detected in mouse kidneys using RT-qPCR analysis. As demonstrated in Fig. 2B, the mRNA levels of TGFβ1 and FN were significantly upregulated in the DN group compared with the control group, (P<0.05) which were markedly decreased following treatment with L-NAME (DN vs. DN+L-NAME). Compared with the control group, mRNA levels of TGFβ1 and FN were significantly upregulated in the DN+L-NAME group (P<0.05). These findings suggest that there may be an association between TGFβ1 and ASncmtRNA-2 levels. The expression of ASncmtRNA-2 was compared with that of TGFβ1 in mice with DN sacrificed at week 24 (n=9), which indicated that they were positively correlated, with a coefficient of determination (r²) as 0.8716 (Fig. 2C). These results suggest that ASncmtRNA-2 is significantly overexpressed in DN and positively correlated with expression of pro-fibrotic factor TGFβ1.

Figure 3. ASncmtRNA-2 is upregulated in HG-treated HRMCs. (A) HRMCs with the indicated treatments were harvested at 0, 8, 16 or 24 h. mRNA levels of ASncmtRNA-2 at each time point were recorded. (B) Relative ROS fluorescence levels were detected in HG and HRMCs with or without L-NAME treatment using dichloro-dihydro-fluorescein diacetate probes. (C) mRNA levels of TGFβ1 and FN were analyzed via RT-qPCR assay at 24 h post-treatment. *P<0.05 vs. LG; #P<0.05 vs. LG. ASncmtRNA-2, antisense mitochondrial non-coding RNA-2; TGFβ1, transforming growth factor β1; HRMC, human renal mesangial cell; LG, low glucose; HG, high glucose; L-NAME, NG-nitro-L-Arginine methyl ester; RT-PCR, revere transcription polymerase chain reaction.

ASncmtRNA-2 is upregulated in cultured HRMCs treated with HG. Based on the experimental observations thus far, the expression levels of ASncmtRNA-2 in cultured HRMCs were studied. Cells were seeded into six-well plates and cultured with LG or HG medium, with or without additional L-NAME treatment. mRNA levels of ASncmtRNA-2 were measured at different time intervals. At 8 h post-treatment, expression of ASncmtRNA-2 remained unchanged in all groups, as compared with 0 h. However, the level of ASncmtRNA-2 mRNA increased significantly in HG and HG+L-NAME-treated cells compared with LG-treated cells from 16 h onwards (P<0.05). Although a significant increase was also observed in the ASncmtRNA-2 mRNA level in the HG+L-NAME group vs. the control group, this was markedly reduced compared with the HG group (Fig. 3A). Relative ROS levels in HRMCs were detected with the non-fluorescent probe DCFH-DA. DCFH reacts with cellular ROS to generate the fluorescent product DCF, which can be easily traced within cells. Therefore, intracellular ROS levels were determined using a DCFH-DC probe as described previously (27). As shown in Fig. 3B, ROS fluorescence was significantly upregulated in HG-treated cells (P<0.05), but was suppressed by 37.5% when HG cells were co-incubated with L-NAME, suggesting that L-NAME effectively inhibits ROS production. Levels of ASncmtRNA-2 mRNA in HG and HG+L-NAME-treated cells were studied. Cells were cultured in LG or HG medium, with or without specific ASncmtRNA-2-silencing shRNA transfected into HRMCs in vitro and a scramble shRNA was transfected for control. Cells were cultured in LG or HG conditions with or without the specific shRNA. mRNA levels of ASncmtRNA-2, TGFβ1 and FN were determined via reverse transcription-quantitative polymerase chain reaction. *P<0.05, HG vs. LG; #P<0.05, HG+shASncmtRNA-2 vs. HG. ASncmtRNA-2, antisense mitochondrial non-coding RNA-2; TGFβ1, transforming growth factor β1; HRMC, human renal mesangial cell; LG, low glucose; HG, high glucose; shRNA, short hairpin RNA; FN, fibronectin.

Figure 4. Knockdown of ASncmtRNA-2 inhibits the expression of TGFβ1 in HG-treated HRMCs. Specific ASncmtRNA-2-silencing shRNA were transfected into HRMCs in vitro and a scramble shRNA was transfected for control. Cells were cultured in LG or HG conditions with or without the specific shRNA. mRNA levels of ASncmtRNA-2, TGFβ1 and FN were determined via reverse transcription-quantitative polymerase chain reaction. *P<0.05, LG vs. HG; #P<0.05, HG+shASncmtRNA-2 vs. HG. ASncmtRNA-2, antisense mitochondrial non-coding RNA-2; TGFβ1, transforming growth factor β1; HRMC, human renal mesangial cell; LG, low glucose; HG, high glucose; shRNA, short hairpin RNA; FN, fibronectin.

Knockdown of ASncmtRNA-2 inhibits the expression of TGFβ1 in HG-treated HRMCs. The role of ASncmtRNA-2 in regulating the expression of TGFβ1 was evaluated using a specific ASncmtRNA-2-silencing shRNA to knockdown expression of ASncmtRNA-2. As indicated in Fig. 4, the levels of ASncmtRNA-2 mRNA decreased to 60-70% by specific silencing shRNA in LG- and HG-treated cells. Furthermore, the expression of ASncmtRNA-2, TGFβ1 and FN were...
significantly increased in HG conditions as compared with LG (P<0.05). ASncmtRNA-2 was significantly depleted by shASncmtRNA-2 in the cells and mRNA levels of TGFβ1 and FN were significantly decreased by 34.8 and 44.4% (P<0.05), respectively. These findings suggest that knockdown of ASncmtRNA-2 is able to decrease the expression of TGFβ1, which in turn indicates that ASncmtRNA-2 positively regulates pro-fibrotic factors during DN progression.

Discussion

There are many complications associated with DM, including DN, which may lead to renal failure and eventual death (28). Overproduction of oxidative products, such as ROS, induces oxidative damage to the cellular and extracellular structures of the kidneys, particularly in HRMCs (29). Therefore, the present study used HRMCs to evaluate the expression of ASncmtRNA-2 upon HG and L-NAMe stimulation. ROS are able to induce injury to human kidneys through the following mechanisms: i) Inducing lipid peroxidation, protein cross-linking and the formation of DNA adducts, leading to tissue damage; ii) inducing direct damage to cellular DNA; and iii) activating multiple cellular signaling pathways, including the nuclear factor-xB and TGFβ1 signaling pathways. These mechanisms induce further generation of ROS, secretion of cytokines, synthesis and deposition of extracellular matrix components, which induce more severe damage to kidneys (30,31). Therefore, it is necessary to identify a novel method to regulate ROS generation to reduce renal damage in DN in a clinical setting.

LncRNAs are typically defined as non-coding RNA >200 nt in length that have been revealed to have roles in various diseases, typically acting as scaffolds for multiple proteins and forming complexes to regulate protein expression (12,13). LncRNA UCA1 has previously been shown to contribute to cardiomyocyte apoptosis by suppressing the expression of P27, whose overexpression in heart injury is associated with enhanced ROS production and increased cell apoptosis (31). However, it remains to be elucidated whether other IncRNAs serve a role in mechanisms of oxidative stress.

Recently, studies of mitochondrial IncRNAs and their role in oxidative stress-induced damages have been conducted (9,18). It has been demonstrated that mitochondrial dysfunction is associated with high ROS levels and vice versa (18); therefore, mitochondrial IncRNAs are considered to be a viable target for the regulation of ROS production. A recent pilot study demonstrated that mitochondrial IncRNA ASncmtRNA-2 was increased in aging and replicative senescence in human endothelial cells (18), suggesting that mitochondrial ASncmtRNA-2 may be associated with ROS generation. The present study further demonstrates that ASncmtRNA-2 is overexpressed in DN in vivo. Inhibition of ROS production through administering L-NAMe significantly ameliorated renal damage in mice and decreased the expression of ASncmtRNA-2, suggesting that ASncmtRNA-2 may be a substrate of ROS and may execute the ROS-mediated effects in DN. In the HG-cultured HRMCs, ROS was increased and the expression of ASncmtRNA-2 exhibited a similar pattern, further suggesting that ROS are able to induce the expression of ASncmtRNA-2. This hypothesis is further supported by the fact that inhibition of ROS via L-NAMe administration decreased ASncmtRNA-2 expression. These in vivo and in vitro data suggest that ASncmtRNA-2 is a substrate of ROS that may have critical roles in DN progression.

The present study demonstrated that increased expression of ASncmtRNA-2 may be associated with higher extracellular matrix deposition in glomeruli, suggesting that ASncmtRNA-2 may regulate the glomerular fibrosis process during DN. Therefore, a key pro-fibrotic factor, TGFβ1, and its down-stream target FN, were evaluated. It was discovered that mice with DN exhibited severe glomerular fibrosis with significant increases in TGFβ1 and FN expression levels. mRNA expression levels of ASncmtRNA-2 were positively correlated with that of TGFβ1 in the DN samples, and the in vitro data also revealed that HG induced increases in ASncmtRNA-2 and TGFβ1 levels. Knockdown of ASncmtRNA-2 by specific shRNA induced significant decreases in mRNA levels of TGFβ1 and FN. These data suggest that TGFβ1 is regulated by ASncmtRNA-2 in DN, and ROS-induced expression of ASncmtRNA-2 may contribute to DN fibrosis via regulating the pro-fibrotic factor TGFβ1. However, the detailed mechanisms of how ASncmtRNA-2 regulates TGFβ1 remain to be elucidated. Bianchessi et al. (18) recently suggested that ASncmtRNA-2 may function as a non-canonical precursor of microRNA (miRNA), has-miR-4485 and has-miR-1973, as they originate from a mitochondrial transcript. However, further research is required to elucidate the regulatory network between TGFβ1, ASncmtRNA-2 and miRNA in DN.

In conclusion, ASncmtRNA-2 expression is upregulated in DN. ROS-induced expression of ASncmtRNA-2 may contribute to DN fibrosis through regulating TGFβ1; however, the mechanisms by which ASncmtRNA-2 regulates TGFβ1 remain to be elucidated. Therapeutic agents targeting ASncmtRNA-2 may prove to be effective for the prevention and treatment of DN.

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