Downregulation of IncRNA-SRA participates in the development of cardiovascular disease in type II diabetic patients

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Abstract. Long non-coding RNA steroid receptor RNA activator (IncRNA-SRA) has been proven to regulate vascular smooth muscle cell (VSMC) proliferation, indicating its possible involvement in cardiovascular disease. Diabetes is a major cause of cardiovascular disease. The aim of the present study was to investigate the involvement of IncRNA-SRA in type II diabetic cardiovascular disease. The plasma levels of IncRNA-SRA were identified to be significantly lower in patients with type II diabetic cardiovascular disease compared with those in type II diabetic patients without any obvious complications and in healthy controls. A 5-year follow-up study revealed that low vs. high expression levels of IncRNA-SRA were associated with an increased incidence of cardiovascular disease in type II diabetic patients. High-glucose treatment did not significantly affect the expression of IncRNA-SRA in human VSMCs \textit{in vitro}. However, ectopic overexpression of IncRNA-SRA increased the viability of human VSMCs in a high-glucose environment. It was concluded that downregulation of IncRNA-SRA may participate in the development of cardiovascular disease in type II diabetic patients.

Introduction

Diabetes mellitus, as one of the most frequently diagnosed metabolic disorders, affects ~7% of the population worldwide (1). The high-glucose environment in diabetic patients affects the normal function of major organs, leading to the occurrence of a series of diabetic complications (2). Diabetes mellitus may be classified into three major types, including type I, type II and gestational diabetes. In has been reported that type II diabetic patients have a 2-6-fold increased risk of death from cardiovascular complications compared with that of healthy people (3). The prevention of cardiovascular disease in patients with type II diabetes is critical for their survival (4).

A high blood glucose concentration globally affects the expression of genes, including a large set of long non-coding RNAs (IncRNAs) (5), which are critical factors in physiological and pathological processes (6). Those IncRNAs exhibit upregulated or downregulated expression during the development of diabetes to promote or inhibit the progression of diabetes and its associated complications (7-9). The IncRNA steroid receptor RNA activator (SRA) has been proven to participate in multiple human diseases (10,11). Genetic variants of IncRNA SRA are closely correlated with the risk of breast cancer (10). Furthermore, overexpression of IncRNA SRA was indicated to promote hepatic steatosis through the inhibition of adipose triglyceride lipase (11). IncRNA-SRA was recently proved to promote the proliferation of vascular smooth muscle cells (VSMCs) (12), which have pivotal roles in the pathogenesis of cardiovascular diseases (13). The present study was performed to assess the role of IncRNA-SRA in cardiovascular disease in patients with type II diabetes mellitus and in VSMCs under high-glucose conditions \textit{in vitro}. The results may indicate that IncRNA-SRA is implicated in the pathogenesis of diabetic cardiovascular disease by regulating the viability of VSMCs.

Materials and methods

Patient groups and clinical samples. A total of 108 patients with type II diabetic cardiovascular diseases were diagnosed by laboratory tests and stress perfusion cardiovascular magnetic resonance imaging at Nanning Second People's Hospital (Nanning, China) between January 2012 and January 2013. Among those patients, 34 cases were included in the present study according to strict inclusion and exclusion criteria to serve as the diabetic cardiovascular disease group (DCD group). The inclusion criteria were as follows: i) The patients were diagnosed for the first time; ii) no treatment prior to admission; iii) informed consent. The exclusion criteria were i) other diabetic complications; ii) other severe diseases; iii) chronic diseases; iv) patient age of >70 years. At the same time, 178 type II diabetic patients without any obvious complications were included to serve as the diabetic group (D group) and 44 age- and gender-matched healthy controls were included as the control group (C group). The DCD group included 19 males and 15 females, with an age range of 29-69 years and...
a mean age of 47.2±5.1 years. The D group included 93 males and 85 females, with an age range of 25-69 years and a mean age of 46.2±6.2 years. The C group included 27 males and 17 females, with an age range of 26-68 years and a mean age of 46.7±5.5 years. Whole blood (10 ml) was extracted from each participant on the day of admission, and was used to isolate the plasma using a routine method. The plasma was stored in liquid nitrogen until analysis. The present study was approved by the Ethics Committee of Nanning Second People’s Hospital (Nanning, China), and all participants provided written informed consent.

Follow-up. The 178 type II diabetic patients without any obvious complications were followed up every 2 months for 5 years. The occurrence of cardiovascular disease was recorded. A total of 172 patients completed the follow-up procedure. A total of 6 patients died during the follow-up.

Cell culture and transfection. Human VSMCs were purchased from Lonza Group Ltd. (cat. no. CC-2583; Basel, Switzerland). VSMCs were cultured in medium 231 (cat. no. M231500; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with smooth muscle growth supplement (cat. no. S00725; Gibco; Thermo Fisher Scientific, Inc.) and maintained at 37°C in a humidified atmosphere containing 5% CO₂. Full-length lncRNA-SRA cDNA was amplified via polymerase chain reaction (PCR) using primers with a Nhe1 restriction site at the 5’end. The cDNA used in PCR reaction was synthesized using total RNA extracted from plasma samples obtained from patients, which was mentioned in next section. Full-length lncRNA-SRA cDNA was cloned into Nhe1 linearized pEGFP-C3 vector (Clontech, Palo Alto, CA, USA) to generate the lncRNA-SRA expression vector. VSMCs (5x10⁴) were transfected with 10 nM lncRNA-SRA expression vector using Lipofectamine 2000® reagent (Invitrogen; Thermo Fisher Scientific, Inc.,), according to the manufacturer's protocol. Untransfected cells were used as the control group and cells transfected with empty vector were used the negative control group. For D-glucose treatment, 0, 10, 30 or 50 mM D-glucose was added to the culture medium and VSMCs were cultured for 6, 12 or 18 h following transfection prior to any subsequent experimentation.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from VSMCs or plasma samples using TRIZol® reagent (Invitrogen; Thermo Fisher Scientific, Inc), according to the manufacturer's protocol. The RNA concentration was measured using a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). Total RNA was reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, Inc.) using the following reaction conditions: 54°C for 30 min and 75°C for 5 min. qPCR was subsequently performed using the PowerUp SYBR™ Green Master mix (cat. no. A25743; Applied Biosystems; Thermo Fisher Scientific, Inc.). The following primer pairs were used for qPCR: lncRNA-SRA forward, 5'-GCTAGGGCACATTAGTGCTGC-3' and reverse, 5'-CGCCTGACACTGCTGAGAAC-3'; β-actin forward, 5'-GACCTCTATGCGCAACACTG-3' and reverse, 5'-AGTACTTGCGCTGAGGAGA-3'. The following thermocycling conditions were used for qPCR: Initial denaturation at 95°C for 50 sec; 40 cycles of 95°C for 15 sec and 57°C for 40 sec. lncRNA-SRA was quantified using the 2⁻ΔΔCq method (14) and normalized to β-actin and 18S rRNA.

MTT assay. After transfection, the expression of lncRNA-SRA in VSMCs was detected by RT-qPCR. Subsequent experiments were performed only in case of the overexpression rate of lncRNA-SRA reaching 200%. VSMCs were collected and cell suspensions were prepared with a final density of 5x10⁴ cells per ml. A total of 0.1 ml cell suspension was added to each well of a 96-well plate, followed by the addition of D-glucose at a final concentration of 10, 30 or 50 mM. The plate was incubated at 37°C with 5% CO₂ for 6 h. Following incubation, 10 µl MTT was added to each well and cells were cultured for a further 4 h at 37°C. Following MTT incubation, DMSO (10 µl/well) was added to dissolve the purple formazan crystals. The optical density (OD) was measured at 570 nm using a microplate reader (BioTek™ 800™ TS; BioTek Instruments, Inc., Winooski, VT, USA). The OD value of control cells treated with 0 mM D-glucose was set as 100% and the viability of the cells in the treated groups was expressed as the percentage of their OD value vs. that in the control group.

Statistical analysis. GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA, USA) was used for all statistical analyses. Gene expression and cell viability data were reported as the mean ± standard deviation and compared by one-way analysis of variance followed by Fisher's least-significant difference test. The incidence of diabetic cardiovascular disease was compared using the Student’s t-test. Correlation analyses were performed by determining Pearson's correlation coefficient. Receiver operating characteristic (ROC) curves were drawn and the area under the curve (AUC) was determined to assess the ability of the lncRNA to distinguish between the different groups. P<0.05 was considered to indicate a statistically significant difference.

Results

LncRNA-SRA is significantly downregulated in patients with type II diabetic cardiovascular disease. The expression level of lncRNA-SRA was detected by RT-qPCR in the plasma of patients in the DCD, D and C groups. The clinicopathological features of these patients are summarized in Table I. As presented in Fig. 1, the plasma levels of lncRNA-SRA were significantly decreased in patients with type II diabetic cardiovascular disease compared with type II diabetic patients without any obvious complications and healthy controls (P<0.05). No significant differences in the plasma levels of lncRNA-SRA were obtained between type II diabetic patients without any obvious complications and healthy controls (P>0.05). Of note, the plasma levels of lncRNA-SRA in the DCD group were significantly and inversely correlated with the systolic blood pressure (r=-0.82, P<0.0001), the levels of low-density lipoprotein...
(r=-0.79, P<0.0001) and the levels of triglycerides (r=-0.75, P<0.0001), and were significantly and positively correlated with the levels of high-density lipoprotein (r=0.71, P<0.0001) (data not shown).

Downregulation of lncRNA-SRA distinguishes patients with type II diabetic cardiovascular disease from healthy controls and type II diabetic patients without any obvious complications. ROC curve analysis was performed to evaluate the diagnostic value of lncRNA-SRA for diabetic cardiovascular disease. With the healthy controls as a reference, the area under the curve (AUC) was 0.9041, with a standard error of 0.03625 and 95% confidence interval of 0.8830-0.9752 (Fig. 2A). With the type II diabetic patients without any obvious complications as a reference, the AUC was 0.8679, with a standard error of 0.04222 and a 95% confidence interval of 0.7851-0.9507 (Fig. 2B).

Low plasma levels of lncRNA-SRA are associated with a high incidence of cardiovascular disease in type II diabetic patients. A total of 172 type II diabetic patients without any obvious complications on the day of admission completed the 5-year follow-up. According to the median relative plasma level of lncRNA-SRA (1.44), these patients were divided into a high (n=86) and a low (n=86) expression group. During the follow-up, cardiovascular disease occurred in 41 cases, including 29 cases in the low expression group and 12 cases in the high expression group. As presented in Fig. 3, the incidence of cardiovascular disease was significantly higher in the low expression group compared with that in the high expression group (P<0.05).

A high-glucose environment has no significant effect on lncRNA-SRA expression in VSMCs. VSMCs were cultured with 0 mM (control), or with 10, 30 or 50 mM D-glucose in culture medium for 6, 12 or 18 h. Subsequently, the expression of lncRNA-SRA in the VSMCs was detected by RT-qPCR. As presented in Fig. 4, treatment with high glucose at different concentrations for different durations did not significantly affect lncRNA-SRA expression in VSMCs (P>0.05).

lncRNA-SRA overexpression improves the viability of VSMCs under high-glucose treatment. An MTT assay was performed to assess the impact of lncRNA-SRA on the viability of VSMCs under high-glucose treatment for 12 h. lncRNA-SRA overexpression was achieved by plasmid transfection (Fig. 5A). High glucose (10, 30 or 50 mM D-glucose) significantly reduced the viability of VSMCs compared with the control group (P<0.05; Fig. 5A). In addition, lncRNA-SRA overexpression significantly increased the viability of VSMCs compared with the untransfected control cells and negative control-transfected cells in the

### Table I. Clinicopathological parameters of patients within the 3 groups.

| Clinical parameter                        | Group C (n=44) | Group D (n=178) | Group DCD (n=34) |
|------------------------------------------|----------------|----------------|-----------------|
| Age (years)                              | 46.7±5.5       | 46.2±6.2       | 47.2±5.1        |
| Sex (n, %)                                |                |                |                 |
| Male                                     | 27 (61.4)      | 93 (52.2)      | 19 (55.9)       |
| Female                                   | 17 (38.6)      | 85 (47.8)      | 15 (44.1)       |
| BMI (kg/m²)                              | 21.3±1.9       | 24.4±2.6       | 24.7±2.3        |
| Systolic blood pressure (mmHg)           | 116.7±3.4      | 117.1±3.7      | 135.5±7.2       |
| Diastolic blood pressure (mmHg)          | 76.7±5.9       | 77.2±6.7       | 79.2±9.9        |
| Oral glucose tolerance test blood glucose level (mmol/l) | 6.2±1.8       | 14.4±2.7       | 14.9±2.6        |
| LDL (mg/dl)                              | 77.5±22.1      | 84.5±19.7      | 88.7±22.2       |
| HDL (mg/dl)                              | 57.4±9.2       | 49.2±8.8       | 44.5±11.8       |
| Triglycerides (mg/dl)                    | 137.8±89.2     | 150.2±78.4     | 177.5±100.8     |

Data were expressed as the mean ± standard deviation. Groups: C, healthy controls; D, patients with type II diabetes; DCD, patients with type II diabetic cardiovascular disease. LDL, low-density lipoprotein; HDL, high-density lipoprotein; BMI, body mass index.
presence of D-glucose, but not in the absence of glucose (P<0.05; Fig. 5B).

Discussion

lncRNA-SRA is involved in regulating the proliferation of VSMCs (13), indicating its potential involvement in the pathogenesis of cardiovascular diseases (15). The key result of the present study is that lncRNA-SRA is specifically down-regulated in diabetic patients with cardiovascular disease, and the downregulation of lncRNA-SRA may serve as a potential diagnostic marker for this disease.

The development of diabetes is associated with the occurrence of a series of complications (16,17). Previous studies suggested that IncRNAs are key factors in the development of diabetic complications. Sun et al (16) reported that lncRNA Erb-b2 receptor tyrosine kinase 4-intron region (Erbb4-IR) was upregulated in a mouse model of diabetic kidney injury, and that the upregulation of lncRNA Erbb4-IR is closely associated with disease progression. A study by Zhuo et al (17) revealed that lncRNA H19 is down-regulated in diabetic cardiomyopathy and overexpression of lncRNA H19 inhibits autophagy by epigenetically silencing DIRAS family GTPase 3. However, most IncRNAs involved in diabetic complications are regulated by high glucose and have no diagnostic value to distinguish patients with a specific diabetic complication from diabetic patients without this complication (16,17). lncRNA-SRA has pivotal roles in different types of human disease. Genetic variants of lincRNA SRA have been proved to be closely associated with the risk of breast cancer (10). lncRNA-SRA also inhibits the expression of adipose triglyceride lipase, thereby promoting hepatic steatosis (11). However, the involvement of lncRNA-SRA in diabetic cardiovascular disease has remained elusive. It has been reported that lncRNA-SRA promotes the proliferation of VSMCs, which have pivotal roles in the pathogenesis of cardiovascular diseases (12,13). The present study demonstrated that the plasma levels of lncRNA-SRA may serve as a potential diagnostic biomarker for type II diabetic cardiovascular disease.
disease. However, the expression of lncRNA-SRA has been reported to be affected by multiple diseases. Therefore, the diagnostic specificity should be further investigated.

A high-glucose environment affects the expression of certain lncRNAs, and altered expression of those lncRNAs participates in diabetes-associated pathological processes (18,19). Normal blood glucose levels are ~5 mM (20). In the present study, a high-glucose environment was created by the addition of D-glucose (10, 30 or 50 mM) to the cell culture medium. However, high-glucose treatment had no significant effect on lncRNA-SRA expression in VSMCs. Therefore, lncRNA-SRA may not participate in the initiation of cardiovascular disease in diabetic patients, while indirect effects cannot be excluded. lncRNA-SRA expression may be altered by the presence of cardiovascular disorders during the development of diabetes. Of note, ectopic overexpression of lncRNA-SRA increased the viability of VSMCs in a high-glucose environment. Therefore, lncRNA-SRA overexpression may serve as a potential therapeutic strategy for the treatment of diabetic cardiovascular disease.

Various factors contribute to the development of diabetic complications (21,22). The present 5-year follow-up study revealed that low plasma levels of lncRNA-SRA were associated with a significantly increased incidence of cardiovascular disease in patients with type II diabetes. Therefore, detection of plasma lncRNA-SRA may provide guidance for the prevention of diabetic cardiovascular disease. Of note, the present study has certain limitations. For instance, the molecular mechanisms of the role of lncRNA-SRA in diabetic cardiovascular disease were not elucidated. In addition, no in vivo experimental validation was performed and therefore this will be examined in future studies. Furthermore, future studies may be required to examine the expression of lncRNA-SRA in patients with cardiovascular disease without diabetes.

In conclusion, the present study suggested that down-regulation of lncRNA-SRA is involved in the pathogenesis of diabetic cardiovascular disease. Plasma lncRNA-SRA may serve as a potential diagnostic biomarker for this disease.

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Availability of data and materials
The datasets used and/or analyzed in the present study are available from the corresponding author on reasonable request.

Authors’ contributions
SR and YQ designed the experiments. SR, YZ and BL performed experiments. KB, LW, YL and YYL prepared the materials and analyzed the data. YQ interpreted the data and drafted the manuscript. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate
This study passed the review of the Ethics Committee of Nanning Second People’s Hospital (Nanning, China) and all participants provided written informed consent.

Patient consent for publication
Not applicable.
Competing interests

The authors declare that they have no competing interests.

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