Involvement of long non-coding RNA ZNF503 antisense RNA 1 in diabetic retinopathy and its possible underlying mechanism

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ABSTRACT

ZNF503 antisense RNA 1 (ZNF503-AS1) is a newly identified long non-coding RNA (lncRNA) that regulates retinal pigment epithelium differentiation. To study its role in diabetic retinopathy, we performed RT-qPCR to measure plasma ZNF503-AS1 levels of 298 diabetic patients immediately after the diagnosis, during the follow-up, and at the end of follow-up. Plasma IncRNA ZNF503-AS1 expression in 96 healthy participants was also detected by RT-qPCR. Transforming growth factor beta 1 (TGF-β1) expression after ZNF503-AS1 overexpression was detected by Western blot. Cell proliferation and apoptosis were detected by cell proliferation and apoptosis assays, respectively. We found that ZNF503-AS1 was not differentially expressed in healthy participants and diabetic patients. High plasma IncRNA ZNF503-AS1 level was correlated with a high incidence of diabetic retinopathy. Plasma IncRNA ZNF503-AS1 level was higher in patients with diabetic retinopathy than in patients with other complications (p < 0.05). ZNF503-AS1 overexpression inhibited proliferation, promoted cell apoptosis, and upregulated TGF-β1 expression (p < 0.05). We concluded that ZNF503-AS1 might participate in diabetic retinopathy by activating TGF-β signaling.

Highlights

- ZNF503-AS1 predicts diabetic retinopathy.
- ZNF503-AS1 upregulates TGF-β1.
- ZNF503-AS1 regulates cell proliferation and apoptosis.

Introduction

A high glucose environment in diabetic patients induces a series of pathological changes through oxidative stress [1]. Diabetic retinopathy, a common complication that develops in the eyes of diabetes patients, affects more than 3% of adults aged over 40 years old in the United States [2]. The incidence of retinal complications increases with a prolonged course of diabetes. It has been reported that a considerable portion of diabetic patients will experience vision-threatening retinal changes [3]. In some patients, blindness may occur, leading to a reduced quality of life [4]. At present, early diagnosis and treatment remain to be the key factor for the treatment of diabetic retinopathy.

Molecular alterations, such as altered gene expression and methylation, play critical roles in diabetic retinopathy [5]. Transforming growth factor beta (TGF-β) plays a pivotal role in mediating high glucose-induced pathological changes in diabetic patients [6]. A previous study has shown that prolonged abnormally upregulated blood glucose levels can increase TGF-β expression in ocular tissues [7]. Activated TGF-β signaling induces capillary basal lamina thickening, reflecting pathological changes...

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in preclinical diabetic retinopathy at early stages [8]. A recent study has shown that ZNF503 antisense RNA 1 (ZNF503-AS1) is a newly identified long non-coding RNA (lncRNA) that participates in the differentiation of retinal pigment epithelium [9]. Therefore, it is reasonable to hypothesize that ZNF503-AS1 may participate in diabetic retinopathy and interact with TGF-β signaling. This research explored the potential involvement of ZNF503-AS1 in diabetic retinopathy and analyzed its interaction with TGF-β signaling. We found that ZNF503-AS1 participates in diabetic retinopathy by inhibiting proliferation, promoting apoptosis of human retinal pigment epithelial cells, and activating TGF-β signaling.

Materials and methods

Patients and follow-up

A total of 298 diabetics (type 2) patients without significant complications, including 188 males and 110 females (44.3 ± 6.1 years), were enrolled from January 2010 to January 2013. Diagnosis of diabetes was performed according to the criteria established by the Chinese Medical Association in 2009 with a blood glucose level >7.0 mmol/l. All those patients were newly diagnosed cases. Patients who suffered from other severe diseases were excluded. In addition, 98 healthy individuals, including 60 males and 38 females (46.1 ± 6.6 years), were also included to serve as the controls. The diabetic patients were followed up for 6 years (ended in January 2019). Among them, 396 patients participated, but only 298 patients completed the follow-up. During the follow-up, hemoglobin A1c blood test, cholesterol and blood pressure tests, foot exams, eye exams, kidney disease tests, tooth and gum exams, diabetic ketoacidosis urine tests, and thyroid tests were routinely performed to detect potential diabetic complications. The occurrence of diabetic complications, including diabetic retinopathy, diabetic lung diseases, diabetic heart disease, and diabetic nephropathy were recorded. Diagnostic criteria for diabetic retinopathy were as follows: (1) typical microaneurysm was observed in the fundus, such as exudation, hemorrhage, macular edema, and neovascularization, (2) exclusion of retinopathy caused by other causes. Ethics approval was obtained from Affiliated Hospital at Changchun University of Chinese Medicine, and informed consent was provided. Table 1 shows the clinical data of both patients and healthy controls.

| Variables       | Levels | Diabetes | Control |
|-----------------|--------|----------|---------|
| Age, year       | Mean ± SD | 44.3 ± 6.1 | 46.1 ± 6.6 |
| Sex             | Male | 188 | 60 |
|                 | Female | 110 | 38 |
| Hypertension    | Negative | 136 | 98 |
|                 | Positive | 162 | 0 |
| Disease duration, year | Mean ± SD | 7.8 ± 3.1 | NA |
| HbA1c, %        | Mean ± SD | 7.15 ± 1.01 | 4.72 ± 0.63** |
| BMI             | Mean ± SD | 24.18 ± 1.01 | 22.78 ± 1.23** |

Notes: NA, not available; **, p < 0.01.

Blood extraction and preparation of plasma

Ten milliliters (ml) of whole blood was extracted from the elbow vein of each diabetic patient and healthy control under fasting condition immediately after the diagnosis of diabetic complications during follow-up and at the end of follow-up. Blood was kept at room temperature in BD Vacutainer® PPT™ Plasma Preparation Tube for 30 min. After that, blood was centrifuged at 1600 g for 20 min to collect plasma.

qRT-PCR

Total RNAs were extracted using Trizol (Invitrogen, USA), prepared as cDNA samples using X kit at 55°C for 15 min and 80°C for 10 min, and subjected to PCR using primers 5'-TAACTGGTGAAGCCCGGAAG-3' (forward) and 5'-TCGACTGGTCATTAAA TGCT-3' (reverse) for IncRNA ZNF503-AS1 and 5'-GACCTCATGCAACACAG-3' (forward) and 5'-AGTACCTGGCCTCAGGAGG-3' (reverse) for β-actin. The 2^ΔΔCt method was used to normalize Ct values [10].

Cells and transient transfection

Retinal pigment epithelial cell-line ARPE-19 from ATCC (USA) was cultured in DMEM: F12 medium (Catalog No. 30–2006, ATCC). Plasmid ZNF503-AS1 was constructed with pEGFPC3 (Clontech, Palo Alto, CA, USA) as
the backbone vector. The vector (µg) and Lipofectamine 2000 (µl) were mixed at a ratio of 1:3 to form vector-reagent complexes and incubated with ARPE-19 cells at 37°C for 6 h to achieve transfection.

**Cell proliferation assay**

After transfection, cells were collected, prepared as a single cell suspension (4x10⁴ cells per µl), and cultured in 96-well plates. After the addition of CCK-8, OD values at 450 nm were measured at 4 h later.

**Cell apoptosis assay**

After transfection, cells were collected and prepared as a single cell suspension (4x10⁴ cells per µl). Cells were then cultured in media with 30 mM D-glucose for 6 h and stained for 15 min using Annexin V-FITC/PI detection kit (BD Biosciences). After that, cells were sorted on a flow cytometer (BD Biosciences).

**Western blot**

Proteins were isolated using a RIPA buffer separated on 10% SDS-PAGE gels and transferred onto PVDF membranes. After blocking, the membranes were incubated first with primary antibodies against TGF-β1 (1: 2000, ab92486, Abcam), TGFBR1 (1: 2000, ab31013, Abcam), Bcl2 (1: 2000, ab59348, Abcam), Bax (1:2000, ab32503, Abcam) and GAPDH (1: 1000, ab9485, Abcam) and then with anti-rabbit IgG-HRP secondary antibody (1:1000, MBS435036, MyBioSource). Signals were produced using ECL and analyzed with Image J software.

**Statistical analysis**

Data were compared using unpaired t-test (two groups) and ANOVA LSD test (multiple groups). The incidences of diabetic retinopathy and other complications were compared by Chi-square test. P < 0.05 was statistically significant.

**Ethical approval and informed consent**

The Ethics Committee of the Affiliated Hospital of Changchun University of Chinese Medicine approved this study. All subjects signed informed consent. Participants did not receive a stipend.

**Results**

**Comparison of plasma IncRNA ZNF503-AS1 levels between healthy participants and diabetic patients**

Differential expression of a gene determines its function. To this end, we first measured the levels of IncRNA ZNF503-AS1 in healthy participants and diabetic patients. As shown in Figure 1, plasma IncRNA ZNF503-AS1 levels were not significantly different between healthy participants and diabetic patients. Therefore, ZNF503-AS1 is unlikely to be a critical player in diabetes. It is worth noting that Chi-square analysis showed that ZNF503-AS1 was not closely associated with patients’ age, gender, hypertension, disease duration, HbA1c, and BMI in both patient and control groups (data not shown).

**Plasma IncRNA ZNF503-AS1 levels and their correlations with the incidence of diabetic retinopathy and other diabetic complications**

To analyze the potential role of ZNF503-AS1 in the occurrence of diabetic complications, patients were divided into low and high ZNF503-AS1 expression groups with the median plasma ZNF503-AS1 level as the cutoff point (n = 136).
During the follow-up, diabetic retinopathy was diagnosed in 34 cases (24 cases and 10 cases in high and low ZNF503-AS1 expression group, respectively), diabetic lung diseases in 23 cases (13 cases in high and 10 cases in low-expression groups, respectively), diabetic heart disease in 35 cases (11 cases and 14 cases in high and low ZNF503-AS1 expression groups, respectively), and diabetic nephropathy in 44 cases (20 cases and 24 cases in high and low ZNF503-AS1 expression groups, respectively). As shown in Figure 2, the incidence of diabetic retinopathy was significantly higher in the high ZNF503-AS1 level group (Figure 2a) (p < 0.05) than in the low ZNF503-AS1 level group. Moreover, the incidences of diabetic lung diseases (Figure 2b), diabetic heart disease (Figure 2c), and diabetic nephropathy (Figure 2d) were not significantly different between high and low ZNF503-AS1 expression groups (p > 0.05). Therefore, ZNF503-AS1 may participate in the occurrence of diabetic retinopathy.

**Comparison of plasma ZNF503-AS1 levels between patients with different diabetic complications**

In total, 298 patients completed the 6 years of follow-up. Among them, 136 patients developed single diabetic complications and 45 patients developed multiple complications, and 17 patients

![Figure 2](image-url)

**Figure 2.** High plasma IncRNA ZNF503-AS1 levels were correlated with the incidence of diabetic retinopathy but not other diabetic complications. This figure shows the comparison of incidences of diabetic retinopathy (a), diabetic lung diseases (b), diabetic heart disease (c), and diabetic nephropathy (d) between high and low ZNF503-AS1 expression groups. Notes: *, p < 0.05.
were unable to confirm without complications. All these patients were excluded from this study. The remaining 100 diabetic patients were diagnosed with no obvious complications. To further confirm the role of ZNF503-AS1 in the occurrence of diabetic retinopathy, plasma ZNF503-AS1 levels before admission and after follow-up were compared in each patient group. As shown in Figure 3a, plasma ZNF503-AS1 levels were not significantly different among patients with different diabetic complications on the day of admission (p > 0.05). In contrast, plasma ZNF503-AS1 levels were significantly higher in patients with diabetic retinopathy than in patients in other groups during the follow-up (Figure 3b, p<0.05).

**ZNF503-AS1 was upregulated by high-glucose and ZNF503-AS1 overexpression promoted TGF-β1 expression**

To explore the role of blood glucose in regulating ZNF503-AS1 expression, cells were treated with different dosages of glucose, and ZNF503-AS1 expression was determined by RT-qPCR. D-glucose treatment increased ZNF503-AS1 level in a dose-dependent manner from 5 mM (normal blood glucose level) to 15 mM (Figure 4a, p<0.05). Moreover, ZNF503-AS1 overexpression was achieved in human retinal pigment epithelial cells ARPE-19 with transfection of the ZNF503-AS1 expression vector (Figure 4b, p<0.05). TGF-β signaling plays a pivotal role in the pathogenesis of diabetic retinopathy [8]. In our study, ZNF503-AS1 overexpression increased the accumulation of TGF-β1 and TGF-β receptor1 (TGFBR1) in ARPE-19 cells (Figure 4c, p<0.05). In contrast, application of TGF-β1 (Sigma-Aldrich) showed no impact on ZNF503-AS1 expression (Figure 4d, p<0.05). Original Western Blot images were presented in supplemental File 1.

**ZNF503-AS1 overexpression inhibited proliferation and promoted apoptosis of ARPE-19 cells**

Cell proliferation and apoptosis are involved in diabetic retinopathy. To this end, the proliferation and apoptosis of ARPE-19 cells after ZNF503-AS1 overexpression were evaluated by CCK-8 assay and cell apoptosis assay, respectively. The results showed that ZNF503-AS1 overexpression significantly inhibited cell proliferation (Figure 5a) and increased cell apoptosis (Figure 5b). In addition, TGF-β inhibitor LY2109761 (100 nM, LY, Sigma-Aldrich) attenuated these effects (Figure 5b). Therefore, ZNF503-AS1 may regulate cell proliferation and apoptosis in diabetic retinopathy through TGF-β signaling.

**Discussion**

ZNF503-AS1 is a newly identified lncRNA that plays a pivotal role in the differentiation of retinal pigment epithelium [9]. However, its functions in other diseases are still unknown. Our study found that lncRNA ZNF503-AS1 participates in the pathogenesis of diabetic retinopathy and its action is likely to

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**Figure 3. Comparison of plasma ZNF503-AS1 levels between patients with different diabetic complications.** This figure shows the comparison of plasma ZNF503-AS1 levels between patients with different diabetic complications on the day of admission (a) and at the end of follow-up (b).

Notes: *, p < 0.05; D, diabetes only; DR, diabetic retinopathy; DL, diabetic lung diseases; DH, diabetic heart disease; DN, diabetic nephropathy.
be achieved by activating TGF-β, which has been proven to promote diabetic retinopathy [8].

Both temporal and sustained high blood glucose environments in the human body change the expression patterns of a large set of lncRNAs [11,12], a subgroup of non-coding RNAs that play pivotal roles in both pathological and physiological processes. A recent study reported 303 lncRNAs with an altered expression pattern in the retinas of mice with early diabetic retinopathy [13]. However, abnormal expression of these lncRNAs is likely to be induced by high glucose environments and whether they are diabetic retinopathy specific remains unknown. Our study showed that plasma ZNF503-AS1 levels were significantly higher in diabetic retinopathy patients than in diabetic patients without obvious complications, but not in diabetic patients with other complications. Therefore, ZNF503-AS1 induces retinopathy in diabetic patients. Although diabetic retinopathy and age-related macular degeneration have similar effects on visual loss [14], opposite ZNF503-AS1 expression patterns in these two groups may indicate their different pathogenic mechanisms.

Figure 4. ZNF503-AS1 overexpression promoted TGF-β1 expression. This figure shows the effects of high glucose environments on ZNF503-AS1 expression in ARPE-19 cells (a). ZNF503-AS1 overexpression was achieved in human retinal pigment epithelial ARPE-19 cells (b), upregulation of TGF-β1 and TGF-β receptor1 (TGFBR1) protein after ZNF503-AS1 overexpression (d) and ZNF503-AS1 expression in ARPE-19 cells treated with exogenous TGF-β1 (d).

Notes: *, p < 0.05; C, cells without transfection, NC, empty vector transfection; ZNF503-AS1, cells transfected with the ZNF503-AS1 expression vector.

High plasma ZNF503-AS1 levels were related to a high incidence of retinopathy in diabetic patients, indicating the potential application of plasma ZNF503-AS1 in the prediction of diabetic retinopathy occurrence. However, although higher plasma ZNF503-AS1 levels were observed in diabetic retinopathy patients than in diabetic patients
with other complications and diabetic patients without obvious complications by the time of diagnosis, no significant differences in plasma ZNF503-AS1 levels were found among these groups of patients on the day of admission. These inconsistent results may be caused by the small size of patients with diabetic retinopathy (n = 34), and further studies with a larger sample size are needed.

Polymorphism of the TGF-β1 encoding gene has been proven to be a risk factor for diabetic retinopathy [15]. Besides that, previous studies have also shown that TGF-β signaling can achieve its biological roles by interacting with lncRNAs [16,17]. The interactions between TGF-β signaling and lncRNAs have also been observed in other diabetic complications, such as diabetic cardiomyopathy [18–20]. Our results proved that lncRNA ZNF503-AS1 is likely an upstream activator of TGF-β signaling. This conclusion is made based on the following observations: 1) ZNF503-AS1 overexpression upregulated TGF-β1 expression; 2) addition of exogenous TGF-β1 had no significant effects on ZNF503-AS1 expression; 3) TGF-β inhibitor attenuated the effects of ZNF503-AS1 overexpression on cell apoptosis and proliferation. Overall, our data thus uncovered a new signaling transduction pathway in the pathogenesis of diabetic retinopathy.

It is known that miR-663 can target TGF-β1 [21]. We performed bioinformatics analysis and
found that ZNF503-AS1 is highly likely a sponge of miR-663 (data not shown). Therefore, ZNF503-AS1 may sponge miR-663 to upregulate TGF-β1. However, more studies are needed to further verify this possibility. With the increased understanding of the role of lncRNAs in diabetic complications, lncRNAs are expected to serve as potential targets to treat these diseases [22,23].

Our study failed to perform in vivo animal model experiments to further analyze the roles of ZNF503-AS1 in the apoptosis retinal pigment epithelial cells induced by high-glucose conditions. In addition, the mechanism that mediates the interaction between ZNF503-AS1 and TGF-β signaling remains unclear. Our future studies will try to perform deeper analyses to further confirm our conclusion.

This study is limited by the small sample size. In addition, all patients are Han Chinese. Moreover, only type 1 diabetes was investigated. Our future studies will include more patients from different races and with type 1 diabetes to further confirm our conclusions.

In conclusion, ZNF503-AS1 is correlated with the high incidence of diabetic retinopathy. ZNF503-AS1 may inhibit proliferation and promote apoptosis of retinal pigment epithelial cells by activating TGF-β signaling.

Author contributions
TH and DN designed this study. TH and WL conducted the experiments. HZ helped with data analyses. TH wrote the manuscript, and DN revised the manuscript. All authors have read and approved the manuscript.

Availability of data and material
The data that support the findings of this study are available on request from the corresponding author.

Disclosure statement
No potential conflict of interest was reported by the author(s).

Ethics approval and consent to participate
This study passed the review board of the Ethics Committee of the Affiliated Hospital at Changchun University of Chinese Medicine. Informed consent was obtained from all individual participants included in the study.

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