#### Original Research

# **Expression and Regulatory Ability of Long Non-Coding RNADLX6 Antisense RNA 1 in Gestational Diabetes Mellitus**

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#### Abstract

**Background**: Gestational diabetes mellitus (GDM) is characterized by elevated blood glucose during pregnancy, which may affect both the fetus and the pregnant woman. This study introduced the expression and regulatory ability of long non-coding RNA (lncRNA) DLX6 Antisense RNA 1 (DLX6-AS1) in patients with GDM, aiming to reveal the action potential and diagnostic value of DLX6-AS1. **Methods**: This study included 70 pregnant patients with GDM and 50 healthy pregnant women. DLX6-AS1 levels were determined using real-time quantitative polymerase chain reaction (RT-qPCR), and the diagnostic value of DLX6-AS1 was evaluated by receiver operating characteristic (ROC) curve. The GDM cell model was constructed using human chorionic trophoblast cells, and the cell proliferation capacity was assessed using cell counting kit-8 (CCK-8) method. Cell apoptosis was analyzed by flow cytometry. Moreover, luciferase assay was performed to evaluate the relationship between DLX6-AS1 and miR-497-5p. **Results**: DLX6-AS1 and blood glucose levels were markedly increased in GDM patients, and a positive correlation was observed between both levels (r = 0.7072, p < 0.0001). GDM affected the cell activity, while DLX6-AS1 silencing enhanced the proliferation activity, and suppressed cell apoptosis in GDM cell model via directly targeting miR-497-5p. miR-497-5p expression was low in GDM, and its content was affected by DLX6-AS1 silencing (p < 0.001). Furthermore, DLX6-AS1 exhibited a promising diagnostic function in GDM (area under the curve (AUC) = 0.937, sensitivity = 92.9%, specificity = 86.0%). **Conclusions**: DLX6-AS1 was positively expressed and mediated GDM through sponge miR-497-5p, suggesting it may be used as a diagnostic factor to predict the occurrence of GDM.

Keywords: gestational diabetes; DLX6-AS1; miR-497-5p; blood glucose; GDM cell model; diagnosis

#### 1. Introduction

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Gestational diabetes mellitus (GDM) refers to diabetes developed during pregnancy in indivuals with normal glucose metabolism or potential impaired glucose tolerance before pregnancy [1]. Patients with GDM account for 2%–5% of all pregnant women, and this incidence is increasing year by year [2]. The incidence rate of GDM in China is approximately 7.7% [3]. GDM is prone to cause amniotic fluid infection in pregnant women, premature delivery, and may cause gestational diseases, such as gestational hypertension and type 2 diabetes mellitus. In addition, it can also affect the development and health of the fetus [4,5]. Notably, patients with GDM have no significant symptoms, which may lead to missed diagnosis and delayed treatment. Therefore, a deeper understanding of the pathological mechanism of GDM and determination of effective diagnostic markers are crucial to prevent and control the risk of GDM in pregnant women.

With continuous advances in genetic engineering, long non-coding RNAs (lncRNAs) have become a research hotspot. LncRNAs play a corresponding regulatory role in multiple tumors and other common diseases [6,7]. For example, recent studies have reported that lncRNA TUG1 acts as a sponge for miR-328-3p and regulates the extracellular signal-regulated kinase (ERK) signaling pathway to downregulate insulin resistance in mice with GDM [8]. DLX6 Antisense RNA 1 (DLX6-AS1) is found on chromosome 7 (7q21.3) in humans and is a promising lncRNA [9,10]. Ghafouri-Fard et al. [11] proposed that DLX6-AS1 is overexpressed in various cancers and has oncogenic properties. The importance of DLX6-AS1 in cancer therapy was demonstrated by Zhao et al. [10]. In addition, DLX6-AS1 was reported to aggravate the progression of diabetic nephropathy in mice by upregulating the miR-346/GSK- $3\beta$  pathway [12]. However, the expression and molecular mechanism of DLX6-AS1 in GDM are not yet studied. It is unclear whether DLX6-AS1 improves the condition of patients with GDM by targeting microRNA (miRNA).

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Table 1. General data analysis of GDM patients and healthy control pregnant women.

| Parameters                 | Participants       |                  | <i>n</i> -value |
|----------------------------|--------------------|------------------|-----------------|
|                            | Control $(n = 50)$ | GDM (n = 70)     | p vulue         |
| Age (years)                | $30.41 \pm 4.73$   | $30.54\pm3.70$   | 0.862           |
| BMI (kg/m <sup>2</sup> )   | $21.68\pm2.73$     | $23.05\pm2.11$   | 0.002           |
| Pregnancy cycle (weeks)    | $25.63\pm2.55$     | $25.02 \pm 1.46$ | 0.098           |
| FBG (mmol/L)               | $4.51\pm0.49$      | $7.41 \pm 1.09$  | < 0.001         |
| 1 h blood glucose (mmol/L) | $6.10 \pm 1.68$    | $9.01\pm2.17$    | < 0.001         |
| 2 h blood glucose (mmol/L) | $5.29 \pm 1.15$    | $8.65 \pm 2.87$  | < 0.001         |

Noted: BMI, body mass index; FBG, fasting blood glucose; GDM, gestational diabetes mellitus. Data are expressed as n or mean  $\pm$  SD (standard deviation). The control and GDM groups were analyzed by the Student's *t*-test.

In this study, we evaluated the serum DLX6-AS1 levels in patients with GDM and explored its target in order to understand the molecular mechanisms of DLX6-AS1 in GDM. Moreover, a GDM cell model was constructed to reveal the influence of abnormal expression of DLX6-AS1 on cell behavior, in order to provide new insights for the diagnosis and preclinical treatment of GDM.

## 2. Materials and Methods

#### 2.1 Included Samples

Patients with GDM (n = 70) and healthy pregnant women (n = 50) of similar age were randomly selected from the Second Affiliated Hospital of Guangxi Medical University. Blood samples were taken from all pregnant women in the morning after fasting for 12 hours, and blood glucose levels were measured with a glucose meter (Accu-Chek, Roche Diagnostic, Berlin, Germany). The blood samples were centrifuged for 10 minutes (3000 r/min) to obtain serum, which was stored in the refrigerator (Haier, Qingdao, Shandong, China) at -80 °C. The clinical data of all participants were collected and recorded (Table 1). The study was supervised by the Ethics Committee of the hospital (Ethics number: 2022-KY-0113), and all patients who participated in this study were informed about the purpose of the study and voluntarily signed the consent form.

The GDM patients included met the diagnostic criteria of the International Diabetes and Pregnancy Study Group [13] and were diagnosed by two experts in our hospital. In addition, the inclusion criteria for GDM patients were as follows: all participants were singleton pregnant women with natural pregnancy, and GDM patients met the above diagnostic requirements. Exclusion criteria: the patients had other serious diseases before and after pregnancy, or the patients had a history of drug allergy.

#### 2.2 Real-Time PCR (qPCR)

TRIZOL reagent (Invitrogen, Walthamm, MA, USA) was applied to extract RNA from the serum samples, and RNA quality was verified using a spectrophotometer (Agilent, Santa Clara, CA, USA). Further, cDNA was

obtained using a reverse transcription kit manufactured by Applied Biosystems. The PCR reaction system was then configured by the SYBR Green PCR Kit (Applied Biosystems, Waltham, MA, USA), including 2× SYBR GREEN qPCR Super Mix (5 µL), cDNA sample and primer (2 µL each), and  $ddH_2O$  (1 µL). Assays were performed on the ABI 7500 Real-time PCR system (Applied Biosystems, Foster City, CA, USA) with 40 cycles of denaturation at 95 °C for 2 min and amplification at 95 °C for 15 s, followed by annealing at 60 °C for 30 s and extension at 72 °C for 30 s. Finally, DLX6-AS1 and miR-497-5p were quantified by internal reference genes GAPDH and U6. The sequence of primers involved are as follows: DLX6-AS1 forward 5'-GGTGGGGGAACCTCCAAAC-3', reverse 5'-ATTGACATGTTAGTGCCCT-3'; miR-497-5p forward 5'-CTTCAGCAGCAGCACACTG-3'. reverse 5'-CAGTGCAGGGTCCGAGG-3'; GAPDH 5'-GTCAGCCGCATCTTCTTTG-3', forward reverse 5'-GCGCCCAATACGACCAATC-3'; U6forward 5'-CTCGCTTCGGCAGCACA-3', reverse, 5'-ACGCTTCACGAATTTGCGT-3'.

#### 2.3 Cell Culture

Human chorionic trophoblast cells HTR-8/Svneo were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 medium (Gibco, Waltham, MA, USA) with 10% fetal bovine serum (FBS, Invitrogen, Waltham, MA, USA) at 37 °C. The GDM cell model was obtained by inducing HTR-8/SVNEO cells with 25 mM glucose. The GDM cell model was treated with lipofectamine 3000 (Thermo Fisher, Waltham, MA, USA) and cotransfected with si-DLX6-AS1 or negative control (si-NC). The sequences of siRNA involved are as follows: si-DLX6-AS1 forward 5'-CGCUUGUCUUACUUAA-3', reverse 5'-AGUAAGACAAGCGGCT-3'; si-NC for-5'-UUCUCCGAACGUGUCACGU-3', ward reverse 5'-ACGUGACACGUUCGGAGAA-3'.

HTR-8/Svneo cell line was certified by short tandem repeat (STR) authentication. The cell line was verified in our laboratory by the LookOut Mycoplasma PCR detection



Fig. 1. DLX6-AS1 (DLX6 Antisense RNA 1) level was enhanced in GDM patients and was positively proportional to blood glucose concentration. (A) DLX6-AS1 levels in control serum (n = 50) and GDM serum (n = 70). (B) The blood glucose content of the healthy pregnant women compared to GDM patients (\*\*\*p < 0.001), groups were analyzed by the Student's *t*-test. (C) DLX6-AS1 was positively correlated with blood glucose concentration (r = 0.7072, p < 0.0001). (D) DLX6-AS1 may serve as a diagnostic factor for GDM (area under the curve (AUC) = 0.937, sensitivity = 92.9%, specificity = 86.0%).

kit (Sigma-Aldrich, St. Louis, MO, USA) after purchase and during subculture, and no contamination occurred. The cell line was then preserved and subcultured for study in 2022 in our laboratory without any mycoplasma contamination. If the cell line is ever contaminated with mycoplasma, the cells may appear blurred in outline, clumping, and discoloration of the medium, which can be observed by scanning electron microscopy.

#### 2.4 Proliferation Assay

The successfully transfected cells were selected and cultured in 96-well plates. Cell counting kit-8 (CCK-8) solution (Beyotime, Shanghai, China) was added at the corresponding time points. After incubation at 37 °C, optical density (OD) value was measured at 450 nm by a microplate reader (Thermo Fisher, Waltham, MA, USA).

#### 2.5 Assessment of Apoptosis

The cell apoptosis levels were assessed according to the Annexin V FITC/PI apoptosis detection assay kit (Invit-

rogen, Waltham, MA, USA). Transfected cells were stained with Annexin V and PI buffer, and subjected to flow cytometry (Thermo Fisher, Waltham, MA, USA) after washing the cells with phosphate-buffered saline (PBS, 0.01 M).

#### 2.6 Luciferase Reporter Assay

The binding fragments of DLX6-AS1 and miR-497-5p were cloned and inserted into the pmirGLO reporter vector and designated as wild type-DLX6-AS1 (WT-DLX6-AS1) and mutant type-DLX6-AS1 (WUT-DLX6-AS1), respectively. They were co-transfected with mimic NC, miR-497-5p mimic or miR-497-5p inhibitor into GDM cell models for 48 hours, and luciferase activity was evaluated.

#### 2.7 Statistical Analysis

The data were processed by GraphPad Prism 7.0 software (GraphPad Software, Inc., San Diego, CA, USA), and the measurement data were represented by mean  $\pm$  standard deviation (SD). The differences between two groups were measured by Student's *t*-test, and three or more groups were



Fig. 2. Knockdown of DLX6-AS1 affected the biological functions of the cells. (A) After transfection with si-DLX6-AS1, DLX6-AS1 levels were decreased in the cells. (B,C) Behavioral assessment of GDM cell models (\*p < 0.05, compared with the controls; #p < 0.05, compared with the GDM model. One-way ANOVA (analysis of variance) combined with the Tukey's post hoc test was used to analyze the differences between the different groups). OD, optical density; NC, negative control.

Α



Fig. 3. DLX6-AS1 directly targets miR-497-5p. (A) DLX6-AS1 and miR-497-5p have potential binding sites. (B) The increase of miR-497-5p reduced the luciferase activity of (wild type-DLX6-AS1) WT-DLX6-AS1. \*\*p < 0.01, \*\*\*p < 0.001, compared with the controls. One-way ANOVA combined with the Tukey's post hoc test was used to analyze the differences between the different groups. MUT-DLX6-AS1, mutant type-DLX6-AS1.

analyzed by one-way ANOVA combined with the Tukey's post hoc test. Correlation analysis was conducted by Spearman's method. The receiver operating characteristic (ROC) curve implied the possibility of DLX6-AS1 in the diagnosis of GDM. Each set of independent experiments was performed more than three times, and p < 0.05 was considered significant.

## 3. Results

#### 3.1 General Information of the Participants

As shown in Table 1, the age of GDM patients included was  $(30.54 \pm 3.70)$  years, and the gestational cycle was  $(25.02 \pm 1.46)$  weeks, while the age of healthy control group was  $(30.41 \pm 4.73)$  years, and the gestational cycle was  $(25.63 \pm 2.55)$  weeks. There was no significant difference between the two groups (p > 0.05). However, the mean values of body mass index (BMI), fasting blood glucose (FBG), 1 h and 2 h blood glucose in GDM patients were significantly higher than those in healthy pregnant women (p < 0.05).

#### 3.2 DLX6-AS1 and Blood Sugar Content in GDM

DLX6-AS1 was actively expressed in GDM patients compared with healthy control pregnant women (1.75  $\pm$ 0.28, p < 0.001; Fig. 1A). The measurement of blood glucose content in pregnant women also displayed that blood glucose levels were higher in GDM patients than in controls as shown in Fig. 1B (7.41  $\pm$  1.09, p < 0.001). Spearman's correlation analysis in Fig. 1C further revealed that DLX6-AS1 level was positively correlated with blood glucose content in GDM patients (r = 0.7072, p < 0.0001), suggesting that elevated DLX6-AS1 may rise blood glucose level in GDM patients. The drawing of the ROC curve in Fig. 1D illustrated the predictive value of DLX6-AS1 with a cutoff value of 1.426, and the high sensitivity and specificity (92.9% and 86.0%) indicted the potential of DLX6-AS1 in diagnosing GDM.

#### 3.3 The Regulation of DLX6-AS1 Knockdown on Cell Proliferation and Apoptosis

The biological function of DLX6-AS1 was explored in a GDM cell model by silencing DLX6-AS1, and the transfection efficiency is shown in Fig. 2A. Cell growth was sig-



Fig. 4. miR-497-5p was downregulated in GDM. (A) miR-497-5p in healthy serum (n = 50) and GDM serum (n = 70). (B) miR-497-5p level was significantly upregulated after transfection with si-DLX6-AS1. p < 0.05, p < 0.001, compared to healthy controls; p < 0.05, compared with the GDM model. The control and GDM groups were analyzed by the Student's *t*-test. One-way ANOVA combined with the Tukey's post hoc test was used to analyze the differences between the different groups.

nificantly reduced in the GDM model, whereas cell proliferation was restored after DLX6-AS1 silencing. Moreover, the apoptosis rate of GDM model was increased, while downregulation of DLX6-AS1 reduced the apoptosis of cells (Fig. 2B,C).

#### 3.4 DLX6-AS1 Sponges miR-497-5p

The potential binding sites of DLX6-AS1 and miR-497-5p are shown in Fig. 3A. Luciferase assay revealed that the miR-497-5p mimic clearly reduced the luciferase signal of WT-DLX6-AS1, while WUT-DLX6-AS1 had no significant effect (Fig. 3B). This indicated that DLX6-AS1 directly targeted miR-497-5p. Moreover, miR-497-5p was downregulated in the GDM patients ( $0.46 \pm 0.16$ , \*\*\*p < 0.001), as shown in Fig. 4A. Lastly, miR-497-5p levels were low in GDM model cells, and DLX6-AS1 silencing enhanced miR-497-5p expression (Fig. 4B).

### 4. Discussion

With the improvement in economy and change in ideological cognition, late marriage and late childbearing have become the general trend. In recent years, human birth rate has significantly decreased, and the female reproductive age has been rising [14–16]. Age-related diseases during pregnancy, such as GDM, gestational hypertension, and recurrent spontaneous abortion, are on the rise. Among them, GDM has attracted great attention as a metabolic disease with high incidence during pregnancy [17]. Due to the complex changes of glucose metabolism in pregnant women during pregnancy, increased glucose demand or insufficient insulin secretion may induce GDM. However, the symptoms of GDM are non-specific, increasing the difficulty in the diagnosis of GDM. Therefore, searching new diagnostic markers for GDM can help to understand the pathological mechanism of GDM.

The changes in the expression of lncRNAs are related to the cause and progression of many diseases [18]. In GDM, the plasma levels of lncRNA MEG8 were confirmed to be elevated, and the incidence of kidney injury increased with the upregulation of MEG8 [19]. LncRNA RPL13P5 was prominently expressed in GDM and was found to mediate insulin resistance through the phosphatidylinositol 3kinase-Akt (PI3K-Akt) pathway [20]. The differential levels of DLX6-AS1 were revealed earlier in tumors. In fact, DLX6-AS1 was enriched in liver cancer [21], pancreatic cancer [22] and cervical cancers [23], which may provide promising therapeutic strategy in certain cancers. Moreover, DLX6-AS1 was positively expressed in mice with Parkinson's disease (PD), and DLX6-AS1 knockdown improved the neurological function and attenuated inflammation levels in PD mice [24]. Ye et al. [25] described the increase of DLX6-AS1 levels in diabetic retinopathy and its predictive potential. In the present study, DLX6-AS1 was upregulated in the serum samples of GDM and was closely correlated with the blood glucose concentration of patients. Importantly, blood glucose content is an important index to evaluate the level of glucose metabolism, and a clinical marker to assess GDM [26]. Meanwhile, with the increase of blood glucose, the biological functions of trophoblast cells may be inhibited. In the constructed GDM cell model, DLX6-AS1 was overexpressed and the cell growth was suppressed. After transfection with si-DLX6-AS1, the cell proliferation ability was elevated, and the apoptosis levels were decreased.

While exploring the mechanisms of various diseases, increasing evidence revealed that lncRNAs along with miR-NAs can play a regulatory function [27,28]. MiR-497-5p is located on chromosome 17p13.1 and is involved in the progression of various diseases [29]. Lu et al. [30] stated that miR-497-5p was decreased in cervical cancer, and LINC00511 regulated the miR-497-5p/MAPK1 axis to accelerate the progression of cervical cancer. Zhang et al. [31] reported that high glucose decreased the miR-497-5p level in HK-2 cells, and that the lncRNA MIR503HG/miR-497-5p/CCL19 regulatory network alleviated inflammation in diabetic nephropathy. Previous evidence mentioned that miR-497-5p was reduced in GDM, and knockdown of lncRNA XIST, that directly targets miR-497-5p, negatively affected cell viability [32]. Similar to previously mentioned, miR-497-5p was downregulated in the serum samples of GDM and in the GDM model cells in the present study, and it was negatively regulated by DLX6-AS1. Luciferase reporting assay and ROC analysis revealed that DLX6-AS1 silencing sponged miR-497-5p and mediated GDM progression. Therefore, DLX6-AS1 may be considered a diagnostic biomarker for GDM. However, more samples and more comprehensive studies are needed to support our results.

## 5. Conclusions

In summary, DLX6-AS1 was clearly increased in GDM and was proportional to blood glucose levels. GDM suppressed the growth of human chorionic trophoblast cells, and DLX6-AS1 knockdown mediated miR-497-5p to promote cell proliferation. This study demonstrated the potential of DLX6-AS1 as a biomarker for GDM and provided a new direction in the diagnosis and treatment of patients with GDM.

# Availability of Data and Materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

# **Author Contributions**

RL, QHH and LCT designed the research study. XHM, MLW, YQ and QW performed the research. RL, QHH, LCT, JJL, XXL and XD analyzed the data. QHH and LCT wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

# **Ethics Approval and Consent to Participate**

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The study was approved by the Bioethics Committee of the Second Affiliated Hospital of Guangxi Medical University (Ethics number: 2022-KY-0113), and all patients who participated in this study were informed about the purpose of the study and voluntarily signed the consent form.

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# **Conflict of Interest**

The authors declare no conflict of interest.

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