miR-720 Regulates Insulin Secretion by Targeting Rab35

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Research

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Abstract

**Background:** miRNAs pose a good prospect in the diagnosis and treatment of type 2 diabetes (T2D). This study aimed to investigate whether miR-720 targets Rab35 to regulate insulin secretion in MIN6 cells and its molecular mechanism, and the clinical value of miR-720 as a specific biomarker of T2D.

**Methods:** Fifty-five samples of new diagnosis T2D patients and normal control were collected. Levels of miR-720, fasting blood glucose, insulin and other indicators of glucose and lipid metabolism were determined. We increased and decreased miR-720 expression using miR-720 mimic and inhibitor to identify the effect of miR-720 on insulin secretion in MIN6 cells, respectively. Then we used miR-720 mimic, miR-720 inhibitor and dual luciferase reporter gene assays to prove miR-720 regulate insulin secretion by targeting Rab35 in MIN6 cells. In addition, we overexpressed and silenced of Rab35 gene to detect the expression of PI3K, Akt and mTOR in MIN6 cells by RT-PCR and western blot.

**Results:** Circulating miR-720 was significantly higher in T2D group than control group, and miR-270 was positive correlated with fasting blood glucose, while negatively correlated with insulin. Overexpression of miR-720 inhibited insulin secretion and miR-720 downregulation promoted insulin secretion. miR-720 regulated insulin secretion by targeting Rab35 in MIN6 cells. Compared with the control group, the expression of PI3K, Akt and mTOR was significantly decreased by overexpression of Rab35 gene, while silencing Rab35 gene could induce the expression of PI3K, Akt and mTOR.

**Conclusions:** We conclude that miR-720 may as a potential biomarker for the diagnosis of T2D. Increase of miR-720 reduced Rab35 expression then activate PI3K/Akt/mTOR signal pathway, thus inhibiting insulin secretion.

Background

Type 2 diabetes (T2D) is a homeostasis disorder of glucose metabolism caused by insulin resistance (IR) and/or pancreatic β-cells dysfunction, which involves the interaction of genetic, environmental and behavioral factors\(^{(1, 2)}\). Previous studies on the pathogenesis of T2D mainly focused on pancreatic β-cells function and insulin secretion regulation. However, recent studies have confirmed that epigenetic mechanisms play an important role in the pathophysiological process of T2D\(^{(3)}\).

MicroRNA (miR) is a highly conserved non-coding small RNA widely distributed in eukaryotic cells, 18–25 nucleotides long, being capable of affecting the biological functions of other genes by binding to the 3'UTR end of its target gene\(^{(4, 5)}\). More and more evidences\(^{(6–8)}\) show that miRNAs are involved in the regulation of glucose metabolism, insulin synthesis and secretion, which are the core links in the development of T2D. There is no doubt that miRNAs provide new molecular clues for the pathological study of T2D, and miRNAs are expected to be specific diagnostic markers and therapeutic targets for T2D\(^{(9, 10)}\). miR-720 has been widely studied since its discovery, which is closely related to the pathophysiological process of various tumors. For example, the expression of miR-720 was decreased in
breast cancer, and it was associated with lymph node metastasis (11). The expression of miR-720 increased in cervical cancer (12). In colorectal cancer, the expression level of miR-720 in tumor tissues is higher than that in normal tissues (13). Compared with healthy people, the expression of miR-720 in serum of colorectal cancer patients was significantly increased, and its expression level was related to male gender and lymph node metastasis of colorectal cancer patients. Some studies suggest that miR-720 may be a prognostic marker for these specific types of cancer (14). The research on miR-720 in T2D is still very limited yet.

Rab proteins are known to be important participants in insulin secretion by pancreatic β cells. Rab functions are accompanied by cyclical activation and inactivation of GTP-bound and GDP-bound forms between the cytosol and plasma membrane that are regulated by upstream regulators (15). In our previous study (16), we found that Rab35 is closely related to the insulin secretion in pancreatic β cells, and Rab35 might be the target of miR-720 in pancreatic β cells.

Therefore, the aim of the current study was to investigate whether miR-720 targets Rab35 to regulate insulin secretion in MIN6 cells and its molecular mechanism, and the clinical value of miR-720 as a specific biomarker of T2D. The results are expected to obtain reliable evidence that miR-720 regulates insulin secretion, and provide a scientific basis for the application of miR-720 and its target as a new diagnostic index and treatment of T2D.

**Materials And Methods**

**Ethical compliance**

This study was approved by the Ethics Committee of The First Affiliated Hospital of Jinan University, and written informed consents were provided by all the participants.

**Experimental materials and reagents**

All the participants were recruited from the first affiliated hospital of Jinan University between January 2019 and July 2020 who were further classified into two groups: healthy control group and T2D group.

T2D was confirmed in accordance with the World Health Organization (WHO) criteria (17): fasting blood glucose (FG) levels ≥ 7.0 mmol/L, or a 2-hour oral glucose tolerance test (OGTT) ≥ 11.1 mmol/L in the presence of symptoms and glycated hemoglobin (HbA1c) levels > 6.5%. The 50 T2D patients were diagnosed for the first time, and no diabetes drugs were used before the diagnosis. Exclusion criteria: patients with other severe malnutrition; long-term use of glucocorticoids and other hormone drugs; patients with severe heart, liver and kidney dysfunction; patients with severe pneumonia, nephritis and other systemic infectious diseases.

**Reverse transcription polymerase chain reaction (RT-PCR)**
About 10 ml venous blood was extracted and totals RNA was isolated using PAXgene Blood miRNA Kit (Qiagen) according to the manufacturer's recommendation. ABgene Reverse-iT One-Step RT-PCR Kit (ReadyMix Version) and a Mastercycler gradient thermocycler (Eppendorf, Germany) using the following primers for: miR-720 primers (forward primer, GCGTGCTCTCGCTGGGG; reverse primer, GTGCAGGGTCCGAGGT); PI3K primers (forward primer, AGCAGGTACCTGGTGATTG; reverse primer, AGAAGGACAGGTCCAGAGA); Akt primers (forward primer, ACTCATTCCAGACCCACGAC; reverse primer, CCGTACACCACGTCTCTCT); mTOR primers (forward primer, GGGCAGCAACAGTGAAGT; reverse primer, ACGGAAGAGCCTTGAGCAG); β-actin primers (forward primer, AAATCTGGCACCACCTTC; reverse primer, GGGGTGTTGAAGGTCTCAAA) were synthesized (18).

**Western blotting**

Western blot analysis was performed as described previously (5). Briefly, proteins were separated using SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked in Tris-buffered saline with 5% milk and 0.05% Tween and incubated overnight at 4 °C with primary antibodies including rabbit anti-Rab35 (1:1000, USA), rabbit anti-PI3K (Abcam), rabbit anti-Akt (Abcam), rabbit anti-mTOR (Abcam), rabbit anti-GFP (Abcam) and rabbit anti-PTPRN2 antibodies (Sigma). After being washed 3–5 times with TBST, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Jackson ImmunoResearch) and visualized using enhanced chemiluminescence reagents.

**Cell Line and Culture**

The mouse insulinoma cell line MIN6 was obtained from BoGu Biotechnology Co. Ltd.(Shanghai, China). High-glucose (4500 mg/L) Dulbecco's modified Eagle's medium (DMEM) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Fetal bovine serum (FBS) was purchased from Biological Industries (Cromwell, CT, USA). The MIN6 cells were maintained in high-glucose DMEM supplemented with 12% FBS, 10 µL/L β-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), 100 U/ml penicillin, and 100 µg streptomycin mixture (Solarbio, Beijing, China) at 37 °C in 5% CO₂.

**Insulin Enzyme-linked immunosorbent assay (ELISA)**

After transfection for 48 h, supernatants from MIN6 cells were assayed for insulin. Insulin was quantified according to the manufacturer's protocol using 96-well plates and an Insulin Mouse ELISA Kit (catalog# EMINS, Invitrogen Corporation, CA).

**Statistical analysis**

Results were shown as mean ± standard deviation (SD). The statistical analysis was performed using the SPSS 18.0 (SPSS Inc., Chicago, Illinois) and the data were visualized using the GraphPad 7. The independent t test was employed for comparison between two groups. The correlation between miR-720 and FBG, and miR-720 and FINS were assessed by one-way ANOVA, followed by Fisher's least significant difference test. Two-tailed P-values < 0.05 were considered statistically significant.
Results

Clinical characteristics of the subjects

Fifty healthy control subjects and 50 T2D subjects were enrolled and the relevant clinical data were shown in Table 1. Gender and age distribution showed no statistical difference (P > 0.05) in two groups, whereas body mass index (BMI), FBG, c peptide (C-P), fasting insulin (FINS), HbA1c, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), total cholesterol (TC) and triglyceride (TG) differed significantly in T2D group when compared with healthy control group.

| Factors         | T2D group       | Control group   |
|-----------------|-----------------|-----------------|
| Gender (M/F)    | 50 (24/26)      | 50 (27/23)      |
| Age (years)     | 57 ± 8.2        | 55 ± 7.8        |
| BMI (kg/m²)     | 26.2 ± 4.1**    | 23.1 ± 3.8      |
| FBG (mmol/L)    | 13.68 ± 2.81**  | 4.96 ± 0.86     |
| C-P (ng/ml)     | 2.52 ± 0.77*    | 2.09 ± 0.72     |
| FINS (mU/L)     | 13.15 ± 5.47*   | 10.12 ± 6.41    |
| HbA1c (%)       | 9.89 ± 2.74**   | 3.21 ± 1.27     |
| LDL-C (mmol/L)  | 3.28 ± 0.37**   | 2.38 ± 0.68     |
| HDL-C (mmol/L)  | 1.18 ± 0.61*    | 1.37 ± 0.34     |
| TC (mmol/L)     | 4.70 ± 0.52**   | 4.12 ± 0.85     |
| TG (mmol/L)     | 2.49 ± 1.06**   | 1.50 ± 0.58     |

Note: Data are presented as number (percentage) for categorical data or mean ± standard deviation (SD) for parametrically distributed data.

Abbreviations: BMI, body mass index; T2D, type 2 diabetes; FBG, fasting blood glucose; C-P, c peptide; FINS, fasting insulin; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride.

*: P < 0.05 compared to control group

**: P < 0.01 compared to control group.

Circulating miR-720 in type 2 diabetes
Circulating miR-720 was significantly higher in T2D group compared with control group (Fig. 1A). There was a positive correlation between circulating miR-720 and FBG (Fig. 1B). The expression of miR-720 was negatively correlated with FINS (Fig. 1C). These results suggested that the secretion of miR-720 is closely related to T2D events.

**miR-720 effects on insulin secretion in MIN6 Cells**

To identify the effect of miR-720 on insulin secretion in MIN6 cells, we increased and decreased miR-720 expression using the miR-720 mimic and inhibitor, respectively, and detected the level of insulin secretion after stimulation with 5.7 and 16.7 mM glucose. The ELISA results showed that miR-720 overexpression inhibited insulin secretion under highglucose stimulation \((P<0.01)\) (Fig. 2A), and miR-720 downregulation promoted insulin secretion under highglucose stimulation \((P<0.01)\) (Fig. 2B). Regardless of whether miR-720 was up- or down-regulated, there was no effect on insulin secretion under basal-glucose stimulation (Figs. 2). These results indicated that miR-720 plays a negative regulatory role in GSIS, but not in insulin secretion at physiological blood glucose levels.

**Rab35 is the target of miR-720**

miR-720 mimic could down regulated Rab35 mRNA and protein expression in MIN6 Cells (Fig. 3A, 3B); miR-720 inhibitor could up regulated Rab35 mRNA and protein expression in MIN6 Cells (Fig. 3C, 3D). Relationship between miR-720 and its target Rab35 based on dual luciferase reporter gene assays. The results showed overexpression of miR-720 significantly inhibited the firefly luciferase activity of Rab35 wild type compared with miR-NC group \((P<0.05)\). However, there was no significant effect on the firefly luciferase activity of Rab35 mutant (Fig. 3E).

Subsequently, we wanted to investigate whether Rab35 had a negative effect on insulin secretion compared with miR-720 in MIN6 cells. Therefore, we constructed a miR-720 insensitive Rab35 expression vector (pcDNA3.0-Rab35). The vector only contains Rab35 coding sequence on the basis of pcdna3.0-vector to avoid the target of miR-720. Successful overexpression of Rab35 was evaluated by Western blot (Fig. 3F). Then, we analyzed the effect of Rab35 expression on insulin secretion in MIN6 cells. Compared with the control group transfected with empty vector pcdna3.0-vector, Rab35 overexpression promoted insulin secretion (Fig. 3G). The results suggest that Rab35 has an opposite effect on insulin secretion in MIN6 cells compared with miR-720, which further suggests that Rab35 may be a functional target of miR-720 in MIN6 cells. It was confirmed that miR-720 regulate insulin secretion by targeting Rab35 in MIN6 cells.

**Rab35 regulates PI3K/AKT/mTOR signaling pathway**

After overexpressed and silenced of Rab35 gene, the expression of PI3K/Akt/mTOR signaling pathway related molecules in MIN6 cells was detected by RT-PCR and Western blot respectively. The results showed that compared with the control group, the expression of PI3K, Akt and mTOR was significantly decreased by overexpression of Rab35 gene (Fig. 4A, 4B), while silencing Rab35 gene could induce the expression of PI3K, Akt and mTOR (Fig. 4C, 4D).
Discussion

Circulating miRNAs have become ideal and non-invasive molecular markers to evaluate the pathophysiological status of various diseases due to their stable changes in diseases, stable existence in serum, plasma, urine, saliva and other body fluids, and easy to obtain and preserve clinically\(^\text{(19)}\). According to some previous studies, the levels of miR-126, miR-15a and miR-223 in peripheral blood were decreased several years before the onset of T2D; compared with patients with diabetes mellitus (DM) and metabolic syndrome (MS), the serum levels of miR-9, miR-29, miR-30d, miR-34a, miR-124a, miR-146a and miR-375 were higher in newly diagnosed T2D patients\(^\text{(20)}\); compared with the control group, the serum levels of miR-375 and miR-9 were higher in patients with pre diabetes mellitus. These two miRNAs were directly related to the presence of pre diabetes and T2D, and miR-375 was independently related to the occurrence of T2D. miR-375 alone or combined with miR-9 can be used as early detection markers of pre diabetes and T2D\(^\text{(21)}\). In addition, studies have shown that miR-378, miR-126-3p and miR-223 in blood are indicators of disease staging and prognosis in elderly patients with T2D\(^\text{(22)}\). In conclusion, serum miRNAs are noninvasive and ideal indicators for the diagnosis and prognosis of T2D\(^\text{(23)}\). However, no miRNA has been recognized as a specific diagnostic marker for T2D. In this study, we found that the serum miR-720 level of 50 newly diagnosed T2D patients was significantly higher than that of healthy control group\((P<0.05)\), and miR-720 was positively correlated with blood glucose level and negatively correlated with insulin level\((P<0.05)\). This suggests that miR-720 is highly expressed in T2D patients and is related to blood glucose and insulin levels. This suggests that miR-720 may reflect the disorder of glucose metabolism in T2D patients to a certain extent, and may become a potential biomarker for the diagnosis of T2D.

Insulin release from pancreatic β cells is a necessary condition for maintaining normal glucose homeostasis in humans and many other animals. miRNAs are known to be involved in the regulation of insulin signaling pathway, but the mechanism is still unclear. Therefore, it may have important clinical value to explore the mechanism of miRNAs regulating insulin secretion by pancreatic beta cells and to understand the significance of miRNAs in T2D. Evidence suggests that miRNAs, as key regulators of gene expression, play an important role in the production, transport and secretion of insulin. The change of miRNAs expression can lead to insulin secretion dysfunction and IR, destroy insulin signaling pathway and various physiological processes, and then lead to the occurrence and development of DM\(^\text{(24)}\). As a result, the potential role of miRNAs in the treatment of T2D has been widely concerned\(^\text{(25,26)}\).

The production of insulin is the key function of pancreatic beta cells, and the release of insulin is necessary to maintain glucose homeostasis. Among the known miRNAs that play an important role in insulin secretion and glucose homeostasis, miR-375 is the most concerned\(^\text{(27)}\). Some studies suggest that miR-375 is an important regulator of pancreatic beta cells function\(^\text{(28,29)}\); overexpression of miR-375 can reduce the number and viability of pancreatic beta cells\(^\text{(29,30)}\). It can regulate the insulin secretion by directly targeting genes related to exocytosis\(^\text{(28,31)}\). In addition, miR-103 and miR-107 can regulate insulin and glucose homeostasis in vivo. They play an important role in insulin sensitivity and may be
potential targets for the treatment of T2D (20). However, the mechanism of miR-720 in regulating insulin secretion by pancreatic beta cells is still unclear. Many studies have explored the function and target of miR-720 in disease progression. Known targets of miR-720 include TWIST1 in breast cancer (11), Rab35 in cervical cancer (12), StarD13 in colorectal cancer (13) and CCND1 in pancreatic cancer (14).

Other studies have shown that miR-720 can negatively regulate p63 and promote epithelial development (32); miR-720 participates in the control of human dental pulp cell stem cell phenotype by directly inhibiting NANOG’s level (33); targeting miR-720 can help restore the immunity of patients with chronic hepatitis B (34). Our study found that overexpression of miR-720 inhibited glucose stimulated insulin secretion in MIN6 cells, while down-regulation of miR-720 promoted insulin secretion. Subsequently, we found that Rab35 was the target of miR-720 in MIN6 cells by luciferase reporter gene.

Rab proteins are also known to be important participants in exocytosis and secretion of insulin by pancreatic β cells. Rab functions are accompanied by cyclical activation and inactivation of GTP-bound and GDP-bound forms between the cytosol and plasma membrane that are regulated by upstream regulators (16, 35). In our previous study, we found that Rab35 is closely related to the exocytic and secretory function of pancreatic β cells (16). PI3K/Akt/mTOR signaling pathway regulates the life activities of many kinds of cells, including cell growth, proliferation and differentiation. Over activation of PI3K/Akt/mTOR is involved in diabetic retinopathy, diabetic nephropathy and IR (36). PI3K/Akt/mTOR is a signal pathway closely related to insulin signal transduction (6, 24). The expression of PI3K and phosphorylation of Akt in kidney, liver, skeletal muscle and adipose tissue of DM rats were significantly decreased; in the development of DM, persistent hyperglycemia can promote the activation of PI3K / Akt signaling pathway, and ultimately accelerate the development of DM (37). This study found that overexpression of Rab35 inhibited PI3K/Akt/mTOR signaling pathway and promoted insulin secretion; when Rab35 was inhibited, PI3K/ Akt/mTOR pathway was activated and insulin secretion was down regulated. Therefore, we speculate that Rab35 can regulate PI3K/Akt/mTOR signaling pathway and thus affect insulin secretion.

Conclusions

Insulin is the most important hormone regulating glucose metabolism in vivo, and the normal secretion of insulin can maintain the homeostasis of glucose metabolism (38). Studies at home and abroad have confirmed that miRNAs can regulate insulin secretion and peripheral blood miRNAs can be used as biomarkers in the diagnosis and prognosis of T2D (21). However, the research on miR-720 in T2D is still very limited. Therefore, this research fills the gap in this field to a certain extent and has research significance. In conclusion, we conclude that miR-720 may become a potential biomarker for the diagnosis of T2D; the increase of miR-720 level can inhibit Rab35 protein expression and then activate PI3K/Akt/mTOR signal pathway related to insulin signal, thus inhibiting insulin secretion of pancreatic β cells (Fig. 5).
Abbreviations

T2D: type 2 diabetes; IR: insulin resistance; miR: microRNA; FBG: fasting blood glucose; HbA1c: glycated hemoglobin; BMI: body mass index; C-P: c peptide; FINS: fasting insulin; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol; TC: total cholesterol; TG: triglyceride; DM: diabetes mellitus; MS: metabolic syndrome

Declarations

Ethical Approval and Consent to participate

All of the procedures were conducted according to the guidelines of the Medical Ethics Committees of the Health Bureau of the Guangdong Province of China. This study was approved by the Ethics Committee of the first affiliated hospital of Jinan University.

Consent for publication

Not applicable.

Availability of supporting data

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

Competing interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Authors' contributions

ZJL conceived and guided the research; DW and YLF analyzed the data; CTL wrote the manuscript and identified the research and editorial manuscript; LF reviewed manuscript. All authors read and confirmed the manuscript.

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miR-720 was closely related to type 2 diabetes. A. Circulating miR-720 in type 2 diabetes patients; B. There is a positive correlation between miR-720 and FBG in T2D patients; C. The expression of miR-720 was negatively correlated with FINS **: P < 0.01.
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Figure 2

Effects of miR-720 on insulin secretion by MIN6 Cells. Levels in MIN6 cells after transient transfection with miR-720 mimics (A) and inhibitor (B) under basal-glucose (5.7 nM) and high-glucose (16.7 mM) stimulation, Data are shown as mean±SD. ##: P < 0.01, compared with 5.7mM glucose group **: P < 0.01, compared with miR-720 NC group NC: negative control.
Figure 3

miR-720 regulate insulin secretion by targeting Rab35 in MIN6 cells. A. miR-720 mimic could down regulates Rab35 mRNA expression B. miR-720 mimic could down regulates Rab35 protein expression C. miR-720 inhibitor could up regulates Rab35 mRNA expression D. miR-720 inhibitor could up regulates Rab35 protein expression E. Relationship between miR-20a and its target Rab35 based on dual luciferase reporter gene assays F. Western blot showed that the constructed vector pcdna3.0-rab35 had a high expression level G. Compared with the control group transfected with empty pcdna3.0-vector, Rab35 overexpression promoted insulin secretion. ∗: P < 0.05
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Rab35 regulates PI3K/AKT/mTOR signaling pathway A. Western blot was used to detect the expression levels of PI3K, Akt and mTOR after Rab35 overexpression B. RT-PCR was used to detect the expression levels of PI3K, Akt and mTOR after Rab35 overexpression C. Western blot was used to detect the expression levels of PI3K, Akt and mTOR after Rab35 silencing D. RT-PCR was used to detect the expression levels of PI3K, Akt and mTOR after Rab35 silencing ∗: P < 0.05
Figure 4

Rab35 regulates PI3K/AKT/mTOR signaling pathway A. Western blot was used to detect the expression levels of PI3K, Akt and mTOR after Rab35 overexpression B. RT-PCR was used to detect the expression levels of PI3K, Akt and mTOR after Rab35 overexpression C. Western blot was used to detect the expression levels of PI3K, Akt and mTOR after Rab35 silencing D. RT-PCR was used to detect the expression levels of PI3K, Akt and mTOR after Rab35 silencing ∗: P < 0.05
Figure 5

miR-720 regulates insulin secretion by targeting Rab35/PI3K/AKT/mTOR signaling pathway
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