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Original Article

MiR-34a and miR-34c are involved in the pathogenesis of type-2 diabetes by modulating the cell cycle of pancreatic beta-cell

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Abstract: Beta-cell dysfunction is one of the main characters of type 2 diabetes; however the mechanism under this phenomenon is not well understood. It has been reported disturbed miRNA profile is related to the pathogenesis of diabetes and miR-34a overexpression is related to impaired beta cell proliferation in rats. In this study, we first detected the level of miR-34a, b and c in the serum of T2DM patients and found a higher level of miR-34a and c. Subsequently, the potential targets of miR-34 were predicted using bioinformatics tools. Using dual-luciferase assay and immunoblotting, we confirmed that CCNE2 is a direct target of miR-34a and c, and the target region is conserved in mice. Finally, we identified that miR-34a and c can induce G0/G1 arrest and apoptosis in mouse pancreatic beta-cells. Thus, our data collectively suggest that higher expression of miR-34a and miR-34c may be related to the pathogenesis of T2DM, and the serum level of miR-34a and miR-34c has a potential to be used as a biomarker for T2DM diagnosis.

Keywords: Type 2 diabetes, miRNA, CCNE2, cell cycle, apoptosis

Introduction

The number of patients suffered from diabetes is continuously increasing all over the world and among the peoples with diabetes, more than 90% have type 2 diabetes (T2DM). T2DM is characterized by insulin resistance and β-cell dysfunction. Till now, a number of studies have consistently shown that β-cell function is diminished in people with T2DM [1, 2]. Further study has shown that β-cell apoptosis is increased in patients with T2DM, whereas neither β-cell replication nor neogenesis is decreased, suggesting that increased β-cell loss is the main cause of β-cell dysfunction [3]. Research from rats also provides the evidence that ageing induced β-cell dysfunction which was associated with altered miRNAs expression profile, is involved in the pathogenesis of diabetes [4].

MiRNA is a group of endogenous, short noncoding RNAs, which regulates genes expression through targeting the 3'UTR of mRNA. MiRNAs have been found in various organisms, and many of them are evolutionary conserved. Meanwhile, it is estimated that more than a half of all human protein-coding genes are potentially regulated by miRNAs [5]. Recently, disturbed miRNAs profiles in serum and monocyte have been found in the patients with T2DM [6, 7]. Meanwhile, single-nucleotide polymorphisms in the coding regions of some miRNAs have been found to be related to the susceptibility to T2DM [8].

In this study, we first detected the expression of 3 miR-34 family members in the serum samples of patients suffered with type 2 diabetes. Subsequently, predicted and confirmed the two targets of miR-34. The biological functions of miR-34 were also examined.

Materials and methods

Subjects

A cohort of 65 subjects diagnosed with T2DM, according to the criteria of The Expert Committee on the diagnosis and classification of Diabetes Mellitus [9], were recruited from

Chinese PLA General Hospital, China. 65 healthy controls with similar ethnical and social background, neither suffering from T2DM nor other important medical disorders served as controls. Informed consents were obtained from all the participants. All study protocols and designs were approved by the ethics committees of the Chinese PLA General Hospital.

Blood samples were collected from each participant in EDTA-anticoagulated tubes. Portion of blood sample was processed for plasma separation which was stored at -80°C until used.

RNA isolation and qRT-PCR

Total RNA was extracted from 100 μl serum by using Trizol LS Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The expression level of miRNAs was detected by TaqMan miRNA RT-Real Time PCR. Single-stranded cDNA was synthesized by using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and then amplified by using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) together with miRNA-specific TaqMan MGB probes (Applied Biosystems, Foster City, CA, USA). The serum miR-16 level was quantified for normalization. Each sample in each group was measured in triplicate and the experiment was repeated at least three times for the detection of miRNAs.

Cell culture

HEK293T and HeLa cells were cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 IU/ml penicillin and 10 mg/mL streptomycin. All cells were maintained at 37°C under an atmosphere of 5% CO_2.

Islet isolation and cell culture

Islets were isolated by collagenase digestion and cultured in RPMI 1640 [10]. Dissociated islet cells were obtained by trypsin digestion.

Cell cycle analysis

Cells were seeded onto 12-well plates (1×10^5 per well). After transfection for 48 hours, cells were harvested and fixed in 70% ethanol and stored overnight at 4°C. For analysis, 1 mL of freshly prepared PBS staining solution [200 μg/mL RNase A, 20 mg/mL propidium iodide, and 0.1% Triton X-100] was added to the cells. DNA content was analyzed the same day by flow cytometry. ModFit Cell Cycle Analysis software was used to determine the cell cycle phase.

Cell apoptosis assay

Flow cytometry (FCM) was performed 48 h post-transfection in 6-well plates. The cells were resuspended in binding buffer containing Annexin V-fluorescein isothiocyanate and propidium iodide (PI) according to the manufacturer's instructions (KeyGen Biotech Co., Ltd., Nanjing, China). The samples were analyzed using a Beckman Coulter Flow Cytometer (Beckman Coulter, Fullerton, CA, USA) and experiments were performed in triplicate.

Dual luciferase assay

A segment of 1862 bp CCNE2 3'UTR segment was cloned into downstream of firefly luciferase coding region in pmirGLO plasmid (Promega, Madison, WI, USA) to generate luciferase reporter vector. For luciferase reporter assays, HEK293T cells were seeded in 48-well plates. miRNAs mimics or antagonists and luciferase reporter vector were co-transfected by using lipofectamine 2000 (Invitrogen, Carlsbad, CA USA). Two days later, cells were harvested and assayed with the Dual-Luciferase Assay kit (Promega, Madison, WI USA). Each treatment was performed in triplicate in three independent experiments. The results were expressed as relative luciferase activity (Firefly LUC/Renilla LUC).

Western blotting

Protein extracts were boiled in SDS/β-mercaptoethanol sample buffer, and 20 μg samples were loaded into each lane of 10% polyacrylamide gels. The proteins were separated by electrophoresis, and the proteins in the gels were blotted onto PVDF membranes (Amersham Pharmacia Biotech, St. Albans, Herts, UK) by electrophoretic transfer. The membrane was incubated with mouse anti-INSR monoclonal antibody (Abcam, Cambridge, MA, USA), or rabbit anti-CCNE2 monoclonal antibody (Abcam, Cambridge, MA, USA) or mouse anti-β-actin monoclonal antibody (Santa Cruz Biotechnolo-

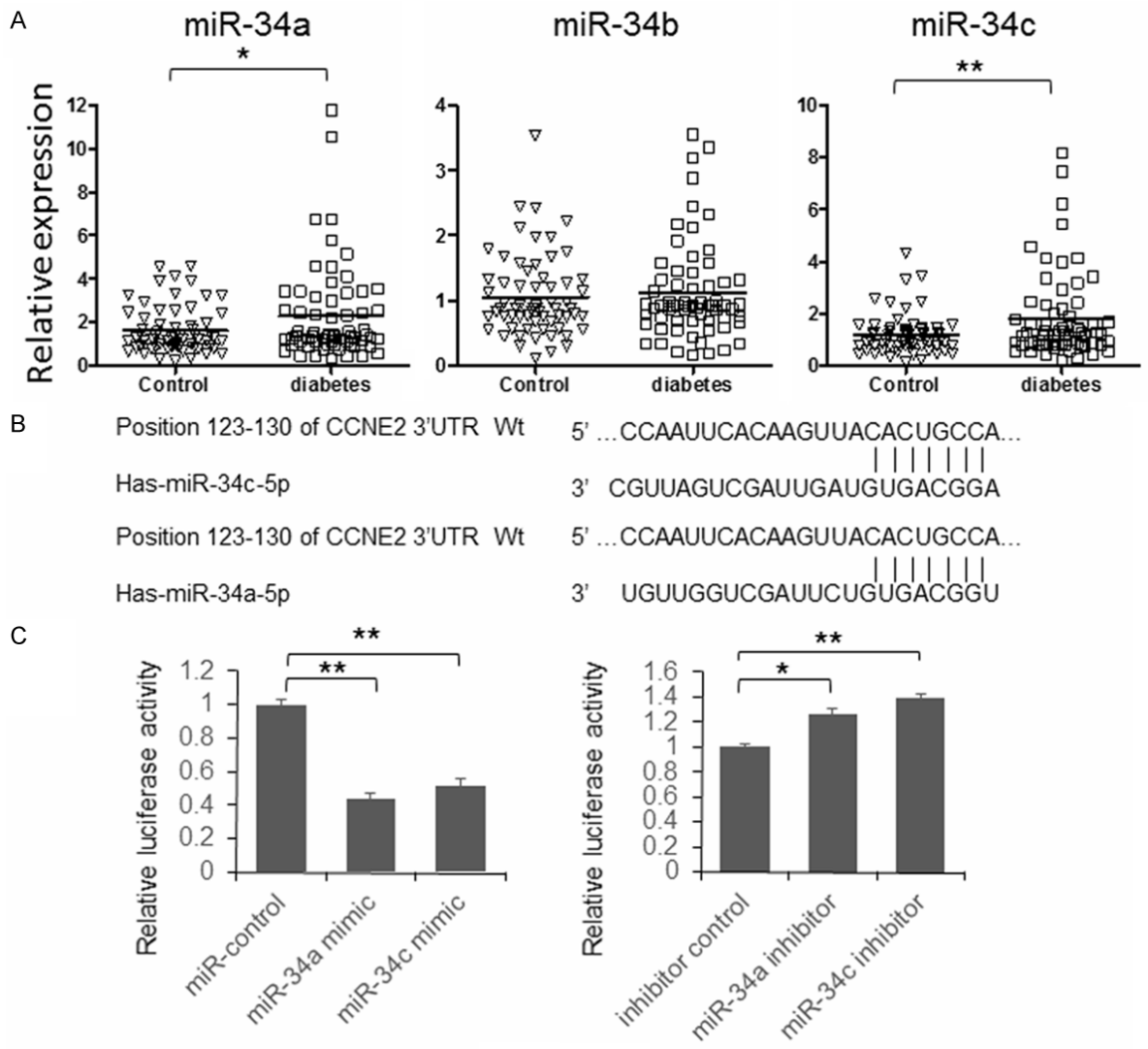

Figure 1. miR-34a and c overexpressed in serum of T2DM patients. A. Serum total RNA was isolated using Trizol and the expression of miR-34a, b and c was detected by qRT-PCR. The results were analyzed by student's t-test and $P<0.05$ was considered statistically significant. B. The schematic diagram of interaction between miR-34a/c and 3'UTR of CCNE2. C. Dual-luciferase assay to examine the interaction between miR-34a/c and CCNE2 mRNA. The results were exhibited as relative luciferase activity (firefly luciferase/renilla luciferase). Results were analyzed by student's t-test and $P<0.05$ was considered statistically significantly. $^{*}P<0.05$, $^{**}P<0.01$.

gy Inc., Santa Cruz, CA, USA) for 2 h at 37°C. The specific protein-antibody complex was detected by using horseradish peroxidase conjugated goat anti-rabbit or rabbit anti-mouse IgG. Detection by the chemiluminescence reaction was carried using the ECL kit (Pierce, Appleton, WI, USA). The β-actin signal was used as a loading control.

Statistical analysis

All the results were analyzed by using SPSS Statistical Package version 16. The data of two groups were analyzed by student's t-test and the correlation analysis was processed by χ^2-analysis. $P < 0.05$ was considered statistically significant.

Results

In this study, we first detected the expression of miR-34 family members in the serum of patients suffered from T2DM. As shown in **Figure 1A**, miR-34a and miR-34c have significant higher expression in patients' serum samples. To further explore the role of miR-34a and miR-34c during the pathogenesis of T2DM, the direct targets of them were predicted using

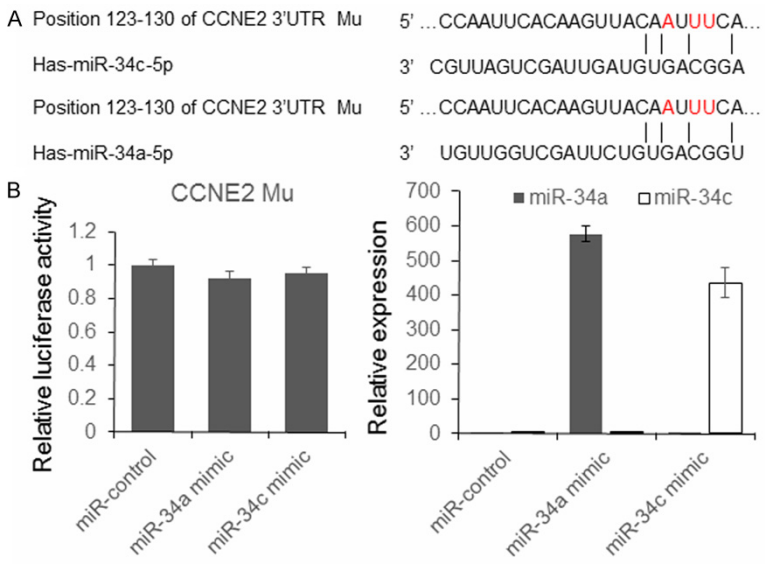

Figure 2. Examine the miR-34a/c target region of CCNE2 mRNA. A. Schematic diagram of mutant reporter vector construction. Mutated nucleotides were shown as red characters. B. miR-34a or c co-transfected with mutated reporter vector into 293T cells. The luciferase activity was detected 48 hours after transfection. The relative miR-34a and c levels were detected by qRT-PCR.

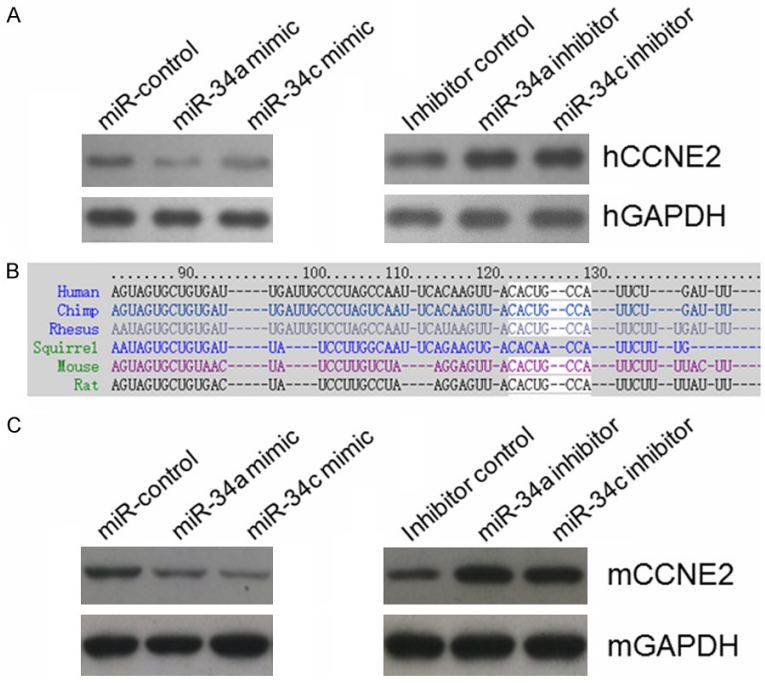

Figure 3. miR-34a and c repress CCNE2 expression in human and mouse. A. HeLa cells were transfected with miR-34a/c mimic or inhibitor. The expression of CCNE2 was detected by immunoblotting. GAPDH was used as loading control. B. Sequence conservation between human and mouse. C. Mouse pancreatic beta-cells were transfected with miR-34a/c mimic or inhibitor. The expression of CCNE2 was determined by western blot.

online bioinformatics tool: TargetScan. As shown in **Figure 1B**, there is an 8-mer predicted target region in the 3'UTR of CCNE2 messenger RNA. So we cloned the full length of 1862 bp 3'UTR of CCNE2 into pmirGLO vector, before the stop codon of firefly luciferase. Subsequently, the interaction between miR-34a/c and CCNE2 3'UTR was examined by dual-luciferase assay. As shown in **Figure 1C** (left panel), the relative luciferase activity was significantly repressed by miR-34a and miR-34c mimics and up-regulated by miR-34a or miR-34c inhibitor. When 3 nucleotides in the predicted target region were mutated (**Figure 2**), the luciferase activity was reduced in the cells transfected with miR-34a or miR-34c mimic. These results indicated that miR-34a and miR-34c can repress the expression of firefly luciferase expression by targeting 3'UTR of CCNE2.

To further examine whether endogenous CCNE2 was repressed by miR-34a and miR-34c or not, HeLa cells were transfected with miR-34a or miR-34c, with sequence scrambled short RNA as control. The cells were lysed 48 hours after transfection and the expression of CCNE2 was examined by immunoblotting. As shown in **Figure 3A**, the expression of CCNE2 is significantly repressed by miR-34a and miR-34c. Meanwhile, the inhibitor of miR-34a or miR-34c can up-regulate CCNE2 expression.

By searching the results of conservation analysis between several species on Tar-

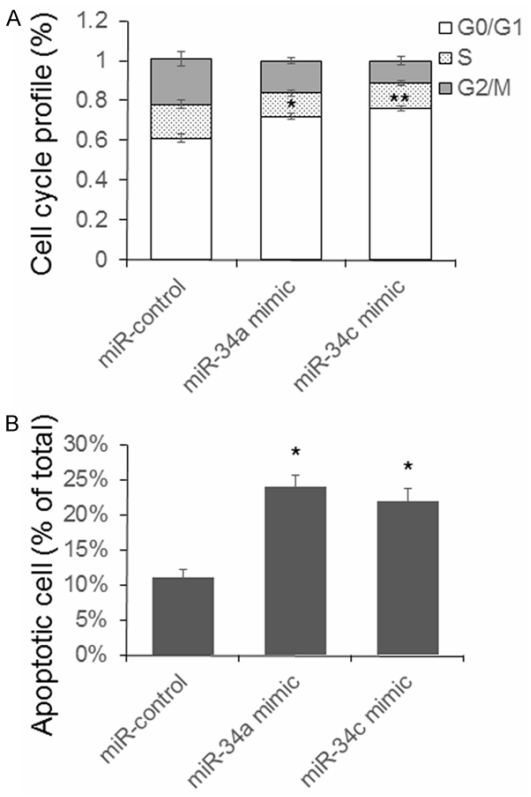

Figure 4. miR-34a and c induce beta-cell G0/G1 phase cell cycle arrest and apoptosis. Mouse pancreatic beta-cells were transfected with miR-34a or c for 48 hours. Cells were fixed and stained, and then, cell cycle or cell apoptosis were determined by flow cytometry. Results were analyzed by student's test and $P<0.05$ was considered statistically significant. $^{*}P<0.05$, $^{**}P<0.01$.

getScan, we find that the miR-34a and miR-34c target region in CCNE2 3'UTR is conserved between human and mouse (**Figure 3B**). So we detected whether overexpressed miR-34a and miR-34c can repress mouse CCNE2 expression in mouse pancreatic beta-cells. Primary mouse beta-cells were transfected with miR-34a/c mimic or inhibitor using lipofectamine 3000, the expression of CCNE2 was detected 48 hours after transfection. As shown in **Figure 3C**, the expression of CCNE2 was repressed by miR-34a/c mimics and up-regulated by miR-34a/c inhibitors.

To explore the biological function of overexpressed miR-34a and miR-34c in beta-cells, the cell cycle of mouse beta-cells was detected after miR-34a and miR-34c mimic transfection. As shown in **Figure 4A**, a significant G0/G1 arrest has been found in the cells transfected with miR-34a and miR-34c. Since miR-34a has been reported to be related to beta cell apoptosis [4], we test the effect of miR-34a and miR-34c on beta-cell apoptosis. As shown in **Figure 4**, the number of apoptotic cells raised in the miR-34a and miR-34c group.

Discussion

Beta-cell dysfunction is one of the main characters of type 2 diabetes, however the mechanism under this phenomenon is not well understood. It has been reported disturbed miRNA profile is related to the pathogenesis of diabetes and miR-34a overexpression is related to impaired beta cell proliferation in rats [4, 11]. In this study, we first detected the level of miR-34a, b and c in the serum of T2DM patients and found a higher level of miR-34a and c. Subsequently, the potential targets of miR-34 were predicted using bioinformatics tools. Using dual-luciferase assay and immunoblotting, we confirmed that CCNE2 is a direct target of miR-34a and c, and the target region is conserved in mice. Finally, we identified that miR-34a and c can induce G0/G1 arrest and apoptosis in mouse pancreatic beta-cells. However, the reason of why miR-34a and c is up-regulated is still unknown. Maybe, some factors as genetic variation and miRNA processing disturbance are involved in this process. In addition, the expression of miR-34 family members and CCNE2 were also need to be detected in the clinical tissue samples to confirm our conclusion.

In conclusion, our data collectively suggest that higher expression of miR-34a and miR-34c may be related to the pathogenesis of T2DM, and the serum level of miR-34a and miR-34c has a potential to be used as a biomarker for T2DM diagnosis.

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Disclosure of conflict of interest

None.

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