Original Article
Overexpression of miR-34c inhibits high glucose-induced apoptosis in podocytes by targeting Notch signaling pathways

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Abstract: Recent findings have shown that microRNAs play critical roles in the pathogenesis of diabetic nephropathy. miR-34c has been found to inhibit fibrosis and the epithelial-mesenchymal transition of kidney cells. However, the role of miR-34c in diabetic nephropathy has not been well studied. The current study was designed to investigate the role and potential underlying mechanism of miR-34c in regulating diabetic nephropathy. After treating podocytes with high glucose (HG) in vitro, we found that miR-34c was downregulated and that overexpression of miR-34c inhibited HG-induced podocyte apoptosis. The direct interaction between miR-34c and the 3'-untranslated region (UTR) of Notch1 and Jagged1 was validated by dual-luciferase reporter assay. Moreover, Notch1 and Jagged1 as putative targets of miR-34c were downregulated by miR-34c overexpression in HG-treated podocytes. Overexpression of miR-34c inhibited HG-induced Notch signaling pathway activation, as indicated by decreased expression of the Notch intracellular domain (NICD) and downstream genes including Hes1 and Hey1. Furthermore, miR-34c overexpression increased the expression of the anti-apoptotic gene Bcl-2, and decreased the expression of the pro-apoptotic protein Bax and cleaved Caspase-3. Additionally, the phosphorylation of p53 was also downregulated by miR-34c overexpression. Taken together, our findings suggest that miR-34c overexpression inhibits the Notch signaling pathway by targeting Notch1 and Jagged1 in HG-treated podocytes, representing a novel and potential therapeutic target for the treatment of diabetic nephropathy.

Keywords: Diabetic nephropathy, podocytes, Notch, microRNAs, cell apoptosis

Introduction

Diabetic nephropathy, a severe and common complication of diabetes, has become the leading cause of end stage renal disease [1]. Diabetic nephropathy not only affects the quality of life of patients, but also endangers their lives [2]. Understanding the underlying mechanism of diabetic nephropathy pathogenesis could provide novel insights for the development of effective therapeutics for diabetic nephropathy. Podocytes, attached to the outer surface of the glomerular basement membrane, have been suggested to play an important role in the development of diabetic nephropathy [3, 4]. High glucose (HG) has been found to contribute to podocyte injury, which is characterized by increased albumin filtration and decreased expression of podocin, nephrin and slit diaphragm-associated proteins [5, 6]. It has been reported that HG induces podocyte apoptosis in vitro and in vivo [7]. Loss of podocytes has been suggested to be the early mechanism of the pathogenesis of diabetic nephropathy [7, 8]. Therefore, targeting podocytes may provide new renal-protective drugs for diabetic nephropathy.

The initiation factors of diabetic nephropathy include glucose and lipid metabolism disorder, oxidative stress and inflammatory mediators that activate various signaling pathways [9-12] including the Notch pathway [13]. The Notch pathway is an important signal for cell differentiation and cell survival [14, 15], and is known to play critical roles in kidney development [16]. Notch proteins, including Notch1-Notch4, are transmembrane receptors and bind many
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ligands including Delta-like (Dll) 1, Dll3, Dll4, Jagged1 and Jagged2 [17]. The activation of Notch results in the sequential proteolytic cleavage of the Notch receptor, which releases Notch intracellular domain (NICD) into the nucleus, which in turn activates downstream gene transcription including that of the Hes and Hey genes [18, 19]. Nowadays, there is increasing evidence that the Notch signaling pathway is extensively involved in HG-induced podocyte apoptosis [20, 21]. Therefore, targeting the Notch pathway may provide novel insights into the mechanism underlying diabetic nephropathy and possible targets for drug development.

MicroRNAs (miRNAs) with ~24 nucleotides are non-coding RNAs that regulate gene expression by targeting the 3'-untranslated region (UTR) of the target gene to inhibit protein translation [22-24]. In this way, miRNAs have been found to be involved in various cellular processes including cell survival, differentiation and apoptosis [22]. Compelling studies have suggested that miRNAs play an important role in podocytes [25-28]. miR-93 has been found to be decreased in diabetic mice and HG-treated podocytes [29]. miR-29c is upregulated in diabetic mice and HG-treated podocytes, where it contributes to Rho kinase activation and podocyte apoptosis by targeting Sprouty homolog 1 [30]. With the development of technology, miRNAs have become key targets for disease therapy. Thereby, anti-miRNAs have already been developed in clinical trials for diseases such as hepatitis C [31]. Given the tremendous role of miRNA in disease, targeting the key miRNAs in the pathogenesis of diabetic nephropathy is expected to provide novel and potentially therapeutic strategies.

Using bioinformatic algorithms, we determined that Notch1 and Jagged1, which are critical molecules in the Notch pathway [32], are the predicted target genes of miR-34c. Given the critical roles of the Notch pathway in diabetic nephropathy, we aimed to investigate the role of miR-34c in HG-induced podocyte apoptosis and the potential underlying mechanism. Here, we demonstrate that miR-34c plays an important role in HG-induced podocyte apoptosis by interacting with the 3'-UTR of Notch1 and Jagged1 to inhibit Notch signaling activation and abrogate apoptotic and p53 pathways.

Materials and methods

Cell culture and treatment

Mouse podocytes were purchased from the Cell Resource Center of Peking Union Medical College (Beijing, China) and conditionally immortalized using a temperature-sensitive SV40 large T-cell antigen (tsA58 Tag). To induce cell proliferation, podocytes were grown in Dulbecco’s modified eagle medium (DMEM)-F12 (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Sigma, St. Louis, MO, USA) containing 10 U/ml γ-interferon, at 33°C and 5% CO2 atmosphere (growth permissive conditions). Then, cells were cultured in fresh DMEM-F12 supplemented with 10% FBS without γ-interferon and maintained at 37°C and 5% CO2 atmosphere for 10-14 days to induce quiescence (growth restrictive conditions) [33]. After that, podocytes were cultured in serum-free medium containing 30 mM D-glucose (high glucose, HG) or 5 mM D-glucose (normal glucose, NG) for 24, 48 or 72 h. pre-miR-34c (GenePharma, Shanghai, China) was administered with lipofectamine 2000 as per the supplier’s instructions.

Apoptosis assay

Cell apoptosis was examined by annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. After treatment of pre-miR-34c for 48 h, the cultured podocytes were collected and washed three times with ice-cold phosphate-buffered saline (PBS). Then, the cells were trypsinized and centrifuged at 200 g for 5 min. The pellets were collected, washed with PBS, and resuspended in binding buffer. Annexin V stock solution was added and the cells were incubated for 30 min at 4°C then stained with PI for 5 min in the dark. Finally, apoptotic podocytes were quantified by fluorescence-activated cell sorting scan (BD Biosciences, San Jose, CA, USA).

RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) or a one-step primerscript miRNA cDNA synthesis kit.
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(Takara, Dalian, China). Complementary DNA was produced using M-MLV reverse transcriptase (Life Technologies, Carlsbad, CA, USA). Gene expression was detected by RT-qPCR as follows. A mix containing the cDNA template, corresponding primers and SYBR Green qPCR Master Mix (Thermo Fisher, Shanghai, China) was subjected to RT-qPCR quantification (94°C for 4 min; 94°C for 20 s, 55°C for 30 s, and 72°C for 20 s for plate reading for 35 cycles; and melt-curve analysis from 65 to 95°C). The primers used were as follows: miR-34c (Forward: GCTGCTGATTGCACTGATTAG, Reverse: CTCAACTGGTGTCGTGGAGTC); U6 SnRNA (Forward: CTCGCTTCGGCAGCACA, Reverse: AACGCTTCACGAATTTGCGT); Notch1 (Forward: GTGGATGACCTAGGCAAGTCG, Reverse: GTCTCCTCTTGTTCTGCTC); Jagged1 (Forward: AGAAGTCAGAGTTCAGAGGCGTCC, Reverse: AGTAGAAGGCTGTCACCAAGCCAAC); Hes1 (Forward: CACGACACCGGACAAACCA, Reverse: GC-CGGGAGCTATCTTTCTTAAGT); Hey1 (Forward: AAGACGGAGAGGATCTGACAGCAG, Reverse: CAGACTCCGTTCTCTGAGGCAA); β-actin (Forward: TTCTCTCTTTGGATGGAAT, Reverse: GAGCAATGATCTTGATCTTCTTC). β-actin and U6 SnRNA were used as internal references for quantification of the relative expression of mRNA and miRNA, respectively, using the 2^ΔΔCt method.

**Western blot analysis**

Cells were lysed in cell lysis buffer (Beyotime, Haimen, China) and the protein concentration was measured by using the Bradford method. A total of 25 μg proteins were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electrophoretically transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membranes were blocked by 2.5% nonfat milk for 1 h at 37°C, and then incubated with primary antibodies at 4°C overnight. After incubating with horseradish peroxidase-conjugated secondary antibody (Boster Corporation, Wuhan, Hubei, China) for 1 h at room temperature, protein bands were visualized using an enhanced chemiluminescence detection system (Amersham, Little Chalfont, UK). The following primary antibodies were used:

**Figure 1.** The expression of miR-34c in HG-treated podocytes. Podocytes were incubated with HG (30 nM) and the miR-34c expression was analyzed by RT-qPCR at the indicated times (0-72 h). Statistical analysis was calculated by one-way ANOVA. N = 3, *P < 0.05, **P < 0.01 vs. control (0 h).

**Figure 2.** Effect of miR-34c on HG-induced podocyte apoptosis. A. Cell apoptosis was detected by flow cytometry after HG stimulation for 48 h. Podocytes treated with normal glucose (5 nM) was used as the control. Podocytes stimulated with HG were treated with PBS (Blank), control pre-miRNAs (pre-miR Ctrl) or pre-miR-34c. Podocytes were harvested and stained with Annexin V and PI before apoptotic cells were examined. B. Representative histograms show the fractions of apoptotic cells. Statistical analysis was calculated by one-way ANOVA. N = 3, **P < 0.01 vs. Normal, &&P < 0.01 vs. Blank or Pre-miR Ctrl.
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were used: antibodies against Notch1, Hes1, p53, Bcl-2, Bax and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA); antibodies against NCID were purchased from Abcam (Cambridge, UK); antibodies against Hey1 were purchased from Bios (Beijing, China); antibodies against p-p53 and cleaved Caspase-3 were purchased from Cell Signaling Technology (Danvers, MA, USA).

Dual-luciferase reporter assay

Fragments from the 3’-UTR of Notch1 and Jagged1 containing the predicted binding sequences for miR-34c were amplified and sub-cloned into pGL3 luciferase promoter vector (Promega, Madison, WI, USA). The site-directed mutagenesis of miR-34c binding sites in the 3’-UTR of Notch1 and Jag1 was performed using a Site-Directed Mutagenesis Kit (Stratagene, Hangzhou, China). pGL3 vectors (0.1 μg) containing wild type or mutated 3’-UTR fragments were co-transfected with pre-miR-34c (50 nM) in human embryonic kidney 293 (HEK293) cells using lipofectamine 2000 and cultured for 48 h. After the cells were harvested and lysed, the luciferase activity was measured using the dual-luciferase reporter assay kit (Promega) as per the supplier’s instructions.

Figure 3. miR-34c directly targets the 3’-UTR of Notch1 and Jagged1. The predicted binding sequences of the 3’-UTR of Notch1 (A) and Jagged1 (B) mRNA with miR-134. (B) Examination of the interaction between miR-34c and the 3’-UTR of Notch1 (C) and Jagged1 (D) mRNA. Cells were transfected with pGL3 reporter plasmids containing either the wild type or mutant versions of the 3’-UTR of Notch1 or Jagged1 in the presence of pre-miR-34c and cultured for 48 h before being harvested for analysis. Pre-miR Ctrl was used as the control. Statistical analysis was calculated Student t test. N = 3, **P < 0.01 vs. Pre-miR Ctrl.
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Statistical analysis

Data are presented as mean ± standard deviation (SD). Statistical analysis was performed using SPSS version 11.5 (SPSS Inc., Chicago, IL, USA) and statistical significance was calculated by one-way analysis of variance (ANOVA) among multiple groups, or Student t test between two groups. Differences were regarded statistically significant at \( P < 0.05 \).

Results

Expression of miR-34c is decreased in HG-treated podocytes

To gain an insight into the role of miR-34c in HG-treated podocytes, we firstly examined the expression profiles of miR-34c in podocytes post HG challenge via RT-qPCR. The results showed that the expression of miR-34c was significantly decreased in podocytes after stimulation with HG for 24 h, continuously decreased after 48 and 72 h (Figure 1). The data suggested a critical role for miR-34c in HG-treated podocytes.

**Overexpression of miR-34c inhibits HG-induced cell apoptosis in podocytes**

To investigate the contribution of miR-34c to HG-induced cell apoptosis in podocytes, the effect of miR-34c overexpression on cell apoptosis induced by HG was examined by flow cytometry. The results showed that HG-treated podocytes exhibited a significant increase in

Figure 4. Effect of miR-34c on the expression of Notch1 and Jagged1. (A) RT-qPCR analysis of the mRNA expression of Notch1 and Jagged1 in different groups. Podocytes treated with pre-miR-34 were stimulated with HG for 48 h before being collected for analysis. N = 3, **\( P < 0.01 \) vs. Normal, &\( P < 0.05 \) vs. Blank or Pre-miR Ctrl. (B) Western blot analysis of Notch1 and Jagged1 protein expression levels in different groups with indicated antibodies. \( \beta \)-actin was used as the control. Protein levels of Notch1 (C) and Jagged1 (D) were quantified using Image-Pro Plus 6.0 software. Statistical analysis was calculated by one-way ANOVA. N = 3, **\( P < 0.01 \) vs. Normal; &\( P < 0.05 \) vs. Blank or Pre-miR Ctrl.
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apoptotic cells as compared with normal glucose-treated podocytes, whereas pre-miR-34c treatment significantly decreased HG-induced cell apoptosis in podocytes (Figure 2A and 2B). These results imply that miR-34c is associated with HG-induced cell apoptosis in podocytes.

miR-34c inhibits the expression of Notch1 and Jagged1 by targeting the 3'-UTR

To investigate the potential mechanism of miR-34c in regulating HG-induced podocyte apoptosis, we screened the putative targets of miR-34c using bioinformatic algorithms. Interestingly, we found that Notch1 and Jagged1, which are the core molecules in the Notch signaling pathway and play critical roles in HG-induced podocyte apoptosis [32], were the predicted targets of miR-34c (Figure 3A and 3B). To validate whether miR-34c directly targets the 3'-UTR region of Notch1 and Jagged1 mRNA, a dual-luciferase reporter assay was performed. The results showed that co-transfection of pGL3-Notch1-3'-UTR with pre-miR-34c in HEK293 cells significantly decreased luciferase activity in comparison with the control group, whereas co-transfection of pre-miR-34c with pGL3-Notch1-Mut-3'-UTR containing mutations in the predicted consensus sequences for miR-134 had no apparent effect on luciferase activity (Figure 3C). Similar results were obtained using pGL3-Jagged1-3'-UTR and pGL3-Jagged1-Mut-3'-UTR with pre-miR-34c (Figure 3D).

Next, to verify whether miR-34c regulates the expression of Notch1 and Jagged1 in podocytes, we treated cells with pre-miR-34c and examined the mRNA and protein expression of Notch1 and Jagged1 by RT-qPCR and western blot analysis, respectively. The results showed that overexpression of miR-34c significantly decreased the mRNA expression levels of Notch1 and Jagged1 (Figure 4A). Consistently, western blot results (Figure 4B) showed that the protein expression of Notch intracellular domain (NICD), Hes1 and Hey1 was markedly decreased by miR-34c overexpression. These findings suggest that Notch1 and Jagged1 are direct targets of miR-34c.

Overexpression of miR-34c suppresses the activation of Notch signaling pathway

To determine the effects of miR-34c on the Notch pathway, we measured the transcription levels of Hes1 and Hey1, which are downstream genes of the Notch pathway, via RT-qPCR. The results showed that the elevated mRNA expression levels of Hes1 and Hey1 induced by HG in podocytes were significantly decreased by miR-34c overexpression (Figure 5A). Furthermore, the protein expression of Notch intracellular domain (NICD), Hes1 and Hey1 was examined by western blot analysis (Figure 5B). Consistently, the protein expression levels of
NICD, Hes1 and Hey1 were found to be markedly increased in HG-treated podocytes, but were reversed by miR-34c overexpression (Figure 5C). These results suggest that miR-34c is capable of inhibiting the Notch pathway.

**Overexpression of miR-34c inhibits apoptotic and p53 pathways**

To further delineate the molecular mechanism of miR-34c in HG-induced podocyte apoptosis, we detected the effect of miR-34c on Bcl-2, Bax, cleaved Caspase-3, phosphorylation level of p53 (p-p53), and total p53 protein levels in HG-treated podocytes. Western blot analysis revealed that HG stimulation markedly decreased the protein levels of Bcl-2, but significantly increased the protein levels of Bax and cleaved Caspase-3 (Figure 6A and 6B). Furthermore, overexpression of miR-34c significantly inhibited the p-p53 protein levels induced by HG in podocytes (Figure 6C and 6D). However, there was no difference in total p53 protein levels among the different groups. These results indicate that miR-34c inhibits the apoptotic and p53 pathways, which are activated by HG in podocytes.

**Discussion**

It has recently been reported that miR-34c can inhibit kidney fibrosis and the epithelial-mesenchymal transition [34]. The results of the present study provide evidence that the expression of miR-34c is affected by HG treatment in podocytes and that overexpression of miR-34c inhibits Notch signaling and apoptotic pathways by targeting Notch1 and Jagged1 in HG-treated podocytes. These results suggest an important role of miR-34c in regulating podocyte apoptosis.

The role of the Notch signaling pathway in diabetic nephropathy has been widely studied in recent years. Previous studies found that the Notch ligand Jagged1 and its downstream effector, Hes1, were detected in renal biopsies and tubuli of kidneys from diabetic nephropathy patients, but not in healthy controls, implying an important role of Notch pathways in the
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pathogenesis of diabetic nephropathy [13]. Niranjan et al. reported that NCID was overexpressed in glomerular epithelial cells in diabetic nephropathy and that overexpression of NCID induced podocyte apoptosis in vitro and in vivo through activation of p53 [35]. Notch1 signaling was found to be significantly increased in HG-treated podocytes and inhibition of Notch1 decreased vascular endothelial growth factor expression and increased nephrin expression, and ameliorated HG-induced podocyte apoptosis [20]. More recently, Gao et al. reported that the expression of Notch1, NCID, jagged1, Hes1 and Hey1 was highly upregulated in HG-treated podocytes and that a chemical inhibitor or specific short hairpin RNA of Notch1 effectively inhibited HG-induced podocyte apoptosis by suppressing Bcl-2 and p53-dependent apoptotic pathways [32]. Notch1, jagged1 and Hes1 were upregulated in HG-treated glomerular mesangial cells, leading to transforming growth factor-beta signaling pathway activation, which was involved in the pathogenesis of diabetic nephropathy [36]. A recent study reported that coactivator-associated arginine methyltransferase 1 degradation induced by HG facilitated Notch1-mediated podocyte apoptosis [37]. These studies have indicated that modulation of Notch1 signaling may provide novel strategies for diabetic nephropathy therapy. Here, we have demonstrated that inhibition of the Notch signaling pathway by miR-34c overexpression inhibited HG-induced podocyte apoptosis. Our data further confirm the critical role of the Notch signaling pathway in HG-induced podocyte apoptosis, revealing that miR-34c is a novel inhibitor of the Notch signaling pathway.

We found that miR34c inhibited the Notch signaling pathway by directly interacting with the 3′UTR of Notch1 and Jagged1, which are critical for Notch signaling pathway activation. The interaction between miR34c and Notch signaling pathways has been reported in other cellular processes. Bae et al. found that miR-34c directly targeted Notch1, Notch2 and Jagged1 to regulate osteoclast differentiation and bone homeostasis [38]. miR-34c was reported to attenuate the epithelial-mesenchymal transition of kidney cells by downregulation of Jagged1 [34]. Here, we found that miR-34c directly targets the 3′UTR of Notch1 and Jagged1 by dual-luciferase reporter assay. Moreover, overexpression of miR-34c inhibited the expression of Notch1 and Jagged1 in HG-treated podocytes. The activation of the Notch signaling pathway indicated by increased expression of NCID, Hes1 and Hey1 was also blocked by miR-34c overexpression.

In the current study, we found that overexpression of miR-34c inhibited the apoptotic and p53 pathway. This finding was consistent with the reports of Gao et al., who demonstrated that inhibition of the Notch pathway apparently inhibited HG-induced podocyte apoptosis by downregulation of the Bcl-2 and p53 pathways [32]. In lung cancer cells, miR-34c was found to contribute to resistance to paclitaxel-apoptosis by inhibiting p53 [39]. However, there have also been studies reporting that miR-34c has a positive role in promoting cell apoptosis [40, 41]. The apparent discrepancies between studies indicate that miR-34c may have different biological functions in different cells following different types of stimulation. Nonetheless, we have provided evidence that miR-34c attenuates HG-induced podocyte apoptosis by targeting Notch1 and Jagged1 to inhibit Notch signaling pathway activation. Given that the Notch pathway contributes to HG-induced podocyte apoptosis, miR-34c can be expected to be a novel and effective therapeutic target for the treatment of diabetic nephropathy.

Disclosure of conflict of interest

None.

Abbreviations

HG, high glucose; 3′-UTR, 3′-untranslated region; Dll, Delta-like; NICD, Notch intracellular domain; FITC, isothiocyanate; PI, propidium iodide; DMEM, Dulbecco’s modified eagle medium; FBS, fetal bovine serum.

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References

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