MicroRNA-451 regulates p38 MAPK signaling by targeting of Ywhaz and suppresses the mesangial hypertrophy in early diabetic nephropathy

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ABSTRACT

Diabetic nephropathy (DN) is a major diabetic complication. However, the initiating molecular events triggering DN are unknown. In this study we focused on microRNA-451 (miR-451), which is downregulated during early DN. We found that miR-451 negatively regulated the expression of Ywhaz through Ywhaz 3' UTR and that Ywhaz was required for the miR-451-mediated downregulation of p38 MAPK signalling. Moreover, over-expression of miR-451 inhibits glomerular mesangial cell proliferation in vitro and in vivo. These findings suggest that the growth-inhibitory effect of miR-451 may be explained in part by miR-451-induced suppression of Ywhaz and p38 MAPK signalling, providing evidence for the potential role of miR-451 in early DN.

1. Introduction

Diabetes is the leading cause of end-stage renal failure, and much of the morbidity and mortality of diabetes can be attributed to nephropathy. Diabetic nephropathy (DN) is characterized early in its course by glomerular hypertrophy and, importantly, mesangial hypertrophy, which is associated with the eventual glomerulosclerosis [1]. Despite the great progresses that have been made in recent decades, the mechanism involved in mesangial hypertrophy is not fully understood.

MicroRNAs (miRNAs) are a group of small, non-coding RNAs that negatively regulate gene expression by imprecisely binding to complementary sequences in the 3' untranslated region (UTR) of their target mRNAs. Imperfect base pairing between a miRNA and its targets suggests that miRNAs have a large regulatory potential, and miRNAs are estimated to regulate 30% of genes in the human genome [2]. Recently, it has been reported that miRNAs play a role in diabetes mellitus and its complications [3]. The pancreatic islet-specific miRNA, miR-375, plays a negative role in insulin secretion, and it is also a key determinant of blood homeostasis [4]. The overexpression of miR-29 aggravates insulin resistance [5]. MiR-133 promotes diabetic cardiopathy by inhibiting kaliun ion channel of ERG gene, indicating the participation of miRNAs in diabetes mellitus and its complications [6]. Moreover, in our previous studies, we found the deregulation of some microRNAs was a frequent event in early DN, including microRNA-451 (miR-451) [7]. However, the role of miR-451 in mediating mesangial hypertrophy of DN has never been studied.

In this study, we investigated the potential role of miR-451 in mesangial hypertrophy during early DN in vitro and in vivo. We utilized computational and experimental approaches for prediction and verification of miR-451 targets. By bioinformatics analysis, we discovered five potential target genes for miR-451. We also provided experimental evidence that the growth-inhibitory effect of miR-451 may be explained in part by miR-451-induced suppression of Ywhaz and p38 MAPK signalling.
in vitro and in vivo. Overall, these data indicate that miR-451-mediated regulatory pathways are complex and play a critical role in DN.

2. Materials and methods

2.1. MiR-451 target prediction

MiR-451 target site prediction was performed by using miRGen software [8]. The intersection of the three algorithms: miRanda (microrna.org), PicTar, TargetScan, were chosen.

2.2. Constructs, transfections, and assays

Mesangial cells of db/db DN mice (db/db-MC) were transfected with miR-451 mimics or a mimics control (Guangzhou RuiBio Corp., Guangzhou, China) [9], using Lipofectamine 2000 (Invitrogen, CA, USA), as previously described [10]. Mesangial cells of db/m mice (db/m-MC) were also transfected with miR-451 inhibitor or an inhibitor control (Guangzhou RuiBio Corp., Guangzhou, China), where all the nucleotides in the inhibitors contain 2′-OMe modifications at every base. Forty-eight hours after transfection, cells were harvested for Western blot analysis.

The 3′UTR of the Ywhaz gene was amplified by PCR from the total RNA extracted from db/db DN mesangial cells, which was extracted with a Genomic DNA Extraction kit (TaKaRa Bio Inc, Tokyo, Japan). Primers for Ywhaz 3′UTR were as follows: Ywhaz-F: 5′-CCGCTGAG CCGCTTCCAACCTTTGTTC-3′; Ywhaz-R: 5′-GAATGCCGCAG-TCC CATCATGATATTTATGACATTTAC-3′. The PCR products were excised with XhoI and NotI and cloned into pmIR-RB-REPORT™ vector (Guangzhou RuiBio Corp., Guangzhou, China). This plasmid contains a hRLuc (synthetic Renilla luciferase gene) encoding Renilla luciferase as the reporter and hLuc (synthetic firefly luciferase gene) encoding firefly luciferase as the internal control. The recombinant plasmid pmIR-RB-Ywhaz-3′UTR was confirmed by restriction enzyme digestion and DNA sequencing. A Dual-Luciferase Reporter Assay System (Promega) was used to examine the effects of miR-451 on Ywhaz expression in mesangial cells and in db/db mice, were measured by real time RT-PCR (details shown in Supplementary data).

2.5. Real time reverse transcription (RT)-PCR

RNA was extracted by Trizol reagent (Invitrogen). MiR-451 expression in mesangial cells and in db/db mice, were measured by real time RT-PCR (details shown in Supplementary data).

2.6. Growth inhibition test

MTT Cell Proliferation Assay Kit (Invitrogen) was employed to analyze cell proliferation (details shown in Supplementary data).

2.7. Glomerular morphological observation

Sieved renal glomeruli taken from mice were observed under the microscope and measured with a micrometer. The average diameter of 100 glomeruli in a group was counted in three different visual fields.

2.8. Western blot analysis

Ywhaz, p-p38 MAPK and p-MKK3 proteins were detected by Western blot using rabbit polyclonal anti-Ywhaz (sc-1019, Santa Cruz, CA, USA), mouse monoclonal anti-p-p38MAPK (sc-7973, Santa Cruz, CA, USA) and rabbit polyclonal anti-(p-MKK3) (sc-293107, Santa Cruz, CA, USA), respectively (details shown in Supplementary data).

2.9. Immunofluorescence

Expression of Ywhaz and co-expression of p-p38 MAPK and p-MKK3 proteins in mice were detected by immunofluorescence (details shown in Supplementary data).

2.10. Statistical analysis

All statistical tests were performed using SPSS software (SPSS, Chicago, IL). Comparisons between groups were carried out by ANOVA or Kruskal-Wallis tests, as appropriate, according to the Gaussian or non-Gaussian distribution of the data. Changes in each group were analyzed using one-way ANOVA or non-parametric tests. P-values < 0.05 were considered statistically significant. All data are presented as means ± standard deviation (S.D.).
3. Results and discussion

3.1. Bioinformatic analysis for miR-451 target prediction

The purpose of computational analysis was to seek potential targets for mmu-miR-451. miRGen [8], an integrated database, provides the predicted target groups by using optimized combinations of the widely used target prediction programs. To improve the accuracy of prediction, we chose the intersection of the three most widespread programs: miRanda (microrna.org), PicTar, TargetScan. As a result, we obtained five possible targets for miR-451 (Supplementary Table 1).

3.2. Ywhaz 3'UTR contains a conserved binding site for mmu-miR-451

Among these predicted candidate targets for miR-451, Ywhaz attracted our attention due to its roles on cell proliferation at some pathological conditions [14–16] and its regulation on insulin receptor signal pathway by interacting with IRS1 protein and MAPK pathway by effecting on the activation of JNK1 and p38 MAPK, the important signal pathways related to diabetes and DN [17,18]. Ywhaz (14-3-3ζ) belongs to the 14-3-3 family of proteins, which are a group of highly conserved proteins that are involved in many vital cellular processes such as metabolism, protein trafficking, signal transduction, apoptosis and cell cycle regulation [19,20].

By bioinformatics analysis, a 7-nt match (nucleotides 2–8) to the seed region at the 5' end of mature miR-451 is present in the Ywhaz 3'UTR (Supplementary Fig. 1A), which is considered to be required for the target recognition [21]. By analyzing homology, we found that the putative mmu-miR-451 target site in Ywhaz 3'UTR is highly conserved in all fourteen genomes, including human, mouse, rat, horse, monkey, and so on (Supplementary Fig. 1B). Given the analysis stated above, Ywhaz was chosen for further investigation.

3.3. Ywhaz is a target for miR-451

To verify the targeting of endogenous Ywhaz by miR-451, we transfected miR-451 mimics or NC mimics into mesangial cells of db/db DN mice because miR-451 expression was decreased in the kidney of DN [7]. It showed that the expression of miR-451 was highly increased in miR-451-mimics-transfected db/db DN cells compared with NC mimics-transfected db/db DN cells (Supplementary Fig. 2A). Western blot for Ywhaz protein showed that the expression level of Ywhaz was reduced significantly in db/db mesangial cells transfected with miR-451 mimics, compared with the NC mimics-transfected and non-transfected cells (Fig. 1A and B). No significant differences in the levels of Ywhaz protein were observed between the cells transfected with the mimics control and non-transfected cells. Then, we investigated whether Ywhaz expression is depressed by blocking miR-451 activity in db/m mesangial cells expressing native miR-451. A similar approach has been used to study the relationship between let-7 and RAS [22]. Real-time RT PCR showed that the expression of miR-451 was decreased in miR-451 inhibitor-transfected db/m cells compared with NC inhibitor-transfected db/m cells (Supplementary Fig. 2B). An approximately 50% increase of Ywhaz protein level was observed in db/m mesangial cells transfected with miR-451 inhibitor, compared to NC inhibitor-transfected db/m mesangial cells (Fig. 1A and B). These data indicate that miR-451 can negatively regulate Ywhaz expression.

Negative regulation of Ywhaz protein levels by miR-451 could be explained by the direct interaction between miRNA and mRNA or by an indirect effect. Given that the 3'UTR of Ywhaz contains a miR-451 complementary site, we propose that this regulation is direct. To test...
this, we constructed a luciferase reporter with entire mouse Ywhaz 3'UTR (Supplementary Fig. 3) and cotransfected db/db mesangial cells with each of the luciferase/Ywhaz 3'UTR reporters combined with either miR-451 mimics or the mimics control. As shown in Fig. 1C, transcripts carrying the Ywhaz 3'UTR exhibited a significantly reduction in luciferase activity in the presence of miR-451. In contrast, the mimics control had no significant effect on the luciferase activity (P > 0.05). These findings suggested that 3'UTR of Ywhaz contained regulatory information, allowing miR-451 to regulate Ywhaz. In an effort to confirm this, db/m mesangial cells were cotransfected each of the luciferase/Ywhaz 3'UTR reporters along with miR-451 inhibitor or the inhibitor control. Fig. 1D showed that miR-451 inhibitor did restore luciferase activity of Ywhaz 3'UTR. Conversely, the reporter carrying the Ywhaz 3'UTR was only weakly affected by the inhibitor control (P > 0.05). Our combined results provide a strong support that 3'UTR of Ywhaz is indeed a target of miR-451 and miR-451 negatively regulates the expression of Ywhaz through the Ywhaz 3'UTR.

3.4. Over-expression of miR-451 in vitro and in vivo

miR-451 was over-expressed in db/db DN mesangial cells by miR-451 mimics transfection and the tissues of the kidney of db/db DN mice by pGenesil-miR-451 treatment. Real time RT-PCR displayed that miR-451 was significantly enhanced in db/db-miR-451 mesangial cells compared with NC mimics transfected cells (P < 0.01, Supplementary Fig. 2A). Also, the expression of miR-451 was significantly increased in the kidney tissues of pGenesil-miR-451-treated db/db DN group compared to pGenesil-1-treated db/db DN group (P < 0.01, Supplementary Fig. 4). Together, these data demonstrated that miR-451 was over-expressed in the db/db-miR-451 mesangial cells by transfection and in the kidney tissues of db/db DN mice injected with pGenesil-miR-451.

3.5. Effects of miR-451 on biochemical parameters in vivo

At 7 weeks of age, db/db mice already showed significant hyperglycemia, whereas UAE did not increase compared with db/m mice. At 9 weeks of age, db/db mice without treatment showed significant elevation of UAE (data were not shown). Thus, 7-week-old db/db mice exhibited features similar to the human normoalbuminuric stage, and 9-week-old mice showed features similar to the human early stage of diabetic nephropathy.

Plasmids containing miR-451 (30 mg/kg/d) were injected into 7-week-old db/db mice for 2 weeks. There were no significant differences in body weight, blood glucose and UAE among the miR-451-treated-db/db group, pGennsil-1-treated db/db group and untreated db/db group (data were not shown). Therefore, these data show that there is maybe no effect on body weight, blood glucose and UAE of db/db mice when over-expressed miR-451 in vivo.

3.6. Growth-inhibitory effect of miR-451 in vitro and in vivo

To determine the role of miR-451 in cell proliferation, we examined the cell viability by MTT assay. The proliferation of miR-451 mimics-transfected mesangial cells was significantly inhibited compared with untreated db/db mesangial cells and NC mimics-transfected cells (Supplementary Fig. 5). The result is in agreement with previous data showing that over-expression of miR-451 suppresses cell growth in vitro [23]. Moreover, we evaluated the growth suppression effect of miR-451 in vivo. The diameter of glomeruli of 9-week-old mice was significantly greater in the pGenesil-1-treated and untreated db/db groups than that in the
Fig. 3. Immunofluorescence staining of the expression of Ywhaz (red), p-p38 MAPK (green) and p-MKK3 (red) in renal glomerular tissue of db/db DN mice. (A) The expression of Ywhaz was tested by immunofluorescence staining. Representative photographs were taken from renal tissues of untreated db/db (A1), pGenesil-1-treated db/db (A2), miR-451-treated db/db (A3) groups and untreated db/m controls (A4). The expression of Ywhaz in renal glomerular tissue of miR-451-treated group was reduced compared with those in the pGenesil-1-treated and untreated db/db groups. (B) The co-expression of p-p38 MAPK and p-MKK3 in renal glomerular tissue were tested by dual-fluorescence staining. Merging of both images (yellow) showed co-localization of p-p38 MAPK (green) and p-MKK3 (red) in renal tissues of db/db DN mice. Representative photographs were taken from renal tissues of untreated db/db (B1), pGenesil-1-treated db/db (B2), miR-451-treated (B3) and the normal control (B4) groups. The expression of p-p38 MAPK and p-MKK3 were co-decreased in the renal tissues in the miR-451-treated group compared with those in the pGenesil-1-treated and untreated groups. Arrows indicate renal glomerular tissue. Figures are shown at 400x magnification.
control group. Interestingly, it was smaller in miR-451-treated group than in the pGenesil-1-treated and untreated groups (Supplementary Fig. 6). Together, these data confirm that over-expression of miR-451 inhibits the glomerular mesangial cell proliferation in vitro and in vivo.

3.7. miR-451 contributes to the restoration of p38 MAPK signaling by targeting of Ywhaz in vitro

As stated above, we verified that Ywhaz is a target for miR-451. Notably, it has been reported that Ywhaz related to cell proliferation and regulates the insulin receptor signal pathway and p38 MAPK signal pathway [14,17,18], suggesting that Ywhaz maybe have a relationship with mesangial hypertrophy in early stage of DN. Therefore, it is tempting to speculate that over-expression of miR-451 might have an effect on some DN related genes via down-regulation of Ywhaz.

Moreover, p38 MAPK signaling is known to promote inflammatory and profibrotic responses and has been associated with other cellular functions such as glucose uptake, cell differentiation, apoptosis and proliferation [24–26], and highly associated with the glomerular mesangial cell proliferation and extracellular matrix deposition in early period of DN [27]. The expression and activity of p38MAPK were increased in the kidney tissues of early diabetic nephropathy rat [28] and were also enhanced in the mesangial cell cultured with high glucose [29], suggesting that p38MAPK pathway plays an important role in DN in vitro and vivo.

To test this hypothesis, we examined the protein levels of p38 MAPK signaling: p-p38 MAPK and p-MKK3. A decrease in Ywhaz and the coordinated reduce in p-p38 MAPK and p-MKK3 were observed in db/db-miR-451 mesangial cells, compared with untreated db/db mesangial cells and control db/db mesangial cells, as tested by Western blot (Fig. 2A and B). Therefore, it indicates that over-expression of miR-451 in db/db-miR-451 mesangial cells can decrease the expression of p-p38 MAPK and p-MKK3. However, the exact mechanism of miR-451-mediated depression of p38 MAPK and MKK3 is not fully clear, and further studies are needed to clarify the issue.

3.8. Effect of miR-451 on Ywhaz and p38 MAPK signaling expression in vivo

To determine the in vivo relevance of these observations, we tested the expression of Ywhaz, p-p38 MAPK and p-MKK3 from the kidney tissue of db/db DN mice. Consistent with results in vitro, the reductions of Ywhaz, p-p38 MAPK and p-MKK3 level were detected by Western blot in the kidney tissue of pGenesil-miR-451-treated db/db mice, when compared with pGenesil-1-treated db/db mice and untreated db/db mice (Fig. 2C and D). Immunofluorescence assay revealed that Ywhaz was reduced, and p-p38 MAPK and p-MKK3 were co-decreased in the kidney tissue of miR-451-treated db/db mice, when compared with pGenesil-1-treated db/db mice and untreated db/db mice (Fig. 3 A and B). Taken together, these in vivo data strongly support our in vitro findings that miR-451 represses Ywhaz expression and concomitantly reduces the expressions of p-p38 MAPK and p-MKK3 in early DN.

In conclusion, miR-451 prevented mesangial hypertrophy by targeting the reduction of Ywhaz and p38 MAPK signaling in vivo and in vitro. Therefore, miR-451 might play an important role in the pathogenesis of DN and offers a potential target for intervention and prevention of DN. Further investigations are required, including a larger number of specimens, and more studies on the “crosstalk” of the network over the deregulated miRNAs in DN are also needed.

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Appendix A. Supplementary data


References


