Original Article

MicroRNA-22 alleviates murine lupus nephritis by targeting NLRP3

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Abstract: MicroRNAs (miRNAs, miRs) have been found as a novel class of gene expression modulators. For patients with systemic lupus erythematosus (SLE), lupus nephritis (LN) is the predominant cause of morbidity and mortality. The NOD-like receptor (NLR) family, pyrin domain containing 3 (NLRP3) is a well-known member of inflammasomes, which is an essential element in promoting inflammation and autoimmunity. By carrying out microarray analysis, we found that miR-22 was deregulated in mesangial cells of LN patients compared with normal mesangial cells (MCs). Bioinformatics study showed that NLRP3 is one of the targets of miR-22. By carrying out western blot and other assays, we found that NLRP3, ASC and active caspase1 were suppressed by miR-22. Together with the results of decreased IL-1β, we confirmed that miR-22 attenuated LN by downregulating the expression of NLRP3 related genes, so as the activation of NLRP3 inflammasome.

Keywords: Lupus nephritis, microRNA, mesangial cells, inflammasome, IL-1β

Introduction

As an autoimmune disease, systemic lupus erythematosus (SLE) could affect many organs. Lupus nephritis (LN) is one of severe complications of SLE, which is characterized by immune complex-mediated renal inflammation [1]. Almost 50% incidence of morbidity and mortality is caused by LN in lupus patients [2]. Although considerable advances have been reached in therapeutic strategy, many LN patients are going through end-stage renal disease [3, 4]. Therefore, it is urgent to find out the underlying mechanisms of LN.

Increased number of mesangial cells and infiltration of inflammatory cells have been observed in LN patients. It has been found that inflammasome is motivated during inflammation [5]. The NOD-like receptor (NLR) family, pyrin domain containing 3 (NLRP3) is a well-known member of inflammasomes and essential element in promoting inflammation and autoimmunity [6, 7]. Many researchers found that immune complexes can give rise to the NLRP3 inflammasome activation in LN patients [8].

MicroRNAs (miRNAs, miR) are a group of short, endogenous, non-coding RNAs of about 22 nucleotides long [9, 10]. By binding to the 3’-untranslated region (3’-UTR) of the messenger RNA (mRNA), miRNAs induce translational repression or mRNA degradation and regulate many biological processes [11]. Recently, more and more evidence has suggested that the expression of miRNAs is related to the pathogenesis of LN.

By carrying out microarray, we found that miR-22 is deregulated in LN. Furthermore, NLRP3 is one of target genes of miR-22. The present study aimed to identify the role of miR-22 in LN and its regulatory function in modulating NLRP3 expression and activation.

Material and methods

Mice and treatment protocol

MRL/lpr mice (12 week-old, female) were purchased from Shanghai Laboratory Animal Company (Shanghai, China) and maintained under specific pathogen-free conditions in the Laboratory Animal Center. All animal experi-
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Experiments were performed with the approval of National Institute of Health Guide for Care and Use of Animals. MRL/lpr mice were divided into two groups randomly: group A (MRL/lpr mice without intervention) (n=9); group B (miR-22 injection) (n=9); The sex- and age-matched C57BL/6 mice as the control group (Ctrl) (n=6). The mice of group B were injected with 10 μg of pCMV-miR-22 plasmid (Origene Technologies, Rockville, MD), and the control group was injected with empty pCMV vector. This injection was carried out via 50 μl transfection reagent (Polyplus-transfection Inc, New York, USA) and performed once a week for a total of 8 weeks. All animals were sacrificed after various treatments. Kidney tissues were harvested and fixed in 10% neutral-buffered formalin.

Biochemistry

Blood urea nitrogen (BUN) levels in blood samples were analyzed using a commercial autoanalyzer (Beckman Coulter, Inc., USA) at the end of the experiment. Mice were placed in metabolic cages for 24-hour urine collection biweekly, starting when the mice were 12 weeks. With Multistix 10SG reagent strips (Bayer Health Care, Elkhart, IN), urinary protein excretion was tested and graded on a scale of 0-4, where 0=none, 1=30-100 mg/dl, 2=100-300 mg/dl, 3=300-2,000 mg/dl, and 4 > 2,000 mg/dl).

Histopathology and immune complex deposition

The paraffin sections of kidney were stained with reagents of hematoxylin and eosin (HE), the severities of the glomerular and renal vascular lesions were graded blindly on a scale of 0 to 3 according to the histopathology findings.

For detection of immune complex deposition, frozen kidney sections were stained for mouse immunoglobulins and C3 with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (Santa Cruz, CA, USA), or FITC-conjugated goat IgG fraction to mouse complement C3 (Cedarlane, Hornby, ON), followed with 10% fetal bovine serum blocking. To detect immune complex deposition, the fluorescence intensity was calculated and scored from 0 to 3.

Enzyme-linked immunosorbent assay (ELISA)

The level of serum anti-dsDNA (anti-double-stranded DNA) was determined by ELISA (Enzyme-linked immunosorbent assay). For details, 96-well plates were pre-coated with 5 μg/ml calf thymus dsDNA. Mice serum was added into the 96-well plates, and absorbance was measured at 450 nm.

To prepare a reference standard curve, a mouse anti-dsDNA mAb was utilized. According to the standard curve, with normal mouse IgG employed as negative control, the anti-dsDNA concentrations were quantified. Serum and kidney IL-1β level were analyzed by using commercial ELISA kits according to the manufacturer’s instructions. Optical density was measured with an ELISA plate scanner (CA94089, Molecular Devices, Sunnyvale, Canada).

Western blotting

Fresh kidney tissues was lysed in cell lysis buffer (Cell Signaling Technology, Beverly, MA), homogenized and centrifuged. The extracted protein was mixed with 2x sodium dodecyl sulfate (SDS) sample buffer at a 1:1 ratio and boiled. The supernatant of the cell lysis was collected for western blotting analysis. Separated the protein using acrylamide gel electrophoresis and transferred the protein to polyvinylidene difluoride membranes (Millipore, USA). The membranes were incubated with primary antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) for NLRP3 (1:3,000), ASC (1:2,000), caspase-1 p20 (1:2,000), and β-actin (1:4,000) at 4°C overnight. After being washed, the membranes with target protein were incubated with an HRP-conjugated secondary antibody (1:2,000), followed by visualized and densitometric analysis. The relative expression of each protein were normalized against β-actin as the loading control.

Luciferase reporter assay

The genomic miR-22 region was amplified by PCR and the PCR product was cloned into the pMIR-GLO luciferase vector, as pMIR-NLRP3-3’UTR. The Mut-NLRP3-3’UTR recombinant has a mutation in the putative miR-22 seed region. HEK-293 cells were plated in 96-well plates. When the cells group 80% confluence, cells
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A, B. Detection of NLRP3, ASC, and caspase-1 p20 by western blot. Quantitative expression was normalized against β-actin, as the endogenous control. C, D. Renal and serum IL-1β levels. ELISA was carried out and each experiment was performed three times. Values are presented as mean ± SD. *P < 0.05, **P < 0.01 vs. control groups.

Figure 2. miR-22 attenuated renal injury in MRL/lpr mice. A. Proteinuria level of mice. B. BUN levels of mice in control or miR-22 treated groups. C. D. Histopathologically scores showed the damage index of kidneys by HE staining of kidney sections. Results are presented as mean ± SD. *P < 0.05, **P < 0.01 vs. Ctrl groups.

were co-transfected with reporter construct and miR-22. Control cells were co-transfected with a non-specific sequence of miRNA and pMIR-GLO-NLRP3-3'-UTR plasmid. Cells were...
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Figure 3. Serum anti-dsDNA antibody level and renal immune complex deposition. A. By ELISA, serum anti-dsDNA antibody was assessed. B. Immune complex deposition in the kidney was scored based on fluorescence intensity by testing FITC-conjugated anti-C3 and anti-IgG antibodies. Each experiment was performed in 3 times. Results are presented as mean ± SD. *P < 0.05, **P < 0.01 vs. Ctrl groups.

Figure 4. The survival rate of MRL/lpr mice was increased with the treatment with miR-22. Treatment began at 12 weeks old, and observed for 18 weeks. Each experiment was performed three times. Results are presented as mean ± SD. *P < 0.05.

Statistical analysis

For statistical analysis, a two-tailed Student t test or one-way analysis of variance was used. All data are shown as Mean ± standard deviation (SD). Software package SPSS version (16.0) was used. To compare the survival rates, Kaplan-Meier method and log-rank analysis were employed. P < 0.05 was considered statistically significant.

Results

miR-22 inhibited NLRP3 inflammasome activation

The effects of miR-22 on NLRP3 inflammasome activation has been examined in 12-week-old MRL/lpr mice and in age-matched C57BL/6 mice as normal controls (n=6 mice per group). Enhanced protein expression of NLRP3 and ASC was observed in the kidneys from MRL/lpr mice compared with the kidneys from normal control mice (Figure 1A).

The effects of miR-22 on activation of the NLRP3 inflammasome in MRL/lpr mice were tested. Western blot analysis showed that 8 weeks of treatment with miR-22 resulted in suppressed renal expression of NLRP3, ASC, and caspase 1-p20 (P < 0.05) (Figure 1A). Furthermore, renal and serum levels of IL-1β were significantly reduced after treatment (P < 0.01) (Figure 1C, 1D).

miR-22 alleviated dysfunction in renal and injury of MRL/lpr mice

To verify the effects of miR-22 on renal function, several indicators were tested. Both the MRL/lpr group and miR-22 treated group displayed an increase in urinary protein over time, starting from week 12. As
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Sybr green RT-PCR was used to detect the effect of candidate miRNAs and confirmed miR-22 as an important molecular for forward study. To investigate the effect of miR-22, the mortality of MRL/lpr mice was measured. There is a significant survival benefit in miR-22 treated group compared with the mice in control group (Figure 4).

miR-22 is a negative regulator of NLRP3 expression

We used bioinformatics software to predict the potential targets of miR-22. We find out that NLRP3 is one of the targets of miR-22. By bioinformatical analysis, we found that the 3’UTR of NLRP3 contained the highly conserved target region for miR-22 (Figure 5). The mutation in seed sequence of the target region leads to a complete loss of regulation by miR-22 on NLRP3 (Figure 5).

Discussion

Although significant improvements in LN therapy have been achieved, the prognosis is still poor [12]. The pathogenesis of LN remains unclear. It is urgent to clarify the underlying mechanisms and to find out new means of treatment for LN.

Recent years, it is recognized that microRNA mediated post-transcription regulation of genes

Figure 5. Bioinformatics analysis of the targets of miR-22 and luciferase reports. The luciferase reporter vector containing WT NLRP3-3’-UTR or mutant 3’-UTR was cotransfected with miR-22 or control into HEK293 cells. 48 h post-transfection, firefly luciferase activities were determined and normalized to Renilla luciferase. Results are presented as mean ± SD. *P < 0.05, **P < 0.01.
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and this kind of regulation plays an important role in controlling mRNA expression. By decreasing target mRNA amounts, miRNAs exert their regulatory function in many biological processes, such as autoimmune responses. Inflammation is involved in the pathogenesis of LN. The inflammasome is found to start the inflammatory responses to various signals [13, 14]. Inflammation and injury in kidney can be caused by immune complex deposition in mesangial cells [15]. The upregulation of NLRP3 mRNA was the key step for the activation of NLRP3 [16]. The activation of NLRP3 inflammasome can be regulated at transcript levels [17]. The present study suggested that miR-22 significantly reduced the expression of NLRP3. NLRP3 is the key molecular for NLRP3 inflammasome activation [18].

In our study, significant down-regulation of miR-22 in the kidneys of MRL/lpr mice, in close association with increased IL-1β production and renal damage. MiR-22 prevented the development of LN, and also resulted in a reduction in proteinuria and BUN levels. MiR-22 decreased the levels of circulating anti-dsDNA antibodies. Thus, the results of this study indicate that miR-22 may be a way for therapy in SLE.

Diminished IL-1β secretion was also observed with miR-22 overexpression. Increased IL-18 and IL-1β implied the activation of NLRP3 inflammasome [19]. Besides, increased NLRP3 expression resulted in more cleavage of caspase-1 and release of IL-1β. By inducing the production of other cytokines, such as TNF-α, IL-17 exerts strong proinflammatory effects [20] and facilitating T-cell infiltration [21, 22]. The cytokine IL-1β is an important pro-inflammatory mediator and involved in the development of LN [23]. The production of IL-1β is controlled by the activation of the inflammasome, which is related with the expression of NLRP3 [4]. The NLRP3 inflammasome/IL-1β signaling pathway has been reported to be implicated in LN [24]. The expression of NLRP3 is increased in patients with LN [25], as well as ASC [26]. By linking NLRP3 and procaspase-1, ASC activates caspase-1 to promote IL-1β production [27]. At the same time, dsDNA and anti-dsDNA antibodies induce IL-1β production by activating the NLRP3 inflammasome [28].

In this study, miR-22 administration significantly inhibited NLRP3 inflammasome activation, as evidenced by the downregulation of NLRP3, ASC, and caspase-1 p20 expression, as well as the production of IL-1β. These results suggest that miR-22 has the effects of attenuating LN.

In conclusion, our data have shown that miR-22 effectively ameliorated LN by inhibiting NLRP3 inflammasome activation. We suppose that miR-22 may be a promising therapeutic strategy to prevent LN progression by affecting the NLRP3 inflammasome activation, which might composed a reasonable therapeutic venture.

Disclosure of conflict of interest

None.

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