

Original Article

MicroRNA-9 influence lupus nephritis by targeting STK3 related MAPK signaling transduction pathway

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Received November 20, 2016; Accepted November 28, 2016; Epub March 1, 2017; Published March 15, 2017

Abstract: Lupus nephritis (LN) is a typical characteristic of systemic lupus erythematosus. MicroRNAs (miRs) are reported to be involved in the onset and development of LN. The major purpose of the current study was to uncover the function of miR-9 in the pathogenesis of LN. Expression statuses of miR-9 and its potential target, STK3 in clinical samples were investigated. Then a dual firefly luciferase assay was conducted to reveal the regulating pattern of miR-9 on STK3. Human renal mesangial cells (HMCs) were employed to detect the effect of miR-9 down-regulated expression on viability and apoptosis in nephritis cells. The mechanism of miR-9 affecting LN was explored by measuring activities of STK3, ERK, JNK, and p38 in HMCs. It was found that miR-9 level was positively correlated with LN occurrence while STK3 was negatively correlated. Firefly luciferase assay demonstrated a negative regulation of miR-9 on STK3 transcription. Reduction of miR-9 significantly down-regulated cell viability and induced apoptosis in nephritis cells. The phenotypic changes in HMCs were accompanied by suppression of all the four studied indicators. The promoting function of miR-9 on LN was firstly elucidated in our study, and the molecule mechanism was the directly inhibition of STK3 transcription, which subsequently deactivated the downstream MAPKs.

Keywords: Lupus nephritis, MAPK, miR-9, STK3

Introduction

More than 60% patients of systemic lupus erythematosus (SLE) will suffer from impairments of lupus nephritis (LN), which represents a severe clinical manifestation of SLE [1]. LN is characterized by a higher prevalence in childhood-onset SLE cases [2], and has shown resistance to traditional biological drugs for autoimmune disorders to some extent: most patients of LN can be effectively treated with current immunosuppressants in the short term while the long-term outcome is not so satisfactory [2, 3]. The fact that some LN patients do not respond to immunosuppressive therapy is surprising in that all autoimmune diseases depend on anti-presenting cells that initiate the activation and proliferation autoreactive lymphocyte subsets. Thus, exploring factors that distinguish the pathogenesis of LN (SLE) from other autoimmune disorders are of great value for the development of novel therapies to improve the outcome of this disease. Immune complex accumulation in kidneys of SLE pa-

tients is the earliest step in the development of LN and these immune complexes trigger a series of events that result in inflammation and injury in kidneys. In recent years, studies associated with genome-wide techniques have demonstrated that numerous different genetic aberrances are present in renal disorders including patients suffering from LN [2] and propose several mechanisms to account for glomerular immune complex accumulation [4]. Although the functions of the most biomarkers for LN were only verified in certain studies, some of these pathways might have true impact on the pathogenesis of LN.

MicroRNAs (miRs), which are small non-coding RNAs and a major part of epigenetics regulation, have been shown to play an important role in pathogenesis of LN [5-7]. By partially binding to 3'UTR of targeted mRNAs, miRs regulate gene expression and functional gene network endogenously [8-11], i.e., cell developmental timing, cell cycle control, apoptosis, and carcinogenesis [12, 13]. Moreover, based on recent

studies, miRs not only contribute to the regulation of immune cell development but also to the modification of innate and adaptive immune response, which infer the determinant function of miRs in the attack of autoimmune diseases [14]. Research of Lu et al. reported that miR-145a in glomerular tissues from LN patients was up-regulated while the level of miR-145a in LN tubulointerstitial tissue was not overexpressed [15]. In addition, elevated tubulointerstitial production of miR-638 was found to be positively correlated with proteinuria and SLE Disease Activity Index (SLEDAI) score in the same study [15]. Kato et al. demonstrated that TGF- β activated Akt in glomerular mesangial cells through a miR-215a and miR-217-dependent manner, which further revealed the involvement of miRs in mesangial cell activation and in kidney disorders [16].

Taken together, miRs are being recognized as potential therapeutic targets in the treatment of LN and other autoimmune diseases. Thus, in the current study, we focused on the function of another member of miRs, miR-9, in the development of LN. The factor is reported to be down-regulated in multiple types of cancers [17]. In vertebrates, miR-9 exerts its function in different cell-autonomous effects on the proliferation, migration and differentiation of neural progenitor cells by modulating different mRNA targets [18]. Therefore, miR-9 is eliciting emerging interest for its critical role in regulating development and disease. However, to the best of our knowledge, few studies are performed to determine the function of miR-9 in the onset and development of LN. Toward this, the expression of miR-9 and its potential targeted gene STK3 (serine/threonine kinase 3) in clinical LN samples were investigated. The latter one is a key member of MAPK pathway and a novel tumor suppressor [19, 20]. Then the regulation pattern of miR-9 on STK3 was determined. The production of miR-9 in human renal mesangial cells (HMCs) were specifically regulated to determine the role of miR-9 in the onset of LN, also, to preliminarily reveal the possible mechanism through which miR-9 exerted its function in this severe autoimmune disorder.

Materials and methods

Chemicals and cell cultures

Antibodies against STK3, p38, phosphorylated-p38 (p-p38), ERK, phosphorylated-ERK (p-ER-

K), JNK, phosphorylated-JNK (p-JNK), and GAPDH were purchased from Promega (Madison, WI, U.S.A.). Mimics of miR-9 and a non-targeting version of the mimics were constructed by GenePharma (Shanghai, China). Plasmids encoding wild STK3 3'UTR sequence (psiCHECK-2-STK3) and mutant STK3 3'UTR sequence (psiCHECK-2-mutant) were constructed by Sangon Biotech (Shanghai, China). Human renal mesangial cells (HMCs) and 293T cells were purchased from cell chamber of modern analysis and test center Central in Zhongnan university (Hunan, China) and cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in an atmosphere of 95% air and 5% CO₂ at 37°C. For experimental use, cells from three to six passages were employed.

Patients and clinical sample collection

To investigate the expression patterns of miR-9 and STK3 in clinic, 20 LN kidney tissue samples and the corresponding kidney tissues samples were collected. Further, 20 blood samples of LN patients and corresponding blood samples of health human were collected as well. The sample collection was conducted from June 2013 to April 2015 in Shandong Provincial Hospital affiliated to Shandong University. All the LN patients enrolled in the current study had active LN symptom based on the ACR criteria [37] and possessed detailed information of clinicopathological and prognostic characteristics. All the controls in the current study were individually matched for age, gender and area of residence with LN patients. The study was approved by Shandong Provincial Hospital affiliated to Shandong University ethnics committee. The ethics committee approved the relating screening, inspection, and data collection of the patients, and all subjects signed a written informed consent form. All works were undertaken following the provisions of the Declaration of Helsinki.

Cell culture and transfection

In the current study, HMCs were transfected with inhibitor of miR-9 and a non-targeting inhibitor using Lipofectamine 2000 reagent according to the manufacturer's instruction (Lipofectamine 2000). Afterwards, health HMCs and inhibitor transfected HMCs were cultured for 72 h to stimulate cell proliferation. Each treatment was represented by 15 replicates. And

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every 24 h during the incubation, cells in five randomly selected wells of each treatment were collected for determination of cell viability.

Real-time quantitative PCR (RT-qPCR)

Whole RNA was extracted using Trizol method according to the manufacturers' instruction (Takara Bio Inc., Otsu, Shiga, Japan). *GAPDH* was selected as the reference gene. cDNA templates were achieved by reversely transcribing RNA using reverse transcriptase kit (DBI Bioscience, Shanghai, China). The final 20 μ L RT-qPCR reaction mixture contained 10 μ L of Bestar® SybrGreen qPCR masterMix, 0.5 μ L of each primers (*miR-9*, forward: 5'-CTCAAC-TGGTGTCTGGAGTCGGCAATTCAGTTGAGTCAT-ACAGC-3', reverse: 5'-ACACTCCAGCTGGGTCTT-TGGTTATCTAG-3'; *STK3*, forward: 5'-TGGATGG-CTCCTGAGGTGATT-3', reverse: 5'-TGTTGGTGGT-GGATTTGTGGG-3'; *ERK*, forward: 5'-TTCCAA-ATGCTGACTCCAA-3', reverse: 5'-TCGGGTCGTA-ATACTGCTCC-3'; *JNK*, forward: 5'-AAGCCATGA-GGCAAGAACTAT-3', reverse: 5'-AATACAAGCA-TCTTCTCCAGCA-3'; *p38*, forward: 5'-GAACAAG-ACAATCTGGGAGGTG-3', reverse: 5'-TTCGCATG-AATGATGGACTGAAA-3'; U6 forward: 5'-CTCGC-TTCGGCAGCACA-3', reverse: AACGCTTACGA-ATTTGCGT; *GAPDH* forward: 5'-ACACCCACTCC-TCCACCTT-3', reverse, 5'-TTACTCCTTGAGGGC-CATGT-3'), 1 μ L of the cDNA template, and 8 μ L of Rnase free H₂O. Thermal cycling parameters for the amplification were set up as following: a denaturation step at 94°C for 2 min, followed by 40 cycles at 94°C for 20 s, 58°C for 20 s and 72°C for 20 s. PCR reaction was conducted on a traditional PCR thermocycler (ABI 9700, Applied Biosystems) and relative expression level of targeted gene was calculated according to the expression of 2^{- $\Delta\Delta$ ct}.

Western blotting assay

Protein product was extracted using Whole Protein Extraction Kit according to the manufacturers' instruction (WLA019, Wanleibio, China) and GAPDH was used as reference protein. The concentration of the extracted protein samples was determined using Pierce™ BCA Protein Assay Kit (No. 23227) according to the manufacturers' instruction. For western blotting assay, 20 μ g protein sample was subject to a 13% sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subse-

quently transferred proteins onto polyvinylidene difluoride (PVDF) sheets. The membranes were washed in TBS for three times, 5 min for each time and then incubated primary antibody against STK3 (1:1500), ERK (1:1500), p-ERK (1:1500), JNK (1:1500), p-JNK (1:1500), p38 (1:1000), p-p38 (1:500) or GAPDH (1:1000) with 5% skim milk powder solution for 1 h. Afterwards, the membranes were subjected to three time 5-min TBS washing and incubated with HRP Goat anti-Rabbit IgG (secondary antibodies) (1:20000) for 40 min at 37°C. After the final three washes with TBS, the blots were developed using Beyo ECL Plus reagent and the results were observed in the Image-Pro Plus 6.0 system. The relative expression levels of STK3 in different groups were calculated with Gel-Pro-Analyzer (Media Cybernetics, USA).

ELISA

Serum and red blood cells in blood samples were separated by centrifugation at 3000 rpm for 30 min. Then the supernatants were collected for subsequent ELISA detection using a STK3 ELISA detection kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction. The production of STK3 in different samples was determined according to the OD values at 450 nm measured using a Microplate Reader (Model Benchmark).

Dual-luciferase reporter assay

To determine the regulation of miR-9 on the transcription of STK3, 293T cells were cultured to approximately 80% confluence in a 24-well plate and then co-transfected with different combinations of dual-luciferase reporter plasmids (psiCHECK-2-STK3 and psiCHECK-2-mutant) and inhibitor (miR-9 and NC) for 48 h. The activities of firefly and Renilla luciferases were determined using a Dual-Luciferase Reporter Assay System (Promega cat. no. E1910), and activity of firefly luciferase was normalized to that of Renilla luciferase.

CCK-8 assay

Cell viabilities of HMCs under different treatments at different time points were measured using a CCK-8 method. 100 μ L CCK-8 solution was added to the cultures and incubated at 37°C for 1 h. The OD values at 450 nm of differ-

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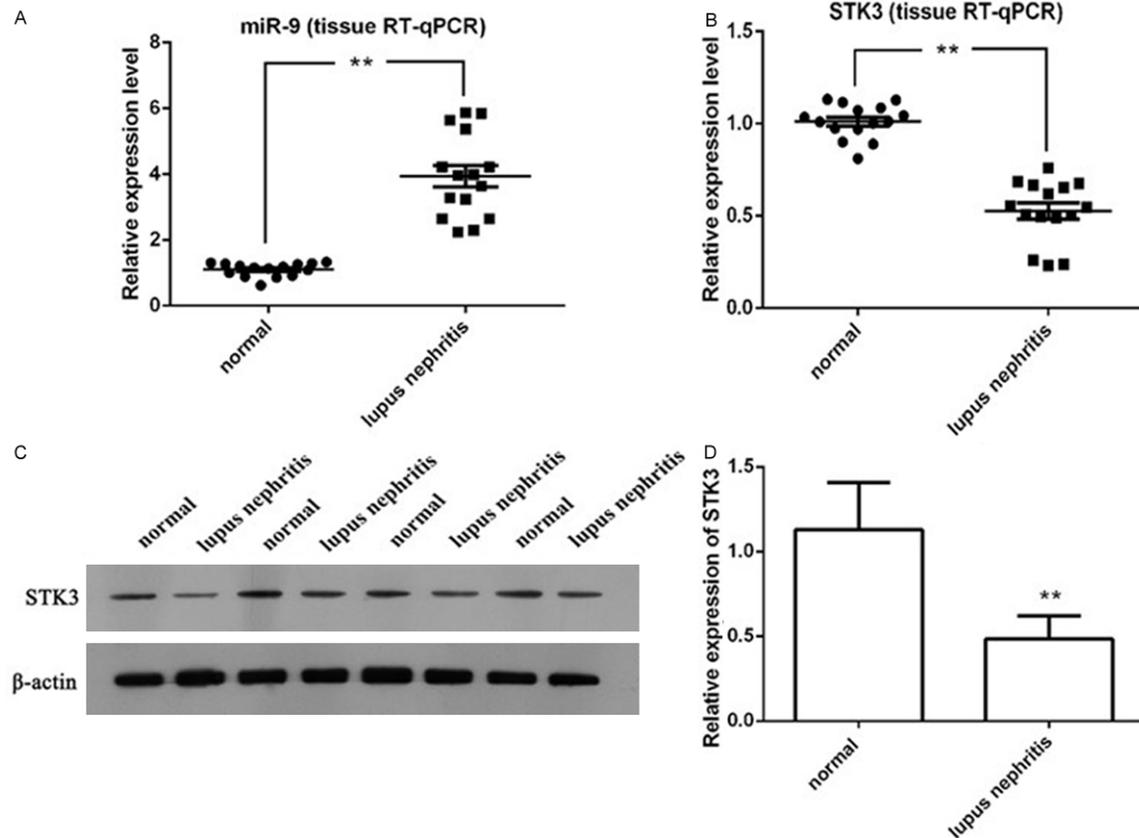


Figure 1. Production of miR-9 and STK3 in clinical LN tissues compared with normal tissues. A: Production of miR-9 in LN tissue samples were significantly up-regulated compared with control samples, the detection was performed using RT-qPCR. B: Production of STK3 in LN tissue samples were significantly down-regulated compared with control samples, the detection was performed using RT-qPCR. C, D: Production of STK3 in Part of LN tissues were significantly down-regulated compared with control samples, the detection was performed using western blotting. “***”, significantly different, $P < 0.01$.

ent treatments were recorded using a Mircoplate Reader.

Flow cytometry

Cell apoptotic rates of different treatments were determined with flow cytometry. For cell samples cultured for 48 h with 20% FBS, 5 μ L Annexin V were added into the cultures and incubated for 10 min at room temperature and then resuspended with 1 \times Binding buffer supplemented with 10 μ L propidium iodide (PI). The apoptotic rates were detected using a flow cytometer (Accuri C6, BD, USA). Apoptotic cell rate (UR+LR-all apoptosis cell percentage) was equal to the sum of the late apoptotic rate (UR, upper right quadrant-advanced stage apoptosis cell percentage) and the early apoptotic rate (LR, lower right quadrant-prophase apoptosis cell percentage).

Statistical analysis

All the data were expressed in the form of mean \pm SD. Student t-test, one-way ANOVA and post-hoc multiple comparisons using a LSD method were conducted using SPSS version 19.0 (IBM, Armonk, NY, USA). A P value less than 0.05 was accepted as statistically significant.

Results

Production of miR-9 and STK3 were up-regulated in LN tissue samples

The statuses of production of miR-9 and STK3 in LN patients and control cases were investigated with three methods. As illustrated in **Figure 1A**, the expression level of miR-9 in kidney tissues from LN patients was significantly higher than that in health people ($P < 0.01$).

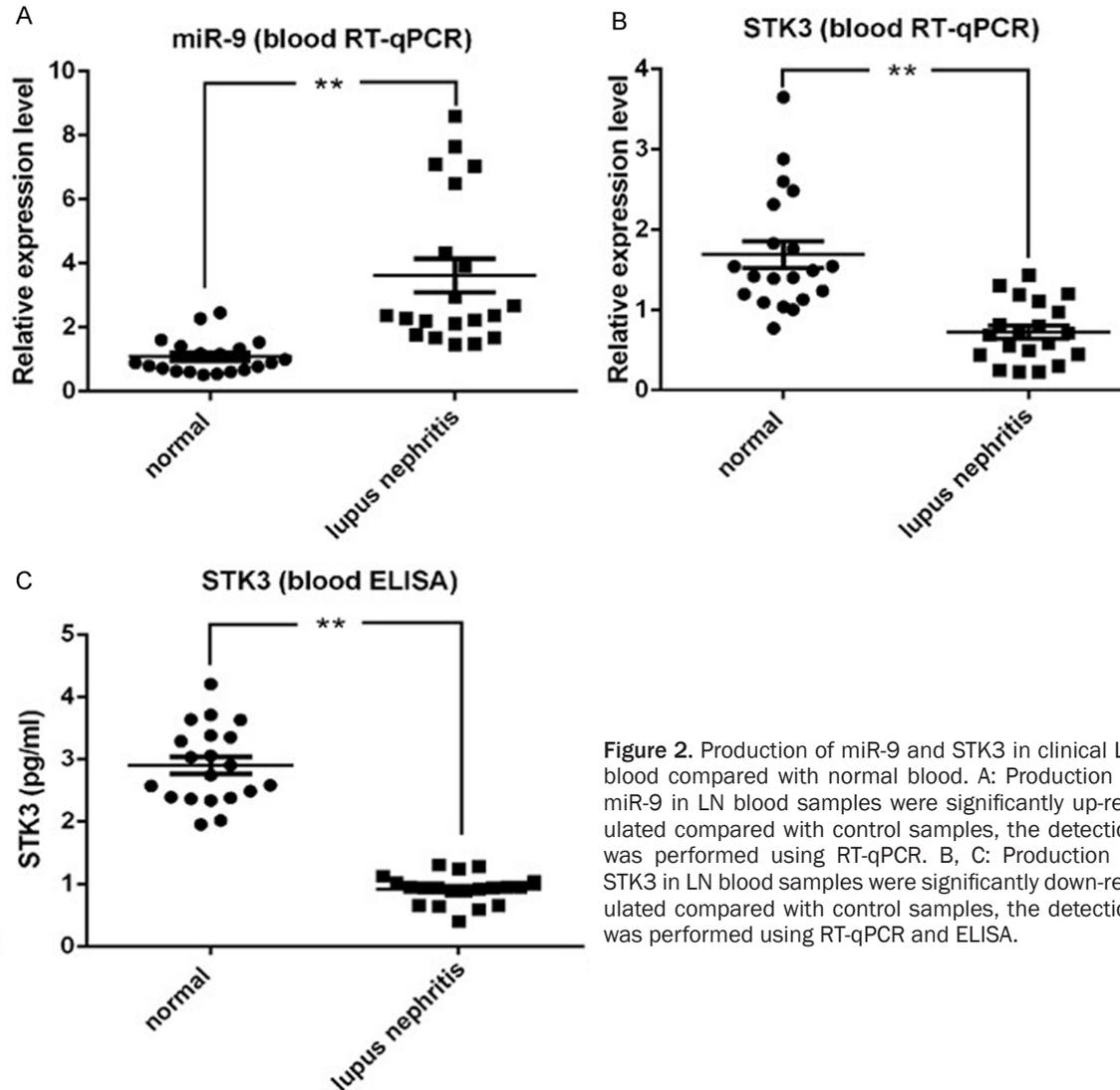


Figure 2. Production of miR-9 and STK3 in clinical LN blood compared with normal blood. A: Production of miR-9 in LN blood samples were significantly up-regulated compared with control samples, the detection was performed using RT-qPCR. B, C: Production of STK3 in LN blood samples were significantly down-regulated compared with control samples, the detection was performed using RT-qPCR and ELISA.

Similar conclusion could also be drawn from the detection using blood samples (**Figure 2A**), which further support the hypothesis that miR-9 could serve as a novel therapeutic target for LN. For the investigation of STK3, the expression pattern of the molecule was exactly opposite to that of miR-9. Production of STK3 was down-regulated both at mRNA and protein levels in kidney and blood samples from LN patients (**Figures 1B-E, 2B, 2C**), indicating the function of STK3 in promoting LN.

MiR-9 directly regulated the transcription of STK3

By using online biology software Targetscan (<http://www.targetscan.org/>), we hypothesized that STK3 may be a target gene of miR-9

(**Figure 3A**). To further verify the association between the expression of miR-9 and production of STK3, a dual luciferase assay was conducted. HMCs were co-transfected with different combinations of report plasmids and RNA mimics. It was found that only in cells transfected with psiCHECK-2-STK3 and miR-9 mimics, the activity of firefly luciferase was inhibited, and the difference compared with the other three groups was statistically significant ($P < 0.01$) (**Figure 3B**). Considering the fact that cells transfected with psiCHECK-2-mutant and miR-9 mimics didn't exhibit any change in activity of firefly luciferase, it was concluded that miR-9 directly regulated the expression of STK3 by suppressing its transcription. Meanwhile, these results were demonstrated by qPCR. As show in **Figure 3C**, when down-regulated miR-9

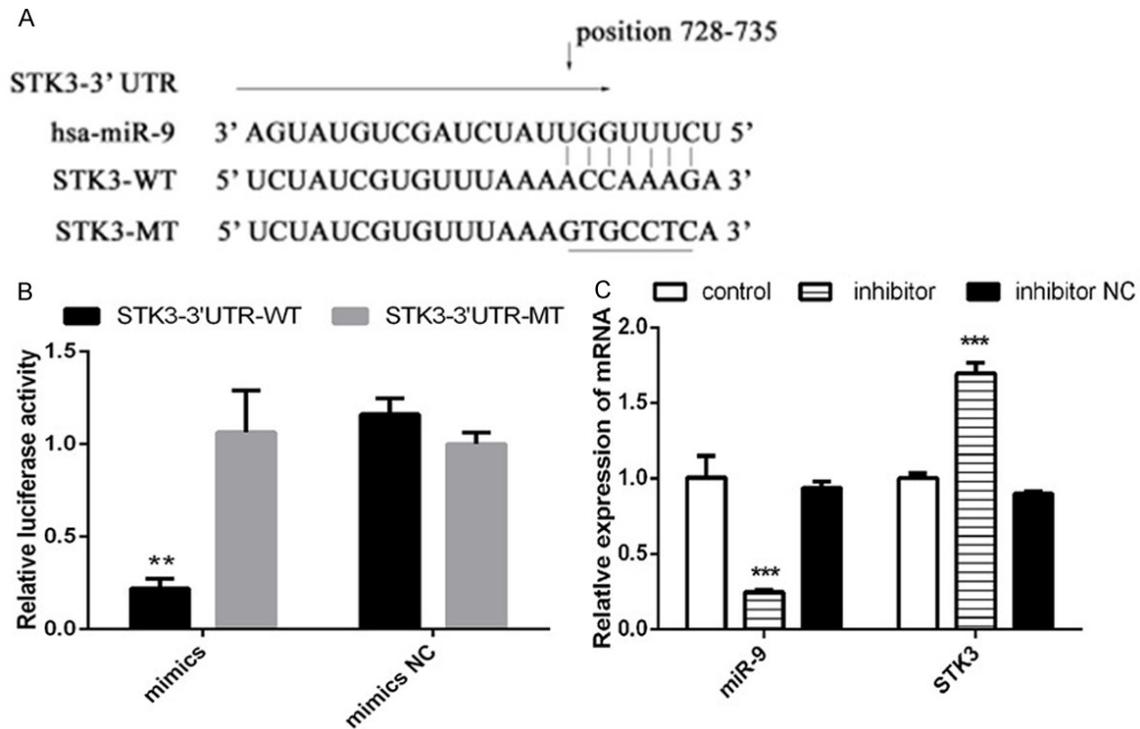


Figure 3. MiR-9 was capable of directly regulating the transcription of STK3 gene. A&B: MiR-9 mimics could reduce the activity of firefly luciferase in 293T cells transfected with wild type of STK3 3'UTR while had no influence on the firefly luciferase activity in 293T cells transfected with mutant STK3 3'UTR. C: Production of STK3 in miR-9 over-expression HMCs were significantly up-regulated compared with control group, the detection was performed using RT-qPCR. “***”, “***”, significantly different, $P < 0.01$, $P < 0.001$.

by inhibitor in HMCs, The STK3 was significantly up-regulated in both mRNA and protein levers ($P < 0.01$).

Transfection of miR-9 inhibitor attenuated the viability and induced apoptosis in HMCs

The cell viability of HMCs under different treatments was quantified using CCK-8 assay. In **Figure 4A**, compared with NC and control group, transfection of miR-9 inhibitor decreased the OD_{450} value in HMCs since the 24th hour of the assay, and the differences between miR-9 inhibitor group and control or NC group were statistically significant for all the sampling points ($P < 0.05$). Furthermore, based on the results of CCK-8 assay, cell samples collected from 48 h was employed for subsequent apoptosis and molecular detection. And post-transfection of miR-9 inhibitor, the apoptotic rate of HMCs (10.2%) increased to four-fold compared with control HMCs (2.7%) and HMCs transfected with NC inhibitor (3.0%) (**Figure 4B, 4C**), which was synchronous with change of cell viability.

MiR-9 antagonized HMCs through a MAPK pathway dependent manner

To explore the mechanism through which miR-9 altered the biological features in HMC cells, the production and activation of STK3, ERK, JNK, and p38 were quantified with RT-qPCR and western blotting assay. It was found that down-regulation of miR-9 levels in HMC activated the MAPK pathway (**Figure 5A-C**). For p38 and JNK, transfection of miR-9 inhibitor not only raised the production of total mRNA but also the phosphorylation of protein, based on the quantitative analyses, the difference between miR-9 inhibitor groups and control or NC groups was statistically significant ($P < 0.01$, $P < 0.001$). The mRNA production of ERK was inhibited, and significant influence on the phosphorylation of the total protein was detected ($P < 0.05$, $P < 0.01$). Considering the key role of all these four molecules in MAPK pathway, it was hypothesized that miR-9 could promoted the onset of LN through a MAPK pathway dependent manner.

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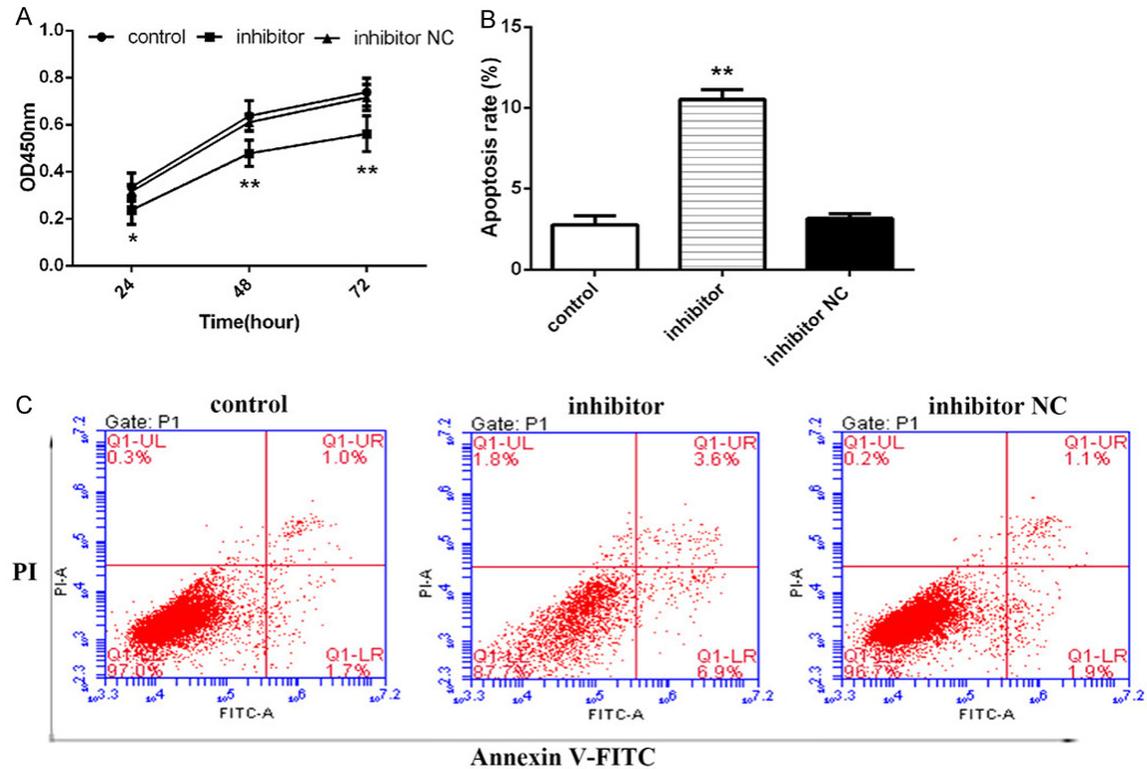


Figure 4. Transfection of miR-9 inhibitor attenuated the proliferation and induced cell apoptosis in HMCs. A: Quantitative analysis results of CCK-8 assay, at all the three time points, the OD₄₅₀ value of miR-9 inhibitor group was significantly lower than other two groups. B: Quantitative analysis results of apoptotic rates. C: Representative images of flow cytometry, the apoptotic rate of miR-9 inhibitor group was higher than the other groups. “*”, P < 0.05. “**”, P < 0.01.

Discussion

The functional significance of miR-9 is verified not only by its conservative nucleotide composition from flies to human beings but also by its diverse cell-autonomous effects on multiple biological processes [18]. Current studies regarding the function of miRs have correlated the expression pattern of miR-9 with the oncogenesis of types of tumors, and the corresponding targets involved in the regulation of miR-9 on these cancers have also been identified [21-24]. The crucial function of miR-9 in cancer development has already made the factor a hot subject, but based on recent findings, the molecule might also play a key role in other severe disease. A computational analysis focusing on lupus susceptibility genes in human beings and mice revealed that numerous target sites for over 140 conserved miRs existed in most susceptibility genes related to onset and development of lupus [25], which inferred the possible regulating effect on pathogenesis of LN by miRs

such as miR-9. With intensive effort made to verify the possibility, several LN-associated miRs, including miR-145, miR-146a, miR-638, etc, were found [26]. Although the involvement of miR-9 in LN attack has not been validated by previous studies, it was reasonable to perform a comprehensive investigation on the role of miR-9 in this serious autoimmune disorder.

In the current study, the expression status of miR-9 in clinical LN patients and control cases was detected. The results clearly demonstrated a positive association between miR-9 production and occurrence of LN, which inferred that miR-9 could serve as novel predictor for LN. Except for miR-9, the expression of STK3 was also quantified with clinical samples. Bioinformatics software prediction results showed that STK3 might be a potential target of miR-9. The molecule is also defined as mammalian STE20-like protein kinase 2 (MST2) and a members of the germinal center kinase group II (GCK II) family of mitogen-activated protein

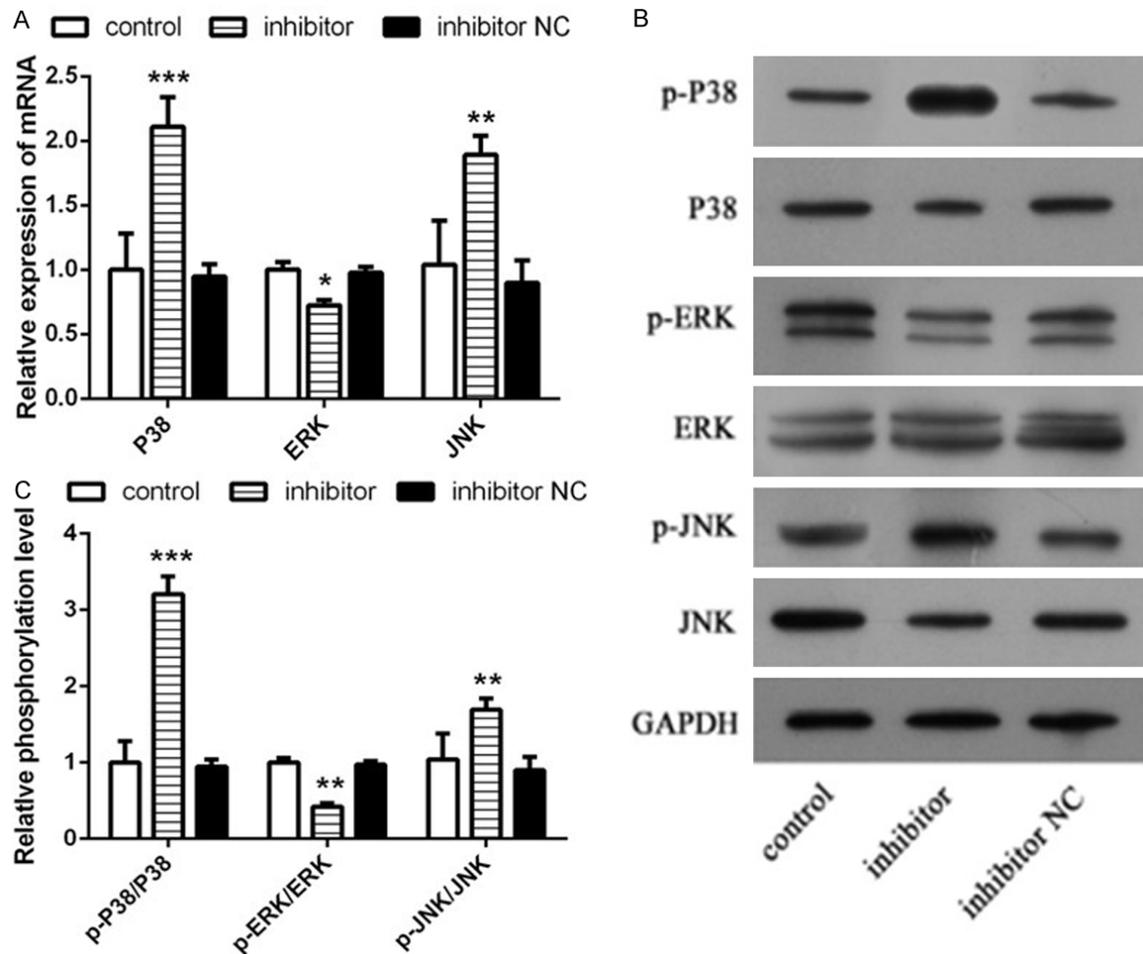


Figure 5. Interference expression of miR-9 influence the activity of MAPK pathway. A: RT-qPCR was used to detect the total production of JNK, ERK and p38. The mRNA expression of JNK, ERK and p38 was significantly up-regulated in miR-9 inhibitor groups compared with the other two groups. The total production of ERK was down-regulated by transfection of miR-9 inhibitor. B: Western blotting validation of the effect of miR-9 on the activity of MAPK pathway. For ERK, overexpression of miR-9 reduced the total production of the indicator; For JNK and p38, interference of miR-9 up-regulated the total production and phosphorylation of the indicators. C: Quantitative analysis results of western blotting. “*”, $P < 0.05$. “**”, $P < 0.01$. “***”, $P < 0.01$.

kinase (MAPK)-related kinases [19]. Previous studies have validated the inducing effect of STK3 on the activation of ERK1/2, JNK, and p38 [27, 28]. Therefore, determining the effect of miR-9 on STK3 might provide a preliminary explanation on the mechanism through which miR-9 exerted its function in the formation of LN. Contrary to the detecting results of miR-9, the transcription and expression of STK3 in LN were both down-regulated. The findings with clinical samples inspired us to conduct subsequent experiments to reveal the detailed function of miR-9 in LN and its interaction with STK3.

A series of *in vitro* experiments were conducted to further explore the role of miR-9 in LN and

the underlying mechanism related to its effect. The direct regulation on transcription of STK3 by miR-9 was first validated using a dual luciferase reporter assay, which explained the expression pattern of the two molecules detected with clinical samples. Afterwards, HMCs were transfected with different inhibitor RNAs to evaluate the potential of miR-9 in LN. According to the results of CCK-8 assay and flow cytometry, down-production of miR-9 dramatically attenuated cell viability and induced cell apoptosis in HMCs. Moreover, the influence of miR-9 on MAPK pathway was also determined. As one of the central pathways involved in numerous biological processes, MAPK pathway also contributed greatly to the proliferation and apoptosis of mesangial cells [29]. Several previous stud-

ies have shown that activation of JNK and P38 MAPKs, and deactivation of ERK in renal tissue are important elements of apoptosis [30-32]. JNK pathway can activate a variety of apoptotic, such as p53, Bcl-2 and Bcl-xl [33, 34]. P38 signaling pathways can phosphorylation growth retardation and DNA damage gene transcription factor 153 (GADD153) [35]. It can also regulate transcription the NF-kappa B pathway related gene [36]. In the current, the production and activation of JNK, and p38 were activated, and ERK were deactivated post transfection of miR-9 inhibitor, which further supported the findings in previous studies. As above mentioned, STK3 was capable to induce the activation of MAPK members [35]. Furthermore, the interaction between miR-9 and MAPK might also provide a possible explanation for the mechanism of other anti-LN therapies.

In conclusion, the potential as a LN predictor of miR-9 was firstly elucidated in the current study. Moreover, it was also proposed that the factor can directly down-regulating the transcription of STK3, which would subsequently activate the downstream JNK and P38, deactivate ERK MAPKs. However, it should be aware of that our data were only obtained from *in vitro* system and only three members of MAPK pathway were detected. Other effectors of MAPK signaling associated with the function of miR-9 in LN and whether our findings existed *in vivo* still need to be further explored.

Acknowledgements

The work is supported by National Natural Science Foundation of China (No. 81202824).

Disclosure of conflict of interest

None.

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