MiR-103a-3p antagonism attenuates pneumonia via inactivating the PI3K/AKT/NF-κB signaling pathway by targeting PTEN

CURRENT STATUS: POSTED

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DOI:
10.21203/rs.2.20468/v1

SUBJECT AREAS
General Cell Biology & Physiology Molecular Biology

KEYWORDS
miR-103a-3p, pneumonia, PTEN, PI3K/AKT/NF-κB signaling pathway
Abstract

Pneumonia accounts for approximately 15% mortalities in adolescents worldwide. MicroRNAs (miRNAs) regulate numerous diseases including pneumonia. miRNA and mRNA expression levels were detected by real time polymerase chain reaction (RT-qPCR). Protein expression levels were determined by enzyme-linked immunosorbent assay (ELISA) and western blot. The interaction between phosphatase and tensin homolog on chromosome ten (PTEN) and miR-103a-3p was explored by dual luciferase reporter assay. Cell viability and cell apoptosis were detected by cell Counting Kit-8 (CCK-8) and flow cytometry. Herein, we discovered that PTEN was decreased and miR-103a-3p was overexpressed in Ana-1 cells of in vitro pneumonia model. miR-103a-3p downregulated the expression levels of PTEN. AntagomiR-103a-3p reversed the increased cell apoptosis and decreased cell viability and inflammatory cytokine expression levels (tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6) induced by LPS in Ana-1 cells by PTEN. AntagomiR-103a-3p inhibited the activation of PTEN/PI3K/AKT/NF-κB signaling pathway induced by LPS in Ana-1 cells. Taken together, our findings exhibited that miR-103a-3p attenuated LPS induced pneumonia by blocking the activation of PTEN/PI3K/AKT/NF-κB signaling pathway and the following cell apoptosis as well as release of proinflammatory cytokines, suggesting that miR-103a-3p might serve as a novel therapeutic target for the treatment of pneumonia.

Background

In adolescents, pneumonia brings about 15% mortalities globally (1). In 2013, there were approximately 950,000 children who were less than 5 years old suffered with pneumonia (2). Vaccines against viruses, for instance, Haemophilus influenzae type b and Streptococcus pneumoniae, are introduced in a number of countries; whereas, in developing countries, it remains challenging to achieve effective protection against the viruses (2). Therefore, it is urgent to investigate novel therapeutic methods for patients with pneumonia.

MiRNAs are a type of small, non-coding RNAs which regulate mRNA expression at the transcriptional and post-transcriptional levels by targeting their 3’UTR (3, 4). MiRNAs function in numerous biological processes (5, 6). Additionally, miRNAs regulate genes which are involved in the immune system (7),
including macrophages (8); and dysfunction of miRNAs contributes to pulmonary diseases (9).

Mounting evidences prove that miRNAs are correlated with the pathogenesis of pneumonia, for instance, miR-124-3p (10), miR-125-3p (11), and miR-155 (12), etc.

PTEN was reported to be related with pneumonia, for instance, lipoxin A4 promotes the activation of alveolar epithelial sodium channel γ by miR-21/PTEN (13); miR-371b-5p induces cell proliferation in lung alveolar progenitor type II cells through targeting PTEN (14); inhibition of miR-92a inhibits LPS-induced pulmonary inflammation by targeting PTEN (15). Whether there are other miRNAs that can target PTEN are left to be investigated in the present study.

The current study demonstrated that, in LPS induced in vitro model of pneumonia, miR-103a-3p attenuated inflammation by inactivating PTEN/PI3K/AKT/NF-κB signaling pathway, indicating that miR-103a-3p has a potential to be a novel therapeutic target for the treatment of pneumonia.

Results

**PTEN was downregulated in pneumonia**

After the establishment of the in vitro pneumonia model by LPS in Ana-1 murine macrophages, the PTEN mRNA and protein levels were measured by RT-qPCR and western blot, respectively. We found that, in comparison with the control group, PTEN mRNA (Fig. 1A) and protein levels (Figure 1B&C) were significantly downregulated by LPS.

 Afterwards, LPS treated Ana-1 murine macrophages were applied for the following experiments.

**miR-103a-3p targeted PTEN 3’UTR**

MiR-103a-3p was predicted to target the position 1082-1089 of PTEN 3’UTR with the context ++ score percentile of 98 by TargetScan version 7.1 (http://www.targetscan.org/vert_71/), the corresponding prediction figure was presented in Fig. 2A. The interaction between miR-103a-3p and PTEN 3’UTR was verified by the dual-luciferase reporter assay. As for the Ana-1 murine macrophages transfected with PTEN-WT-3’UTR, there was significantly upregulated luciferase activity in the antagomiR-103a-3p group in comparison with the antagomiR-NC group (Fig. 2B), whereas, as for the Ana-1 murine macrophages transfected with PTEN-MUT-3’UTR, there was no significant difference in luciferase activity between the antagomiR-103a-3p group and the antagomiR-NC group (Fig. 2B).
miR-103a-3p inhibited PTEN expression

After the establishment of the in vitro pneumonia model by LPS in Ana-1 murine macrophages, the miR-103a-3p level was measured by RT-qPCR. The results exhibited that, in comparison with the control group, miR-103a-3p was significantly upregulated by LPS (Fig. 3A).

To verify the successful transfection of antagomiR-103a-3p into the Ana-1 murine macrophages, the difference of miR-103a-3p level between antagomiR-NC group and antagomiR-103a-3p group was detected by RT-qPCR. Results exhibited that, in comparison with the antagomiR-NC group, there was significantly downregulated miR-103a-3p level in the antagomiR-103a-3p group (Fig. 3B). Afterwards, the effects of antagomiR-103a-3p on PTEN mRNA and protein levels were measured by western blot and RT-qPCR, respectively. We found that, in comparison with the antagomiR-NC group, there were significantly upregulated mRNA and protein levels of PTEN in the antagomiR-103a-3p group (Fig. 3C-E).

miR-103a-3p was correlated with PTEN mRNA in the serum of patients with pneumonia

PTEN mRNA level was significantly decreased in the serum of in patients with pneumonia compared to the healthy volunteers (Fig. 4A). miR-103a-3p level was significantly increased in the serum of in patients with pneumonia compared to the healthy volunteers (Fig. 4B). In the serum of in patients with pneumonia, miR-103a-3p was negatively correlated with PTEN mRNA level (Fig. 4C).

AntagomiR-103a-3p rescued LPS-induced decrease of cell viability in Ana-1 murine macrophages by targeting PTEN

To explore the function of miR-103a-3p on cell viability of Ana-1 murine macrophages, cell viability was measured by CCK-8. In comparison with the siRNA group, siRNA1 PTEN and siRNA2 PTEN significantly decreased the mRNA (Fig. 5A) and protein level (Fig. 5B&C) of PTEN. On account of the more important inhibitory effects on PTEN expression, siRNA2-PTEN was used in the subsequent experiments, and named as siRNA PTEN. Compared to control group, there was significantly down-regulated cell viability of Ana-1 murine macrophages in LPS group, which was significantly rescued by antagomiR-103a-3p but not antagomiR-103a-3p+siRNA PTEN (Fig. 5D).

AntagomiR-103a-3p rescued LPS-induced cell apoptosis in Ana-1 murine macrophages by
targeting PTEN

Compared to control group, there was significantly up-regulated cell apoptosis of Ana-1 murine macrophages in LPS group, which was significantly rescued by antagomiR-103a-3p but not antagomiR-103a-3p+siRNA PTEN (Fig. 6A&B).

**AntagomiR-103a-3p rescued LPS-induced overactivation of PTEN/PI3K/AKT/NF-κB signaling pathway by targeting PTEN**

To explore the function of miR-103a-3p on signal pathway in pneumonia, PI3K/AKT phosphorylation and NF-κB activity were measured by western blot. In comparison with the control group, LPS significantly upregulated p-PI3K and p-AKT level (Fig. 7A&B) as well as p-p65 level (Fig. 8A&B), which were markedly downregulated by antagomiR-103a-3p but not siRNA PTEN+antagomiR-103a-3p.

**AntagomiR-103a-3p rescued LPS-induced increase of TNF-α, IL-1β and IL-6 levels by targeting PTEN**

To explore the function of miR-103a-3p in inflammation of pneumonia, RT-qPCR and ELISA were applied for the measurement of mRNA and protein level of inflammatory cytokines, including TNF-α, IL-1β and IL-6. As for mRNA level, RT-qPCR results exhibited that, in comparison with the control group, there were significantly upregulated mRNA levels of TNF-α, IL-1β and IL-6 in the LPS group, which were significantly downregulated by antagomiR-103a-3p but not antagomiR-103a-3p+siRNA PTEN (Fig. 9A). As for protein level, ELISA results exhibited that, in comparison with the control group, there were significantly upregulated protein concentration of TNF-α, IL-1β and IL-6 in the LPS group, which was significantly downregulated by antagomiR-103a-3p but not siRNA PTEN+antagomiR-103a-3p (Fig. 9B).

**Discussion**

PTEN plays a pivotal role in various cellular processes, for instance, inflammation (16). miRNAs function by targeting mRNAs (3, 4). In the present study, PTEN 3’UTR was found to be targeted by miR-103a-3p; in addition, PTEN was downregulated while by miR-103a-3p was upregulated by LPS, indicating the involvement of miR-103a-3p in pneumonia. As previously reported, miR-103a-3p plays a role in numerous cancers, for instance, miR-103a-3p regulated the progression of human gastric...
cancer (17) and glioma (18). Currently, we further enriched the function of miR-103a-3p in human diseases, we also demonstrated the potential role of miR-103a-3p in pneumonia. Interestingly, during our conduction of the study, there was also a novel report showing the increased level of miR-103a-3p in the serum of children with pneumonia (19), which further verified our findings in pneumonia regarding the role of miR-103a-3p.

Cell apoptosis is involved in the process of pneumonia. Inhibition of cell apoptosis suppresses the progression of pulmonary fibrosis (20) and LPS-induced acute pneumonia (21, 22). Herein, we found that, LPS induced cell apoptosis in Ana-1 murine macrophages was reduced by miR-103a-3p/PTEN axis, with the potential molecules and/or signaling pathways unelucidated.

PTEN inhibits the activation of PI3K/AKT signaling pathway, which was also related with cell apoptosis (17). Meanwhile, PTEN also regulates the activation of PI3K/AKT signaling pathway in pneumonia. Such as, PTEN regulated AKT pathway in LPS-induced inflammatory lung injury (13); controlled PI3K/AKT pathway in lung alveolar progenitor type II cells (14); inactivated AKT/NF-κB pathway in pulmonary inflammation (15). Moreover, mounting studies are showing the involvement of PI3K/AKT signaling pathway in pneumonia, such as, knockdown of HAGLROS inhibits cell apoptosis and inactivates PI3K/AKT signaling pathway (22); inhibition of PI3K/AKT signaling pathway attenuates the reinfection of streptococcus pneumoniae (23). Consistently, the p-PI3K and p-AKT levels were measured in the present study, LPS induced upregulation of p-PI3K and p-AKT, which was reduced by antagomiR-103a-3p.

PI3K/AKT signaling pathway serves as an upstream activator of the NF-κB signaling pathway (24). Furthermore, studies also have indicated the involvement of AKT/NF-κB pathway in LPS-induced inflammatory responses in murine macrophages (15, 25). In addition, the activation of NF-κB is a prerequisite for production of various inflammatory cytokines, including TNF-α, IL-1 and IL-6 (26), generating a much more severe inflammatory response in stimulated macrophages (27). Expression levels of proinflammatory cytokines including IL-6, TNF-α and IL-1β are correlated with pneumonia (28), which are also reported to be induced by LPS in the in vitro pneumonia model in A549 cells or monocytes cells (29, 30). Consequently, NF-κB p-p65, IL-6, TNF-α and IL-1β expression levels were
measured in the present in vitro pneumonia model. miR-103a-3p was found to attenuate pneumonia, evidenced by the downregulation of cytokine release induced by LPS including TNF-α, IL-1β and IL-6 as well as the inactivation of NF-κB p-p65 induced by LPS from Ana-1 cells following antagomiR-103a-3p.

Conclusions
miR-103a-3p suppressed cell apoptosis and inflammation in pneumonia by inhibiting the activation of PTEN/PI3K/AKT/NF-κB signaling pathway induced by LPS in macrophages.

Materials And Methods

Cell culture
The Ana-1 murine macrophages were purchased from American Type Culture Collection (ATCC). Ana-1 murine macrophages were incubated in RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 1% penicillin and streptomycin in 95% humidified atmosphere and 5% CO₂ at 37°C.

For the establishment of an in vitro pneumonia model, the Ana-1 murine macrophages were induced by 1 µg/ml LPS (10; 31), and they were randomly separated into 4 groups, including control group, LPS group, LPS+antagomiR-103a-3p group and LPS+antagomiR-103a-3p+siRNA PTEN group.

Serum samples
The blood samples were obtained from 29 healthy children (16 males and 13 females, aged from 3 to 13 years old) and 29 patients with pneumonia (19 males and 10 females, aged from 3 to 13 years old) in Guizhou Provincial People’s Hospital (Guizhou, China) between Mar, 2016 and Feb, 2018. Serum samples were obtained via centrifugation at the speed of 1,000 x g for 10 min, then the supernatant was collected for the subsequent experiments. The present study was approved by the Ethical Committee of Guizhou Provincial People’s Hospital (approval no. 2016011605). All the parents or the legal guardians of the participants provided written informed consent prior to the conduction of the present study.

Transient transfection
antagomiR-103a-3p, antagomiR-negative control (NC), siRNA, siRNA1 PTEN and siRNA2 PTEN were
synthesized by GenePharma Corporation (Shanghai, China). Cell transfection of antagomiR-103a-3p, antagomiR-NC and/or siRNA, siRNA PTEN was conducted using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer’s protocols.

**Cell viability assay**

Cell viability was determined by a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) in accordance with the manufacturer’s protocols. Ana-1 murine macrophages (5x10^3 cells/well) were placed in 96-well plates and incubated for 24 h at 37°C. After that, 10 µl CCK-8 solution was added to each well of the 96-well plates. Ana-1 murine macrophages were incubated for 2 h at 37°C. The absorbance at 450 nm was recorded.

**Cell apoptosis assay**

Cell apoptosis was detected by Annexin-V/Dead Cell Apoptosis kit (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were then diluted in 100 µl 1X Annexin-binding buffer to 1x10^6 cells/ml. Annexin V (5 µl) and propidium iodide (1 µl) were added to the above cell suspension. Cells were placed at room temperature for 15 min. Afterwards, annexin-binding buffer (400 µl) was added. Stained cells were analyzed by BD FACSCalibur flow cytometer (BD Biosciences).

**ELISA**

The Ana-1 murine macrophages (5x10^5 cells/well) in 4 groups were plated in their corresponding 24-well plate, followed by incubation in 95% humidified atmosphere and 5% CO₂ at 37°C overnight. Afterwards, the protein levels of TNF-α, IL-6 and IL-1β from the supernatants of Ana-1 murine macrophages were assessed by ELISA kits for mouse TNF-α (BMS607-3) which was purchased from Invitrogen (Carlsbad, CA, USA), and ELISA kits for mouse IL-6 (RAB0308) and IL-1β (RAB0274) which were purchased from Sigma-Aldrich (St. Louis, MO, USA).

**RT-qPCR**

TRIZol (Invitrogen, Carlsbad, CA, USA) and mirVana kit (Applied Biosystems, Thermo Fisher Scientific, CA, USA) were used for the extraction of RNA in the detection of RNA and miRNA, respectively. TaqMan Gene Expression Assays kit and TaqMan MicroRNA Reverse Transcription kit (Applied
Biosystems, Thermo Fisher Scientific, CA, USA) were applied for the reverse transcription of RNA and miRNA, respectively. RT-qPCR reactions were carried out with the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). The expression level of miR-103a-3p and the mRNA levels of IL-6, TNF-α and IL-1β were normalized to U6 and GAPDH, respectively. The data was analyzed with the quantification $2^{-\Delta\Delta Cq}$ method (32).

**Western blotting**

The Ana-1 murine macrophages were first subjected to RIPA (Roche Diagnostics, Basel, Switzerland) for the extraction of total proteins. Then, the proteins were subjected to electrophoresis on 8% SDS-polyacrylamide gels and transferring to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked by 5% non-fat milk at room temperature for 1 h, and incubated with primary antibodies against GAPDH (#5174, 1: 1000), PTEN (#9188, 1: 1000), p-PI3K (#17366, 1: 1000), PI3K (#4249, 1: 1000), p-AKT (#4060, 1: 1000), AKT (#4685, 1: 1000), NF-κB p-p65 (#3033; 1: 1,000) and NF-κB p65 (#8242; 1: 1,000), which were purchased from Cell Signaling Technology (Boston, MA, USA) at 4°C overnight. GAPDH served as an internal control. The membranes were incubated with anti-rabbit IgG (7074, 1:1,000, Cell Signaling Technology, Boston, MA, USA). The protein bands were visualized by the enhanced chemiluminescence detection system (PerkinElmer Life and Analytical Sciences, Boston, USA). Densitometric analysis was performed with Quantity One 4.62 (Bio-Rad).

**Dual luciferase reporter assay**

miR-103a-3p was first predicted to target PTEN 3’UTR by TargetScan version 7.1 (http://www.targetscan.org/vert_71/).

Thereafter, PTEN wild-type (WT) 3’UTR containing complementary sequences for the seed sequence of miR-103a-3p was amplified by PCR and cloned into the psi-CHECK-2 Vector (Promega, Madison, MI, USA), while a mutant type (MUT) 3’UTR of PTEN was generated by QuikChange II Site-Directed Mutagenesis Kit (Stratagene, USA). The promoters of miR-103a-3p were cloned and inserted into the upstream of luciferase gene in the pGL3-Basic vector (Promega).

As for luciferase activity, the Ana-1 murine macrophages were plated into 96-well plates and co-
transfected with 400 ng of either psi-CHECK-2-PTEN-WT-3’UTR or psi-CHECK-2-PTEN-MUT-3’-UTR and 50 ng antagomiR-103a-3p or antagomiR-NC by Lipofectamine 2000 (Invitrogen). At 48 h later, the firefly luciferase activity was detected by the dual luciferase assays system (Promega). And Renilla luciferase activity served as the internal control.

**Statistical analysis**

Data were analyzed by GraphPad Prism software version 5.04 (San Diego, CA, USA). The differences between two groups were analyzed by student's t-test, while the differences among 4 groups were analyzed by one-way analysis of variance followed by Bonferroni's post hoc test. Data were presented as the mean ± standard error of the mean. Experiments were performed at least 3 times. P<0.05 indicated a statistically significant difference.

**List Of Abbreviations**

MiRNAs MicroRNAs

RT-qPCR real time polymerase chain reaction

ELISA enzyme-linked immunosorbent assay

UTR untranslated region

PTEN phosphatase and tensin homolog on chromosome ten

CCK-8 Cell Counting Kit-8

TNF-α tumor necrosis factor-α

IL-1β interleukin-1β

LPS lipopolysaccharide

PI3K phosphatidylinositol kinase

AKT protein kinase B

NF-κB nuclear factor kappa-B

ATCC American Type Culture Collection

NC negative control

WT wild-type

MUT mutant
Declarations

**Ethics approval and consent to participate**

The present study was approved by the Ethical Committee of Guizhou Provincial People’s Hospital (approval no. 2016011605).

**Consent for publication**

All the parents or the legal guardians of the participants provided written informed consent prior to the conduction of the present study.

**Availability of data and materials**

The data are available from the corresponding author upon request.

**Competing interests**

There was not any type of conflict of interests in current study.

**Authors’ contributions**

Conceived the study: XYZ, CZ;

Carried out the experiments and analyzed the data: LX, SQY, ZHOY;

Wrote the paper: CZ.

All the authors have received the final version of the manuscript and approved the submission.

**Funding**

Current study was funded by the Respiratory Disease Clinical Research Center in Guizhou Province (2016-2907) and Science and Technology Project of Guizhou Province (2017-1100; 2019-1195).

**Acknowledgements**

Not applicable.

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Figures
Figure 1

PTEN was markedly downregulated in LPS treated Ana-1 murine macrophages. PTEN mRNA level (A) and protein levels (B and C) were significantly downregulated by LPS compared to the control group. ** p<0.01, *** p<0.001 vs. control.
miR-103a-3p targeted PTEN 3'UTR (A). The interaction between miR-103a-3p and PTEN was proved by dual-luciferase reporter assay (B). ** p<0.01 vs. antagomiR-NC.
miR-103a-3p inhibited PTEN levels. miR-103a-3p level was significantly upregulated by LPS compared to the control group (A). Compared with the antagomiR-NC group, there was significantly lower miR-103a-3p level in antagomiR-103a-3p group (B). Compared with the antagomiR-NC group, antagomiR-103a-3p significantly upregulated the mRNA level (C) and protein levels (D and E) of PTEN. ** p<0.01 vs. control or antagomiR-NC.
miR-103a-3p negatively correlated with PTEN in patients with pneumonia. PTEN mRNA level was significantly decreased (A) while miR-103a-3p level was significantly increased (B) in the serum of in patients with pneumonia compared to the healthy volunteers. In the serum of in patients with pneumonia, miR-103a-3p was negatively correlated with PTEN mRNA level (C). ** p<0.01 vs. healthy volunteers.

Figure 4
Inhibition of miR-103a-3p reversed LPS-induced decrease of cell viability by PTEN. In comparison with the siRNA group, siRNA1 PTEN and siRNA2 PTEN significantly decreased the mRNA (A) and protein level (B and C) of PTEN. AntagomiR-103a-3p significantly rescued LPS-induced down-regulation of cell viability, which was reversed by siRNA PTEN (D). * p<0.05 LPS vs. control; ** p<0.01 siRNA1 PTEN, siRNA2 PTEN vs. siRNA; # p<0.05 LPS+antagomiR-103a-3p vs. LPS; & LPS+siRNA PTEN+antagomiR-103a-3p vs. LPS+antagomiR-103a-3p.
Inhibition of miR-103a-3p reversed LPS-induced cell apoptosis by PTEN. Compared to control group, there was significantly up-regulated cell apoptosis of Ana-1 murine macrophages in LPS group, which was significantly rescued by antagomiR-103a-3p but not antagomiR-103a-3p+siRNA PTEN (A and B). *** p<0.01 LPS vs. control; ## p<0.05 LPS+antagomiR-103a-3p vs. LPS; && LPS+siRNA PTEN+antagomiR-103a-3p vs. LPS+antagomiR-103a-3p.
Inhibition of miR-103a-3p reversed LPS-induced activation of PI3K/AKT signaling by PTEN. AntagomiR-103a-3p significantly rescued LPS induced up-regulation in p-PI3K and p-AKT protein levels, which were reversed by siRNA PTEN (A and B). ** p<0.01 LPS vs. control; # p<0.05 LPS+antagomiR-103a-3p vs. LPS; & LPS+siRNA PTEN+antagomiR-103a-3p vs. LPS+antagomiR-103a-3p.
Figure 8

Inhibition of miR-103a-3p reversed LPS-induced activation of NF-κB signaling by PTEN

AntagomiR-103a-3p significantly rescued LPS induced up-regulation in p-p65 protein levels, which were reversed by siRNA PTEN (A and B). ** p<0.01 LPS vs. control; # p<0.05 LPS+antagomiR-103a-3p vs. LPS; & LPS+siRNA PTEN+antagomiR-103a-3p vs. LPS+antagomir-103a-3p.
Inhibition of miR-103a-3p reversed LPS-induced increase of TNF-α, IL-1β and IL-6 levels by PTEN AntagomiR-103a-3p significantly rescued LPS induced up-regulation in mRNA (A) and protein levels (B) of TNF-α, IL-1β and IL-6, which were reversed by siRNA PTEN. ** p<0.01,
*** p<0.001 LPS vs. control; # p<0.05 LPS+antagomiR-103a-3p vs. LPS; & LPS+siRNA
PTEN+antagomiR-103a-3p vs. LPS+antagomiR-103a-3p.