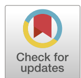
Jornal de Pediatria 2023;**99(2)**: 187−192

Jornal de
Pediatria
www.jped.com.br

ORIGINAL ARTICLE

The immunogenic involvement of miRNA-492 in mycoplasma pneumoniae infection in pediatric patients



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Received 16 February 2022; accepted 7 July 2022
Available online 1 October 2022

KEYWORDS
Mycoplasma pneumoniae pneumonia (MPP);
miRNA-492;
IL-6;
IL-18;
HMGB1;
Inflammatory responses

Abstract

Objective: This study aimed to evaluate the role of miRNA-492 in the progression of mycoplasma pneumoniae (MP) infection in pediatric patients.

Methods: Forty-six children admitted to the present study's hospital and diagnosed with mycoplasma pneumonia were recruited as the study group from March 2018 to August 2019, and 40 healthy children were selected as the control group.

Results: The expression levels of miRNA-492, TNF-α, IL-6 and IL-18 in the study group were significantly higher than those in the control group ($p < 0.05$). There was no significant correlation between miRNA-492 and most of the immune-correlated indicators in the study group, except for IL-6, IL-18 and HMGB1. Meanwhile, overexpression of miRNA-492 increased IL-6 secretion in PMA-activated monocytes ($p < 0.01$).

Conclusion: The present study's results suggested that miRNA-492 might play a role in the pathogenesis of mycoplasma pneumoniae pneumonia in children by regulating the secretion of immune-inflammatory factors such as IL-6 and IL-18 in the mononuclear macrophages.

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Introduction

Mycoplasma pneumoniae pneumonia (MPP) is a type of primary atypical pneumonia, which is one of the most serious infectious diseases among children.[1] It is mainly caused by mycoplasma pneumoniae (MP) infection.[2] In recent years, the number of children infected with MPP has increased significantly, and the incidence has shown a significant upward trend. MP invasion activates the immune system to produce multiple inflammatory factors, which accumulate locally and gradually spread from the upper respiratory tract to the lower respiratory tract.[3] For example, the manifestations of pharyngitis and nasopharyngitis are caused by ascending infection; while descending infection can cause lower respiratory tract infection with the clinical manifestations of bronchiolitis, pneumonia, etc.[4] In severe cases, it can affect the brain, heart, liver, and kidneys of children, causing complications such as encephalitis, myocarditis, and hepatitis.[5]

At present, the specific pathogenesis of MPP in children is not clear. The adsorption mechanism of respiratory epithelial cells is currently recognized as the direct route of MP

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https://doi.org/10.1016/j.jped.2022.07.010
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infection. MP directly invades and destroys epithelial cells, and combines with antigen components to form immune complexes, thereby activating the complement system and immune cells to suppress immune function and destroy immune regulation.[2,6] In addition, the humoral immune theory and cellular immune theory of MPP are gradually being revealed.[7] CD3, CD4 and CD8 molecules are the surface antigens of lymphocytes, which are mainly responsible for cellular immunity and function to resist viruses and regulate the immune system. CD3+ cells are mature T lymphocytes, indicating the immune function status of human cells; CD4+ T cells are inducible T cells/helper T cells, which are important hub cells for regulating immune responses; CD8+ T cells are suppressor T cells/cytotoxic T cells, which are direct killer cells during immune responses. Under normal circumstances, the number of T cells and their subgroups are relatively stable. However, activation of immune system antigens leads to reduced numbers of CD3 +, CD4 + and CD4 + /CD8 + T cells and disrupted immune regulation.[8] IgA, IgG, IgM, and their complements C3 and C4 are activated by B lymphocytes, differentiated into plasma cells, and secreted to produce immunoglobulins, which are the main substances of the humoral immune system.[9] White blood cells (WBC) and C-reactive protein (CRP) are sensitive indicators of systematic inflammation. Cytokines are produced by immune cells and play a role in the inflammatory response and immune response during immune damage caused by MPP, and can regulate the function of immune cells.[10]

MicroRNA (miRNA) is involved in regulating post-transcriptional gene expression in animals and plants.[11] A large number of studies have shown that miRNA-492 can regulate the expression of a variety of proteins including CD147, and play an important role in tumorigenesis, growth and metastasis, as well as insulin resistance, angiogenesis and other biological processes.[12,13] More importantly, the target protein CD147 of miRNA-492 plays an important role in multiple immune-related biological processes such as T lymphocyte development, humoral immunity, and cellular immunity.[14] At present, there are few studies on miRNA-492 in MPP. Therefore, understanding the involvement of miR-492 in the immune-inflammatory mechanism caused by MP infection can provide new ideas for exploring the intervention methods against MPP.

Methods

Study subjects

A total of 46 children (30 males, 16 females) with MPP admitted to Zibo Central Hospital from March 2018 to August 2019 were included in this study, with an average age of 6.4 ± 2.9 years. The diagnostic criteria were in line with the 8th edition of Practical Children science. During the same period, 40 healthy children (28 males and 12 females) in the Pediatric Care Department of the hospital were selected as the control group, with an average age of 6.2 ± 2.6 years. All subjects included in this study had no serious diseases such as heart, liver, kidney and tumor, no congenital malformations, and no recent history of infection. This study has been approved by the hospital ethics committee, and all subjects have obtained parental consent and signed informed consent.

Inclusion criteria: children with respiratory symptoms such as fever, cough, dyspnea, and pulmonary rales; children with unilateral or bilateral abnormal changes in chest imaging; laboratory test positive for MP-specific IgM antibody (microparticle agglutination method, the acute phase titer $>$1:80 is positive, and the MP antibody titer in the recovery phase and acute phase is 4 times or more increased or decreased).

Exclusion criteria: children with other mixed infections, such as viruses and bacteria; children with congenital lung diseases; children with other respiratory diseases, such as tuberculosis and bronchial asthma; children with blood system diseases; children with tumor diseases and immune diseases; the guardian declined to participate in the study.

The general information of the enrolled children, including gender, age, length of hospital stay, and past medical history, were collected. The clinical characteristics of the enrolled children were collected, including fever peak, cough, lung auscultation, and the presence or absence of pleural effusion and other internal and external pulmonary complications. The laboratory test indicators were collected on the day of admission, including routine blood and C-reactive protein levels. On the day of admission, chest radiograph, electrocardiogram, etiological examination, myocardial enzymes, and liver and kidney function indexes were routinely performed.

Specimen collection

Fasting anticoagulant blood (2 mL) was collected on the morning of the next day after admission. Within 2 h after collection, the blood was centrifuged at 2500 rpm for 10 min at 4 °C, and the upper layer of yellow plasma was aspirated for freezing storage at −80 °C. The remaining blood cells were separated by the erythrocyte lysis method to obtain white blood cells. After that, 1 mL Trizol was added to obtain the white blood cell Trizol mixture, which was frozen at −80 °C for subsequent use.

Quantitative PCR

The white blood cell Trizol mixtures of 46 cases in the study group and 40 cases in the control group were dissolved on ice. The RNA extraction kit (Qiagen, Germany) was used to extract and purify leukocyte RNA. The UV spectrophotometer was used to determine the absorbance (A) value at 260 nm to calculate the RNA concentration, which was controlled to be between 0.15 and 1.00. Reverse transcription was performed afterward, which strictly followed the instructions of the mi-Script II reverse transcription kit. The reaction product was diluted with enzyme-free water and stored in a refrigerator at −20 °C for later use. Relevant primers were designed and synthesized by Qiagen, and the reaction mixture was prepared according to the instructions of the mi-Script SYBR Green Kit. Each reaction system was 15 μL, and the reaction conditions were as follows: 95 °C pre-activation for 15 min, 94 °C denaturation for 15 s, 55 °C annealing for 30 s, 70 °C for 30 s, a total of 45 cycles. U6 and GAPDH were used as the internal controls. Each reaction

was duplicated with 3 wells, and the internal reference was used for normalization. The primers were as follows:

miRNA-492:
F: 5'-GGGGTACCCCCTGGCTGGAACAGAAGAT-3';
R: 5'-CCCAAGCTTCCCTGGTCTTGGCTGGGATC-3'
HMGB1:
F: 5 ’ -CGGGATCCGATGGGCAAAGGAGATCCTAAAA-3′
R: 5′-CCCTCGAGTTCATCATCATCATCTTCTTCTT-3′.
U6:
F: 5’-GTGCGTGTCGTGGAGTCCG-3’;
R: 5’-AACGCTTCACGAATTTGCGT-3’;
GAPDH:
F: 5’-ATGCCTCCTGCACCACCAACTGCTT-3’;
R: 5’-TGGCAGTGATGGCATG- GACTGTGGT-3’.

ELISA detection

ELISA kit for tumor necrosis factor (TNF)-a (Cat# BMS223-4), IL-6 (Cat# BMS213-2), and IL-18 (Cat# BMS267-2) were obtained from eBioscience (San Diego, USA) and the assay was performed according to the manufacturer’s recommended procedures. All samples were assayed in triplicates. Levels of cytokines in each sample were extrapolated from standard curves generated in parallel using kit-provided standards.

Monocyte experiment

THP-1 cells were cultured following the vendor’s suggested procedure. Lentivirus vectors for overexpressing miRNA-492 were constructed and stably co-transfected into THP-1 cells. Empty vectors were transfected as the control. Phorbol 12-myristate 13-acetate (PMA, 10 μM) was added prior to the transfection in order to differentiate the monocytes into macrophages. After the miRNA-492 overexpression was verified by real-time PCR, the secretion of pro-inflammatory factor IL-6 in mononuclear macrophages was detected by ELISA.

Statistical methods

SPSS 25.0 software package was used for statistical analysis, and all normally distributed data were expressed as mean $\pm$ standard deviation (S.D.) and Student’s t-test was used for inter-group comparisons. Meanwhile, non-normally distributed data were expressed as median and the Mann-Whitney U rank sum test was used for inter-group comparisons. Enumeration data were expressed as percentages or rates, and the Chi-square test or Fisher’s exact probability method was used for inter-group comparisons. Spearman rank correlation analysis was used for correlation analysis. All tests were conducted bilaterally with the level of alpha = 0.05.

Results

Clinical information

A total of 56 children who met the diagnostic criteria for MMP and 54 healthy controls were included in this study, of which 24 were excluded due to underlying diseases or family members’ rejection. In the end, forty-six children with Mycoplasma pneumoniae and 40 healthy controls were included. Among them, there were 30 males and 16 females in the MPP group, with an average age of 6.4 $\pm$ 2.9 years, and 28 males and 12 females in the healthy control group, with an average age of 6.2 $\pm$ 2.6 years. There was no significant difference in gender and age between the control group and the MPP group ($P > 0.05$). The hospitalization days of the children in the MPP group ranged from 5 to 12 days, and 45 patients had cough symptoms, accounting for 97.82% (29 males and 16 females). A total of 16 patients (10 males and 6 females) had rales on lung auscultation during the hospitalization of the 46 MPP children, accounting for 34.78%. Some children had signs such as pulmonary rales during the development of the disease, and 2 infants had wheezing on lung auscultation. Among the children with MPP, 11 patients (6 males and 5 females) had internal and external pulmonary complications, accounting for 23.91%, and 2 of them had a small amount of pleural effusion. Three children had digestive system symptoms such as nausea, vomiting, and anorexia, and one of them was accompanied by abnormal liver function. One patient presented with chest tightness and shortness of breath. His cardiac auscultation revealed arrhythmia, and auxiliary examinations showed abnormal ECG and myocardial enzymes. He received oxygen therapy. All the children in the MPP group had different degrees of

Table 1 Laboratory indicators and their comparison between the two groups.

Indices	Study Group (*n* = 46)	Control group (*n* = 40)	*t*	*p* value
WBC ($\times 10^9$/L)	7.24 (6.05−8.56)	6.83 (5.42−7.53)	6.921	0.186
CRP (mg/L)	10.44 (5.09−12.59)	0.04 (0.01−0.58)	1.178	< 0.001[c]
IgA (g/L)	1.16 (0.79−1.48)	0.74 (0.54−0.95)	1.974	0.007[b]
IgG (g/L)	8.37 (7.05−9.26)	8.18 (6.74−9.52)	10.483	0.932
IgM (g/L)	1.34 (1.12−1.73)	0.86 (0.72−1.23)	1.397	< 0.001[c]
CD3+ (%)	68.21 (63.02−75.08)	68.87 (66.52−73.03)	9.325	0.673
CD3+CD4+ (%)	33.41 (27.01−37.79)	35.86 (34.00−39.53)	3.856	0.042[a]
CD3+CD8+ (%)	25.41 (21.58−28.75)	24.85 (24.05−27.39)	8.261	0.568
NK cell (%)	12.21 (9.02−15.12)	15.78 (11.85−21.87)	2.293	0.024[a]

[a] significant different with $p < 0.05$.
[b] significant different with $p < 0.01$.
[c] significant different with $p < 0.001$; WBC, white blood cells; CRP, C-reactive protein; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; NK cell, natural killer cell.

Table 2 The level of miRNA-492 and cytokines and the comparison.

Groups	n	TNF-α (pg/mL)	IL-6 (pg/mL)	IL-18 (pg/mL)	HMGB1 ($\times 10^{-3}$)	miRNA-492 ($\times 10^{-3}$)
Study Group	46	539.03 $\pm$ 153.26	356.87 $\pm$ 106.52	449.06 $\pm$ 86.37	6.87 $\pm$ 4.59	7.48 $\pm$ 1.83
Control Group	40	51.29 $\pm$ 6.58	38.53 $\pm$ 4.06	87.34 $\pm$ 13.51	3.45 $\pm$ 0.98	3.65 $\pm$ 0.78
t		1.937	1.526	1.722	2.953	3.026
p value		< 0.001[b]	< 0.001[b]	< 0.001[b]	0.003[a]	0.008[a]

[a] significant different with $p < 0.01$.
[b] significant different with $p < 0.001$; The expression levels of HMGB1 and miRNA-492 were presented as the proportion to the reference.

fever, and the fever degree was 37.8–39.5 °C. The clinical data were shown in Table S1.

Comparison of laboratory indicators between children in the study group and those in the control group

In this study, 46 children diagnosed with mycoplasma pneumonia in the hospital from March 2018 to August 2019 were recruited as the study group, and 40 healthy children were selected as the control group. Immune indexes including WBC, CRP, immunoglobulin (Ig) A; IgG, IgM, percentages of CD3+, CD3+CD4+, CD3+CD8+ T cells, and natural killer (NK) cells were evaluated in both groups. Our results showed that CRP, IgA, and IgM were significantly increased, whereas CD3+CD4+ T cells and NK cells were remarkably decreased in the study group (Table 1). No significant differences were observed in WBC, IgG, CD3+ T cells, or CD3+CD8+ T cells ($p > 0.05$).

Comparison of miRNA-492 and cytokines between the two groups

Subsequently, the authors detected the levels of miRNA-492 and inflammatory cytokines in the peripheral blood of children with MPP (study group) and healthy controls (control group). The present results showed that miRNA-492 was significantly upregulated in the study group ($p = 0.008$, Table 2). In addition, levels of TNF-α, IL-6, IL-18, and HMGB1 were remarkably enhanced in the study group compared to the control group (Table 2).

Correlation analysis between miRNA-492 and laboratory indicators or cytokines

Next, the authors conducted a correlation analysis between miRNA-492 and laboratory indicators or cytokines that were altered in the study group. The present data revealed that the upregulated miRNA-492 was significantly correlated with the elevated IL-6, IL-18, and HMGB1 (Table 3). However, miRNA-492 showed no correlation with the levels of CRP, IgA, IgM, CD3+CD4+ T cells, NK cells, or TNF-α in the peripheral blood of children with MMP.

Overexpression of miRNA-492 induced IL-6 secretion in macrophages

THP-1 is a human peripheral blood mononuclear cell line originally derived from patients with an acute monocytic leukemia, which is widely used in studies of monocytes and macrophages-related mechanisms, signal pathways, nutrition, and drug transport. THP-1 cells can be differentiated into macrophages by phorbol ester (PMA) and can induce M1 polarization through lipopolysaccharide (LPS) and IFN-γ to release cytokines such as TNF-α and IL-6, as a well-established *in vitro* inflammation model. Therefore, the authors first overexpressed empty vector or miRNA-492 in THP-1 cells to detect the difference in IL-6 levels between these two groups of cells after PMA activation and induction. The present results showed that after overexpressing miRNA-492, IL-6 level significantly increased compared to the empty vector (198.75 $\pm$ 12.08 pg/ml v.s. 148.21 $\pm$ 11.69 pg/ml, $t = 2.937$, $p < 0.001$).

Table 3 Correlation analysis between miRNA-492 and laboratory indicators or cytokines.

Indices v.s. miRNA-492	r	p value
CRP	0.145	0.528
IgA	-0.196	0.378
IgM	0.012	0.968
CD3+CD4+	0.224	0.365
NK cells	0.069	0.783
TNF-α	-0.145	0.768
IL-6	0.894	0.039[a]
IL-18	0.821	< 0.01[b]
HMGB1	-1.05	< 0.01[b]

[a] significant different with $p < 0.05$
[b] significant different with $p < 0.01$.

Discussion

Children with MPP are prone to immune dysfunction due to the special surface antigen structure and protein structure of MP, and the abnormalities of relevant immune indexes may persist for as long as one year.[15] Previous studies have also confirmed that disordered immune function is the main pathogenic mechanism responsible for MP.[16] It has also been reported that MP infection inhibits the phagocytosis of neutrophils, and thus causes immune dysfunction and upregulation of IgA.[17] In this study, the authors found that the levels of IgM and IgA, but not IgG increased significantly in children with MPP. IgM increases rapidly in the early stage of MPP, while IgG and IgA only elevate more than 2 weeks after MPP, suggesting that some children with MPP in this study were in the early stage of MPP.

T lymphocytes are crucial for the mediation of human cellular immune response.[18] It has been found that the

cellular immune function is impaired by MP infection and interferon level decreases in patients at the early stage of MP infection.[19] However, the alterations of T lymphocyte subsets during MP infection are still controversial. In most studies, CD3+ CD4+ T lymphocytes decreased in patients with MPP,[20] which is consistent with the results of this study, suggesting that the activation process of T lymphocytes is disturbed. However, some studies also found that the proportion of CD3+ CD4+ T lymphocytes increased in children with MPP, which suggested that MP infection caused excessive activation of cellular immune response in children.[21] NK cells mediate the natural immune response, which can directly kill target cells. MP infection can cause the imbalance in Th1 / Th2 regulation.[22] In this study, the authors also found that the percentage of NK cells decreased in children with MPP compared with the control group, which may be attributed to the inhibition of Th1 cells.

The main functions of miRNA are to silence messenger RNA (mRNA) and regulate post-transcriptional gene expression.[23] MiRNA-492 can regulate the expression of a variety of proteins in tumorigenesis, metastasis, insulin resistance, auto-immunity and other biological processes.[24,25] However, few studies have reported the regulatory role of miRNA-492 in infectious diseases. In this study, the authors demonstrated that the expression of miRNA-492 in the peripheral blood of children with MP was higher than that of healthy children, suggesting that miRNA-492 might be involved in the occurrence and development of MPP in children. The correlation analysis was conducted in this study between the level of miRNA-492 and the laboratory indicators and inflammatory factors that were differentially expressed in children with MP. The authors demonstrated that miRNA-492 was positively correlated with IL-6, IL-18, and HMGB1, whereas showed no correlation with other indicators. The authors speculated that it could be attributed to the delayed regulation of miRNA by a series of complex networks, hence no direct correlation could be observed. Meanwhile, HMGB1 has been considered a late on-set inflammatory factor, which correlated better with the level of miRNA-492. However, it would be quite interesting to study the upstream of HMGB1 in future experiments.

It has been reported that MP infection in monocytes leads to increased secretion of pro-inflammatory factor IL-6 in the culture supernatant, and the increased secretion of IL-6 follows the high expression of miRNA-492, which is consistent with the present findings.[26] Therefore, the authors speculated that miRNA-492 regulates the inflammatory response of monocytes by promoting excessive secretion of inflammatory factors such as IL-6. HMGB1 is a conserved non-histone nuclear protein.[27] Previous studies have shown that HMGB1 is highly expressed in the blood of patients with pneumonia, which may indicate the severity of the disease.[28] In this study, the authors showed that the plasma levels of IL-6, IL-18, and HMGB1 in children with MPP were significantly higher than that in healthy controls, suggesting that HMGB1 was also involved in the pathogenesis of MP pneumonia.

The treatment of pediatric MPP requires comprehensive therapy measures, including antibiotics, immunosuppressants, fiberoptic bronchoscopy, and treatment of refractory mycoplasma pneumonia. Now that the authors have shown that miRNA-492 plays a crucial role in the pathogenesis of MPP in children by regulating the secretion of immune-inflammatory factors in this study, targeting miRNA-492 may be a potential therapeutic strategy for pediatric MPP. miRNAs have become important targets for neurodegenerative diseases and epilepsy. It has been reported that identifying small molecules capable of selectively binding miRNAs will have a dramatic impact on the ability to control the expression of proteins associated with neurodegenerative diseases.[29] Therefore, screening small molecules that target binding and degrading miRNA-492 may have a dramatic impact on the treatment of pediatric MPP. The authors plan to further explore this question in future research.

In conclusion, the authors hereby showed that miRNA-492 might play a crucial role in the pathogenesis of MPP in children by regulating the secretion of immune-inflammatory factors such as IL-6 and IL-18 in the monocyte-macrophage system. The present study may reveal the immunomodulatory mechanism underlying MP infection and provide a new approach for the treatment of MPP.

Conflicts of interest

The authors declare no conflicts of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jped.2022.07.010.

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