



# Clinical significance of serum miR-95 in children with *Mycoplasma pneumoniae* infection complicated with diarrhea

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## Abstract

**Introduction and Objective.** *Mycoplasma pneumoniae* infection (MPI) can lead to extrapulmonary diseases, in which diarrhea occurs frequently. The role and impact of microRNA-95 (miR-95) in MPI and MPI with diarrhea (MPI+DIA) remain elusive. The aim of the study is to investigate miR-95 expression and its functional impact in paediatric MPI with diarrhea.

**Materials and Method.** The study enrolled 80 MPI patients (53 without diarrhea, 27 with diarrhea) and 80 matched healthy controls. Expression of miR-95 was quantified using reverse transcription quantitative polymerase chain reaction (RT-qPCR). Predictors of disease status were analyzed through binary logistic regression. An *in vitro* pneumonia model (MP-BEAS-2B) was generated by infecting BEAS-2B cells with *Mycoplasma pneumoniae* (MP). In parallel, an intestinal epithelial injury model was generated by treating Caco-2 cells with 4 µg/mL lipid-associated membrane proteins (LAMPs). Cell proliferation was measured with cell counting kit-8 (CCK-8), apoptosis by flow cytometry, and interleukin-8 (IL-8) and tumour necrosis factor-alpha (TNF-α) levels were determined via RT-qPCR.

**Results.** The study revealed that miR-95 was significantly elevated in the MPI group, with a further increase observed in MPI patients complicated with diarrhea. Additionally, miR-95 served as a discriminator among healthy controls, MPI patients, and MPI+DIA patients. In MP-BEAS-2B and LAMPs-Caco-2 cells, miR-95 was highly expressed. However, downregulating miR-95 significantly enhanced cell proliferation, inhibited apoptosis, and reduced the expression of IL-8 and TNF-α.

**Conclusions.** MiR-95 expression was significantly elevated in MPI patients, and further increased in those with MPI complicated by diarrhea. Downregulating miR-95 significantly protected BEAS-2B and Caco-2 cells from MP damage and alleviated cellular inflammation.

## Key words

diarrhea, diagnosis, inflammatory factors, *Mycoplasma pneumoniae* infection, MiR-95

## INTRODUCTION AND OBJECTIVE

*Mycoplasma pneumoniae* (MP), a cell wall deficient bacterium characterized by a small genome and distinctive cellular architecture [1], represents a primary etiological agent of respiratory tract infections in children and adolescents [2]. It causes up to 40% of community-acquired pneumonia cases among children over five years of age, constituting a significant paediatric health threat [3]. *Mycoplasma pneumoniae* infection (MPI) can induce extrapulmonary manifestations affecting multiple systems, including dermatological, gastrointestinal, cardiovascular, musculoskeletal, neurological, and renal systems [2]. Gastrointestinal symptoms – particularly vomiting and diarrhea – represent the most prevalent extrapulmonary presentations according to research evidence [4], with studies indicating that nausea, vomiting, anorexia, diarrhea, or abdominal pain may occur in as many as 40% of MPI patients [3]. Failure to administer prompt, effective treatment in paediatric pneumonia cases may lead to severe complications, including meningitis and enteritis, thereby substantially elevating clinical risks [5].

MicroRNAs (miRNAs) are 18–25 nucleotide non-coding RNA sequences that suppress translational initiation or induce mRNA degradation via sequence-specific binding to the 3' untranslated regions of target transcripts [6]. Individual miRNAs can target hundreds of mRNAs, thereby modulating the expression of numerous functionally interconnected genes within biological pathways [7]. Clinical investigations reveal elevated serum miR-34a levels in paediatric *Mycoplasma pneumoniae* pneumonia (MPP) patients, which correlate positively with disease severity and delayed recovery [8], suggesting its diagnostic utility. Another investigation suggests that miR-16 ameliorates diarrhea-predominant irritable bowel syndrome (IBS-D) through TLR4/NF-κB pathway-mediated XIST downregulation, attenuating apoptosis and inflammation [9], highlighting its therapeutic potential. Furthermore, miRNA-492 may contribute to the pathogenesis of paediatric MPP by regulating the secretion of immunoinflammatory factors in mononuclear macrophages [10]. Substantial evidence indicates that the aberrant expression of miR-95 is implicated in various pulmonary diseases. MiR-95 has been identified as an oncogenic driver and a candidate therapeutic target in non-small cell lung cancer (NSCLC) [11], with supporting evidence in lung adenocarcinoma (LUAD) [12]. Beyond its established role in oncology, miR-95-5p is also implicated in the development of acute lung injury [13]. Despite these

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findings, its expression and function in MPI complicated by diarrhea (MPI+DIA) remain completely unexplored.

Therefore, the aim of the study is to explore the expression of miR-95 in MPI with diarrhea and its impact on this condition, to offer potential diagnostic and therapeutic targets for MPI with diarrhea in children.

## MATERIALS AND METHOD

**Participant recruitment and sample collection.** Eighty children with MPI were enrolled as the study cohort and stratified by diarrheal status: 53 without diarrhea versus 27 with diarrhea comorbidity. Eighty age-matched healthy children served as controls. Inclusion criteria comprised:

- 1) meeting diagnostic standards for MPI and diarrhea [5], presenting primary symptoms of tachypnea, fever, and cough;
- 2) serological confirmation via microparticle immunofluorescence assay showing either single serum IgM  $\geq 1:160$  or IgG  $\geq 1:512$ , or  $\geq 4$ -fold antibody titer increase in paired sera;
- 3) diarrheal frequency  $\geq 5$  episodes/day with curd-like or watery yellow stool consistency;
- 4) availability of comprehensive medical documentation; and
- 5) provision of informed consent by guardians.

Exclusion criteria included:

- 1) congenital cardiac defects, pulmonary hypoplasia, or immunodeficiency disorders;
- 2) concurrent gastrointestinal malformations or immune pathologies;
- 3) severe malnutrition;
- 4) significant renal/hepatic dysfunction.

The study protocol received approval from the Ethics Committee. Patient recruitment commenced on 1 March 2023, and concluded on 30 May 2023, via consecutive sampling at the paediatric outpatient and inpatient departments of the hospital. For blood collection, venipuncture was performed by clinical personnel under strict aseptic technique following enrollment. Venous blood samples were collected in disposable vacuum tubes and centrifuged to obtain serum. The serum samples were stored at  $-80^{\circ}\text{C}$  until analysis.

**Cell culture.** The human normal bronchial epithelial cell line BEAS-2B (Sunncell Biotechnology, SNL-203) was maintained in DMEM/F12 medium. MP strain ATCC 15531 (HuiYing Biotechnology) was introduced into the culture medium at a 1:10 ratio to establish an MPI cellular model (MP-BEAS-2B) [14]. The human colorectal adenocarcinoma cell line Caco-2 was maintained in DMEM medium (Thermo Fisher, 11965092). For the establishment of an intestinal epithelial injury model, cells were seeded into 12-well plates at a density of  $2 \times 10^5$  cells/mL. The cells were then incubated with  $4 \mu\text{g/mL}$  lipid-associated membrane proteins (LAMPs) for 16 hours; and this treatment group was designated as the LAMPs-Caco-2 model [15]. Both culture systems were supplemented with 10% foetal bovine serum,  $100 \mu\text{g/mL}$  streptomycin, and  $100 \text{ U/mL}$  penicillin (Sigma-Aldrich) and incubated at  $37^{\circ}\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$ . Cells in the exponential growth phase were utilized for subsequent experiments.

**Transfection experiments.** MiR-95 mimic (miR-mimic) and inhibitor (miR-inhibitor) were procured from Thermo Fisher Scientific. Plasmid transfections were performed using Lipofectamine 2000 (Invitrogen), with un-transfected cells and those transfected with negative control mimic-NC or inhibitor-NC serving as control groups. Transfection complexes were prepared by co-incubating mimics, inhibitors, or control plasmids with Lipofectamine 2000 reagent at  $37^{\circ}\text{C}$  for 20 minutes, followed by supplementation into respective cell culture media. Functional consequences of miR-95 knockdown or overexpression were assessed in subsequent experiments conducted 48 hours post-transfection.

**Reverse transcription quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated using Trizol reagent (Invitrogen). Subsequently,  $1 \mu\text{g}$  RNA underwent reverse transcription with a commercial cDNA synthesis kit (Takara). RT-qPCR was performed on an ABI 7500 Real-Time PCR System (Applied Biosystems) employing SYBR Premix Ex Taq reagents (Takara), with all samples analyzed in triplicate biological replicates. Relative expression levels were calculated using the  $2^{-\Delta\Delta\text{Ct}}$  method, with U6 and GAPDH as the endogenous reference genes.

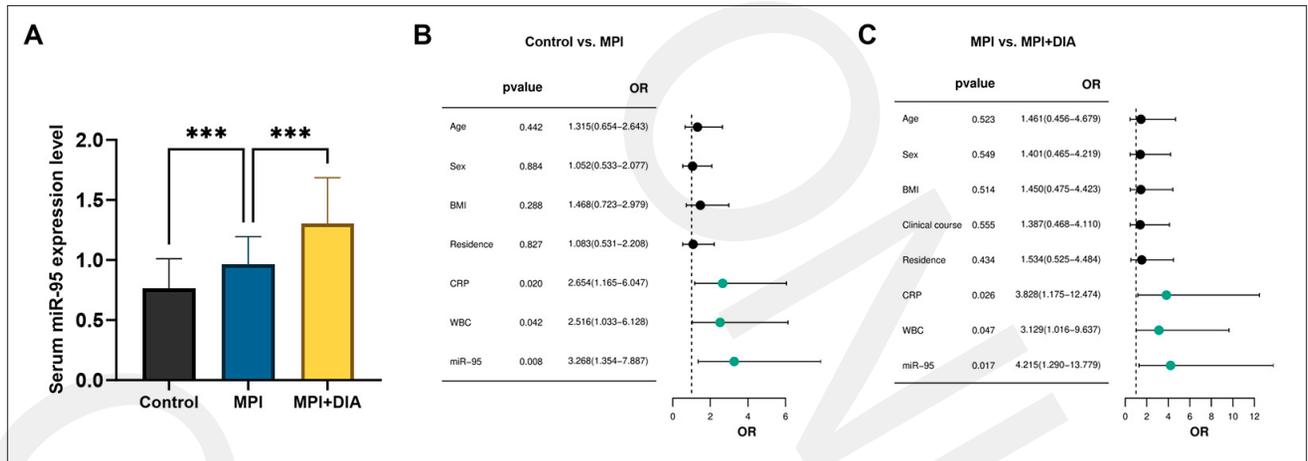
**Cell counting kit-8 (CCK-8) proliferation assay.**  $10 \mu\text{L}$  CCK-8 reagent (Sigma-Aldrich) was introduced to each well, followed by a 2-hour incubation at  $37^{\circ}\text{C}$  under 5%  $\text{CO}_2$ . Optical density was measured at 450 nm using a microplate reader (Thermo Fisher Scientific).

**Flow cytometry.** Cells in logarithmic growth phase were harvested and seeded into 6-well plates at  $1 \times 10^5$  cells/well. Cell suspensions were prepared in binding buffer according to the Annexin V-FITC Apoptosis Detection Kit protocol (Beyotime Biotechnology, C1063S), transferred to flow cytometry tubes, and analyzed using a BD FACSCalibur system with CellQuest software for data acquisition and processing.

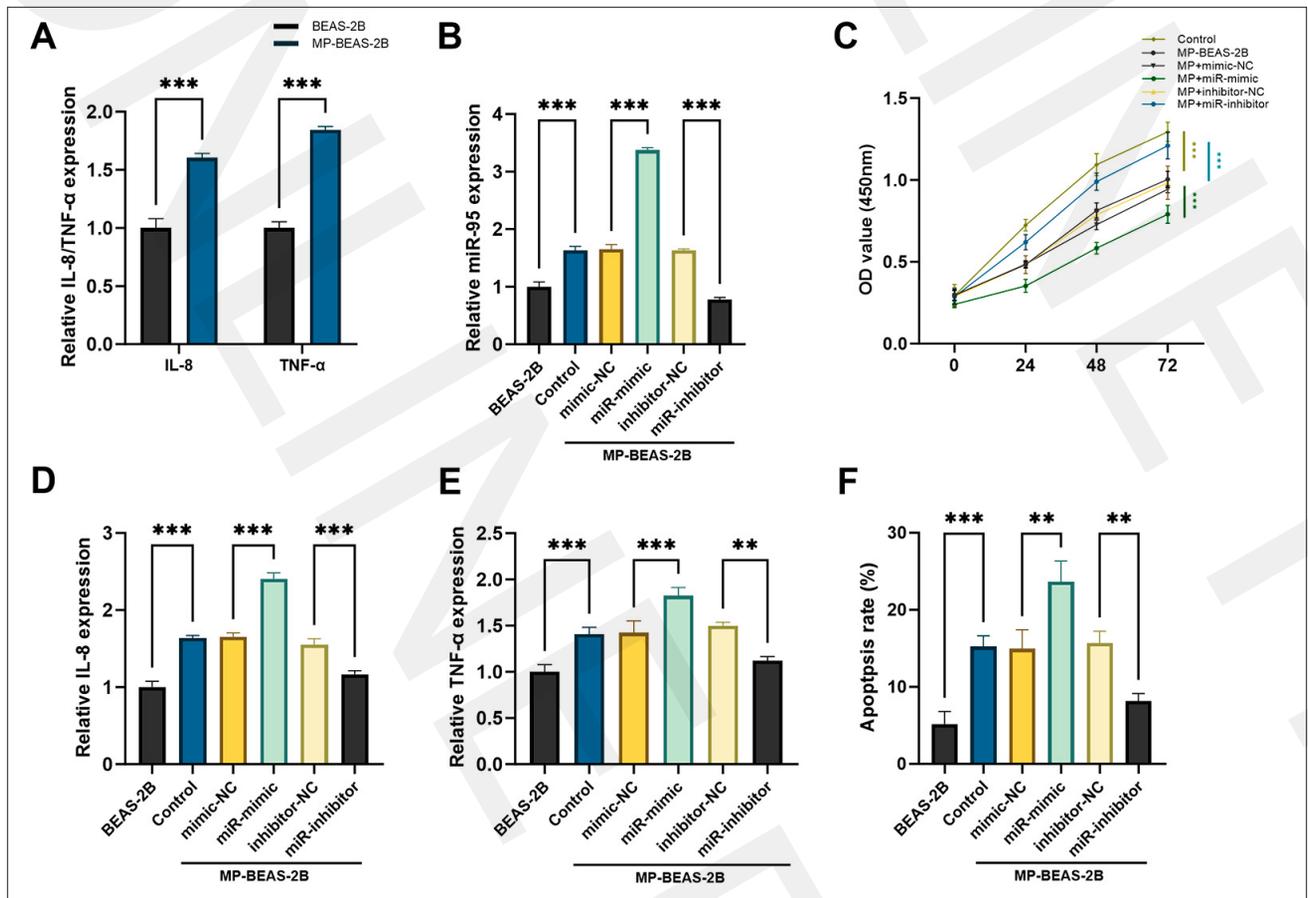
**Statistical analysis.** Data were expressed as mean  $\pm$  standard deviation (SD) derived from  $\geq 3$  independent experiments. Statistical analyses were performed using GraphPad Prism 9.3.1 software, with intergroup comparisons assessed by unpaired Student's t-test, chi-square analysis, and non-parametric test. The multigroup analyses were conducted via one-way analysis of variance (ANOVA). Binary logistic regression was conducted using IBM SPSS Statistics 23. Statistical significance was defined at  $p < 0.05$ .

## RESULTS

**Diagnostic potential of miR-95 in paediatric MPI with diarrhea.** A comparative analysis of baseline characteristics and biochemical indicators was conducted among healthy controls, patients with MPI without diarrhea, and MPI patients with diarrhea. The results revealed no significant differences in age, gender, body mass index (BMI), clinical course, or residence among the three groups. In contrast, significant differences were observed in C-reactive protein (CRP) and white blood cell count (WBC), 2 established risk factors for MPI [16] (Tab. 1). Regarding miR-95 expression, its levels were significantly elevated in the MPI group compared



**Figure 1.** Serum miR-95 expression and diagnostic value analysis. A) miR-95 levels in the serum of 3 groups: control, MPI, and MPI+DIA. B) Binary logistic regression analysis between control and MPI groups. C) Binary logistic regression analysis between MPI and MPI+DIA groups. \*\*\* $p < 0.001$



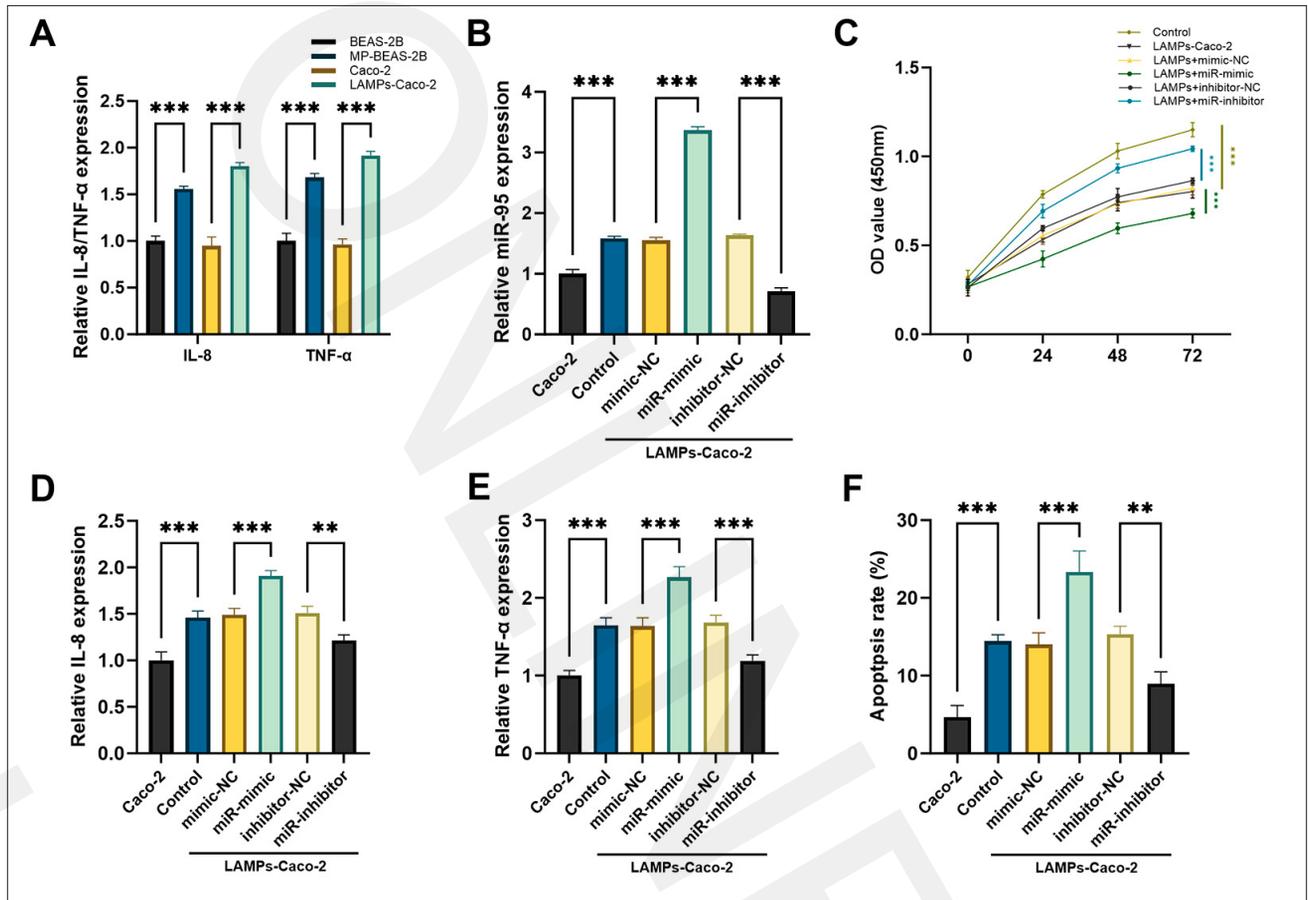
**Figure 2.** Effects of miR-95 on the proliferation, apoptosis, and levels of IL-8 and TNF- $\alpha$  in MP-BEAS-2B cells. A) Expression levels of the inflammatory factors (IL-8/TNF- $\alpha$ ) in different groups. B) Expression level of miR-95 in different groups. C) Effect of miR-95 on the proliferation of MP-BEAS-2B cells. D) Effect of miR-95 on IL-8 levels in MP-BEAS-2B cells. E) Effect of miR-95 on TNF- $\alpha$  levels in MP-BEAS-2B cells. F) Effect of miR-95 on the apoptosis of MP-BEAS-2B cells. \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

with controls and were further increased in the MPI+DIA group, with statistically significant differences between all groups (Fig. 1A). Binary logistic regression analysis indicated that miR-95 was a predictor for MPI (Figure 1B, OR = 3.278, 95% CI: 1.359–7.910,  $p < 0.01$ ) and also for MPI with diarrhea (Fig. 1) (C, OR = 4.215, 95% CI: 1.290–13.779;  $p < 0.05$ ).

These findings suggested that high miR-95 expression was significantly associated with the risk of both MPI and MPI

with diarrhea, and elevated expression levels may indicate a higher risk of progression from MPI to MPI with diarrhea.

**Regulatory effects of miR-95 downregulation on cellular functions in MP-BEAS-2B cells.** In this study, an MPI cell model was established by mycoplasma infection of BEAS-2B cells, as confirmed by increased expression of IL-8 and TNF- $\alpha$  (Figure 2A). MiR-95 was upregulated in MP-BEAS-2B cells,



**Figure 3.** The impact of miR-95 on the proliferation, apoptosis, and levels of IL-8 and TNF- $\alpha$  in LAMPs-Caco-2 cells. A) The expression levels of the inflammatory factors (IL-8/TNF- $\alpha$ ) across different groups. B) The expression level of miR-95 in each group. C) The role of miR-95 in influencing the proliferation of LAMPs-Caco-2 cells. D) The effect of miR-95 on IL-8 levels within LAMPs-Caco-2 cells. E) The effect of miR-95 on TNF- $\alpha$  levels within LAMPs-Caco-2 cells. F) The role of miR-95 in regulating apoptosis in LAMPs-Caco-2 cells.

\*\* $p < 0.01$ ; \*\*\* $p < 0.001$

**Table 1.** Clinical indicators were compared between the control and MPI groups, as well as between the MPI and MPI+DIA groups

Parameters	Control (n=80)	MPI (n=53)	MPI+DIA (n=27)	$p$ -value control vs. MPI	$p$ -value MPI vs. MPI+DIA
Age (years)	7.31 $\pm$ 2.34	7.58 $\pm$ 2.66	8.37 $\pm$ 2.23	0.535	0.193
Gender, n (%)				0.753	0.816
Male	37 (46.25)	26 (49.06)	14 (51.85)		
Female	43 (53.75)	27 (50.94)	13 (48.15)		
BMI (kg/m <sup>2</sup> )	16.35 $\pm$ 1.99	15.86 $\pm$ 1.88	16.29 $\pm$ 3.45	0.152	0.466
Clinical course (days)	NA	20.30 $\pm$ 4.53	20.59 $\pm$ 4.39	NA	0.785
Residence, n (%)				0.920	0.230
Group 1	31 (38.75)	21 (39.62)	7 (25.93)		
Group 2	49 (61.25)	32 (60.38)	20 (74.07)		
CRP (mg/L)	3.22 $\pm$ 2.19	6.56 $\pm$ 2.19	8.47 $\pm$ 2.70	<0.001***	0.001**
WBC (10 <sup>9</sup> /L)	7.71 $\pm$ 2.12	10.00 $\pm$ 1.83	11.21 $\pm$ 2.09	<0.001***	0.009**
miR-95	0.76 $\pm$ 0.24	0.96 $\pm$ 0.23	1.30 $\pm$ 0.38	<0.001***	<0.001***

MPI – *Mycoplasma pneumoniae* infection; MPI+DIA – *Mycoplasma pneumoniae* infection complicated with diarrhea; BMI – body mass index; Residence, Group 1 – city; Group 2 – countryside; CRP – C-reactive protein; WBC – white blood cell; miR-95 – microRNA-95; NA – not applicable.

\*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

and successful overexpression or knockdown was achieved via transfection (Fig. 2B). Functional assays indicated that miR-95 overexpression further suppressed cell proliferation (Fig. 2C), enhanced the expression of IL-8 and TNF- $\alpha$  (Fig. 2D-E), and promoted apoptosis (Fig. 2F) in MP-BEAS-2B cells. In contrast, miR-95 knockdown reversed these effects,

indicating a role in modulating mycoplasma-induced cellular injury and inflammation.

**Regulatory functions of miR-95 downregulation in LAMPs-Caco-2 cells.** An intestinal epithelial injury model was established by treating Caco-2 cells with 4  $\mu$ g/mL

LAMPs, as validated by elevated IL-8 and TNF- $\alpha$  expression (Figure 3A). In this model, miR-95 was upregulated (Fig. 3B), and its expression was further increased by the mimic or decreased by the inhibitor (Fig. 3B). Functional assays showed that miR-95 overexpression exacerbated LAMPs-induced reductions in cell viability (Fig. 3C), enhanced the expression of IL-8 and TNF- $\alpha$  (Fig. 3D-E), and promoted apoptosis (Fig. 3F). Conversely, miR-95 inhibition alleviated these effects, supporting its role in aggravating intestinal epithelial injury under inflammatory conditions.

## DISCUSSION

Given the young age and comparatively underdeveloped cognitive function of children with MPI, effective nursing care and rehabilitation training are paramount for enhancing clinical outcomes and facilitating recovery. By providing a comfortable hospitalization environment and tailored care plans, healthcare providers can effectively alleviate these negative emotions, thereby supporting the child's psychological well-being and enhancing overall treatment outcomes [17]. In terms of treatment, tetracyclines and fluoroquinolones are associated with severe side-effects in children. Therefore, macrolides have become the preferred choice for treating MPP in children [18, 19]. However, the increasing resistance to macrolide antibiotics is complicating clinical treatment. Long-term use of azithromycin (a macrolide antibiotic) may also raise the risk of adverse reactions, including gastrointestinal symptoms such as nausea, vomiting, abdominal pain, diarrhea, and phlebitis [20]. Given these therapeutic challenges and the importance of early intervention, identifying novel biomarkers could offer a new approach to predicting disease progression and guiding individualized supportive care, which may contribute to improving prognosis and reducing complications in affected children.

MiRNAs serve as multifaceted regulators of protein-coding gene expression in higher eukaryotes [21]. They orchestrate diverse cellular processes. Dysregulation of miRNA clusters constitutes a pivotal mechanism underlying pathological alterations, contributing significantly to various disease etiologies, including carcinogenesis [22]. Based on previous literature, miR-95 was selected as the research target in this study. RNA sequencing and bioinformatics analysis have reported that miR-95 is upregulated in patients with mild and severe pneumonia, suggesting its potential as a biomarker [23]. In oncology-related studies, miR-95-3p is highly expressed in cervical tumour tissues and promotes malignant progression by suppressing VCAM1 [24]. In ASCL1-positive lung adenocarcinoma, miR-95-3p and miR-95-5p were identified as miRNAs closely associated with ASCL1 expression [25]. Furthermore, miR-95 is highly expressed in both NSCLC cell lines and tumour tissues [11]. Downregulation of miR-95 enhances radiosensitivity in NSCLC, promotes apoptosis, and inhibits proliferation. Animal experiments further suggested that silencing miR-95 suppresses tumor growth and reduces radiation resistance [26].

The findings of the current study extend the known biological repertoire of miR-95. Beyond its previously reported associations, the data obtained indicate that serum miR-95 was markedly elevated in children with MPI, and

showed a further significant increase in those who developed diarrhea. This graded expression pattern not only reinforced its potential as a clinical biomarker for distinguishing disease severity, but also suggested a possible role in the systemic progression or extrapulmonary dissemination of MPI. The correlation between elevated serum miR-95 and the gastrointestinal complication implicated its involvement beyond the local pulmonary site, potentially reflecting or mediating systemic inflammatory crosstalk.

The mechanistic implications derived from the authors' cellular experiments were noteworthy. In both respiratory (MP-BEAS-2B) and intestinal (LAMPs-Caco-2) epithelial injury models, inhibition of miR-95 consistently exerted protective effects, which were characterized by enhanced proliferation, suppressed apoptosis, and attenuated secretion of the key pro-inflammatory cytokines IL-8 and TNF- $\alpha$ . This concordant response across 2 distinct but clinically relevant cell types supported the hypothesis that miR-95 may function as a common regulatory node in epithelial barrier dysfunction triggered by MPI. The observed upregulation of miR-95 appeared to exacerbate cellular injury and inflammation, whereas its inhibition promoted cellular repair. The consistent phenotype across models suggested that miR-95 could be a convergent therapeutic target for mitigating epithelial damage in both respiratory and intestinal compartments during MPI. These findings position miR-95 not merely as a biomarker but as a potential functional mediator in the pathophysiological link between pulmonary infection and intestinal compromise.

**Limitations of the study.** First, all cases were recruited from a single clinical centre, and the sample size was relatively limited, which may have introduced selection bias and constrained the generalizability of the findings to broader populations. Validation through multi-centre studies with larger cohorts is therefore warranted. Second, although cellular experiments in this study preliminarily indicated that miR-95 inhibition alleviated MPI with diarrhea-related effects, protected BEAS-2B and Caco-2 cells, and reduced cellular injury, its mechanisms within an integrated pathophysiological context, remain incompletely understood.

The present study employed monoculture models of lung epithelial and intestinal epithelial cells, which do not fully recapitulate the intricate lung-gut axis interactions that exist *in vivo*. Third, although serum miR-95 levels were measured, its precise cellular origin during MPI+DIA remains undefined, and the observed elevation may not be disease-specific. Fourth, while the functional phenotypes associated with miR-95 were evaluated, its direct downstream target genes were not experimentally validated. This represents a gap in elucidating the precise molecular mechanism by which miR-95 exerts its effects.

Finally, translating the observed cytoprotective effects into clinical therapeutic applications presents inherent challenges. Future studies should therefore incorporate animal models to assess efficacy and safety *in vivo*, as well as to explore potential off-target effects.

However, this study provided the first evidence implicating miR-95 as a key molecular link in the development of diarrhea following MPI in children. By demonstrating that its elevated serum levels correlated with clinical complications and that it played a functional role in exacerbating injury in relevant cellular models, it established that miR-95 is both a

promising biomarker and a novel therapeutic target. These results contributed to clarifying the molecular regulatory network underlying MPI-associated diarrhea and provided an initial experimental basis for the subsequent development of miR-95-targeted diagnostic markers or therapeutic interventions.

## CONCLUSIONS

MiR-95 expression was remarkably upregulated in MPI and further increased in those presenting with MPI complicated by diarrhea. Reducing its expression level significantly boosted MP-BEAS-2B and LAMPs-Caco-2 cell proliferation, effectively inhibited apoptosis, and lowered IL-8/TNF- $\alpha$  levels.

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