Postprint: MiR-17-5p Targets Autophagy-Related Protein ATG7 to Regulate Macrophage Anti-Mycobacterium tuberculosis Infection

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Abstract

Objective: To investigate the role and mechanism of miR-17-5p in Mycobacterium tuberculosis-mediated autophagy pathway by studying its targeted regulatory mechanism on the autophagy-related gene ATG7 and its effect on cellular autophagy.

Methods: Bioinformatics analysis identified ATG7 as a target gene of miR-17-5p. The targeting relationship between miR-17-5p and ATG7 was verified by constructing ATG7 wild-type (pMirGLO-ATG7-3' UTR-WT) and mutant vectors, and utilizing the dual-luciferase reporter system and Western blot. Meanwhile, a Mycobacterium tuberculosis (H37Ra) infection model in human THP-1 macrophages was established, and cells with different treatments were divided into three groups: miR-17-5p mimics, miR-17-5p inhibitor, and miR-17-5p nc. The effect of H37Ra infection on miR-17-5p expression was detected by quantitative real-time PCR (qRT-PCR), and LC3 protein expression and autophagosome number were further examined by Western blot and immunofluorescence microscopy.

Results: MTB infection caused downregulation of miR-17-5p, which showed a significant decrease with increasing multiplicity of infection. Bioinformatics prediction indicated that miR-17-5p has targeting potential for ATG7, and dual-luciferase reporter assays and Western blot verified that miR-17-5p could bind to ATG7 and negatively regulate it. Furthermore, Western blot and immunofluorescence microscopy revealed that LC3-II expression and autophagosome formation were downregulated in the miR-17-5p mimics group, while the opposite was observed in the miR-17-5p inhibitor group. Comparison between H37Ra-infected and uninfected groups showed significantly enhanced expression of ATG7 and LC3-II proteins.

Conclusion: miR-17-5p directly targets and binds to the ATG7 3'UTR to inhibit autophagy, thereby playing a role in macrophage defense against MTB.

Full Text

Preamble

MiR-17-5p Targeting Autophagy-Related Protein ATG7 Regulates Macrophage Response Against Mycobacterium tuberculosis Infection

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Abstract

Objective: To investigate the role and mechanism of miR-17-5p in Mycobacterium tuberculosis (MTB)-mediated autophagy by examining its regulatory mechanism on the autophagy-related gene ATG7 and its effect on cellular autophagy.

Methods: Bioinformatics analysis identified ATG7 as a target gene of miR-17-5p. Wild-type (pMirGLO-ATG7-3' UTR-WT) and mutant ATG7 vectors were constructed, and the targeting relationship between miR-17-5p and ATG7 was verified using a dual-luciferase reporter system and Western blot. A human THP-1 macrophage infection model was established using Mycobacterium tuberculosis (H37Ra). Cells were divided into three treatment groups: miR-17-5p mimics, miR-17-5p inhibitor, and miR-17-5p negative control (nc). Quantitative real-time PCR (qRT-PCR) was used to detect H37Ra infection effects on miR-17-5p expression, while Western blot and immunofluorescence were employed to examine LC3 protein expression and autophagosome formation.

Results: MTB infection caused downregulation of miR-17-5p in a multiplicity of infection (MOI)-dependent manner. Bioinformatics predictions revealed targeting sites between miR-17-5p and ATG7, which was confirmed by dual-luciferase reporter assays and Western blot showing that miR-17-5p directly binds to and negatively regulates ATG7. Further Western blot and immunofluorescence analyses demonstrated that miR-17-5p mimics reduced LC3-II ex-

pression and autophagosome formation, whereas miR-17-5p inhibitor had the opposite effect. Notably, ATG7 and LC3-II protein expression were significantly higher in H37Ra-infected groups compared to uninfected controls.

Conclusion: MiR-17-5p directly targets the ATG7 3'UTR to inhibit autophagy, thereby playing a regulatory role in macrophage defense against MTB infection.

Keywords: autophagy; Mycobacterium tuberculosis; miR-17-5p; ATG7

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Introduction

Tuberculosis (TB) ranks among the three major infectious diseases affecting humanity and leads global mortality from infectious diseases, representing the ninth leading cause of death worldwide [1]. According to the WHO Global Tuberculosis Report 2018, approximately 1.7 billion people were latently infected with TB in 2017, with an estimated 10 million new active cases globally—around 889,000 in China alone—and roughly 1.57 million TB-related deaths [2]. These statistics underscore the critical challenges in global TB prevention and control.

Mycobacterium tuberculosis (MTB), the causative agent of TB, primarily targets macrophages, which can eliminate invading MTB through immune defense mechanisms including autophagy [3]. As an innate immune defense against MTB, autophagy represents a promising therapeutic strategy for anti-TB interventions [4].

Autophagy is a protective mechanism that maintains intracellular homeostasis and serves as a host cell' s natural immune defense against MTB invasion [5]. This process involves the formation of double-membrane autophagosomes that sequester proteins and organelles destined for degradation, followed by fusion with lysosomes to form autolysosomes, thereby maintaining cellular homeostasis and providing energy for metabolic demands [6]. Autophagy proceeds through three stages: autophagosome formation, autolysosome formation, and degradation, involving precise regulation by over 30 autophagy-related genes (ATGs). ATG7 functions as a critical autophagy-related protein that plays an essential role in this process [7].

Mature microRNAs are highly conserved, non-coding, single-stranded small RNAs approximately 20-24 nucleotides in length [8] that negatively regulate target gene expression by degrading mRNA or inhibiting translation [9]. Numerous microRNAs can bind complementarily to the 3' untranslated region (3' UTR) of target genes, thereby regulating their expression and participating in diverse biological functions including cell cycle, differentiation, proliferation, apoptosis, lipid metabolism, and autophagy [10,11], as well as pathological processes such as cardiovascular disease, leukemia, TB, and cancer [12,13]. Studies have

shown that microRNA expression levels change following MTB infection and can regulate key autophagy proteins. For example, miR-155 is upregulated in MTB infection and inhibits autophagy by targeting ATG3 [14]; miR-144* exerts anti-TB effects by targeting the autophagy-related protein DRAM2 [15]; miR-125a is highly expressed in MTB-infected macrophages and inhibits autophagy by targeting the BECN1 complex component UVRAG, promoting intracellular MTB survival [16]; and miR-33 can inhibit autophagy by regulating multiple autophagy key proteins [17]. These findings demonstrate close connections among MTB infection, microRNAs, and autophagy.

MiR-17-5p belongs to the miR-17-92 family, a highly conserved miRNA cluster located on the third intron of the primary transcript C13orf25 gene on human chromosome 13 [18]. However, the role of miR-17-5p in MTB infection remains unclear.

Building upon previous research on TB, microRNAs, and autophagy, we sought to explore the role and mechanism of miR-17-5p in MTB-mediated autophagy pathways. This study investigates miR-17-5p' s impact on autophagy by verifying its targeting of the autophagy-related gene ATG7. Using an MTB-infected cell model, we examined the immune regulatory role of miR-17-5p in autophagy-mediated defense against MTB in macrophages, thereby advancing our understanding of host immune responses during MTB infection.

Materials and Methods

1.1 Materials

Fetal bovine serum and penicillin-streptomycin solution were purchased from Gibco (USA). RPMI1640 medium and PBS were from BI. Restriction enzymes MluI, HindIII, XhoI, SalI, T4 DNA ligase, and 2×SYBR Green I mix were from TaKaRa (Dalian). Plasmid mini-prep kits and DNA gel extraction kits were from Tiangen Biotech. Lipofectamine™ 2000 transfection reagent, Small RNA extraction kit (RNAiso for Small RNA), and qRT-PCR reverse transcription kits were from Thermo Fisher. Dual-luciferase assay kits and reverse transcription kits were from TransGen Biotech. Synthetic miR-17-5p negative control (miR-17-5p nc), miR-17-5p mimics, and miR-17-5p inhibitor were from Shanghai GenePharma. Trizol reagent was from Invitrogen. Protein extraction kits and BCA protein quantification kits were from KeyGen Biotech. Rabbit anti-ATG7 and anti-LC3 antibodies were from Abcam. GAPDH antibody and HRP-conjugated goat anti-rabbit secondary antibody were from Proteintech. PVDF membranes were from Millipore. Primers for miR-17-5p and ATG7, and DNA sequencing services were from Shanghai Sangon Biotech. The pMirGLO plasmid vector, THP-1 cells, and TOP10 competent bacteria were laboratory stocks.

1.2 Methods

1.2.1 Cell Culture and Differentiation Human THP-1 monocytes were resuspended in 5 ml RPMI1640 medium containing 10% fetal bovine serum (FBS) and 100 mg/L penicillin-streptomycin, then cultured at 37°C with 5% CO for approximately 48 h. Cells were passaged by centrifugation at 800 rpm for 5 min and continued in culture until reaching optimal proliferation for subsequent experiments. For differentiation, cells were seeded in 6-well plates at a density of 6×10 cells/well and treated with 100 ng/ml phorbol 12-myristate 13-acetate (PMA) for 24 h at 37°C with 5% CO to induce differentiation into macrophages. Successful differentiation was confirmed when cells transitioned from single round suspension cells to adherent, irregularly shaped cells with pseudopodia.

1.2.2 Establishment of Mycobacterium tuberculosis-Infected THP-1 Macrophage Model THP-1-derived macrophages were infected with H37Ra at a bacteria-to-cell ratio of 10:1 (MOI=10). After 6 h incubation, the supernatant was removed and replaced with 2 ml fresh complete medium, marking the 0-hour time point of infection. Cells were then cultured for an additional 24 h.

1.2.3 Liposome-Mediated Transfection of THP-1-Derived Macrophages Lipofectamine™ 2000 was used to transfect macrophages with 20 mol/L miR-17-5p mimics or miR-17-5p inhibitors, using control miRNA as a negative control. After 10 h incubation at 37°C with 5% CO , the medium was replaced with fresh medium containing 10% FBS, and cells were cultured for an additional 24 h.

1.2.4 Detection of MicroRNA Expression THP-1-derived macrophages were infected with H37Ra at different multiplicities of infection (MOI = 0, 1, 5, 10, 20). Quantitative real-time PCR (qRT-PCR) was used to detect miR-17-5p expression levels, with U6 snRNA as an internal reference. The reaction mixture contained 2 l template, 1 l each of forward and reverse primers, 10 l SYBR Premix, and RNA-free water to a final volume of 20 l. Each group had three replicate wells, and 2^(-ΔΔCT) values were calculated to analyze miR-17-5p expression.

1.2.5 Detection of Relative miR-17-5p Expression Levels Total RNA was extracted from control, miR-17-5p mimics, and miR-17-5p inhibitor groups. cDNA was synthesized according to the reverse transcription kit instructions, and qRT-PCR was performed to analyze miR-17-5p expression in each group.

1.2.6 Bioinformatics Prediction of Target Genes Bioinformatics software including TargetScan and microRNA.org was used to predict miR-17-5p target sequences in the ATG7 3' -UTR region. Primers were

designed and synthesized: wild-type 3' -UTR forward primer: CCCTCGAGCCGTGTAGAGGGCATCGT; reverse primer: GCGTCGACTCAGAGGAGGCAGCAGAAA; mutant-type forward primer: CCCTCGAGCCGTGTAGAGGGCATCGT; reverse primer: GCGTCGACTTTCCCTACTATATCCAGTTACAGTA. Total RNA was extracted from cells, and target genes were amplified using 2 l RNA, 1 l each of ATG7 primers, 25 l mix, and RNA-free water to 50 l. PCR products were separated on 2% agarose gels, stained with EB for 20 min, washed with distilled water, and visualized using a gel imaging system. Target fragments were recovered using a gel extraction kit.

1.2.7 Vector Construction Target genes and pMirGLO plasmids were double-digested with XhoI and SalI at 37°C for approximately 1 h. Digested fragments and plasmids were ligated using T4 ligase at 22°C for 4 h, then at 4°C overnight to generate recombinant plasmids. These were transformed into competent bacteria and cultured for approximately 14 h. Single positive colonies were selected, and recombinant plasmids pMirGLO-ATG7-3' UTR-WT and pMirGLO-ATG7-3' UTR-MUT were identified by PCR and sequencing.

1.2.8 Dual-Luciferase Reporter Assay Successfully constructed recombinant plasmids pMirGLO-ATG7-3'UTR-WT and pMirGLO-ATG7-3'UTR-MUT were co-transfected with miR-17-5p mimics, miR-17-5p inhibitor, or miR-17-5p nc into HEK293T cells. Each experiment was performed in triplicate. After 48 h transfection, dual-luciferase reagent was added to each well, and firefly and Renilla luciferase activities were measured using a microplate luminometer.

1.2.9 Detection of ATG7 and LC3 Protein Expression Levels THP-1-derived macrophages transfected with control, miR-17-5p mimics, or miR-17-5p inhibitor were observed for transfection efficiency after 8 h, then infected or not with H37Ra. Cellular proteins were extracted using a protein extraction kit, and protein concentrations were determined by BCA assay. Western blot was used to detect ATG7 and LC3 protein expression.

1.2.10 Laser Confocal Microscopy for Immunofluorescence THP-1-derived macrophages from control, miR-17-5p mimics, and miR-17-5p inhibitor groups were cultured on coverslips, fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 for 5 min at room temperature, and blocked with 5% BSA for 30 min. Cells were incubated with LC3 antibody at 37°C for 1 h, then overnight at 4°C, followed by incubation with fluorescent secondary antibody at 37°C for 1 h. Coverslips were mounted with anti-fade mounting medium and observed under a laser confocal microscope.

1.3 Statistical Analysis

All data were analyzed using SPSS 20.0 software. Each experiment was repeated three times, and data are presented as mean ± SEM. One-way ANOVA was used

for statistical comparisons. *, $P < 0.05$;* ***, $P < 0.01$;*** , $P < 0.001$; $P < 0.05$ was considered statistically significant.

Results

2.1. MiR-17-5p Expression Following H37Ra Infection of THP-1 Cells

qRT-PCR analysis revealed that miR-17-5p expression was significantly downregulated following H37Ra infection in a MOI-dependent manner. When MOI > 5, the reduction in miR-17-5p was statistically significant compared to uninfected controls (MOI=0). These results suggest that miR-17-5p may participate in the regulatory processes of H37Ra-infected cells.

[Figure 1: see original paper]. Relative expression of miR-17-5p in THP-1 cells infected by H37Ra at different MOI.

2.2. Vector Construction and Sequencing Validation

Total RNA was extracted from THP-1-derived macrophages, and wild-type and mutant ATG7-3' UTR gene fragments were obtained by PCR and inserted into the pMirGLO luciferase reporter plasmid to generate recombinant plasmids pMirGLO-ATG7-3' UTR-WT and pMirGLO-ATG7-3' UTR-MUT. Double digestion and sequencing confirmed successful construction of both recombinant plasmids [Figure 2: see original paper].

[Figure 2: see original paper]. Identification of recombinant luciferase reporter plasmids. A. Double digestion analysis of pMirGLO-ATG7-3' UTR-WT; 1, recombinant plasmid; 2, plasmid digested with XhoI and SalI; 3, DNA marker. B. Partial sequencing results of pMirGLO-ATG7-3' UTR-WT. C. Double digestion analysis of pMirGLO-ATG7-3' UTR-MUT; 1, recombinant plasmid; 2, plasmid digested with XhoI and SalI; 3, DNA marker. D. Partial sequencing results of pMirGLO-ATG7-3' UTR-MUT.

2.3. Validation of miR-17-5p Targeting of ATG7

Bioinformatics analysis using TargetScan predicted that miR-17-5p could bind to the ATG7-3' UTR [Figure 3A: see original paper]. Transfection of FAM-labeled miR-17-5p mimics and inhibitor into HEK293T cells using Lipofectamine™ 2000 achieved approximately 50% transfection efficiency [Figure 3B: see original paper]. qRT-PCR confirmed that miR-17-5p expression was significantly upregulated in the mimics group and downregulated in the inhibitor group compared to controls [Figure 3C: see original paper], validating the overexpression and knockdown models.

Dual-luciferase reporter assays showed that in cells co-transfected with wild-type ATG7 reporter (pMirGLO-ATG7-3' UTR-WT) and miR-17-5p mimics, luciferase activity was significantly reduced compared to controls. Conversely,

co-transfection with miR-17-5p inhibitor increased luciferase activity. However, in the mutant ATG7 3' UTR group, neither miR-17-5p overexpression nor inhibition significantly altered luciferase activity [Figure 3D: see original paper]. These data demonstrate that miR-17-5p specifically targets the wild-type ATG7-3' UTR but not the mutant sequence.

Western blot analysis in THP-1-derived macrophages further confirmed the regulatory effect. Compared to the control group (H37Ra + miR-17-5p nc), ATG7 protein expression was significantly downregulated in the miR-17-5p mimic group (H37Ra + miR-17-5p mimic) and upregulated in the miR-17-5p inhibitor group (H37Ra + miR-17-5p inhibitor) ($P < 0.05$, [Figure 6: see original paper]), indicating that miR-17-5p negatively regulates ATG7 in H37Ra-infected macrophages.

[Figure 3: see original paper]. Identification of miR-17-5p targeting ATG7. A. Partial sequences of mature miR-17-5p and ATG7 wild-type and mutant 3' UTR. B. Transfection efficiency of miR-17-5p mimics and inhibitor (observed by light and fluorescence microscopy, 10×10). C. Relative miR-17-5p expression detected by qRT-PCR after transfection. D. Relative luciferase activity detection. E. ATG7 protein expression detected by Western blot.

2.4. Effect of miR-17-5p on Autophagy Flux and Autophagosome Formation

Laser confocal microscopy was used to monitor autophagy flux dynamics by examining LC3 immunofluorescence. LC3-II is an autophagosome marker protein involved in autophagosomal membrane elongation and serves as a reliable indicator of autophagy flux. LC3-II expression levels correlate positively with autophagosome number, and the LC3-II/LC3-I ratio reflects autophagic activity [20].

As shown in [Figure 4A: see original paper], under H37Ra infection conditions, inhibition of miR-17-5p (miR-17-5p inhibitor) markedly increased punctate LC3-positive autophagosomes in the cytoplasm, whereas miR-17-5p mimics significantly reduced autophagosome formation compared to controls (miR-17-5p nc). Prior to autophagy induction, LC3 primarily exists as LC3-I and distributes uniformly throughout the cytoplasm. Autophagy activation converts LC3-I to LC3-II, which appears as punctate aggregates reflecting autophagosome numbers. Western blot results showed that LC3-II expression and the LC3-II/LC3-I ratio were increased in the miR-17-5p inhibitor group but decreased in the miR-17-5p mimics group [Figure 4B: see original paper].

[Figure 4: see original paper]. Effects of miR-17-5p on autophagy flux and autophagosome formation. A. LC3-II immunofluorescence detection. B. LC3-II protein expression detected by Western blot.

Discussion

Mycobacterium tuberculosis is an intracellular parasite that primarily infects macrophages, capable of both inducing autophagy and evading immune elimination. Current research indicates that MTB infection triggers immune responses and induces autophagy, primarily through activation of pattern recognition receptors such as Toll-like receptors (TLRs) by MTB surface lipoproteins and nucleic acids. TLRs recognize pathogen-associated molecular patterns (PAMPs) and play crucial roles in innate immunity by promoting cytokine synthesis, antigen-presenting cell maturation, and adaptive immune responses [21]. For instance, hypoxia-induced miR-155 upregulation promotes autophagy via the mTOR pathway during autophagosome formation [22], while miR-149 negatively regulates TLR-triggered inflammatory responses by targeting MyD88 [23].

Autophagy serves as a protective mechanism for maintaining intracellular homeostasis and plays a vital role in innate immunity against pathogen invasion. MTB infection induces formation of double-membrane autophagosomes that fuse with lysosomes to degrade engulfed bacilli, making autophagy a primary defense mechanism in macrophages [24]. However, MTB has evolved autophagy evasion mechanisms that enable latent intracellular infection, complicating bacterial survival and disease pathogenesis [24]. Studies have shown that miR-26 inhibits autophagy by targeting KLF4 during MTB infection [25], while miR-30a targets BECN1 to suppress autophagy and is highly expressed in TB patients, decreasing after anti-TB treatment to promote intracellular bacterial survival [26].

MicroRNAs exert fine-tuned regulation during autophagy. Our study demonstrates that miR-17-5p expression is significantly downregulated in MTB-infected human macrophages, suggesting that enhanced autophagy during infection occurs through microRNA downregulation. We investigated the mechanism by which miR-17-5p directly targets the autophagy-related protein ATG7 in human THP-1 macrophages during anti-MTB responses. In our infection model, miR-17-5p expression decreased with increasing MTB infection intensity. Dual-luciferase reporter assays and Western blot confirmed the targeting relationship between miR-17-5p and ATG7, establishing miR-17-5p as a negative regulator of ATG7. Western blot and immunofluorescence results showing elevated LC3-II and autophagosomes in the miR-17-5p inhibitor group demonstrate that miR-17-5p suppresses autophagy.

Both ATG7 and LC3 play crucial roles in autophagy. As an E1-activating enzyme, ATG7 functions during membrane elongation. LC3-I can be conjugated with phosphatidylethanolamine (PE) through the actions of ATG7 and ATG3 to form the LC3-II-PE complex (LC3-II) [27]. Our findings indicate that miR-17-5p inhibits autophagosome-lysosome fusion and LC3 recycling by suppressing ATG7, thereby reducing LC3-II levels and the LC3-II/LC3-I ratio to inhibit autophagosome formation. The significant reduction of miR-17-5p by MTB

suggests that miR-17-5p downregulation represents one mechanism by which autophagy defends against pathogens during infection.

While miR-17-5p downregulation leads to ATG7 upregulation, enhanced autophagosome-lysosome fusion, and increased autophagy, the precise mechanisms linking miR-17-5p-mediated autophagy regulation to MTB survival and evasion require further investigation. MicroRNAs have emerged as major research targets, serving as both therapeutic targets and disease biomarkers. For example, miR-125b is downregulated in systemic lupus erythematosus patients and inhibits autophagy by targeting UVRAG, impairing anti-MTB responses [28], while miR-9a-5p alleviates ischemic injury by targeting ATG5-mediated autophagy [29]. MiR-148/152 can serve as diagnostic markers for acute myeloid leukemia [30].

This study provides a theoretical foundation for further elucidating the immune regulatory role and mechanisms of miR-17-5p in macrophage defense against MTB. Future work will explore the relationship between MTB and macrophage autophagy pathways, investigate MTB infection and pathogenesis mechanisms, and leverage microRNA research to identify novel therapeutic targets and rapid diagnostic markers for TB control.

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Note: Figure translations are in progress. See original paper for figures.