J Med Biosci Suppl 2025;7: S97 - S105 DOI: 10.58837/CHULA.JMBS.7.S1.15

Original article

Identification of candidate microRNAs associated with tuberculosis in Thai cynomolgus macaques (Macaca fascicularis)

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Abstract

***Background**:* Cynomolgus macaques frequently inhabit areas close to human populations, increasing the risk of *Mycobacterium tuberculosis* complex (MTBC) transmission between humans and non-human primates (NHPs). Tuberculosis (TB) is a chronic airborne disease caused by MTBC, leading to high mortality in humans and NHPs. Controlling TB transmission within and between these species remains a significant challenge due to the complexities of diagnosing TB in NHPs, which necessitate the use of multiple diagnostic tools to ensure accurate and sensitive detection. Emerging evidence suggests that microRNAs (miRNAs) exhibit distinct expression patterns in MTBC infection, making them promising biomarkers for disease detection and monitoring.

***Objective**:* This study aimed to identify candidate miRNA biomarkers associated with active tuberculosis in cynomolgus macaques.

***Methods**:* The plasma samples of cynomolgus macaques were obtained from Krabok-Koo Wildlife Rescue Center, Chachoengsao, Thailand. Eight monkeys were selected and divided into 2 groups, including active TB (n = 4) and uninfected TB (n = 4). The extracted miRNAs were constructed for cDNA libraries and single-end (50 cycles) sequenced in duplicate by DNBSEQ-G400 platform (MGI, China). The data analysis was performed using miRdeep2 and DESeq2 pipelines to identify candidate miRNA biomarkers.

***Results**:* Interestingly, there were 10 miRNAs with significant differential expression in active TB compared to uninfected TB including 5 upregulated and 5 downregulated miRNAs. Five upregulated miRNAs might serve as candidate biomarkers, including miR-200c-5p, miR-664, miR-1262-5p, miR-580, and miR-760.

***Conclusion**:* This study identified a panel of differentially expressed miRNAs as candidate biomarkers for TB infection in cynomolgus macaques, suggesting their potential role in TB pathogenesis.

Keywords: Biomarker, cynomolgus macaques, microRNA, tuberculosis.

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Tuberculosis (TB) is a chronic airborne disease caused by the *Mycobacterium tuberculosis* complex (MTBC) as the main causative agent that produces high morbidity and mortality in both humans and nonhuman primates (NHPs). [1] The World Health Organization (WHO) Global TB Report 2024 indicated that an estimated 10.8 million people fell ill, and 1.25 million people died from TB in 2023. Southeast Asia and African regions showed the highest burden, accounting for 45.0% and 24.0% of global TB cases. [2]

Cynomolgus macaques (*Macaca fascicularis*), also known as crab-eating or long-tailed monkeys, are widely distributed in Southeast Asia. [3] In Thailand, the monkeys commonly reside in human settlements, temples, and popular tourist sites. These extensive interactions between humans and monkeys present a high risk of facilitating the transmission of pathogens, including MTBC. In 2018-2022, the prevalence of MTBC infection in 26 populations of wild cynomolgus macaques living in Thailand was surveyed. [4] Thirteen out of 26 populations were naturally MTBC-infected. In 2020, a large cohort of MTBC-infected captive cynomolgus macaques was discovered at Krabok-Koo Wildlife Rescue Center, an organization under the Department of National Parks, Wildlife, and Plant Conservation of Thailand. [5] However, diagnosing TB in NHPs still has many challenges due to asymptomatic infection phases, the potential reactivation, and the necessity of employing multiple diagnostic tools to ensure accurate and sensitive detection. [6] Thus, controlling TB transmission between monkeys and between monkeys and humans remains a significant challenge.

Upon MTBC infection through aerosolization from the host, the Pattern Recognition Receptors (PRRs), such as Toll-like receptors (TLRs), present on the surface of macrophages and dendritic cells of the recipients, can recognize the pathogen-associated molecular patterns (PAMPs) and stimulate microRNA (miRNA) biogenesis. The miRNAs are small non-coding RNAs with approximately 22 nucleotides that regulate genes by binding to 32 UTRs of target mRNAs, inhibiting the expression or degrading mRNAs. [7] The host cells reprogram their immune responses to activate defense mechanisms against MTBC infection. On the other hand, the bacteria can counter these efforts by modifying the host's miRNA profile to undermine immune defenses and promote intracellular survival. [8, 9] Moreover, miRNAs can be secreted extracellularly via apoptosis, necrosis, exosomes, or by binding to high-density lipoproteins (HDL) and Argonaute RISC catalytic component 2 (AGO2) proteins. [7] Consequently, highly stable MTBC-associated miRNA signatures have been detected in various biological fluids and have been recognized as promising biomarkers for the diagnosis of tuberculosis. This study aimed to identify the candidate miRNA biomarkers to indicate active TB tuberculosis in cynomolgus macaques, which will support the TB diagnosis in NHPs.

Materials and methods

Study cohort

This protocol was approved by the Institutional Animal Care and Use Committee (IACUC) (BT-Animal 16/2566) and the Institutional Biosafety Committee (IBC) (BT-IBC 039/2566) of the National Center for Genetic Engineering and Biotechnology (BIOTEC). Adult cynomolgus macaques, including males and females, were recruited from Krabok-Koo Wildlife Rescue Center, Chachoengsao, Thailand. These macaques lived in the same gang cage or the vicinity of MTBC-infected monkeys reported previously [5], and they were counted as suspected TB monkeys. Eight macaques were selected and classified into 2 groups: uninfected TB (UTB) as a control (n = 4) and active TB (ATB) (n = 4). According to the criteria described in the previous study [10], the ATB group tested positive, whereas the uninfected TB group showed negative results for the Xpert Ultra assay (Cepheid, Sunnyvale, CA, USA).

Specimen collection

The biological specimens (whole blood and pharyngeal wash) were collected. The whole blood of macaques was collected by femoral venipuncture with a maximum of 3 ml/kg body weight and drained in EDTA tubes, followed by plasma isolation. After that, plasma samples were stored at -80 °C until used for miRNA extraction. The pharyngeal wash specimens were collected using phosphate-buffered saline (PBS) to wash the pharyngeal cavity for testing with the Xpert Ultra assay.

miRNA extraction

The miRNAs were extracted using the miRNA Isolation Kit (Geneaid Biotech, Taiwan). Then, the concentration of miRNAs was measured using Qubit™ microRNA Assay Kits (Thermo Fisher, US), and the purity was determined by NanoPhotometer (Implen, German).

Small RNA library preparation and sequencing

The extracted miRNAs from 8 plasma samples (4 ATB and 4 UTB) were used to construct cDNA libraries with different indices using MGIEasy Small RNA Library Prep Kit (MGI, China). The cDNA libraries were quantified, pooled with equal concentration, and single-end (50 cycles) sequenced in duplicate on a DNBSEQ-G400 platform (MGI, China).

Bioinformatic analysis

The FASTQ files were pre-processed using Trimmomatic [11] and Cutadapt [12] for adapter trimming, followed by removing low-quality reads (Q-score < 20) and reads shorter than 18 bp. The filtered reads were then aligned to the cynomolgus monkey genome using Bowtie2. [13] MicroRNA identification was performed by mapping the reads to *Macaca mulatta*, a closely related species available in the miRNA database (miRBase) [14], using the miRDeep2 pipeline. [15] Differential expression (DE) analysis was conducted by DESeq2 package [16] in R v.4.2.3 to identify differentially expressed miRNAs (DE-miRNAs). The criteria for candidate miRNA biomarkers were adjusted $P < 0.05$, and |Log2 Foldchange (LFC)| > 1.

Target prediction and pathway enrichment analysis

To predict the target of DE-miRNAs, TargetScan 8.0 [17] was performed using *Macaca mulatta* miRNA as a reference. The predicted targets were validated for the ortholog gene of *Macaca fascicularis* and analyzed for Gene ontology (GO) and enrichment in KEGG pathways using DAVID [18] with default settings.

Results

Summary of sequencing data

To investigate the miRNA profiles associated with TB in cynomolgus macaques. Small RNA sequencing was conducted on 8 samples (4 ATB and 4 UTB). Pre-processed sequencing reads of small RNA (sRNA) were obtained, with an average length of approximately 28 bp. The mean numbers of pre-processed sRNA sequencing reads were 32,337,049 for the ATB group and 27,508,834 for the UTB group. Annotation of sRNA against the *Macaca mulatta* reference miRNA database revealed distinct miRNA read counts. Specifically, the ATB group exhibited an average of 10,354,185 annotated miRNA reads, whereas the UTB group had an average of 8,797,296 annotated miRNA reads **(Table 1).**

Comparative analysis of miRNA profiles in ATB and UTB

MicroRNA identification was performed by mapping sequencing reads to a reference miRNA database. A total of 353 annotated miRNAs were identified, including 315 (89.2%) shared between ATB and UTB, 19 (5.4%) unique to ATB, and 19 (5.4%) specific to UTB **(Figure. 1A).** The particular miRNAs in ATB and UTB exhibited distinct expression patterns in each sample, while slightly different between replicates **(Figure 1B).**

Differential Expression (DE) Analysis of miRNA in ATB and UTB

Differential expression analyses of miRNAs were conducted, identifying 10 miRNAs exhibiting significant differential expression in ATB compared with UTB (adjusted $P < 0.05$, |LFC| > 1). Among these, five DE-miRNAs were upregulated, while five were

Table 1. Sequencing statistics of miRNA read count.

Sample	Group	Average number of raw reads	Average number of annotated reads (miRNAs)	Average number of raw reads per group	Average number of annotated reads per group
KBK041	UTB	47,289,319	17,634,733 (37.0%)	27,508,834	8,797,296
KBK060	UTB	15,668,010	4,005,905 (26.0%)		
KBK064	UTB	21,047,217	5,396,051 (26.0%)		
KBK066	UTB	26,030,789	8,152,495 (31.0%)		
KBK095	ATB	80,663,294	30,414,282 (38.0%)	32,337,049	10,354,185
KBK105	ATB	18,815,504	4,838,643 (26.0%)		
KBK195	ATB	12,834,227	2,400,153 (19.0%)		
KBK294	ATB	17,035,170	3,763,663 (22.0%)		

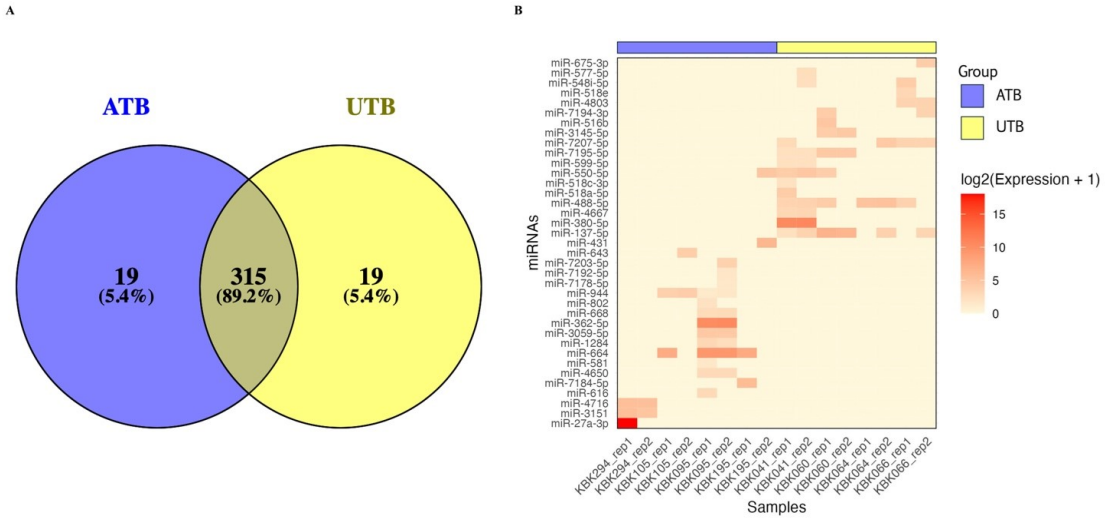

Figure 1. Comparison of miRNA profiles between Active TB (ATB) and Uninfected TB (UTB). (A) A Venn diagram showed 315 collapsed miRNAs between the two groups (gray), along with 19 unique miRNAs in ATB (blue) and 19 unique miRNAs in UTB (yellow); **(B)** Specifically expressed miRNAs showed distinct expression patterns in each sample.

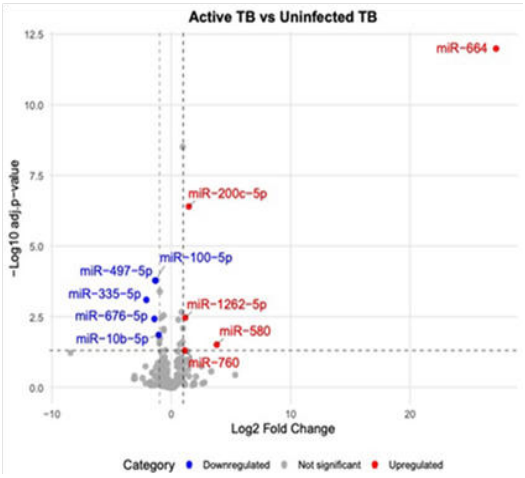

Figure 2. The volcano plot showed DE-miRNAs in ATB compared to UTB.

downregulated. The upregulated DE-miRNAs included miR-200c-5p, miR-580, miR-664, miR-760, and miR-1262-5p. Conversely, the downregulated DE-miRNAs comprised miR-10b-5p, miR-100-5p, miR-335-5p, miR-497-5p, and miR-676-5p **(Figure 2).**

Pathway enrichment analysis

Gene ontology (GO) and KEGG pathway analyses revealed that upregulated DE-miRNAs (miR-200c-5p, miR-580, miR-664, miR-760, miR-1262-5p) were enriched in biological processes (BP) like transcription, symport activity, and GPI-anchor biosynthesis, and linked to cellular components (CC) such as membranes, cytoskeleton, autophagosomes, and receptor complexes. Their molecular functions (MF) included growth factor and cytokine activity, heparin- and DNA-binding. KEGG analysis highlighted their involvement in pathways such as actin cytoskeleton regulation, Ras and Rap1 signaling, and efferocytosis **(Figure 3A, 3C).**

In contrast, downregulated DE-miRNAs (miR-10b-5p, miR-100-5p, miR-335-5p, miR-497-5p, miR-676-5p) were associated with transcriptional regulation, the cell cycle, Ubl conjugation, biological rhythms, and endocytosis. Their targets were enriched in nuclear and extracellular matrix components, with molecular functions involving transferase activity, chromatin regulation, and transcriptional repression. KEGG pathways included key immune-related signals such as TGF-β, NF-κB, Wnt, mTOR, HIF-1, p53, and Hippo **(Figure 3B, 3D).** These results suggest that DE-miRNAs may play essential roles in immune regulation by targeting mRNAs in critical signaling pathways, as presented in **Table 2.**

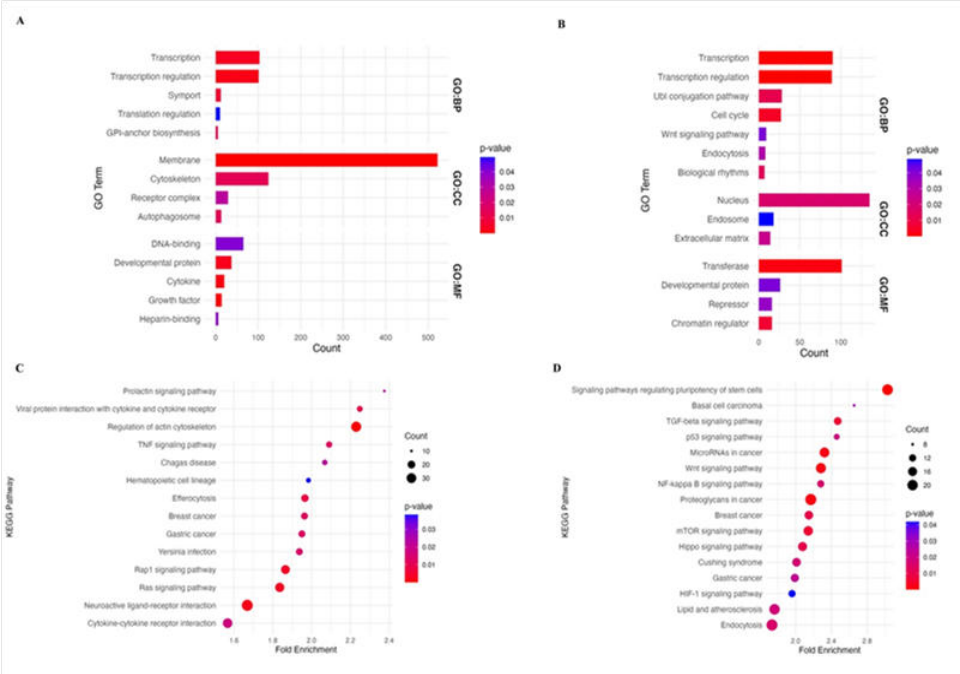

Figure 3. Gene Ontology (GO) enrichment analysis of differentially expressed miRNA (DE-miRNA) targets. **(A)** GO terms in BP, CC, and MF are associated with upregulated DE-miRNAs; **(B)** GO terms in BP, CC, and MF are associated with downregulated DE-miRNAs; **(C)** KEGG pathways enriched among upregulated DE-miRNA targets; **(D)** KEGG pathways enriched among downregulated DE-miRNA targets.

Table 2. mRNA targets of DE-miRNAs enriched in immune biological processes.

DE-miRNA	Expression	Target mRNA	GO-term
miR-200c-5p	Upregulated	CHI3L1	Inflammatory response (GO0006954)
		CRH	
		CRHBP	
		FPR2	
		TAB2	
miR-580	Upregulated	ANO6	Regulation of leukocyte migration
(GO0002685)			
		CCR7	
		CXCL13	
		DNM1L	
		VEGFB	
		ATG12	Autophagy (GO0006914)
		CHMP5	
		RB1CC1	
		RUFY4	
		TMEM41B	
miR-664	Upregulated	C5AR1	Inflammatory response (GO0006954)
		CCL18	
		CCL2	
		CXCL6	
		BMX	Immune response-regulating cell surface
receptor signaling pathway (GO0002768)			
		CD8A	
		PTPN22	
		HRG	Humoral immune response (GO0006959)
		PGLYRP4	
		SLPI	

Table 2. (Cont.) mRNA targets of DE-miRNAs enriched in immune biological processes.

DE-miRNA	Expression	Target mRNA	GO-term
miR-760 (GO0002696)	Upregulated	AMBRA1	Positive regulation of leukocyte activation
		IL6	
		SLC7A1	
miR-10-5p	Downregulated	BCL6	Type 2 immune response (GO0042092)
		GATA3	
miR-335-5p	Downregulated	AHSG	inflammatory response (GO0006954)
		CCR3	
		MAPKAPK2	
miR-676-5p	Downregulated	AIM2	Positive regulation of innate immune response (GO0045089)
		BRCC3	
		CD274	
		IL12A	
		TLR4	
		ADAM10	Leukocyte activation (GO0045321)
		BCL2	
		CXCL6	
		FCER1A	
		HELLS	

Discussion

In this study, we identified candidate miRNAs as potential biomarkers for tuberculosis in cynomolgus macaques. Our findings revealed that the overall miRNA profiles were commonly shared between the active and uninfected TB groups, with only a small subset exhibiting group-specific expression. Ten miRNAs demonstrated significant differential expression in active TB compared to the uninfected group, comprising five upregulated (miR-200c-5p, miR-664, miR-1262-5p, miR-580, and miR-760) and five downregulated miRNAs (miR-10b-5p, miR-100-5p, miR-335-5p, miR-497-5p, and miR-676-5p). These findings suggested that these miRNAs may serve as potential biomarkers for TB in cynomolgus macaques.

Unfortunately, there are limited reports on the role of these miRNAs in tuberculosis within cynomolgus macaques. Five upregulated miRNAs identified in this study lack direct evidence linking them to TB in humans. Previous research showed that hsa-miR-200c-5p represses the viability and invasiveness of colorectal cancer (CRC) cells while promoting apoptosis [19], and hsa-miR-760 suppresses matrix metallopeptidase 2 (MMP2), inhibiting cell proliferation and migration. [20] Meanwhile, hsa-miR-664-3p, hsa-miR-1262, and hsa-miR-580 have not been extensively studied in the context of TB but interact with key immune pathways such as Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (NF-κB) and neutrophil extracellular traps formation in granulomatosis. [21-23] Conversely, some downregulated miRNAs have been associated with TB, particularly hsa-miR-10b-5p and hsa-miR-100-5p. Both miRNAs were found to be upregulated in the plasma of pulmonary TB patients, significantly correlating with cytokine levels such as Tumor Necrosis Factor-Alpha (TNF-α), Interferon-Gamma (IFN-γ), Interleukin-1Beta, 8, 10 (IL-1β, 8, 10), suggesting their potential role in modulating immune responses during infection. However, other miRNAs identified in this study lack direct evidence linking them to TB, though they have been associated with cancer pathways. [24-26] Compared with TB studies in humans, Yurong Fu, *et al.* reported distinct circulating miRNA profiles in active pulmonary tuberculosis patients compared to healthy controls. They identified 59 significantly upregulated (e.g., hsa-miR-744, hsa-miR-574-5p, hsa-miR-218, hsa-miR-29a) and 33 downregulated miRNAs (e.g., hsa-miR-206, hsa-miR-154, hsa-miR-944, hsa-miR-371-3p), particularly miR-29a showed significant upregulation in the active pulmonary tuberculosis group and strong diagnostic potential. [27]

The present study provides insight into the potential biological roles of DE-miRNAs in cynomolgus macaques through GO and KEGG pathway analyses. Our findings demonstrate that both upregulated and downregulated DE-miRNAs are directly involved in key cellular and molecular pathways, particularly those associated with immune regulation. The upregulated DE-miRNAs in active TB compared to uninfected (miR-200c-5p, miR-664, miR-1262-5p, miR-580, and miR-760) were significantly enriched in biological processes related to transcriptional regulation and cellular signaling. Notably, miR-200c-5p has been previously implicated in epithelial-to-mesenchymal transition (EMT). [28] Their targets were localized to membrane and autophagosome components, which are critical in intracellular trafficking. KEGG enrichment analysis demonstrated that upregulated DE-miRNAs target multiple immune signaling pathways, implying their potential role in compromising host immunity through pathway suppression, which may facilitate MTBC survival and active tuberculosis development. For instance, the TNF signaling pathway is a central mediator of inflammation and granuloma formation, both of which are hallmarks of the host defense mechanism against MTB. [29] Similarly, cytokine-cytokine receptor interaction pathways coordinate immune cell communication and recruitment. Dysregulation of cytokine signaling is a critical pathway of TB pathogenesis, where the balance between pro- and anti-inflammatory cytokines determines disease outcome. [30] Efferocytosis, the phagocytic clearance of apoptotic cells, plays an increasingly recognized role in TB pathogenesis by maintaining tissue homeostasis and preventing excessive necrosis that could facilitate bacterial dissemination [31] The enrichment of this pathway suggests that upregulated miRNAs may adjust immune cell turnover, promoting a balanced host response during infection. Moreover, the hematopoietic system generates all blood cells, especially innate immune cells, and adaptive immune cells, supporting immunity and blood balance. [32]

In contrast, downregulated DE-miRNAs such as miR-10b-5p, miR-100-5p, miR-335-5p, miR-497-5p, and miR-676-5p were associated with key processes like cell cycle progression and transcriptional repression. Interestingly, KEGG pathway enrichment analysis of down-regulated DE-miRNAs was enriched in several immune-related signaling pathways, which may be involved in the host response to MTBC infections. Especially, the NF-κB pathway plays a role in promoting the production of pro-inflammatory cytokines (e.g., TNF-α, IL-1β, IL-6) and chemokines critical for granuloma formation. [33] mTOR and HIF-1α signaling critically influence macrophage metabolic reprogramming, thereby determining their capacity to control MTB replication. [34] While some pathways may promote pathogen survival, the TGF-β signaling pathway is an anti-inflammatory cytokine that inhibits T-cell effector functions and suppresses macrophage activation. [35] Wnt signaling plays a role in balancing immune activation and suppression. *Mycobacterium tuberculosis* can manipulate both canonical (β-catenin-dependent) and non-canonical Wnt pathways to inhibit autophagic clearance, promote anti-inflammatory macrophage polarization (M2 phenotype), and evade host immunity. [36] The downregulation of miRNAs targeting both pathway components may represent a host compensatory mechanism to restore tissue damage, although it could potentially create a vulnerability for incomplete pathogen clearance.

However, our study has limitations that should be acknowledged. First, the small sample size in this study may limit the statistical power of our findings. Future studies with larger sample sizes are necessary to validate these findings. Second, some of the identified miRNAs have limited evidence linking them directly to TB, particularly in non-human primates. This highlights a current gap in the field and suggests a need for further studies to explore the potential roles of these miRNAs in host immune responses and TB disease progression. Although this study provided the probability of being targeted by DE-miRNAs and their associated function, it lacks experimental validation to confirm the biological significance of these findings. Future research should focus on functional validation of these miRNAs to confirm the role of these miRNAs in TB pathogenesis, contributing to a deeper understanding of host-pathogen interactions and the development of miRNA-based biomarkers for TB diagnosis and monitoring.

Conclusion

In summary, this study identified a panel of differentially expressed miRNAs as potential biomarkers for tuberculosis in cynomolgus macaques. Ten candidate miRNAs exhibited significant differential expression, suggesting their potential role in TB pathogenesis.

Acknowledgements

The study was supported by the Alliance of International Science Organizations (ANSO) (no. P2350823), and H.M. the King Bhumibhol Adulyadej's 72nd Birthday Anniversary Scholarship. We thank members of the Center of Excellence in Systems Microbiology, Tuberculosis Research Team of National Center for Genetic Engineering and Biotechnology (BIOTEC), National Primate Research Center of Thailand, and Krabok-Koo Wildlife Rescue Center for sample collection.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data sharing statement

Data sharing statement. All data generated or analyzed during the present study are included in this published article. Further details are available for noncommercial purposes from the corresponding author on reasonable request.

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