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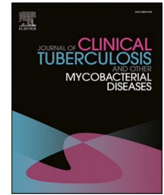

Exosomal microRNAs associated with tuberculosis among people living with human immunodeficiency virus

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ABSTRACT

Objective: To investigate the diagnostic value of selected exosomal miRNAs for Tuberculosis (TB) among people living with human immunodeficiency virus (PLHIV).
Methods: A total of 43 adult HIV patients, including 20 diagnosed with TB and 23 controls, were enrolled. The levels of six exosomal miRNAs (miR-20a, miR-20b, miR-26a, miR-106a, miR-191, and miR-486) were measured using qRT-PCR.
Results: The levels of these six exosomal miRNAs (miR-20a, miR-20b, miR-26a, miR-106a, miR-191, and miR-486) were significantly higher in the plasma of TB patients compared to controls among PLHIV. The Receiver Operating Characteristic (ROC) curve of these six miRNAs showed a fair performance in distinguishing TB patients from controls, with Area Under Curve (AUC) values of 0.78 (95 %CI 0.63–0.93), 0.81 (95 %CI 0.67–0.95), 0.77 (95 %CI 0.61–0.93), 0.84 (95 %CI 0.70–0.98), 0.82 (95 %CI 0.68–0.95) and 0.79 (95 %CI 0.65–0.93), respectively. These miRNAs showed higher AUC values for extrapulmonary tuberculosis compared to pulmonary tuberculosis. An analysis of subgroups was performed based on CD4 + T cell count (< 200 and ≥ 200 cells·μL^{-1}). In the high CD4 count group, all these six exosomal miRNAs appeared to have higher AUC values compared to the low CD4 count group.
Conclusions: These six exosomal miRNAs could serve as potential biomarkers for diagnosing TB among PLHIV.

1. Introduction

Tuberculosis (TB), an infectious disease caused by Mycobacterium tuberculosis (Mtb), is a significant global health issue. Approximately a quarter of the global population is infected with Mtb, with a 5–10 % lifetime risk of developing active TB [1]. In 2022, it was estimated that 7.5 million people were newly diagnosed with TB. China accounted for 7.1 % of global TB cases, ranking as the third highest TB burden country worldwide. TB was the second leading infectious cause of death after COVID-19, resulting in 1.3 million deaths worldwide [2]. Human immunodeficiency virus (HIV) infection is a major risk factor for developing TB [2]. Individuals infected with HIV are over 20 times more likely to develop active TB than those who are not HIV-infected [3]. Additionally, TB can increase viral replication, potentially accelerating HIV disease progression. The interplay between HIV and TB significantly heightens mortality. The mortality rate is higher in TB cases with HIV compared to those without HIV [4]. Therefore, an early diagnosis of TB among HIV patients is crucial for initiating treatment promptly to improve prognosis.

Current TB diagnosis methods, such as smear microscopy, bacterial culture, and GeneXpert MTB/RIF, each have specific limitations. Smear microscopy has low sensitivity, cultures take weeks to yield results, and GeneXpert MTB/RIF is costly and impractical in settings with limited resources [2]. Culture and GeneXpert MTB/RIF, despite their high sensitivity and specificity for smear-positive cases, have decreased diagnostic accuracy in smear-negative cases, among people living with HIV (PLHIV), individuals with extrapulmonary tuberculosis (EPTB), and children [5]. Given these limitations, there is an urgent need for new biomarkers to improve TB diagnosis, especially in specific populations, such as PLHIV.

Exosomes, 30–100 nm vesicles containing RNA and proteins, are released by living cells into the bloodstream. These vesicles facilitate cell communication and immune regulation by delivering their contents to target cells. Recently, the potential of exosomal microRNAs (miRNAs) as biomarkers for various diseases has been investigated due to their abundance, ease of sampling, stability, and regulatory functions [6–8]. However, research on exosomal miRNAs in TB diagnosis remains limited [9,10]. Hu et al. found that exosomal miRNAs (miR-20a, miR-20b, miR-

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26a, miR-106a, miR-191, and miR-486) could potentially enhance the clinical diagnosis of pulmonary tuberculosis (PTB) and tuberculous meningitis (TBM) [10]. This study, however, did not include PLHIV, leaving the diagnostic efficiency of these exosomal miRNAs within this population uncertain [10]. Therefore, in the study, we aimed to determine the diagnostic efficacy of these six exosomal miRNAs for TB in PLHIV.

2. Methods

2.1. Patients and samples

A total of 43 adult HIV in-patients, with no previous history of TB, were not consecutively recruited from Xixi Hospital of Hangzhou between July 2021 and June 2023. Of these participants, 20 were diagnosed with TB, while the remaining 23 served as the control group. The control group, all screened for TB, were not considered for TB based on clinical assessments and MTB test results. The T-SPOT.TB (TSPOT) test identified 10 individuals in the control group as positive for latent tuberculosis infection (LTBI), while 13 tested negative. TB diagnosis, driven by clinicians, was based on clinical and microbiological indicators and involved sending relevant specimens for testing. Microbiologically confirmed TB was classified as having one or more positive samples from acid-fast bacilli, Xpert, culture, or Next Generation Sequencing (NGS). The HIV status of the subjects was confirmed using an HIV antibody test. All study participants were undergoing antiretroviral therapy (ART). The discarded biological blood samples from these 43 patients were preserved for the detection of exosomal miRNAs. Blood samples were collected before starting anti-TB drug therapy and centrifuged at 1500 × g for 15 min at 4 °C. The plasma was then isolated and stored at − 80 °C until needed. Informed consent was obtained from all participants or their legal guardians. The Ethics Committee of Xixi Hospital of Hangzhou approved the study (2021040).

2.2. Exosome isolation and RNA extraction

Exosomes were extracted from plasma using the Exosome Isolation and Purification Kit (Umibio, Shanghai, China) according to the manufacturer's instructions. Total RNA was extracted from exosomes using the Magnetic Beads Nucleic Acids Extraction Kit (Daan Gene, Guangzhou, China).

2.3. Real-time quantitative polymerase chain reaction (qRT-PCR)

The poly(A)-tailed miRNA qRT-PCR primers, specific for six miRNAs (miR-20a, miR-20b, miR-26a, miR-106a, miR-191, and miR-486), were designed and synthesized by RiboBio (Guangzhou, China). The primer sequences were not disclosed due to the company's patent protection. RNAs were reverse transcribed using the Mir-X miRNA First-Strand Synthesis Kit (Takara, Japan). qRT-PCR was carried out by MA-6000 Real-Time Quantitative Thermal Cycler (Molarray, Suzhou, China) using TB Green Advantage qPCR Premix (Takara, Japan). Expression levels of all the miRNAs were normalized to an U6 internal control using the $2^{-\Delta\Delta Ct}$ method. In this experiment, U6 was stable, and there were no significant differences in U6 levels between the study groups.

2.4. Statistical analysis

Data were presented as mean ± SD and compared using the *t*-test for normal distributions. Alternatively, they were presented as Median (Interquartile Range) and compared using the Wilcoxon rank-sum test for non-normal distributions. Categorical variables, expressed as percentages, were compared using the Chi-square or Fisher's exact test, as appropriate. For each miRNA, a receiver operating characteristic (ROC) curve was generated. The area under the curve (AUC) value and 95 % confidence intervals (CI) were calculated to assess diagnostic performances. A p-value < 0.05 was considered statistically significant. Data analyses were performed using SPSS (IBM SPSS Statistics, version 24.0).

3. Results

The study included a total of 43 HIV-infected patients, with 20 in the TB group and 23 in the control group. Out of the 20 TB patients, 18 had microbiologically confirmed TB, while 2 were clinically diagnosed with PTB. The TB group consisted of 14 PTB and 6 EPTB cases. The 6 EPTB cases included 2 cases each of TBM, lymphatic tuberculosis, and tuberculous pleurisy. All EPTB cases had positive MTB test results from the respective sites. The control group included 10 cases of LTBI.

Table 1 displays the demographic and clinical characteristics of the study population. Compared to the control group, the TB group showed a significantly higher proportion of positive HIV RNA and CD4 count of less than 200 cells·μL^{-1}. There were no significant statistical differences in age, gender, CD4/CD8 ratio, or CD4 count between the TB and control groups.

The expression levels of six exosomal miRNAs (miR-20a, miR-20b, miR-26a, miR-106a, miR-191, and miR-486) were evaluated in patients with TB, PTB, and EPTB compared to controls (Table 2 and Figure S1). All six miRNAs showed significantly increased expression in TB patients, with relative fold changes of 3.04, 2.75, 2.95, 4.15, 3.64, and 3.17, respectively. Moreover, these miRNAs demonstrated significantly higher expression levels in both PTB and EPTB patients compared to controls, with an even larger fold change seen in EPTB.

The diagnostic performances of six exosomal miRNAs (miR-20a, miR-20b, miR-26a, miR-106, miR-191, and miR-486) in distinguishing the TB group from the control group were analyzed (Table 3 and Figure S2). All six miRNAs demonstrated fair accuracy in discriminating TB from controls, with AUCs of 0.78, 0.81, 0.77, 0.84, 0.82, and 0.79, respectively. Using the highest Youden's index as the cut-off value, we obtained the following sensitivity and specificity: miR-20a (95.7 %, 60.0 %), miR-20b (91.3 %, 65.0 %), miR-26a (100.0 %, 65.0 %), miR-106a (95.7 %, 75.0 %), miR-191 (78.3 %, 80.0 %), and miR-486 (87.0 %, 65.0 %). We simultaneously evaluated the diagnostic effectiveness of six exosomal miRNAs in differentiating PTB and EPTB from the control group (Table 3 and Figure S2). The results showed that these miRNAs had larger AUC values for EPTB than for PTB. The miRNAs and their respective AUC values were: miR-20a (0.82 for EPTB vs 0.76 for PTB), miR-20b (0.85 vs 0.80), miR-26a (0.78 vs 0.77), miR-106a (0.99 vs 0.77), miR-191 (0.93 vs 0.76), and miR-486 (0.91 vs 0.74).

Subgroup analysis was conducted based on CD4 count. These were divided into a low CD4 count group (CD4 count < 200 cells·μL^{-1}) (n = 24), and a high CD4 count group (CD4 count ≥ 200 cells·μL^{-1}) (n = 19). In the high CD4 count group, miR-20b, miR-26a, miR-106a, and miR-191 were more highly expressed in TB patients, with relative fold changes of 4.03, 3.02, 6.13, and 4.23, respectively, compared to the low CD4 count group (Table 4 and Figure S3). Furthermore, all six exosomal miRNAs (miR-20a, miR-20b, miR-26a, miR-106a, miR-191, and miR-

Table 1
Demographic and clinical characteristics of study participants.

	TB (n = 20)	Non-TB (n = 23)	p-value
Age (years)	40.6 ± 12.0	44.4 ± 15.9	0.373
Male gender, n(%)	16 (80.0 %)	21 (91.3 %)	0.286
Positive HIV RNA, n(%)	14 (70.0 %)	9 (39.1 %)	0.043
CD4/CD8 ratio	0.3 (0.1–0.4)	0.4 (0.2–0.7)	0.201
CD4 + T cell count (cells·μL^{-1})	104 (18–321)	217 (98–380)	0.214
Low CD4 + T cell count*, n(%)	15 (75.0 %)	9 (39.1 %)	0.018
Positive T-SPOT.TB, n(%)	15 (75.0 %)	10 (43.5 %)	0.037
Positive MTB tests, n(%)	18 (90.0 %)	–	–
Pulmonary TB, n(%)	14 (70.0 %)	–	–

TB: tuberculosis; MTB tests included acid-fast bacilli, Xpert, culture, or Next Generation Sequencing. * CD4 + T cell count < 200 cells·μL^{-1}.

Table 2
Differentially expressed exosomal miRNAs in TB, PTB, and EPTB compared to controls.

miRNA name	TB		PTB		EPTB	
	Fold change	p-value	Fold change	p-value	Fold change	p-value
miR-20a	3.04	0.002	2.49	0.008	4.33	0.016
miR-20b	2.75	<0.001	2.67	0.002	2.92	0.008
miR-26a	2.95	0.002	2.46	0.006	4.09	0.036
miR-106a	4.15	<0.001	3.25	0.006	6.26	<0.001
miR-191	3.64	<0.001	3.27	0.007	4.51	<0.001
miR-486	3.17	0.001	2.62	0.015	4.45	0.001

TB: tuberculosis; PTB: pulmonary tuberculosis; EPTB: extrapulmonary tuberculosis.

486) were assessed to distinguish TB patients from controls (Table 5). The results showed that, compared to the low CD4 count group, all six exosomal miRNAs in the high CD4 count group appeared to have higher AUC values, with 0.89, 0.93, 0.87, 1.00, 0.89, and 0.81, respectively.

4. Discussion

Diagnosing and treating co-infection with TB-HIV is crucial because the interaction between HIV and TB significantly increases mortality rates. Microbiological tests, commonly used for TB diagnosis, are less effective in populations like smear-negative TB, EPTB, and PLWH [5]. Symptom screening, the primary method for detecting tuberculosis worldwide, has low sensitivity in those undergoing ART and low specificity in those not receiving such therapy, making it ineffective for PLWH [11]. Additionally, PLWH often present with a high frequency of smear-negative TB and EPTB, making the accurate diagnosis of TB challenging in this population. Hence, there is an urgent need for new biomarkers to improve TB diagnosis in PLHIV [12]. An ideal biomarker should be cost-effective, easily obtainable from an accessible sample like blood or urine, and measurable using user-friendly, point-of-care equipment [5]. Both pathogen-based and host-based biomarkers have been extensively studied in the TB field, and the value of exosomal miRNAs, a type of host-based biomarker, is being evaluated for TB diagnosis.

In a study by Hu et al., six exosomal miRNAs (miR-20a, miR-20b, miR-26a, miR-106a, miR-191, and miR-486) were identified using microarray and qRT-PCR techniques in a non-HIV Chinese population [10]. Hu et al. found that the combination of these six exosomal miRNAs provided significant diagnostic efficacy for PTB and TBM, achieving an AUC of 0.87 and 0.95, respectively. Moreover, integrating miRNAs with electronic health records data in a machine learning algorithm resulted in the highest diagnostic efficacy, with an AUC up to 0.97 for both PTB and TBM. Among the six exosomal miRNAs, miR-20b, miR-191, and miR-486 all demonstrated significant discriminatory value for PTB and TBM.

In this study, we further assessed the diagnostic performance of these six exosomal miRNAs for TB in PLWH. Our study included 20 HIV patients diagnosed with TB, including 6 with only EPTB and 2 with TBM. We found that all six exosomal miRNAs all had fair accuracy in differentiating TB. The six exosomal miRNAs also appeared to provide greater diagnostic value for EPTB, as indicated by higher AUC values compared to PTB. This finding is consistent with the research conducted by Hu et al. [10]. However, due to the limited sample size, we could not assess the diagnostic value of combining these six exosomal miRNAs for TB. No single exosomal miRNA in this study met the World Health Organization's target product profiles (TTP) of $\geq$ 80 % sensitivity and $\geq$ 98 % specificity in adults with HIV [13]. Future studies may explore combinations of multiple exosomal miRNAs to achieve the TTP goal.

HIV-induced depletion of CD4 + T lymphocytes weakens cellular immunity, making individuals more susceptible to opportunistic infections like TB, even when on ART [14]. Numerous studies indicate that HIV-infected patients with a reduced CD4 count, particularly those with less than 200 cells·μL^{-1}, are at a greater risk of TB [15–17]. In our study, we observed that TB patients among PLWH had a lower CD4 count, with a larger proportion having less than 200 cells·μL^{-1}. Furthermore, a subgroup analysis based on CD4 count demonstrated that these six exosomal miRNAs appeared to be more effective in diagnosing TB in a group with a higher CD4 count (above 200 cells·μL^{-1}). However, this result should be interpreted with caution due to the limited sample size. This phenomenon could be attributed to exosomal miRNAs being influenced by the different immune statuses of HIV patients, which

Table 4
Differentially expressed exosomal miRNAs in TB compared to controls based on different subgroups.

miRNA name	CD4 count < 200 cells·μL^{-1}		CD4 count $\geq$ 200 cells·μL^{-1}	
	Fold change	p-value	Fold change	p-value
miR-20a	2.89	0.048	2.70	0.010
miR-20b	1.92	0.096	4.03	0.003
miR-26a	2.64	0.084	3.02	0.014
miR-106a	3.16	0.048	6.13	<0.001
miR-191	3.41	0.030	4.23	0.010
miR-486	3.45	0.025	3.22	0.044

TB: tuberculosis; PTB: pulmonary tuberculosis; EPTB: extrapulmonary tuberculosis.

Table 5
The diagnostic performance of six exosomal miRNAs in differentiating TB from control subjects by receiver operating characteristic (ROC) curves based on different subgroups.

miRNA name	CD4 count < 200 cells·μL^{-1}		CD4 count $\geq$ 200 cells·μL^{-1}	
	AUC (95 %CI)	p-value	AUC (95 %CI)	p-value
miR-20a	0.75 (0.55–0.95)	0.046	0.89 (0.72–1.00)	0.012
miR-20b	0.71(0.49–0.93)	0.089	0.93 (0.79–1.00)	0.005
miR-26a	0.72 (0.51–0.93)	0.079	0.87 (0.64–1.00)	0.016
miR-106a	0.75 (0.55–0.95)	0.046	1.00 (1.00–1.00)	0.001
miR-191	0.77 (0.58–0.96)	0.030	0.89 (0.73–1.00)	0.012
miR-486	0.78 (0.59–0.97)	0.025	0.81 (0.59–1.00)	0.042

TB: tuberculosis; PTB: pulmonary tuberculosis; EPTB: extrapulmonary tuberculosis; AUC: Area Under Curve.

Table 3
The diagnostic performances of six exosomal miRNAs in differentiating TB, PTB, and EPTB from control subjects by receiver operating characteristic (ROC) curves.

miRNA name	TB vs Non-TB		PTB vs Non-TB		EPTB vs Non-TB	
	AUC (95 %CI)	p-value	AUC (95 %CI)	p-value	AUC (95 %CI)	p-value
miR-20a	0.78 (0.63–0.93)	0.002	0.76 (0.58–0.94)	0.009	0.82 (0.59–1.00)	0.018
miR-20b	0.81 (0.67–0.95)	<0.001	0.80 (0.62–0.97)	0.003	0.85 (0.69–1.00)	0.010
miR-26a	0.77 (0.61–0.93)	0.002	0.77 (0.57–0.96)	0.007	0.78 (0.52–1.00)	0.036
miR-106a	0.84 (0.70–0.98)	<0.001	0.77 (0.58–0.96)	0.006	0.99 (0.97–1.00)	<0.001
miR-191	0.82 (0.68–0.95)	<0.001	0.76 (0.60–0.93)	0.008	0.93 (0.85–1.00)	0.001
miR-486	0.79 (0.65–0.93)	0.001	0.74 (0.56–0.92)	0.016	0.91 (0.79–1.00)	0.003

TB: tuberculosis; PTB: pulmonary tuberculosis; EPTB: extrapulmonary tuberculosis; AUC: Area Under Curve.

requires further exploration in future research.

Mtb infections modify the host's miRNA expression, potentially affecting immune responses [18]. Moreover, specific miRNA expression patterns may serve as diagnostic biomarkers for TB [19]. Hu et al. conducted a functional analysis of the six miRNAs (miR-20a, miR-20b, miR-26a, miR-106a, miR-191, and miR-486), suggesting their possible role in TB immunoregulation [10]. These miRNAs are involved in the regulation of autophagy, inflammatory responses, and apoptosis during Mtb infection [20]. For instance, miR-20a can target autophagy-related genes, ATG7 and ATG16L1, to regulate autophagy and support Mtb survival [21]. miR-26a can reduce immune responses and IFNγ-induced macrophage activation by targeting p300, a component of the IFNγ signaling cascade [22]. The mechanisms how these six exosomal miRNAs participate in Mtb infection warrant further study.

Several limitations should be considered. Firstly, the isolated exosomes in the study were not further verified. However, the commercial Exosome Isolation and Purification Kit (Umibio, Shanghai, China) used in the study has undergone product quality control. Secondly, the study had a limited sample size, indicating that the results should be interpreted with caution. Additionally, the cumulative diagnostic effect of the six exosomal miRNAs on TB has not been evaluated yet.

5. Conclusion

Our findings suggest that exosomal miRNAs (miR-20a, miR-20b, miR-26a, miR-106a, miR-191, and miR-486) may serve as potential biomarkers for diagnosing TB among PLWH, have not yet met the TTP goal. These six exosomal miRNAs appear to have superior diagnostic value in EPTB. However, these findings still need to be verified in larger cohorts.

Ethical approval

This study was approved by the Ethics Committee of Xixi Hospital of Hangzhou (2021040). Informed consent was obtained from all participants or their legal guardians.

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CRediT authorship contribution statement

Yujiao Jin: Writing – review & editing, Writing – original draft, Project administration, Methodology, Data curation, Conceptualization. **Yuan Liu:** Writing – review & editing, Writing – original draft, Validation, Data curation. **Wenyan Yu:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Yan Zhang:** Formal analysis, Data curation. **Kenv Pan:** Writing – review & editing, Writing – original draft, Formal analysis. **Miaochan Wang:** Validation, Formal analysis. **Aifang Xu:** Supervision, Methodology, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jctube.2024.100453.

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