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# Developing a diagnostic method for latent tuberculosis infection using circulating miRNA

Shoji Hashimoto<sup>1</sup>, Hong Zhao<sup>2</sup>, Michiyo Hayakawa<sup>3</sup>, Koichi Nakajima<sup>2</sup>, Y-h Taguchi<sup>4</sup> and Yoshiki Murakami<sup>5\*</sup>

## Abstract

**Background:** *Mycobacterium tuberculosis* is known to cause latent tuberculosis infection (LTBI) in 25–50% of the cases, of whom 10–20% develop active tuberculosis (TB). Notably, no marker currently exists for judging the therapeutic effect of TB; it is currently judged by chest X-ray and clinical symptoms. We attempted to establish a marker for distinguishing LTBI from active TB and to identify the probability of recurrence after TB treatment, using information on circulating miRNA expression.

**Methods:** In total, 32 patients were enrolled in this study: 16 with an onset or recurrence of active TB, and 16 with LTBI showing positive interferon-gamma release assays (IGRA) test and chest X-ray. Total RNA from serum in an exosome-rich fraction was first extracted, followed by miRNA expression analysis using a next-generation sequencer, then, this data were analyzed using miRDeep2.

**Results:** Using the expression information of eight miRNAs, LTBI and TB could be diagnosed with an accuracy of 71.8% (odds ratio: 6.16, *p* value = 3.20e-02).

**Conclusions:** A novel method for efficiently differentiating between LTBI and active TB was established. This method appears to be promising for evaluating the therapeutic effect of TB, as it can be performed in a minimally invasive manner.

**Keywords:** LTBI, TB, miRNA, Next-generation sequencing

## Background

Tuberculosis (TB) is an infectious disease caused by *Mycobacterium tuberculosis* (Mtb) that affects the lungs. It is estimated that nearly 25% of the world's population is affected by TB [1]. The reason behind the uncontrollability of TB infection is not only poverty and coinfection with human immunodeficiency virus but also the lack of adequate information on the transmission and pathogenesis of this disease. Notably, nearly 5–15% of the people infected with TB develop overt infection, whereas most patients have latent tuberculosis infection (LTBI). However, patients with overt infection are at a risk of

reactivation after completing their treatment [2]. Therefore, in TB treatment, it is important to diagnose LTBI and prevent the development of TB from LTBI. Once infected with TB, most people remain asymptomatic and noncontagious. However, 5–15% of cases with LTBI may progress to active TB during their lifetime and become infectious [3]. The risk of progression becomes even higher in younger children [4] and immunocompromised or immunosuppressed patients [5, 6].

Though for a long time tuberculin skin test (TST) had been used for the diagnosis of TB, now blood-based interferon-gamma release assays (IGRAs) are widely used to diagnose TB and LTBI because of their simplicity and high specificity [7], and positive IGRA test, normal chest X-ray and contact history (especially within 2 years in high prevalence area) are required to diagnose

\* Correspondence: [yoshikim@tokyo-med.ac.jp](mailto:yoshikim@tokyo-med.ac.jp)

<sup>5</sup>Department of Molecular Pathology, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan  
Full list of author information is available at the end of the article



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LTBI [8]. Moreover it is difficult to determine whether LTBI, a potential infectious disease has been successfully treated (prevented) [9, 10]. Therefore, an accurate and convenient diagnostic method for LTBI has been desired.

Generally, miRNA is a noncoding type of RNA that has a length of about 20 bases, which regulates the expression of a target gene in a base-sequence-specific manner. Many researchers have reported that abnormal miRNA expression is related to the onset and exacerbation of many diseases for example, infectious diseases, degenerative diseases and cancer [11]. In addition, considering the fact that miRNAs are released extracellularly by cell-derived endoplasmic reticulum such as exosomes, miRNA in exosome reflects cellular information, and it is possible to obtain clinical information similar to that of collecting relevant organs [12]. Infection with *Mtb* first induces an interaction with macrophages, following which macrophages infected with *Mtb* release particular miRNAs packaged in exosomes into the extracellular space [13]. Several researchers have reported that many highly expressed miRNAs (miR-484, miR-425, and miR-96) in exosomes are useful in the TB diagnosis [14]. However, this method for analyzing the miRNA profiles of TB and LTBI has not been applied in the diagnosis of LTBI [15].

Reducing the incidence of active TB requires new diagnostic tools and therapeutic options along with a better understanding of the microbiological and clinical spectrum of TB. In this study, we attempt to establish a diagnostic method using circulating miRNAs to develop a biomarker that can help easily evaluate the diagnostic efficiency and therapeutic effects of LTBI and active TB.

## Methods

### Clinical background

The study subjects comprised 16 patients with active TB, and 16 patients with LTBI (all above 18 years old) who visited or were hospitalized at the Osaka Habikino Medical Center for evaluation and initial treatment. This study was performed according to the principles of the Declaration of Helsinki. All study participants provided informed consent. Moreover, this clinical analysis was ethically reviewed and approved by the Osaka Habikino Medical Center (no. 773–4) and Osaka City University (no. 3747).

### RNA extraction and next-generation sequencing analysis

Using 900  $\mu\text{L}$  of serum, an exosome-rich fraction was extracted using ExoQuick (System Biosciences, Palo Alto, CA, USA), and total RNA was extracted from a 200  $\mu\text{L}$  ExoQuick-treated solution using an RNeasy Mini Kit (Qiagen, Hilden, Germany). The concentration, integrity number, 28S/18S ratio, and sample size of the

extracted RNA were qualified using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

To select an 18–30 nt stripe and recycle, total RNA was size-fractionated using polyacrylamide gel electrophoresis (PAGE) gel. An adaptor sequence was ligated with size-fractionated RNA and amplified. All PCR products were purified using PAGE, and then the recycled products were dissolved in a nuclease free water and the PCR product prepared was labeled to create a library. The library was then quantified, and the qualified libraries were sequenced using an Illumina HiSeq 4000 System (Illumina, San Diego, CA, USA). All sequencing data were deposited in NCBI's Gene Expression Omnibus and are accessible through the GEO accession number GSE149645.

### Statistics

In this study, all miRNAs were selected using the recently proposed unsupervised feature extraction method based on tensor decomposition (TD) [16]. In total, 32 FASTQ files were mapped to the hg38 genome using miRDeep2 (<https://www.mdc-berlin.de/content/mirdeep2-documentation>) under default settings. The generated “miRNAs\_expressed\_all\_samples\_X.csv” (where X is the time stamp) files were collected. Subsequently, the 32 expression profiles were formatted as a tensor,  $x_{ijk} \in \mathbb{R}^{3017 \times 16 \times 2}$ , which represents the expression of the  $i$ th miRNA of the  $j$ th normal controls ( $k=1$ ) or patients ( $k=2$ ). Notably,  $x_{ijk}$  was normalized such that  $\sum_i x_{ijk} = 0$  and  $\sum_i x_{ijk}^2 = N$ . Tensor decomposition was then obtained using higher-order singular value decomposition as follows:

$$x_{ijk} = \sum_{l_1 l_2 l_3} G(l_1, l_2, l_3) u_{l_1 j} u_{l_2 k} u_{l_3 i},$$

where  $G \in \mathbb{R}^{16 \times 2 \times 3017}$  is a core tensor and  $u_{l_1 j} \in \mathbb{R}^{16 \times 16}$ ,  $u_{l_2 k} \in \mathbb{R}^{2 \times 2}$ , and  $u_{l_3 i} \in \mathbb{R}^{3017 \times 3017}$  are singular value orthogonal matrices. To identify the values of  $u_{l_3 i}$  used for miRNA selection, we identified which  $u_{l_3 i}$  represents patient- or normal-control-independent components and which  $u_{l_1 j}$  represents a distinction between healthy controls and normal controls. Subsequently, we verified whether  $l_1 = 1$  and  $l_2 = 2$  represent the required properties. We then tried to find the largest absolute value of  $G(1, 2, l_3)$ , which we found to be  $G(1, 2, 2)$ . This means that  $u_{2i}$  should be used for miRNA selection. Therefore, the  $p$ -values were attributed to the  $i$ th miRNA as follows:

$$P_i = P_{\chi^2} \left[ > \left( \frac{u_{2i}}{\sigma_2} \right)^2 \right],$$

where  $P_{\chi^2}[> x]$  is the cumulated  $\chi^2$  distribution, in which the argument is larger than  $x$ , and  $\sigma_2$  is the standard deviation. The  $p$ -values attributed to the  $i$ th miRNA were

corrected by the *Benjamini-Hochberg* criterion, and nine miRNA probes (hsa-let-7i-5p, miR-122-5p, miR-148a-3p, miR-151a-3p, miR-21-5p, miR-423-5p, miR-451a, and two miR-486-5p probes), associated with adjusted  $p$ -values less than 0.01 were selected. Then, the matrix  $x_{in} \in \mathbb{R}^{9 \times 32}$ , which represents the  $i$ th miRNA within the nine selected miRNAs of the  $n$ th patients/normal controls, was formatted. Then,  $x_{in}$  was normalized such that  $\sum_i x_{in} = 0$  and  $\sum_i x_{in}^2 = N$ . Principal component analysis

was then applied to  $x_{in}$  such that the principal component loading is attributed to the  $n$ th normal controls and patients. Then, the first and fourth principal component loadings were used to discriminate between the 16 normal controls and 16 patients. Linear discriminant analysis was also performed with two selected principal component loadings with leave-one-out cross-validation, and all  $p$ -values were calculated using Fisher's exact test.

**Table 1** Clinical background

Basic patient information					Diagnosis of TB		
Sample ID	Diagnosis	Age	Sex	Complications	PCR	QFT	Sputum culture
LP01	LTBI	44	F		(-)	6.52	(-)
LP02	LTBI	52	M		(-)	5.6	(-)
LP03	LTBI	54	F		(-)	2.03	(-)
LP04	LTBI	52	F		(-)	1.13	(-)
LP05	LTBI	51	F		(-)	0.87	(-)
LP06	LTBI	61	F		(-)	7.8	(-)
LP07	LTBI	57	F		(-)	> 10	(-)
LP08	LTBI	24	F		(-)	7.59	(-)
LP09	LTBI	48	M		(-)	> 10	(-)
LP10	LTBI	50	F		(-)	2.45	(-)
LP16	LTBI	78	M		NP	0.34	(-)
LP17	LTBI	57	M		NP	2.38	(-)
LP18	LTBI	67	F		NP	0.98	(-)
LP22	LTBI	70	M	Gastric cancer	NP	2.45	(-)
LP25	LTBI	75	M	IP	NP	9.5	(-)
LP27	LTBI	81	F		NP	1.84	(-)
TP01	TB	49	F		(-)	-0.05	(-)
TP02	TB	56	M	Colon cancer	NP	2.23	(+)
TP03	TB	62	F	Pleuritis	(-)	3.27	(-)
TP04	TB	86	M		(+)	NP	(+)
TP05	TB	83	M		(+)	T-SPOT (+)	(+)
TP06	TB	75	M		(-)	NP	(-)
TP07	TB	62	M	CLL	(+)	T-SPOT (+)	(+)
TP08	TB	62	M	Tongue cancer	(+)	NP	(+)
TP09	TB	85	F		NP	NP	(+)
TP10	TB	76	M	Miliary TB	(-)	NP	(+)
TP12	TB	35	F		(+)	NP	(+)
TP13	TB	29	M	Lymph node TB	LN (+)	NP	(-)
TP17	TB	29	F		(+)	NP	(+)
TP25	TB	54	F	OC	(+)	NP	(+)
TP33	TB	46	F		(+)	NP	(+)
TP41	TB	51	M	DM	(+)	NP	(+)

**Abbreviations:** TB tuberculosis, LTBI latent tuberculosis infection, QFT QuantiFERON-TB, IP Interstitial Pneumonia, CLL chronic lymphocytic leukemia, DM diabetes mellitus, OC oral cavity cancer, NP not performed

**Table 2** Diagnosis using miRNA information

		Prediction	
		LTBI	Active TB
Result	LTBI	12	5
	Active TB	4	11

**Results**

**Clinical background**

Among the study population, one patient with LTBI had gastric cancer and four patients with active TB had colon cancer, tongue cancer, chronic lymphocytic leukemia, and oral cancer. In addition, one patient with LTBI had interstitial pneumonia and one case of active TB had diabetes. On the other hand, in cases with active TB, extrapulmonary lesions were observed in two cases (miliary TB, lymph node TB; Table 1 and Supplementary Table 1).

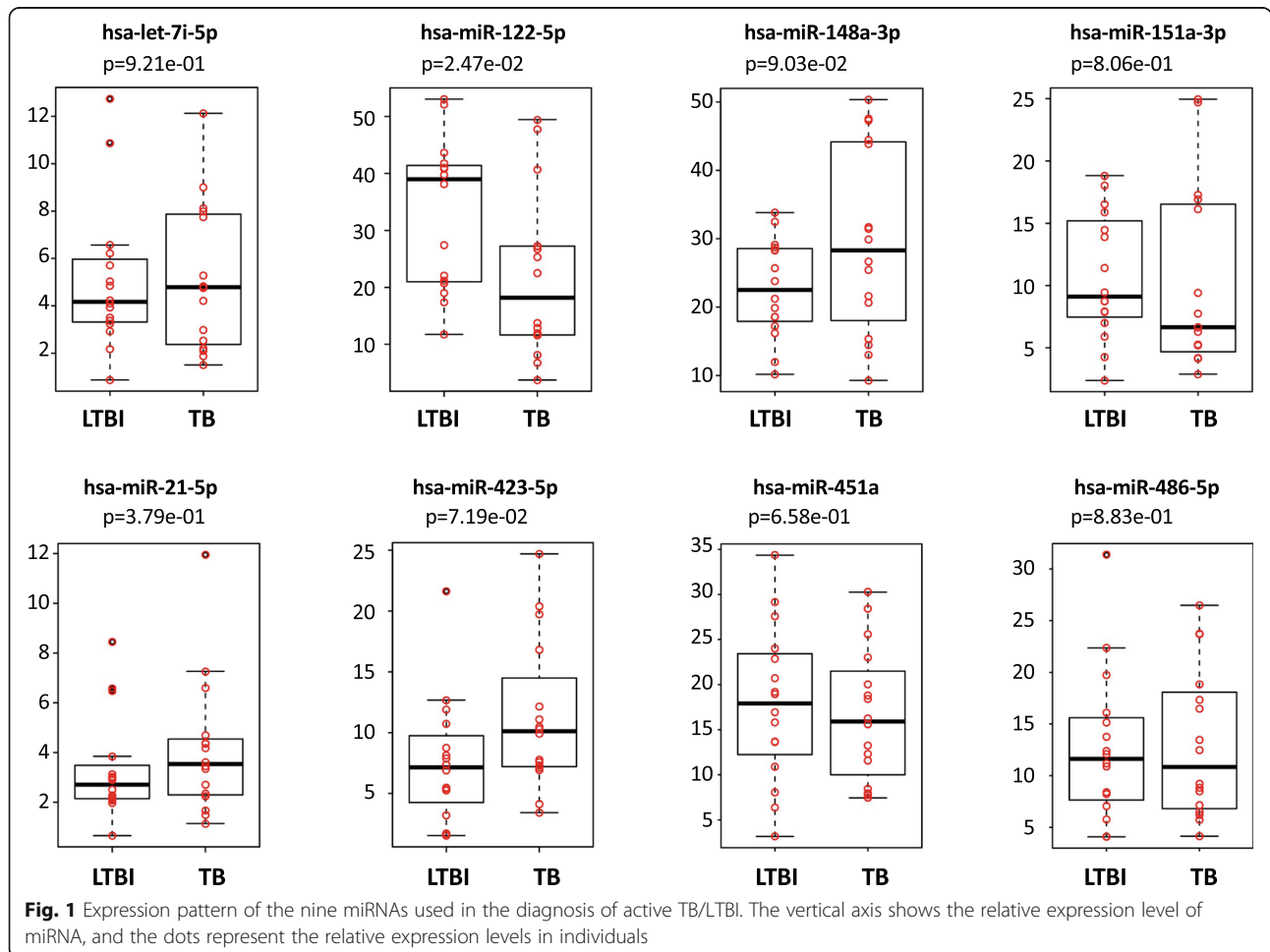
**Comparison of the expression of individual miRNAs**

Using the expression information of eight miRNAs (hsa-let-7i-5p, miR-122-5p, miR-148a-3p, miR-151a-3p, miR-21-5p, miR-423-5p, miR-451a, and miR-486-5p), we were able to classify our patients into 16 patients with LTBI and 16 patients with active TB, with a diagnostic accuracy of 71.8% (odds ratio: 6.16,  $p = 3.20e-02$ ; Table 2).

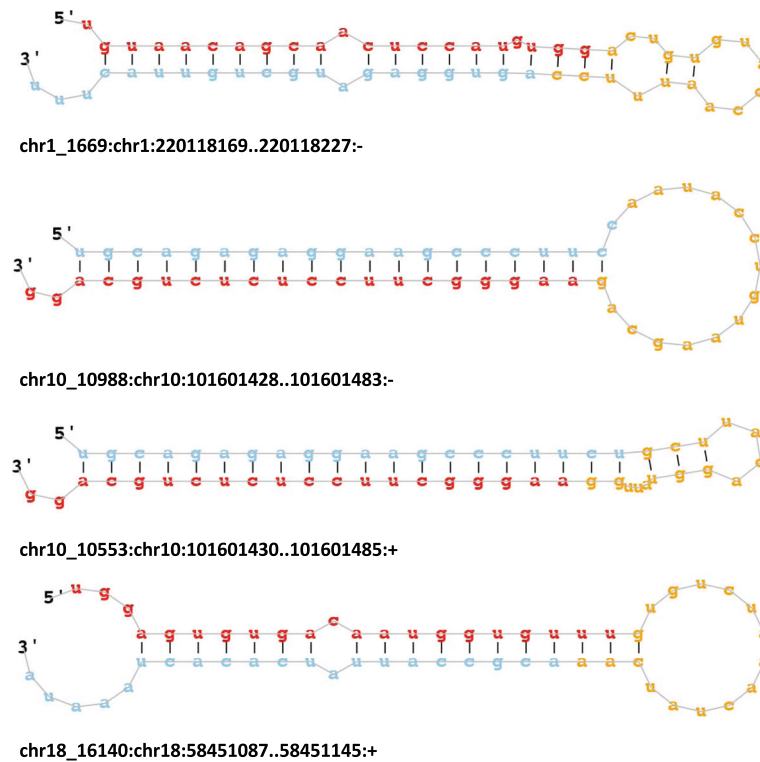
Notably, four miRNAs (hsa-miR-122-5p, miR-151a-3p, miR-451a, and miR-486-5p) were found to exhibit a higher expression in LTBI than in active TB, with a significant difference observed in miR-122-5p ( $p < 0.05$ ). On the other hand, four miRNAs were found to exhibit a higher expression in active TB than in LTBI (hsa-let-7i-5p, miR-148a-3p, miR-21-5p, and miR-423-5p), which showed no significant difference in terms of expression (Fig. 1).

**Universally expressed novel miRNA candidates**

Novel miRNA candidates were analyzed using miR-Deep2 (Fig. 2). In total, four types were expressed in all cases, with the expression of these four miRNA



**Fig. 1** Expression pattern of the nine miRNAs used in the diagnosis of active TB/LTBI. The vertical axis shows the relative expression level of miRNA, and the dots represent the relative expression levels in individuals



**Fig. 2** Virtual structure of novel miRNA candidates. The figure shows the structure of 11 miRNA candidates with significantly different expressions of LTBI and active TB

candidates in LTBI being significantly higher than that in active TB ( $p < 0.05$ ; Table 3).

#### miRNA expression in airway-associated cells

To examine the origin of miRNA in circulating blood, we compared the expression information of miRNA individually in the tracheal epithelial cells, bronchial epithelial cells, and alveolar epithelial cells of healthy subjects. The expression of miRNA was quoted from the miRNA Atlas of RIKEN ([https://fantom.gsc.riken.jp/5/suppl/De\\_Rie\\_et\\_al\\_2017/](https://fantom.gsc.riken.jp/5/suppl/De_Rie_et_al_2017/)) [17]. As shown in Fig. 3, the expression of miR-122-5p and miR-451a is low, the expression of other miRNAs is abundant, and the expression of miR-21-5p in particular is enhanced. The

miRNAs of which expression level was high in TB as compared LTBI, were highly expressed in airway related cells. However, no significant differences were found in the expression of these miRNAs among the three types of epithelial cells mentioned above.

#### Discussion

Though IGRAs are considered to be useful for early diagnosis of LTBI and active TB instead of TST [7], differential diagnosis of LTBI and active TB is often difficult.

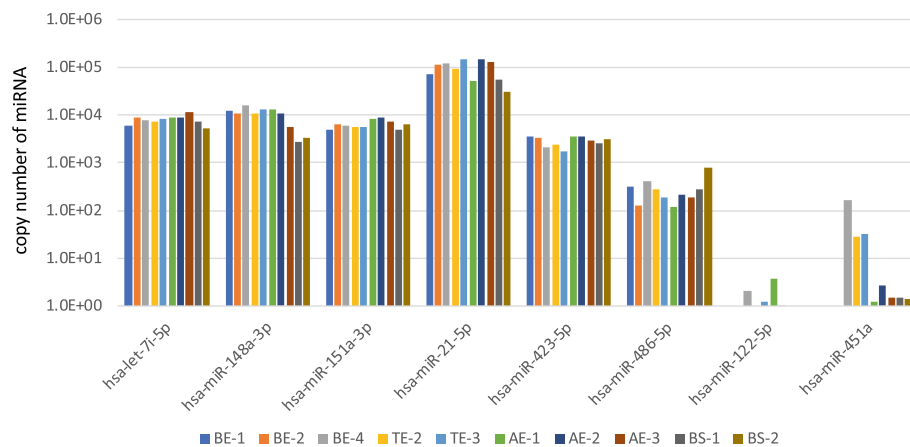
Patients with LTBI show positive IGRA test and normal chest X-ray. Oral isoniazid (INH) treatment for 6 months is considered an effective standard treatment to

**Table 3** List of novel miRNA candidates showing ubiquitous expression

Temporary miRNA name (precursor coordinate)	Mature read count		LTBI/ TP ratio	p-value	miRNA with the same seed
	Average LTBI	Average TB			
chr1:220118169..220118227:-	1600.1	662.6	2.41	1.96e-03	ptr-miR-194
chr18:58451087..58451145:+	1,356,949.3	482,662.4	2.81	5.85e-03	ptr-miR-122
chr10:101601428..101601483:-	3652.0	1607.7	2.27	2.91e-02	None
chr10:101601430..101601485:+	3652.0	1607.7	2.27	2.91e-02	None

Precursor coordinate: existing chromosome, gene start position and end position, sense strand (+), antisense strand (-). p-value: Student's *t*-test. miRNA with the same seed: a known miRNA in which the seed sequence of the new miRNA candidate is identical as analyzed by miRBase ver. 22.1 ([www.mirbase.org](http://www.mirbase.org))

LTBI latent tuberculosis infection, TB tuberculosis



**Fig. 3** miRNA expression levels in airway epithelial cells in next-generation sequencing. The figure shows the expression numbers of the eight miRNAs used in the diagnosis of active TB and LTBI in bronchial epithelium (BE), tracheal epithelium (TE), alveolar epithelium (AE), and bronchial smooth muscle (BS) cells. The miRNA expression profiles of the BE (BE-1, BE-2, and BE-4), TE (TE-2, TE-3), AE (AE-1, AE-2, and AE-3), and BS (BS-1, BS-2) cells were obtained from three, two, three, and two healthy volunteers, respectively

prevent the development of TB in LTBI patients infected within 2 years. Though oral anti-TB treatment reduces the incidence of active TB by about one-third [18], some patients with TB, especially those who originally have liver dysfunction or heavy drinkers, develop liver dysfunction associated with TB treatment. In addition, LTBI and active TB due to INH-resistant *Mtb* increase the rate of treatment failure or relapse from 1.8% [95% confidence interval: 1.2–2.6] to 10.9% [95% confidence interval: 5.9–20] compared to *Mtb* susceptible to all anti-TB drugs [19]. Therefore, three evaluations are important: 1) whether the case is LTBI before the onset of TB, 2) there is no complicated liver dysfunction, and 3) the source of infection is not INH-resistant TB. Since the therapeutic effect cannot be judged using IGRA, it is judged by the improvement or deterioration of chest X-ray and clinical symptoms.

Several reports exist on analysis using miRNA information from body fluids. When healthy individuals and individuals with active TB were diagnosed using miRNAs from exosomes derived from macrophages, an area under the receiver operating characteristic curve of 0.78 was obtained [13]. In addition, the information obtained from the six miRNAs (miR-20a, miR-20b, miR-26a, miR-106a, miR-191, miR-486) in plasma exosomes was useful for differentiating patients with tuberculous meningitis from those with TB [20]. Moreover, exosome miRNAs in pleural effusion were compared between three groups: an active TB group, a healthy control group, and a lung cancer group. Both miR-148a-3p and miR-451a were found to be highly expressed in the active TB group [21]. However, so far, no report exists on the differential diagnosis of active TB and LTBI.

Moreover, no particular correlation was found between circulating miRNA expression and miRNA expression in normal airway epithelial cells. It was also observed that the circulating expression of let-7i-5p, miR-148a-3p, miR-21-5p, and miR-423-5p, which are highly expressed in airway epithelial cells, is higher in cases with active TB than in cases with LTBI. However, the miR-122-5p and miR-451a were found to exhibit a higher expression in LTBI in circulating blood than in active TB. We also obtained multiple novel miRNA candidates that are expressed in all cases. Among these, the expression patterns of LTBI and active TB were found to be significantly different. Although the novel miRNA candidates need to be identified as miRNAs, they are considered as new biomarker candidates.

## Conclusions

We attempted to differentiate LTBI from active TB using information on miRNAs in the circulating blood. Our results showed high diagnostic accuracy. Although the number of subjects in this study was limited, this novel method is promising not only for the differentiation between LTBI and active TB but also for evaluating the treatment effect for both, as compared to conventional IGRAs, chest X-ray, and clinical symptoms.

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s41231-020-00078-7>.

**Additional file 1 : Supplementary Table 1.** Clinical background in detail.

## Abbreviations

LTBI: Latent tuberculosis infection; TB: Tuberculosis interferon-gamma release assays (IGRA) test; Mtb: *Mycobacterium tuberculosis*; TST: Tuberculin skin test; PAGE: Polyacrylamide gel electrophoresis; TD: Tensor decomposition; INH: Isoniazid

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## Authors' contributions

Hong Zhao, Michiyo Hayakawa, and Koichi Nakajima performed the experiments, contributed intellectual input, and provided the discussion. Shoji Hashimoto, Koichi Nakajima, and Y-h Taguchi helped write the manuscript and provided the discussion. Y-h Taguchi performed the next-generation sequencing analysis and data processing. Shoji Hashimoto and Yoshiki Murakami designed the experiments, analyzed and interpreted the data, and wrote the manuscript. The authors read and approved the final manuscript.

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## Availability of data and materials

All data used in the manuscript will be provided upon request to the corresponding author.

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

All the authors agree for the publication.

## Competing interests

The authors have no competing interests to declare.

## Author details

<sup>1</sup>Osaka Habikino Medical Center, Habikino, Osaka 583-8588, Japan.

<sup>2</sup>Department of Immunology, Osaka City University Graduate School of Medicine, Osaka 545-8585, Japan. <sup>3</sup>Department of Pathology and Cell Regulation, Kyoto Prefectural University of Medicine, Kyoto 602-0841, Japan.

<sup>4</sup>Department of Physics, Chuo University, Tokyo 112-8551, Japan.

<sup>5</sup>Department of Molecular Pathology, Tokyo Medical University, 6-1-1 Shinjuku, Shinjuku-ku, Tokyo 160-8402, Japan.

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