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Studies on microRNA in Pediatric Tuberculosis

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ABSTRACT

Background: Pediatric tuberculosis (TB) is still a major problem in the world leads to adverse complications. Research of microRNA(miRNA) conducted to study and identify specific miRNA expression among active TB children specifically in Indonesia. This suggests that miRNA can be one of the diagnostic tools and therapy for pediatric TB that needs to be developed.
Aims: To study spesific miRNA expression in pediatric active TB in Indonesia using microarray profiling.
Methods: This research was an observational analytic study using a cross sectional study design. The study used whole blood of healthy control children who had never received BCG immunization and children diagnosed with active TB. Identified miRNAs expression profile with microarray analysis.
Results: Four Active TB and 2 healthy control children were included in this study. Extraction of RNA with whole blood were done and profiled with microarray methods. Fold change >2 or <-2 with p-val <0.05. Expression of miRNA passed filter criteria are 251 miRNA with 30 (11.95%) miRNA up-regulated and 221 (88.05%) miRNA down-regulated.
Conclusions: Our study suggests that there are a specific miRNAs identified in active TB children in Indonesia and can be one of the tools to diagnosis and monitoring of therapy that needs to be developed.

Keywords: microRNA, pediatric, tuberculosis

INTRODUCTION

Tuberculosis (TB) is a chronic infectious disease caused by infection with Mycobacterium tuberculosis (M.tb). This disease is the second leading cause of death in the world due to infection after HIV / AIDS. In Indonesia, pulmonary TB is still one of the main causes of morbidity and mortality in children (1,2).

Diagnosis of pediatric TB is based on history taking, clinical symptoms, physical examination and support. History of contact, especially with active adult TB sufferers accompanied by a collection of clinical symptoms of children suspected of TB including coughing> 3 weeks, weight loss or not rising for no apparent reason despite having received adequate nutritional treatment, long-standing or recurring fever for no apparent reason > 2 weeks, anorexia and inactivity. However, children infected with TB often show typical symptoms (3,4).

The problem of TB in children is caused by several factors, including limited data on active TB and latent TB infection in children and difficulties in establishing a diagnosis that affects the management of TB therapy in children. The exact diagnosis of TB is made by finding TB germs on direct smear examination and / or culture which is a gold standard examination. However, definitive diagnosis in children is difficult to obtain because of the small number of germs (pausibasiler), the child is difficult to expel phlegm, the location of germs in the parenchyma area far from the bronchus (4-6).

Anamnesis and careful conclusions are needed so as not to cause overdiagnosis or underdiagnosis. Underdiagnosis of TB will result in increased morbidity and mortality whereas overdiagnosis can increase the cost of treatment from TB programs and also potentially increase the incidence of drug resistance. Therefore, the discovery of biomarkers and TB diagnostic testing tools is needed to develop innovative strategies for therapy and managing pediatric TB (7-10).

MicroRNA (miRNA or miR) has become a concern for researchers as biomarkers of diagnosis and therapy in TB. MicroRNA is a ribonucleic acid composed of 19-24 nucleotides, which do not encode proteins, can complement the target genes that encode messenger RNA (mRNA) and function to regulate the level of expression of mRNAs (11-14).

Research on miRNA in several countries has been carried out. This study conducted a preliminary study of miRNA profiling to identify the characteristics of specific miRNA expressed in Indonesian active TB children and contribute in finding a potential biomarker for TB diagnosis in children.

MATERIALS AND METHODS

Ethics Statement

This study was approved by the Ethics Committee of the Faculty of Medicine, Wijaya Kusuma University, Surabaya, Indonesia. Written informed consent was obtained from participants prior to their enrollment in this study.

This study was observational analytic using cross sectional design. Whole blood from 2 healthy control children and 4 children suffering from active TB were obtained. Profiling miRNA expression using microarray then analysed the characterization and profiles. Differentialy expression of miRNA categorized in downregulated and upregulated genes and miRNAs.

Sample Collection

Whole blood samples (2 ml) were obtained from 4 children with active TB and 2 children healthy control aged 0-<18 years recruited from Perak Timur Primary Health Care Surabaya in November 2019 until Januari 2020. Eligibility for entry into this study was based on clinical sign and symptoms of M.tb infection, contact TB, with sugestif TB chest X ray and positive tuberculin. Children with exclusion criteria were not included in this study. Healty control were children who had no clinical sign of TB or LTBI, negative tuberculin test, and never received BCG immunization.

Preparation of Whole Blood Samples

Whole blood samples were taken each of 2 ml from 6 children included in the study inclusion criteria, then stored in a vacutainer tube with K2EDTA anticoagulant (Becton Dickinson). The tubes are stored in refrigerated boxes in upright conditions and sent to a laboratory for total RNA extraction.

Total RNA Extraction from Whole Blood

Extraction of total RNA from whole blood samples using mirVana ™ PARIS ™ RNA and Native Protein Purification Kit (Thermo Fisher Scientific, Invitrogen ™). The total RNA extraction

procedure is as follows: 350 µl whole blood is transferred in a 1.5 ml tube, then 350 µl Denaturing Solution and 700 µl Acid-Phenol Chloroform are added. All samples were vortexed for 1 minute and followed by centrifugation at maximum speed (≥10,000 x g) for 15 minutes at room temperature. The upper phase formed is transferred to a new 1.5 ml tube and recorded in volume. Absolute ethanol is added to the sample in 1: 1 (v / v) volume, then tossing and turning the sample tube until it is well mixed. Move the entire sample volume into the cartridge filter. Centrifugate at maximum speed (≥10,000 x g) for 30 seconds at room temperature, then dispose of the collected fluid in the collection tube.

A total of 700 µl of miRNA Wash Solution 1 was added to the filter cartridge. Centrifugate at maximum speed (≥10,000 x g) for 15 seconds at room temperature, then dispose of the collected fluid in the collection tube. A total of 500 µl Wash Solution 2/3 was added to the filter cartridge. Centrifugate at maximum speed (≥10,000 x g) for 15 seconds at room temperature, then dispose of the collected fluid in the collection tube. Repeat the washing step with Wash Solution 2/3 again. After that, centrifugate at a maximum speed (≥10,000 x g) for 1 minute at room temperature, then discard the collection tube. Attach the cartridge filter to the new collection tube. Add 50 µl Elution Solution at ~ 95oC to the center of the filter. Centrifugate with a maximum speed (≥10,000 x g) for 30 seconds at room temperature. The total RNA is in the collection tube. Transfer the total RNA into the 1.5 ml non-stick tube for storage. The total RNA concentration was measured using a Nano Drop Spectrophotometer at a wavelength of 260 nanometers.

Analysis of MicroRNA Profiles

A total of 6 samples were selected to be analyzed for their microRNA profile by microarray technique. The 6 samples namely the healthy control group (2 samples) and the active TB group (4 samples).

A total of 500 nanograms of total RNA were labeled using FlashTag ™ Biotin HSR RNA Labeling Kits (Thermo Fisher Scientific, Applied Biosystems ™). The labeled RNA was then hybridized to the GeneChip ™ miRNA array 4.0 overnight in the GeneChip ™ Hybridization Oven 645 (Thermo Fisher Scientific, Applied Biosystems ™) with a temperature of 48oC and a rotation speed of 60 rpm for 16-18 hours. Hybridization control (GeneChip ™ Hybridization Control Kit) is included in the hybridization process. After the hybridization process is complete,

the GeneChip ™ miRNA array 4.0 containing the sample is washed and colored using the GeneChip ™ Fluidics Station 450 instrument (Thermo Fisher Scientific, Applied Biosystems ™).

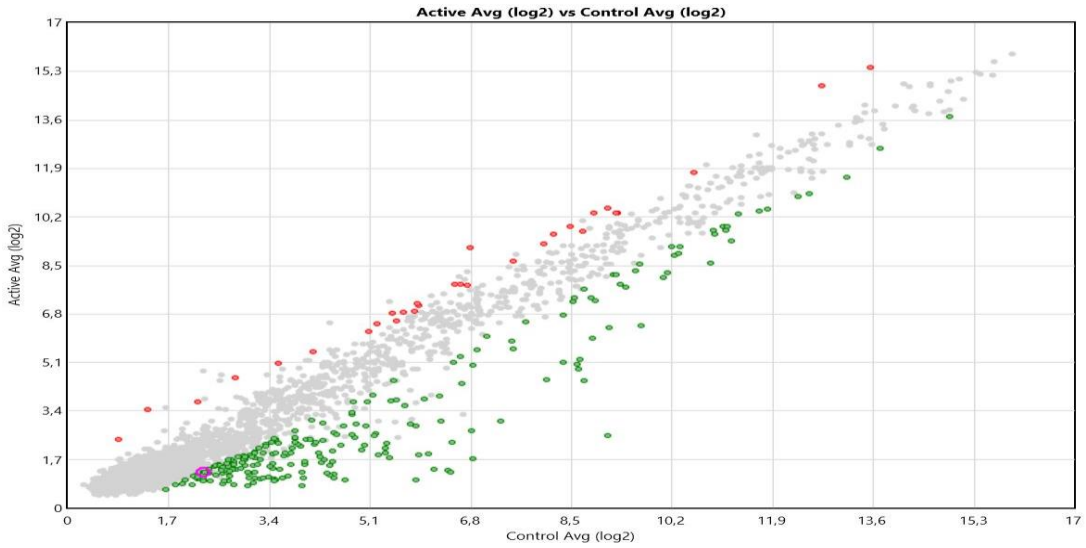
The GeneChip ™ miRNA array 4.0 is then scanned using the GeneChip ™ Scanner 3000 7G (Thermo Fisher Scientific, Applied Biosystems ™) instrument. Each scanned array will produce CEL and CHP files, which will be further analyzed using the Transcriptome Analysis Console (TAC) software version 4.0.1. The quality of hybridization and labeling is observed first before starting the data analysis by observing the Hybridization Control and Spike-In Control signals. If the quality meets the threshold requirements, the analysis is continued by comparing the microRNA expressions in the three sample groups. Only miRNAs that had more than twice the difference in expression in each sample group would be displayed in the final results.

RESULTS

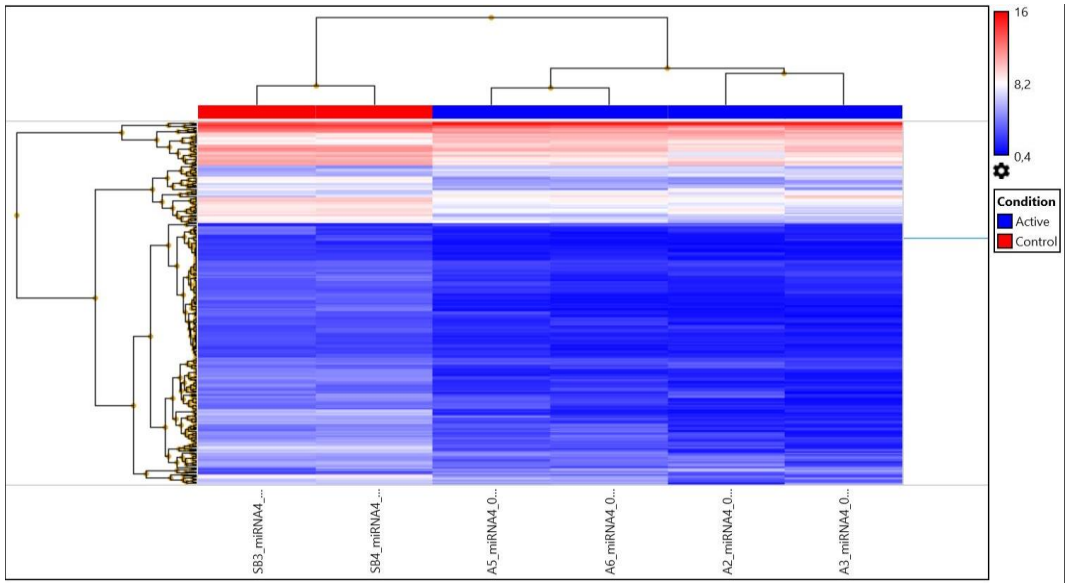
In the active TB group compared with healthy control, the number of active TB subjects was 4 children while the healthy controls were 2 children. The filter criteria in the microarray program according to the detected miRNA ID, with fold change> 2 or <-2, using p-value <0.05. microRNA in accordance with these criteria will be entered and detected in microarray profiling.

In the active TB group and healthy controls there were 6631 total gene counts and as many as 251 miRNAs that could pass filter criteria. From 251 miRNAs there were 30 (11.95%) miRNA up-regulated and 221 (88.05%) miRNAs down-regulation.

Figure 1 (A) illustrates scatter plot miRNAs expression using microarray where red distribution is up-regulation miRNA expression and green distribution shows down-regulation miRNAs expression. Hieralchical clustering of miRNA expression (B) shows the pattern of miRNA expression where blue expresses miRNAs in active TB, red expresses miRNAs in healthy controls.



(A)



(B)

Figure 1. Scatter plot (A) and Hieralchical Clustering (B) of miRNAs Expressed by Microarray in the Pediatric Active TB.

In the active TB group and healthy controls there were 251 miRNA genes expressed, 221 miRNA were down-regulation with the lowest fold change in miRNA-379-5p expression (fold change -81.92) and 30 miRNA up-regulation with the highest fold change in miRNA- 3613-5p (fold change 7.14) as seen in table 1.

Tabel 1.

miRNAs Expression in the Active TB Group Compared with Healthy Control Children

DOWN-REGULATION					
miRNA	**Fold Change**	**miRNA**	**Fold Change**	**miRNA**	**Fold Change**
hsa-miR-379	-81.92	hsa-miR-1306	-4.32	hsa-miR-6894	-2.43
hsa-miR-493	-39.41	hsa-miR-431	-4.26	hsa-miR-323b	-2.42
hsa-miR-381	-32.67	hsa-miR-4725	-4.21	hsa-miR-579	-2.42
hsa-miR-487a	-31.4	hsa-miR-6770	-4.14	hsa-miR-1180	-2.4
hsa-miR-376c	-30.37	hsa-miR-374c	-4.05	hsa-miR-532	-2.38
hsa-miR-337	-29.57	hsa-miR-101	-3.92	hsa-miR-345	-2.36
hsa-miR-433	-23.76	hsa-miR-1273c	-3.84	hsa-miR-744	-2.34
hsa-miR-487b	-20.78	hsa-miR-4479	-3.83	hsa-miR-4758	-2.33
hsa-miR-431	-20.12	hsa-miR-144	-3.83	hsa-miR-4754	-2.33
hsa-miR-99a	-16.27	hsa-miR-1307	-3.8	hsa-miR-34a	-2.33
hsa-miR-1185-1	-15.01	hsa-miR-625	-3.75	hsa-miR-3165	-2.31
hsa-miR-20b	-14.09	hsa-miR-181b	-3.7	hsa-miR-502	-2.31
hsa-miR-134	-13.84	hsa-miR-219b	-3.7	hsa-miR-130a	-2.3
hsa-miR-376a	-13.44	hsa-miR-5001	-3.57	hsa-mir-494	-2.29
hsa-miR-127	-11.97	hsa-miR-299	-3.57	hsa-miR-3691	-2.28
hsa-miR-641	-11.76	hsa-miR-184	-3.55	hsa-miR-452	-2.26
hsa-miR-485	-11.69	hsa-miR-590	-3.51	hsa-miR-668	-2.26
hsa-miR-487a	-11.38	hsa-miR-301a	-3.46	hsa-miR-4747	-2.25
hsa-miR-411	-10.97	hsa-mir-487a	-3.41	hsa-miR-7155	-2.25
hsa-miR-382	-10.42	hsa-miR-423	-3.4	hsa-miR-425	-2.25
hsa-miR-412	-10.26	hsa-miR-3620	-3.36	hsa-miR-491	-2.24
hsa-miR-432	-10.23	hsa-mir-487b	-3.35	hsa-miR-6852	-2.24
hsa-miR-654	-10.06	hsa-miR-3177	-3.3	hsa-mir-93	-2.24
hsa-miR-3909	-9.94	hsa-miR-3064	-3.28	hsa-miR-584	-2.24
hsa-miR-494	-9.91	hsa-miR-6833	-3.26	hsa-miR-4485	-2.23
hsa-miR-483	-9.86	hsa-miR-550b	-3.25	hsa-miR-6826	-2.23
hsa-miR-505	-9.58	hsa-miR-128	-3.22	hsa-miR-18b	-2.21
hsa-miR-654	-9.46	hsa-miR-3939	-3.22	hsa-miR-197	-2.21
hsa-miR-4730	-9.35	hsa-miR-1307	-3.19	hsa-miR-30c-1	-2.21
hsa-miR-1185-2	-9.3	hsa-miR-330	-3.17	hsa-miR-128-1	-2.2
hsa-miR-6716	-9.04	hsa-miR-6807	-3.13	hsa-miR-362	-2.2
hsa-miR-370	-8.77	hsa-miR-1254	-3.12	hsa-miR-8071	-2.18
hsa-miR-495	-8.63	hsa-miR-186	-3.1	hsa-miR-411	-2.16
hsa-miR-589	-8.08	hsa-miR-3158	-3.09	hsa-mir-4508	-2.15
hsa-miR-409	-7.53	hsa-miR-629	-3.05	hsa-miR-93	-2.15
hsa-miR-769	-7.5	hsa-miR-6876	-2.95	hsa-miR-7844	-2.14
hsa-miR-3130	-7.43	hsa-miR-18a	-2.92	hsa-miR-3613	-2.13
hsa-miR-1281	-6.92	hsa-miR-138	-2.92	hsa-miR-6783	-2.13
hsa-miR-543	-6.82	hsa-let-7b	-2.89	hsa-miR-3164	-2.13
hsa-miR-1303	-6.73	hsa-miR-6804	-2.86	hsa-miR-7851	-2.12
hsa-miR-154	-6.68	hsa-miR-4660	-2.84	hsa-miR-6806	-2.11
hsa-miR-409	-6.53	hsa-mir-423	-2.84	hsa-miR-5088	-2.11
hsa-miR-4290	-6.46	hsa-miR-30e	-2.83	hsa-miR-323a	-2.11
hsa-miR-1296	-6.36	hsa-miR-4665	-2.82	hsa-mir-4488	-2.1
hsa-miR-1271	-6.24	hsa-miR-6886	-2.77	hsa-miR-454	-2.1
hsa-miR-329	-6.2	hsa-miR-935	-2.76	hsa-miR-7855	-2.1
hsa-miR-4746	-6.1	hsa-miR-3605	-2.76	hsa-miR-503	-2.1
hsa-miR-483	-5.79	hsa-miR-4726	-2.76	hsa-miR-6820	-2.1

hsa-miR-3120	-5.69	hsa-let-7g	-2.76	hsa-mir-20b	-2.1
hsa-miR-574	-5.62	hsa-miR-378a	-2.75	hsa-mir-323a	-2.1
hsa-miR-539	-5.42	hsa-miR-758	-2.75	hsa-miR-3190	-2.09
hsa-miR-299	-5.41	hsa-miR-324	-2.74	hsa-mir-185	-2.07
hsa-miR-5571	-5.37	hsa-miR-421	-2.73	ACA6	-2.07
hsa-miR-374a	-5.35	hsa-miR-1228	-2.72	hsa-miR-6837	-2.06
hsa-miR-3074	-5.27	hsa-miR-4788	-2.69	hsa-miR-4647	-2.05
hsa-miR-493	-5.25	hsa-mir-487b	-2.67	hsa-mir-299	-2.05
hsa-miR-224	-5.24	hsa-miR-93	-2.66	hsa-miR-17	-2.04
hsa-miR-181a-2	-5.19	hsa-miR-188	-2.61	hsa-miR-20b	-2.04
hsa-miR-940	-5.09	hsa-miR-574	-2.61	hsa-miR-410	-2.03
hsa-miR-424	-5.07	hsa-miR-106b	-2.59	hsa-miR-16-2	-2.03
hsa-miR-3157	-5.04	hsa-miR-5090	-2.59	HBII-85-26	-2.03
hsa-miR-3198	-5.04	hsa-miR-659	-2.54	hsa-miR-4773	-2.03
hsa-mir-381	-4.97	hsa-mir-224	-2.54	hsa-miR-99b	-2.02
hsa-miR-377	-4.9	hsa-miR-7112-5	-2.54	hsa-miR-331	-2.02
hsa-miR-1825	-4.9	hsa-miR-192	-2.52	hsa-mir-3158-1	-2.02
hsa-miR-624	-4.85	hsa-miR-6856	-2.5	hsa-mir-3158-2	-2.02
hsa-miR-1343	-4.85	hsa-miR-3150a	-2.5	hsa-miR-194-3p	-2.01
hsa-miR-485	-4.83	hsa-miR-3148	-2.49	hsa-mir-3181	-2.01
hsa-miR-2278	-4.73	hsa-miR-6767	-2.49	hsa-miR-215	-2
hsa-miR-210	-4.66	hsa-miR-2277	-2.47	hsa-miR-1292	-2
hsa-miR-1304	-4.59	hsa-miR-4667	-2.46	hsa-miR-195	-2
hsa-miR-214	-4.41	hsa-miR-212	-2.45	hsa-miR-6875	-2

UP-REGULATION

miRNA	Fold Change	miRNA	Fold Change	miRNA	Fold Change
hsa-miR-1207	2.01	ENSG00000239154	2.41	hsa-miR-4487	2.79
hsa-miR-7845	2.02	hsa-miR-1260b	2.42	hsa-miR-7641	2.84
hsa-miR-6743	2.06	hsa-miR-183	2.46	hsa-let-7b	3.41
hsa-miR-6805	2.14	hsa-miR-92b	2.46	hsa-miR-486	3.53
hsa-miR-8073	2.23	hsa-miR-6085	2.48	hsa-let-7c-5p	3.97
hsa-miR-6787	2.24	ENSG00000238388	2.54	hsa-miR-4510	4.18
hsa-miR-1273g	2.25	hsa-miR-4492	2.59	hsa-miR-3619	4.2
ENSG00000238414	2.28	U105	2.59	hsa-miR-3200	5.01
hsa-miR-885	2.33	hsa-miR-8075	2.63	hsa-miR-3613	7.14
hsa-miR-6848	2.34	hsa-miR-6134	2.63		
hsa-miR-6726	2.35	hsa-miR-4467	2.66		
hsa-miR-6132	2.41	hsa-miR-183	2.72		

DISCUSSION

This study found in the active TB group and healthy controls found 6631 total gene counts and as many as 251 miRNA expressed. Out of 251 miRNAs expressed, 30 (11.95%) miRNAs were up-regulation and 221 (88.05%) miRNAs were down-regulation. miRNA with down regulation with the lowest fold change was miRNA-379-5p (fold change -81.92) and 30 miRNAs up-regulation with the highest fold change was miRNA-3613-5p (fold change 7.14).

This is different from the study conducted by Fu by miRNA in the circulation of active pulmonary TB patients showing 59 miRNA were overexpressed / up-regulation and 33 miRNA experienced down-regulation compared to controls. The study mentioned high levels of miRNA-361 expression in TB patients compared with healthy patients and was thought to be a reflection of lung lesions due to TB even though this mechanism was under study (15).

Research by Zhou with microarrays obtained from 29 expressed miRNAs contained 15 miRNA up-regulation and 14 miRNA down-regulation. The results of validation with RT-PCR contained 14 very important miRNAs namely miRNA-1, miRNA-155, miRNA-31, miRNA-146a, miRNA-10a, miRNA-125b and miRNA-150 downregulation while miRNA-29 experienced upregulation in children. children with active TB compared to uninfected children (14).

A study by Wang showed that miR-31 was significantly reduced in TB patients, compared to healthy children. The study by Miotto also distinguished 15miRNA, among TB children and healthy controls, and introduced miR-192 as the only candidate, with measurement results showing significant differences in adults and children (8,16).

The discussion above shows some differences in research results. This shows that there are different miRNA characteristics in each country or ethnic group. This is supported by research by Miotto on the ethnic Europa and Africa and found different miRNA expression characters (8).

microRNA is a small and non-coding RNA that has an important function at the post-transcription level and is involved in forming immunity by regulating the repertoire of genes expressed in immune cells. It has been established in recent research that the innate immune response to TB is significantly regulated by miRNA so that the expression of miRNA in TB should be suspected as a picture of the host's immune response to TB. Matters relating to M.tb agents such as strains and pathogenic can also influence the response and expression of miRNA in TB. This requires further studies to be used as a basis for the characterization of specific miRNA in Indonesia with more careful testing (17,18). Other studies encourage further research into miRNA as a biomarker that is appropriate and statistically meaningful by using larger groups and validating potential biomarkers in larger samples (18).

CONCLUSION

In this study, the active TB group and healthy controls obtained 251 expressed miRNAs, 30 (11.95%) miRNAs were up-regulation and 221 (88.05%) had down-regulation miRNAs. From 251

miRNAs that were down-regulation with the lowest fold change, miRNA-379-5p (fold change -81.92) and 30 miRNAs up-regulation with the highest fold change was miRNA-3613-5p (fold change 7.14). Our study suggests that there are a specific miRNAs identified in active TB children in Indonesia and can be one of the tools to diagnose active TB in children that needs to be developed.

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