

## Original Research Article

# Correlation between childhood tuberculosis and abundance of T cell gene transcription and impaired T cell function

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### Abstract

**Purpose:** To investigate the relationship amongst childhood tuberculosis, abundance of T cell gene transcription and impairment of T cell function.

**Methods:** A total of 329 pediatric patients treated for tuberculosis in Central Hospital of Zibo, Zibo, China from 2017 to 2019 were enrolled in the study. Among them, 167 cases of tuberculosis-hospitalized children were assigned to the TB group. Additionally, 162 well- and adequately-treated patients with a previous history of tuberculosis were selected as the control group. The abundance of continuous gene transcripts in the peripheral blood of the children was analyzed. The RNA profiles were analyzed via microarray, while interferon (IFN) level was measured by enzyme linked immunosorbent assay (ELISA). The T cell proliferation was determined by thymidine assay.

**Results:** Within 6 months of the commencement of treatment, the differentially expressed transcripts returned the expression in children in the control group. The abundance of *Talipes equinovarus*, atrial septal defect, robin sequence, and the persistence of the left superior vena cava (TARP) gene transcription in the TB group was lower than in the control group on days 30, 120 and 180 ( $p < 0.05$ ), while *IL1R2* gene transcription abundance in the TB group was higher than in the control group on days 30, 120, 180 ( $p < 0.05$ ). The proliferation of T cells and  $IFN\gamma$  in tuberculosis children (TB group) were lower than in healthy controls ( $p < 0.05$ ). In this study, a total of 129 genes were found to have significant differences in expression, and hence it is speculated that changes in RNA abundance altered the immune pathway.

**Conclusion:** The reduced abundance of T cell gene transcription and renovated T cell function in children with tuberculosis are related to acquired immunodeficiency. The results of this study provide a theoretical basis for the clinical diagnosis and treatment of tuberculosis in children.

**Keywords:** Childrens tuberculosis, T cells, Gene transcription abundance, Immune cells, Impaired function, Acquired immunodeficiency

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## INTRODUCTION

Pediatric tuberculosis has traditionally been relatively neglected, despite the encouraging growth in policy priorities in recent years [1-3].

Pediatric tuberculosis therefore, requires special consideration [4]. Although a lot of studies about the interaction between pathogens have been conducted, most of this information comes from animal model studies or adult tuberculosis

studies. Other studies have shown that the unique transcriptional characteristics of TB in acute diseases not only alter at gene level, but also alter in cell composition, and as well decreases with regards to the number of T cells [5-7]. The developing immune system response in children to *Mycobacterium tuberculosis* infection is unknown, in addition to whether it is different from or similar in adults [8]. Tuberculosis in children usually does not have a strong inflammatory response to acute bacterial infection, and hence it is difficult to diagnose [9]. Many children with tuberculosis have no definite diagnosis at the onset, and are usually diagnosed only after autopsy [10]. Occult manifestations in children are frequently associated with non-responsiveness to tuberculin skin tests, and high rates of extra pulmonary spread indicate that the immune system cannot identify it [11].

However, the immune mechanism is not yet fully understood. Host gene level profiling is particularly suitable as a tool for diseases of the immune mechanism, because it demands small amounts of blood, and requires minimal immediate sample processing [12]. In order to define the general immunological characteristics of childhood tuberculosis, the temporal pattern of the abundance of whole genome RNA transcripts in the peripheral blood of children with tuberculous meningitis was studied, and the correlations between tuberculosis in children and the decrease of gene transcription, in addition to the abundance of T cells and the impairment of T cell function was investigated.

## METHODS

### General information on patients

A total of 329 pediatric patients treated for tuberculosis in Central Hospital of Zibo, Zibo, China from 2017 to 2019 were enrolled in this study. Among them, 167 cases of tuberculosis hospitalized children were assigned to the TB group; Furthermore, 162 well- and adequately-treated patients with a previous history of tuberculosis were placed in the control group. The proportion of male to female was 82:85, and the mean age was  $34.19 \pm 6.11$  months. In the control group, the proportion of male to female was 79:83, and the mean age was  $35.42 \pm 5.94$  months.

### Inclusion criteria

The subjects' ages ranged from 6 to 14 years old; no gender restriction; All the selected cases

met the WHO criteria for tuberculosis and were confirmed by two independent clinicians.

### Exclusion criteria

Age >14 years old; presence of other infections or inflammatory reactions; Chronic diseases of heart, lung, liver and kidney; persons with mental disorders; and those who would not cooperate during the treatment.

### Ethical approval

This study was approved by the institutional medical ethics committee of the hospital where the subjects are from, and the subjects' guardians all signed written informed consent. The study followed the guidelines of Ottawa Group Statement for Clinical Trial Registration.

### Data analysis

The abundance of continuous gene transcripts in blood from a group of children (echelon A) with the most grim form of transmissible tuberculosis (TBM) was analyzed. The study was underway from the time of presentation to the completion of six months of treatment (WHO's standard remedy regimen for TBM). The time from diagnosis to recovery after treatment was analyzed to identify differentially expressed genes associated with the disease. In order to illustrate the time differences and individual differences in RNA expression levels, a linear mixed effect model was used to model the time behavior of each separate probe on the microarray.

The model interpolated each time point and predicted the missing observations, and summarized each probe with a fitting line to describe the average change of expression level over time in all individuals. Then, the slope of these averages was used to test statistical data to detect statistically significant probes. In the time history analysis, the A echelon control was used for the case. In order to verify the results of the time course analysis, the admission time points of echelon A patients with PTB and TBM in echelon B were compared.

The mean expression levels in tuberculosis and tuberculous meningitis were compared with those in healthy Mantoux-negative controls using a logical model and t-test. The average expressions of TBM cases in cohort 1 on day 180 were compared, as well as similar group controls.

## Reverse transcription-polymerase chain reaction (RT-PCR)

### RNA extraction process

Cells or tissues homogenized by denaturing solution were thoroughly mixed with 600  $\mu$ l + 60  $\mu$ l 2 M sodium acetate at pH 4.0, using a vortex oscillator. Phenol, chloroform and isoamyl alcohol (600  $\mu$ l) were thoroughly mixed or oscillated in a whirlpool oscillator for 10 s, then for 10 - 15 min in an ice bath and centrifuged at a speed of 10 000rpm for 20 min at a temperature of 4 °C. The upper layer was vacuumed into a sterile centrifuge tube with isopropyl alcohol in equal volumes, and RNA was precipitated at -70 °C for 30 min. Centrifugation was performed at 10 000 rpm at 4 °C for 20 min. The RNA was precipitated and freeze-dried for 15 min, and the precipitated RNA was re-dissolved in 300  $\mu$ l denatured solution, and thoroughly mixed with 60  $\mu$ l 2M sodium acetate at pH 4.0. Phenol/chloroform/isoamyl alcohol (252/4/1) was thoroughly mixed or oscillated with whirlpool oscillator for 10 s, then for 10~15 min in an ice bath, and the centrifuged at 10,000 rpm for 20 min at 4°C. The upper water phase was sucked into a sterile centrifugal tube. Isopropanol secondary precipitation (-70 for 30 min), 75 % ethanol wash precipitation, and precipitation freeze-drying were carried out. RNA analysis was performed using microarray. The total RNA extracted from standard PaxGene protocol was frozen to Stanford University in California, USA. The quality and quantity of RNA were evaluated using 1.25 % agarose modified gel and Genescan 2 spectrophotometer. Oligo-dT primers with T7-RNA promoter were used, mRNA was reverse-transcribed into cDNA and amplified by linear amplification based on the improved Eberwine-Wang linear amplification program. Thereafter, the 3  $\mu$ g amplified antisense RNA was reverse-transcribed, labeled with Cy5 dUTP (Amersham) and combined with 3  $\mu$ g Cy3 labeled reference cDNA, which was derived from a group of 11 human cell lines.

Samples were washed and concentrated using a MicroCon YM 30 column (Amicon) and then competitively hybridized on a custom-made cDNA microarray containing 37632 elements from cDNA clones representing approximately 18000 unique human genes. AxonScanner II confocal microarray scanner (Axon Instruments) was used to scan the hybrid slides, and each array was normalized separately to adjust the effects of plate, needle and intensity dependent dyes, and to adjust the proportion so that the variances of each array were comparable. Primers used for the reaction are shown in Table 1.

### T cell proliferation and interferon gamma reaction

Heparinized whole blood (180  $\mu$ l, tissue medium 1:10 dilution) was added into the petri dish, which contained 20  $\mu$ l of phytohemagglutinin (PHA). A 5  $\mu$ g / mL or phosphate buffer was used as an unstimulated contrast, then the plate was incubated in the CO<sub>2</sub> incubator at 37 °C. On the third day, the supernatant was removed from the pores containing PHA blood (and replaced with equal volume tissue medium). On the sixth day, the supernatant was removed. Triplicate wells were collected and stored at - 80 °C, and IFN was detected by ELISA. At the last 15 h of incubation and the 15th day after harvest, and on the 4th day of PHA culture, 1  $\mu$  Ci [METHYL-3H] thymidine was added into each well to measure the proliferation response of T cells. Supernatant was added by liquid scintillation measurement. Standardized proliferation response was determined by subtracting the value of the unirritation hole from the PHA hole.

### Statistical analysis

In the non-normal case, non-parametric Mann-Whitney U test was detection to compare continuous variables, otherwise, independent sample t-test was used.  $P < 0.05$  was considered significant.

**Table 1:** RT-PCR primer sequences

Gene	Upstream primers	Downstream primers
AREG	CTGCTGCAAACCTCATGCATT	ACGTTACAACCTTGTGG
CD3D	CCAAATCATGCATCTGCTGT	TTTGACATACGGGCAATCG
CD3G	TCGCATAGTATGACCCTAAGA	GCTATACTCCATGCAAAGG
LCK	TTGACACAACGATCCTCATTT	GAAGGATACTCTCGAACAC
NFATC3	ACGTATTTGGCAACAGTCG	TACGCAACATATTTGGGCG
SLC7A5	CTCTAACGAGGAATCGAAC	TCGACGAAGGACTCTAAAC
TRA	C CGA GC ATT AAC TTTGGT	CAACTCGCTATGGTCAAAG
ZAP70	TCATGTATTAGAGGACCTAT	TCGACGACTCAAGGTAAAC
$\beta$ -actin	C ATT CGAC AAC TTT GGGT	CCCTAGTTAAGTCGATGACAA

## RESULTS

### General subject profiles

In the control group, the ratio of male to female was 18:21, and the average age was  $34.28 \pm 5.14$  months. In echelon B, the proportion of male to female in the observation group was 21:23, with an average age of  $37.42 \pm 7.05$ , while that of the control group was 24:19, with an average age of  $38.19 \pm 6.11$  months. In echelon C, the average age of the observation group was  $44.38 \pm 8.59$  months, and that of the control group was  $42.58 \pm 7.43$  months. These data are shown in Table 2.

### Modeling time variation of gene expression

The thermal map showed the analog expression changes in important gene transcripts is invalid with TBM from the beginning of the diagnosis. Compared with children with a healthy diagnosis, green meant lower transcript abundance, red represented taller transcript abundance, and black represented no disparity in level. The relative degree of the transcript abundance was made known and derived from the average level over time (shown in Methods section). The similar time expression patterns were clustered. The obvious linear change in color was derived from the statistical pattern of the observed time points, such that it could be expressed as a continuum. (Figure 1).

### Analysis of the abundance of TARP gene transcripts

It was found that 262 transcripts showed significant differences between admission and 6 months after. Within 6 months (180 days) of the commencement of treatment, regardless of the direction of regulation, the differentially-voiced

transcripts returned to the expression levels in the children in the control group (Figure 2). The abundance of TARP gene transcription in the case group was lower than in the control group on days 30, 120 and 180 (Table 3) ( $p < 0.05$ ).

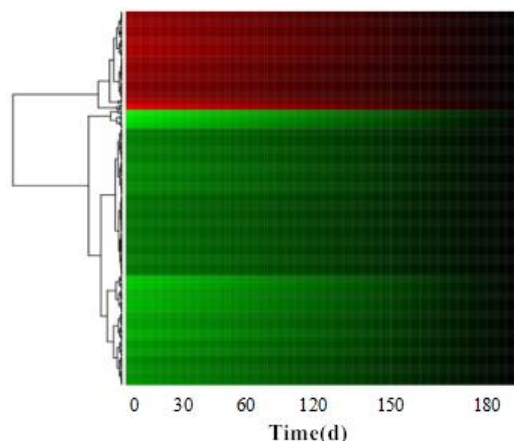


Figure 1: Time modeling of gene expression

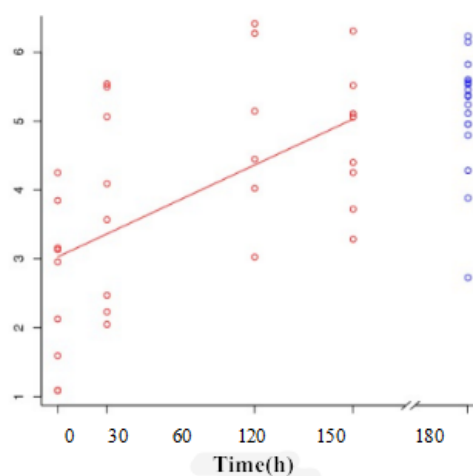


Figure 2: Analysis of the abundance of TARP gene transcripts

Table 2: General conditions of subjects

Variable		Control group	Study group	Chi <sup>2</sup> value	P-value
Echelon A	Sex (male: female)	18:21	20:23	6.04	0.236
	Age (month)	34.28±5.14	36.51±6.05	5.28	0.175
Echelon B	Sex (male: female)	24:19	21:23	6.12	0.228
	Age (month)	38.19±6.11	37.42±7.05	5.35	0.629
Echelon C	Sex (male: female)	37:43	41:39	4.12	0.514
	Age (month)	42.58±7.43	44.38±8.59	6.44	0.375

Table 3: Tarp transcriptional abundance analysis

Group	30	120	180
Control (n=167)	4.73±1.15	5.88±1.36	6.37±1.69
TB (n=162)	3.14±0.53	4.62±0.85	5.65±1.44
t-value	5.224	6.472	5.019
P-value	0.026	0.014	0.005

**Table 4:** Abundance IL1R2 gene transcription

Group	Day 30	Day 120	Day 180
Control (n=167)	1.13±0.12	1.93±0.41	1.87±0.32
TB (n=162)	3.87±0.58	2.86±0.31	2.35±0.44
t-value	5.236	6.124	5.008
P-value	0.026	0.024	0.037

**Table 5:** Gene expression of cell subsets in echelon A

Cell subsets	Gene expression	Not expressed in TBM array	Significant differences
CD4 cell	17	0(%)	0(%)
CD8 cell	25	0(%)	2(8.00%)
T cell	210	8(3.80%)	16(7.60%)
B cell	179	2(1.10%)	3(1.70%)
NK cell	161	3(1.90%)	13(8.10%)
Monocyte	1042	17(1.60%)	19(1.80%)

### Analysis of abundance of IL1R2 gene transcription

The abundance of gene transcripts was analyzed 180 days after reporting to recovery. IL1R2 gene transcription abundance in TB group was higher than control group on days 30, 120 and 180 (Table 4) ( $p < 0.05$ ).

### Gene expression of cell subsets in echelons A

The numbers of CD4 and CD8 T cells, B cells, monocytes and NK cells in the peripheral blood of healthy donors were compared. In addition, the changes in expression were caused by changes in the proportion of cells in the blood of the children with tuberculosis, Celltype Computational Differential Estimation (CellCODE) was used to calculate the relative differences in cells (Table 5).

### Genes with significant difference were regulated by key cytokines

To determine if the gene level patterns of children with acute tuberculosis included IFN -  $\gamma$

and TNF -  $\alpha$  - inducible genes, the gene level profiles of the patients with IFN -  $\gamma$  1 interferon (IFN)  $\alpha$ ,  $\beta$  and IFN induced genes in peripheral blood cells was compared (Table 6).

### Differentially expressed genes in echelon A and echelon B

In order to determine the immune pathways that may be changed by the observed changes in the abundance of RNA, Ingenuity Pathways Analysis (IPA) was used to locate SDE genes in the TBM invalid between the time of admission to 6 months after, and to detect their biological functions and pathways. There were significant differences in the level of 129 genes between echelons A and B. Only partial gene display is shown in the table (Table 7).

### Gene expression changes

This study investigated T cell proliferation in response to mitogen and IFN -  $\gamma$  production in tuberculosis or tuberculosis patients (Figure 3). The proliferations of T cells and IFN $\gamma$  in tuberculosis children was lower than those in healthy controls ( $p < 0.05$ ). (Figure 3, Table 8).

**Table 6:** Genes with significant difference are regulated by key cytokines

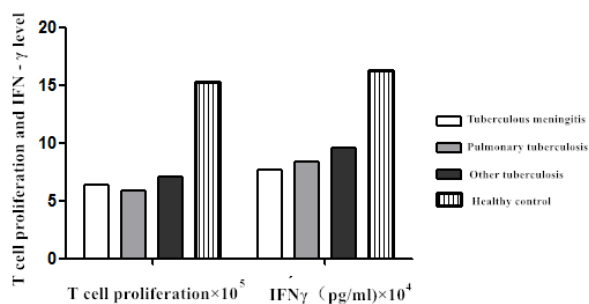
Cell factor	Gene expression	Not expressed in TBM array	Significant differences
IFN $\alpha$	257	6(2.30%)	7(2.70%)
IFN $\beta$	484	11(2.30%)	11(2.30%)
IF $\omega$	377	10(3.70%)	11(2.90%)
IFN $\gamma$	188	3(1.60%)	8(4.30%)
TNF $\alpha$	255	14(5.50%)	8(3.10%)

**Table 7:** Genes with significant differences in gene expression in echelons A and B (parts)

Gene	logFC	Statistic	P-value
GNLY	-1.806	-6.188	0.001
CLC	-1.088	-3.273	0.003
FANCA	2.326	7.756	0.002
IL32	-1.046	-4.798	0.001
IL2RB	-1.804	-6.644	0.001
CD6	-1.377	-5.835	0.004
VNN1	1.8675	5.713	0.003
HLA-DQA1	-0.738	-2.905	0.016
NFATC2	-1.378	-8.179	0.001
...	...	...	...
TNFAIP6	1.401	4.7632	0.003

**Table 8:** T cell proliferation and IFN $\gamma$  level

Project (a echelon)	T cell proliferation $\times 10^5$	IFN $\gamma$ (pg/mL) $\times 10^4$
Tuberculous meningitis	6.37 $\pm$ 0.36	7.62 $\pm$ 0.75
Pulmonary tuberculosis	5.83 $\pm$ 0.23	8.41 $\pm$ 0.88
Other tuberculosis	7.11 $\pm$ 0.42	9.52 $\pm$ 0.67
Healthy controls	15.27 $\pm$ 1.55	16.27 $\pm$ 2.36
F-Value	11.435	10.557
p-Value	0.014	0.026

**Figure 3:** T cell proliferation and IFN $\gamma$  level

## DISCUSSION

Genome transcriptome analysis has shown that the gene level in children with tuberculosis is mainly reduced rather than elevated. It is generally believed that the pro-inflammatory state in tuberculosis is the target of drugs that attempt to ameliorate neuronal damage with steroids. This study also looks forward to finding evidence of T cell activation, as well as evidence of IFN -  $\gamma$  and TNF -  $\alpha$  response profiles. It has been found that the diminished T cell response and the lack of IFN $\gamma$  and TNF responses were consistent with the clinical impression, that is, children's tuberculosis is a "silent" disease in immunology. There is no expected host response to the invasion and dissemination of mycobacteria.

Although the decrease in T cell proliferation observed may be owing to the antigen presentation defects, this study used non-specific T cell mitogen PHA to test proliferation and IFN gamma response. Therefore, the inclusion of

*Mycobacterium tuberculosis* antigens may have found different reactions. The limitation of the analysis of the activated responses is measurement of other cytokines, which can confirm the observation of T cell expression inhibition, because there is sufficient evidence that IFN gamma reaction is inhibited in acute TB [13]. The distribution of expressed genes to activated pathways reveals a significant pattern of reduced transcription levels. This is reflected in the reduced response of T cells to mitogen [14].

These results indicate that childhood tuberculosis go hand in hand acquired immunodeficiency syndrome. This is due to the reduction of a variety of gene products demand for the effective response of cells to pathogen answer. The discovery of decreased gene expression related to T cell cytotoxic reaction provides an understanding of the decrease of serum granulysin concentration in children with acute tuberculosis [15]. These are contrary to the reported gene level studies of adult tuberculosis [16]. The RNA expression characteristics of adult TB have been reported previously, in which increased expression of IFN -  $\gamma$  - induced gene and neutrophil gene is observed [17].

Only 23 significant differentially expressed genes are differentially expressed in the adult TB genome of 312 genes (7.3 %). This may reaction differences of various arrays, but this can be interpret that TB in children occurs after primary infection, before the development of T-cell immunity, and is different from adult TB, which is usually characterized by local lung reactivation

and go hand in hand with an obvious inflammatory responses.

*Mycobacterium tuberculosis* has developed a variety of strategies to evade host immune response. It has been found that inhibition of multiple genes involved in *Mycobacterium* immune recognition and killing infected cells may indicate insufficient understanding of *Mycobacterium*-mediated evasion strategy.

This study have survey decreases in the expressions of critical immune genes in both tuberculous meningitis and tuberculosis, but the decreases are more severe in tuberculosis. In childhood, especially in young children, the difference between the transmission form of tuberculosis meningitis, because many young children have the characteristics of military transmission and organ involvement, and the development of additional lung diseases is more common than adults [18,19]. The findings on the level of gene level in tuberculosis and tuberculous meningitis make clear that patients with diffuse forms of TB have more severe damage to the immune response needed to embody infection. Variations in mycobacterium load may explain the differences in gene level observed in nuclear meningitis and patients with disseminated diseases.

It is well known that acute tuberculosis go hand in hand with suppressed IFN -  $\gamma$  production [20]. Interestingly, after severe illness and septic shock, "immune paraplegia" similar to that of children with TB is also observed; it goes hand in hand with decreased T cell mitotic response, reduced IFN  $\gamma$  and skin non-response [21]. Although the underlying mechanism of immune paralysis in other severe infections is unclear, the decreased level of several key genes in T cells and other immune pathways observed in tuberculosis may represent mechanism of immune paralysis. And a computational method (CellCODE) was used to calculate the relative difference of cell populations.

This shows a decrease in CD4 T lymphocytes in tuberculous meningitis and an increase in neutrophils in tuberculous meningitis, a pathological process induced by the suppression of gene transcription.

## CONCLUSION

The impairment of T cell function in children with tuberculosis are related to acquired immune deficiency. Further studies are needed to determine whether reduced gene level is a mechanism for aggrieved immune function where immune paralysis is surveyed. It is speculated

that *Mycobacterium tuberculosis* may be using a bacterial mechanism to down-regulate host response as an immune escape strategy for intracellular survival.

## DECLARATIONS

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None provided.

### Funding

None provided.

### Ethical approval

None provided.

### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Conflict of Interest

No conflict of interest associated with this work.

### Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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