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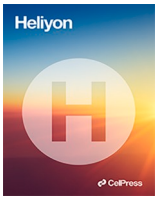

Research article

Integrated sequence-based genomic, transcriptomic, and methylation characterization of the susceptibility to tuberculosis in monozygous twins

Zhi Liu [a], Batu Deligen [b,*], Zhiqiang Han [b], Chaolumen Gerile [c], An Da [b]

[a] Department of Respiratory and Critical Care Medicine, Affiliated Hospital of Inner Mongolia Minzu University, Tongliao, 028007, Inner Mongolia, China
[b] Institute of Mongolian Medicine Pharmacology, Affiliated Hospital of Inner Mongolia Minzu University, Tongliao, 028007, Inner Mongolia, China
[c] Department of Internal Medicine, Xilinguole Meng Mongolian General Hospital, Xilinhaote, 026000, Inner Mongolia, China

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ABSTRACT

Background: Tuberculosis (TB) is a complex disease with a spectrum of outcomes for more than six decades; however, the genomic and epigenetic mechanisms underlying the highly heritable susceptibility to TB remain unclear.
Methods: Integrated sequence-based genomic, transcriptomic, and methylation analyses were conducted to identity the genetic factors associated with susceptibility to TB in two pairs of Mongolian monozygous twins. In this study, whole-genome sequencing was employed to analyze single nucleotide polymorphisms (SNPs), insertions and deletions (InDels), and copy number variations (CNVs). Gene expression was assessed through RNA sequencing, and methylation patterns were examined using the Illumina Infinium Methylation EPIC BeadChip. The gene–gene interaction network was analyzed using differentially expressed genes.
Results: Our study revealed no significant difference in SNP and InDel profiles between participants with and without TB. Genes with CNVs were involved in human immunity (human leukocyte antigen [HLA] family and interferon [IFN] pathway) and the inflammatory response. Different DNA methylation patterns and mRNA expression profiles were observed in genes participating in immunity (HLA family) and inflammatory responses (IFNA, interleukin 10 receptor [IL-10R], IL-12B, Toll-like receptor, and IL-1B).
Conclusions: The results of this study suggested that susceptibility to TB is associated with transcriptional and epigenetic alternations of genes involved in immune and inflammatory responses. The genes in the HLA family (HLA-A, HLA-B, and HLA-DRB1) and IFN pathway (IFN-α and IFN-γ) may play major roles in susceptibility to TB.

1. Introduction

Pulmonary tuberculosis (TB) is a lung parenchymal infection triggered by *Mycobacterium tuberculosis* [1,2]. In Southeast Asia, the

Abbreviations: CNV, copy number variant; InDels, insertions and deletions; NGS, next-generation sequencing; SNPs, single nucleotide polymorphisms; TB, Tuberculosis.

* Corresponding author.
E-mail address: 1658307985@qq.com (B. Deligen).

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incidence rate of TB stands at 246 cases per 100,000 individuals, while in China, it is reported at 52 cases per 100,000 people [1]. The well-established risk factors for TB include exposure to people with TB, younger age, immunosuppression, diabetes mellitus, silicosis, rheumatic diseases, steroid dosing, end-stage kidney disease, exposure to smoke, substance abuse, and healthcare workers [2,3]. One of the crucial features of TB is that only about 5–10 % of immunocompetent persons infected with *M. tuberculosis* will progress *to* active *TB disease* [4–6]. Therefore, in addition to the classical risk factors, host *genetic factors have demonstrated to* play important roles in susceptibility to TB. TB has been considered a complex trait for more than six decades [7]. Genetic susceptibility to this disease has been investigated using a number of twins from the last century, demonstrating a significant excess of monozygous twins with TB compared with dizygous twins and other pairs of siblings [8–10]. Given their virtually identical genetic background, homozygous twins have provided unmistakable evidence of the dominant role of genetic factors in the occurrence and development of TB.

A number of genomic studies have recently been conducted to identify a set of genetic factors underlying susceptibility to TB including *UBE3A* [11,12], chromosome region 8q12–q13 [13], *VDR, IL12, Il12RB1, INFG, MBL, DRB1, SFTPA1/2,* and *NRAMP1* [14–17], *MCP1* [18,19], chromosomes 2q21-2q24 and 5p13-5q22 for PTST-, chromosome 7p22-7p21 for TB [20], *TST1* and *TST2* [21], *TNF1* [22], and *CCL1* [23]. More specifically, genetic *variants* participating directly in human immunity such as interleukin (IL)-10, interferon (IFN)-γ, and nitric oxide synthase 2 play key roles in susceptibility to TB [24–28]. Additionally, previous studies have revealed the important roles of epigenetic modifications of a number of genes including *NRAMP1, IFNG, NOS2A, VDR, ISG15, TACO, TLR1, TLR, IL18R1, PADI, DUSP14,* and *MBL, NLRP-3*, and *MASP-2* in TB susceptibility [29–32].

Most of these studies unraveling the contributions of host genetic factors to TB susceptibility have only been performed using a single gene expression method. To date, no study has integrated sequence-based genomic, transcriptomic, and methylation characterization using monozygous twins, which is critical for understanding how DNA sequence and methylation affect RNA expression.

In this study, we performed a twin study to identify the genetic factors underlying susceptibility to TB by integratively analyzing single nucleotide polymorphisms (SNPs), insertions and deletions (InDels), gene expression profiles, copy number variant (CNVs), and gene methylation.

2. Materials and methods

2.1. Twin study design

Two pairs of Mongolian monozygous twins (participant #1 and #6; participant #2 and #4) were recruited by the Affiliated Hospital of Inner Mongolia University for the Nationalities (Inner Mongolia, China) and The First Hospital of Jilin University (Jilin, China) in 2017. The study was approved by the ethics committee of the Affiliated Hospital of Inner Mongolia University for the Nationalities (Approval No. NM-LL-2016-10-13). Written informed consent was provided by all participants. All methods were performed in accordance with the relevant guidelines and regulations. To avoid the impact of interference factors such as the disease itself and drug treatments on the multiomics analysis during the onset period, only patients who were cured for more than 1 year were included. The families of the two pairs of identical twin Mongolian girls in this study have lived for more than three generations in Inner Mongolia Xing'an Meng Keyouzhong Banner and ChifengBalin Right Banner, without blood relationship histories of intermarriage or histories of other immunological diseases or hereditable diseases. Two girls of each pair of twins (#1 and #2) were diagnosed with TB by sputum smear and computed tomography imaging, while the other girls (#4 and #6) were TB-free but had close or lengthy contact and lived with a person with TB.

2.2. DNA and RNA extraction from blood samples

A volume of 4 mL blood was added to the PAXgene Blood RNA Tube by venipuncture and EDTA-containing tube (2 mL) in January 2017 and stored at −80 °C. Total blood RNA was extracted using the PAXgene RNA Blood Mini Kit (Qiagen, Venlo, The Netherlands), according to the manufacturer's instructions. The amount of RNA was quantified by the spectrophotometric A260 to A280 ratio of 1.8, whereas quality was assessed using denaturing gel electrophoresis using the Agilent 2100 system (Agilent Technologies, Santa Clara, CA, USA). Total blood DNA was extracted using the EDTA DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. The amount of DNA was quantified by the spectrophotometric A260 to A280 ratio of 1.8. All DNA and RNA samples were stored at −80 °C.

2.3. SNP and InDel detection

Next-generation sequencing (NGS) was performed to analyze SNPs, CNVs, and InDels. Genomic DNA libraries were generated as previously described [33,34] for *sequencing* reads using the Illumina HiseqX Ten system (Illumina, Inc., San Diego, CA, USA). The sequences were aligned to the human reference genome (hg19) using GSNAP (https://www.gvst.co.uk/gsnap.htm) with a tolerance of 5 % mismatch [34]. SNPs and InDels were detected with Alpheus software [33,34]. For SNP analysis, data filtering was conducted according to methods outlined in existing literature. Briefly, the data were filtered to validate genuine mutations due to the initial high-throughput sequencing data containing numerous false positive SNVs. Criteria for validation included mutations appearing in more than three reads covering the base position. Additionally, at least 30 % of all reads were required to cover the mutation. The GS20 quality score was set at 20, discernible in reads from both directions. Furthermore, the target RefSeq mRNA sequence was detected in over 90 % of the same reads across the entire length.

2.4. CNV calling

CNVs were identified based on sequence read depth. It employs diverse cluster sizes for guiding read clustering and utilizes a customized cutoff to identify discordant read pairs. The GC% and averaged sequencing depth of every sliding window of the sequenced genome were calculated. Then the values were modeled to a normal distribution with an estimated mean depth and standard deviation (SD) for each level of GC content. The region that had a depth significantly different from that of the whole genome average at the same level of GC content and with flanking sequences that had a depth significantly different from that in the region was considered a potential CNV.

2.5. Gene expression and DNA methylation analysis by microarray

DNA methylation patterns were analyzed using the Illumina Infinium HumanMethylation850 Bead Chip (Illumina, Inc.), according to the manufacturer's instructions. R package minfi was used to perform methylation data preprocessing. Gene expression profiles were analyzed by PrimeView Human Gene Expression Array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's instructions.

Table 1
CNVs in the two pairs of twins.

Name	Individual	Haploid	Type	Confidence
AGER	2#	2.79	Gain	high
	4#	2.51	Normal	high
	1#	1.92	Normal	high
	6#	1.46	Loss	high
HLA-A	2#	1.69	Normal	high
	4#	1.38	Loss	high
	1#	3.05	Gain	high
	6#	1.85	Normal	high
HLA-DMB	2#	1.65	Normal	high
	4#	1.45	Loss	high
	1#	1.87	Normal	high
	6#	1.48	Loss	high
HLA-DOA	2#	2.55	Normal	high
	4#	2.62	Gain	high
	1#	1.71	Normal	high
	6#	1.41	Loss	high
HLA-DQB1	2#	1.76	Normal	high
	4#	1.39	Loss	high
	1#	4.02	Gain	low
	6#	1.82	Normal	low
HLA-E	2#	1.88	Normal	high
	4#	1.44	Loss	high
	1#	2.69	Gain	high
	6#	1.79	Normal	high
HSPA1A	2#	1.56	Normal	high
	4#	1.52	Loss	high
	1#	1.74	Normal	high
	6#	1.5	Loss	high
IFNA2	2#	1.91	Normal	high
	4#	1.43	Loss	high
	1#	2.26	Normal	high
	6#	2.72	Gain	high
LTA	2#	1.98	Normal	high
	4#	1.51	Loss	high
	1#	2.86	Gain	high
	6#	2.03	Normal	high
POU3F3	2#	0.47	Loss	low
	4#	3.36	Gain	low
	1#	2.41	Normal	high
	6#	3.2	Gain	high
PSMB8	2#	2.73	Gain	high
	4#	2.19	Normal	high
	1#	1.55	Normal	high
	6#	1.4	Loss	high
PSMB9	2#	1.98	Normal	high
	4#	2.63	Gain	high
	1#	2.73	Gain	high
	6#	2.41	Normal	high

2.6. Bioinformatics analysis

The differentially expressed genes (DEGs) with official gene symbols (ID) between case and healthy participant in each pair of identical twins were detected using $|\log FC| \geq 1.5$ and maximum gene probe signal ≥ 7 as cutoff thresholds, after which pathway analysis was performed. Differentially methylated genes (DMGs) were identified using the beta difference value > 0.1 or < -0.1 and $p < 0.05$ as a cutoff criterion. Gene-gene interaction analysis was performed by considering genes related to the TB pathway as the total gene. The Search Tool for the Retrieval of Interacting Genes database (https://string-db.org/) was used to extract the interaction relationships among genes in the TB pathways. Cytoscape software (version 3.5.1; www.cytoscape.org) was used to construct the gene interaction network. Meanwhile, the screened genes with differential methylation and differential expression profile interacted with genes in the TB pathways, followed by labeling of the genes according to the upregulation or downregulation relationship.

2.7. Quantitative PCR validation

SNPs and InDels detected with NGS were validated using Sanger sequencing on the ABI 7500 Sanger sequencer (Applied Biosystems, Foster City, CA, USA). The gene expression patterns were further confirmed by quantitative PCR (qPCR). CNVs and methylation were not further validated in the present experiment.

3. Results

3.1. SNPs and InDels

SNPs were detected with NSG in the four participants, which were not further confirmed by Sanger sequencing, indicating that they were false positive. No differential frameshift variant or stop-gain InDel was detected.

3.2. CNV calling

Genomic CNVs (gains, losses, amplification, and homozygous deletion) in the two pairs of twins are summarized in Table 1. Participant #2 showed gain of copy numbers in *AGER* and *PSMB8* and a loss in *POU3F3* and *PSMB9,* whereas participant #4 had gain of copy numbers in *HLA-DOA*, *POU3F3*, and *PSMB9* and a loss in *HLA-A*, *HLA-DMB*, *HLA-DQB1*, *HLA-E*, *HSPA1A*, *IFNA2*, and *LTA*. Participant #1 showed gain of copy numbers in *HLA-A*, *HLA-DQB1*, *HLA-E*, *LTA*, and *PSMB9* and no loss, whereas participant #6 had gain of copy numbers in *IFNA2* and *POU3F3* and a loss in *AGER*, *HLA-DMB*, *HLA-DOA*, *HSPA1A*, and *PSMB8*. The DEGs between participant #1 and participant #6 as well as between participant #2 and participant #4 are presented in Supplementary Tables S1, S2, and S3.

3.3. Methylation analysis

As shown in Table 2, a total of nine DMGs were identified between participant #2 and participant #4 by a non-overlapping sliding window of 10 kb (Table 3) including *BCHE, CARD16, CASP1, CD5L, HLA-DRB1, IRAK4, PDE3B, PRKCZ,* and *TNFAIP3*; and six DMGs including *AMELY, BBS2, HLA-B, IFRD1, IGF2R*, and *PLGC2* were detected between participants #1 and #6.

3.4. mRNA expression profile

The differentially expressed mRNAs are presented in Fig. 1. Compared with participant #4, participant #2 had lower mRNA expression of DMGs *DLAT*, *CXCL8*, and *PTPN22* and higher expression of *MMP9* (Fig. 1A). The mRNA expression of DMGs including *ANXA1, ANXA4, CASP3, DEFA4, FCGR3A, CXCL5, STAT1, STAT2*, and *CCR2* were lower, whereas expression of *CASP1, CARD16, AIF1, ANXA3, CASP4, FCGR1A, SO110, IL15, S100A8, SOCS3, LY96,* and CLEC12A were higher in participant #1 compared with identical twin participant #6 (Fig. 1B). Fig. 2A and B presents the gene-gene interaction network of DEGs leading to TB. A total of 216 DEGs were screened as potential core regulators of TB development in identical twin pairs, indicating very complex gene-gene interactions among all DEGs. Among them, 157 and 59 DEGs were overlapped with TB pathways between participant #1 and #6, and between participant #2 and # 4, respectively. The genes participating in inflammation (IFNA, IL-10R, IL-12B, TLR, and IL-1B), immunity (HLA

Table 2
Gene methylation between participants #1 and #6.

UCSC_REFGENE_NAME	UCSC_REFGENE_GROUP	UCSC_CPG_ISLANDS_NAME
AMELY	TSS1500	
BBS2	Body	
HLA-B	Body	chr6:31323946-31325211
IFRD1	TSS200;5′UTR	chr7:112090122-112091412
IGF2R	Body	
PLCG2	Body	

Table 3
Gene methylation between participants #2 and #4.

UCSC_REFGENE_NAME	UCSC_REFGENE_GROUP	UCSC_CPG_ISLANDS_NAME
BCHE	TSS200;TSS200	
CARD16	TSS200;Body;Body;Body;Body;Body;TSS200	
CASP1	TSS200;Body;Body;Body;Body;Body;TSS200	
CD5L	3′UTR	
HLA-DRB1	Body	chr6:32551851-32552331
IRAK4	1stExon;5′UTR;TSS1500;TSS1500;5′UTR;5′UTR;TSS1500;TSS1500;TSS1500	chr12:44152506–44152922
PDE3B	Body	
PRKCZ	Body; Body; Body	
TNFAIP3	3′UTR	

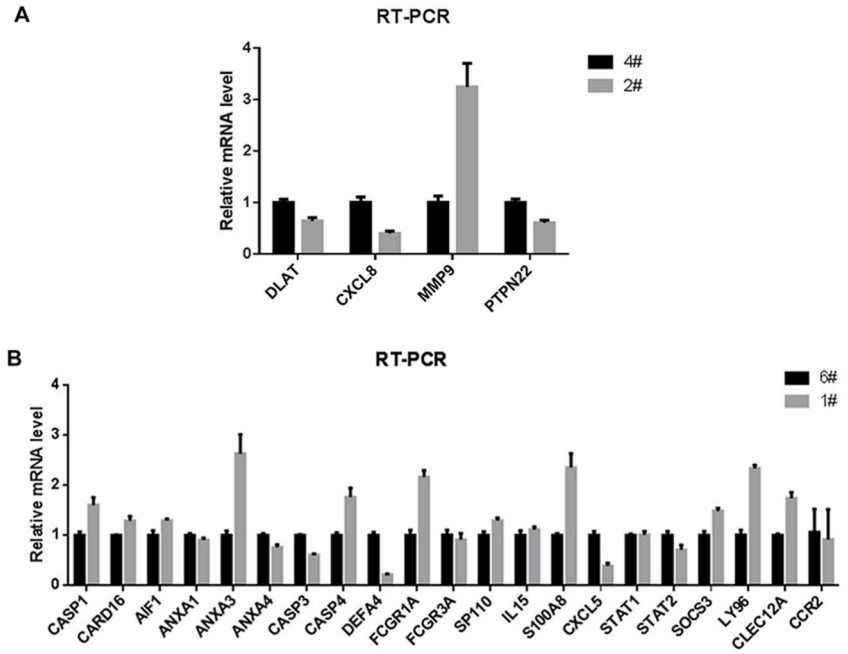

Fig. 1. Differential mRNA expression between participants #2 and #4 (A), and between participants #1 and #6 (B).

family), and the cell cycle (mitogen-activated protein kinase and phosphoinositide 3-kinase) had higher betweenness centrality, degree, indegree, and outdegree, playing crucial roles in TB progression.

4. Discussion

As a complex trait, genetic studies on TB have been extensively ongoing for decades; however, genomic and epigenetic mechanisms underlying inheritable susceptibility to TB remain unclear. Monozygotic twin pairs have long been thought to share 100 % of their genomic information. Genetic differences between monozygotic twins through genomic analyses of CNVs and sequence level variants provide the fundamentals for detection of TB risk genes and variants. In the present study, we performed integrated sequence-based genomic, transcriptomic, and methylation analyses with NSG for two pairs of monozygotic twins to characterize the genetic factors of TB susceptibility. The results revealed associations of inheritable susceptibility to TB with genomic and epigenetic alternations of genes participating in the immune and inflammatory responses.

In this study, we demonstrated several alternations at the SNP and InDel levels in each pair of identical monozygotic twins. However, these alternations were confirmed to be false positives by Sanger sequencing, indicating that *susceptibility* to TB *might not* be due to the gene i*tself, but rather to* gene *expression* affected by the environment and exposure. Alternations of several key genes such as *HLA-DOA*, *POU3F3*, *PSMB9, HLA-A*, *HLA-DMB*, *HLA-DQB1*, *HLA-E*, *HSPA1A*, *IFNA2*, and *LTA* were detected between participants with and without TB in each pair of identical twins at the CNV level, contributing to the immune and inflammatory responses during TB development. The HLA family consists of major histocompatibility complex (MHC) class I and II in humans, and regulates the immunological recognition to play critical roles in the immune responses against many *diseases* and disorders. HLA class I molecules present peptides of *proteins* from within the *cell* to cytotoxic T *cells* for destruction of infected cells, whereas class II proteins present peptides from outside the cells to T cells for defense against pathogens [35]. HLA class I [36–38] and class II [39–42] alleles are

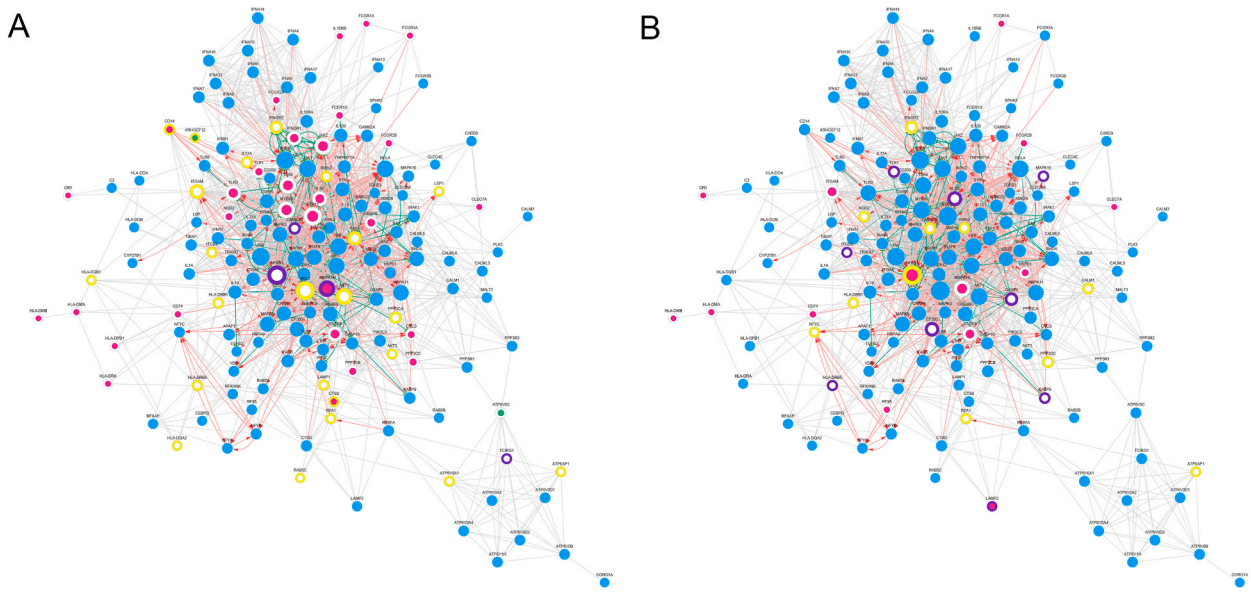

Fig. 2. Gene-gene interaction analysis of DEGs leading to TB between participants #2 and #4 (A) and between participants #1 and #6 (B). A total of 216 DEGs were screened as potential core regulators of TB development in identical twin pairs, indicating very complex gene–gene interactions among all DEGs. The outermost circle refers to methylation, where purple represents upregulation, yellow represents downregulation, and white represents genes that were not differentially methylated. The inner circle refers to the expression profile, where red represents upregulation, green represents downregulation, and white represents genes that were not differentially expressed. Genes with non-significant differences in methylation and expression profiles are shown in light blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

diagnostic markers for TB susceptibility, specifically in *HLA-DQA1, HLA-DQB1,* and *HLA-DRB1*. Accordingly, our study further confirmed associations of genomic alternations in *HLA-DQB1*, *HLA-DQB5*, *HLA-A*, *HLA-DRB5*, *HLA-DRB1, HLA-DOA*, *HLA-DMB*, and *HLA-E*, with inheritable susceptibility to TB. In addition, epigenetic variations in *HLA-DRB1* and *HLA-B* were apparent to be involved in TB.

IFNs are a group of signaling proteins released by host cells to regulate the activity of the immune system in response to the presence of a pathogen [43]. Genetic changes of IFNs are associated with TB susceptibility. Mutations in IFN-γ confer different susceptibilities to different *M. tuberculosis* strains [44–46]. Susceptibility to atypical TB might be associated with mutations in one of the five genes in the IFN-γ pathway [47]. Lee et al. [45] revealed that three IFN-γ polymorphisms confer higher TB risk in Han Taiwanese. In this study, we revealed the association of a SNP in *IFNA8* and a CNV in *IFNA2* with TB in two pairs of Mongolian monozygous twins.

In this study, some factors including chemokine (C motif) ligand (XCL1*),* phosphodiesterase 4 (PDE4) and MUCB5 are also involved in the immune response to TB. XCL1 is a C class chemokine produced by T cells and natural killer T cells during infectious and inflammatory responses, playing an important role in the dendritic cell-mediated cytotoxic immune response [48] and TB [49]. In this study, we detected the association of a SNP in *XCL1* with TB. PDE4 is a target for improving the antibiotic response in TB [50]. A SNP in *PDE4A* was found to be associated with TB in the present study. *MUCB5* [51,52] and *SERPINA1* [53] are reportedly involved in lung disease, but there has been no report of their involvement in TB. We detected the association of *MUCB5* and an InDel in *SERPINA1* with TB for the first time in the present study. *MUCB5* is the major mucin associated with lung disease, whereas *SERPINA1* serves as a serine protease inhibitor that participates in the protection of lungs against neutrophil elastase [53]. The findings of this study provide new insights into other factors that were previously unknown; however, further studies are needed to address the functions of novel factors in TB development and progression.

Our study also demonstrated the potential roles of genes affected by CNVs in TB development including *AGER, HSP1A1, LTA*, and *PSMB8/9*. AGER within MHC class III region is a cell surface transmembrane multiligand receptor that plays an important role in inflammation and lung disease [54]. *HSP1A1* is believed to play a role in modulating the macrophage response to *M. tuberculosis* after silver nanoparticle treatment [55]. Polymorphisms of *LTA* are reported to be involved in the response of TB to treatments [56]. Polymorphisms of *PSMB8* are reportedly associated with intestinal TB [57], whereas *PSMB9* is a major hub gene for TB [58]. The function of *POU3F3* in TB remains unclear. Further study is required to fully characterize the potential functions of these CNV-altered genes in TB.

Previous studies have revealed that the epigenetic changes in TB are very important issue for the diagnosis and treatment of TB [29, 59]. A number of methylated genes are associated with TB including HLA-DRB1 and HLA-B. However, the epigenetic mechanisms of several methylated genes underlying TB are still undetermined and need to be addressed by additional studies. Our study provided the expression profiles of several mRNAs involved in TB. The mechanisms of mRNA regulating TB development are very complex, involving a set of factors such as epigenetics. In the present study, two pairs of monozygous twins with and without TB were compared

to identify differences in transcriptional genes with a high degree of genetic consistency, which could be considered one factor associated with susceptibility to TB or one of the changes caused by TB. However, gene expression in both pairs of identical twins were different in this study. Additional studies are essential to address the factors influencing the expression of these mRNAs.

The findings of this study are limited by the fact that they are from only two pairs of Mongolian monozygous twins. Additional studies are needed to validate the findings in clinics for a better understanding of the genetics underlying the inheritable susceptibility to TB.

5. Conclusions

In conclusion, inheritable susceptibility to TB is associated with transcriptional and epigenetic alternations of genes involved in the immune and inflammatory responses. The genes in the HLA family and IFN pathway play major roles in susceptibility to TB. Further studies using larger twin cohorts and additional DNA sources of cases are needed to confirm our results and overcome the limitations of this study.

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Data availability

Exon sequencing data from twins, with our without tuberculosis infection, obtained through Illumina sequencing during this study, have been deposited in the NCBI SRA database under BioProject ID PRJNA1095406, with accession codes SAMN40722924, SAMN40722925, SAMN40722926, and SAMN40722927. Expression data of mRNA from twins, again with or without tuberculosis infection, can be accessed from the Gene Expression Omnibus database under accession code GSE262613. DNA methylation data are also available from the Gene Expression Omnibus database under accession code GSE263029. For access to the datasets generated and analyzed during the current study, interested parties may contact the corresponding author via reasonable request.

CRediT authorship contribution statement

Zhi Liu: Writing – original draft, Data curation. **Batu Deligen:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Data curation, Conceptualization. **Zhiqiang Han:** Writing – review & editing, Software, Methodology, Formal analysis, Conceptualization. **Chaolumen Gerile:** Writing – review & editing, Software, Methodology, Formal analysis, Conceptualization. **An Da:** Writing – review & editing, Writing – original draft, Investigation.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e31712.

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