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Insights into latent tuberculosis biomarkers from differential gene expression analysis in CD8 memory cells using secondary data *Insilico* approach

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Abstract

Tuberculosis (TB) remains a global health challenge, significantly impacting infectious disease mortality and morbidity. In the quest for effective diagnostic tools and precise treatment strategies, differential gene expression (DEG)-based biomarkers offer a promising avenue. These biomarkers provide specific insights into disease states and treatment responses by deciphering gene alterations within body cells. In this study, we aimed to identify immunological signatures associated with latent Mycobacterium tuberculosis infection in memory T cells. Leveraging transcriptomic analysis, we examined memory CD8 T cells from individuals with latent TB (NCBI-GEO GSM2643205) and healthy controls (NCBI-GEO GSM2643198). Our findings highlight candidate biomarker genes—LDB1, ZNF121, and STAT6—whose differential expression could significantly enhance our understanding of CD8 T cell genetic regulation during latent TB infection. These results hold promise for the development of more accurate biomarkers for diagnosing latent tuberculosis.

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INTRODUCTION

TB is an infectious disease that can cause death for sufferers. Currently, TB is still a top priority for the world and is one of the goals in the SDGs (Sustainability Development Goals). According to the World Health Organization (WHO, 2021), Tuberculosis (TB) is still a health problem in the world today. Referring to the WHO 2021 Global TB Report, Indonesia is the country with the third highest burden of Tuberculosis (TB) after India and China, which have a population of more than 1 billion. Currently, Indonesia is one of eight countries that accounts for 2/3 of TB cases in the world. In 2020, it is estimated that 824,000 people will fall ill and 93,000 will die from TB. From this estimate, in 2020 there were 384,025 cases found or around 47%. This case discovery rate decreased by 178,024 from 2019 due to the impact of the COVID-19 pandemic. This situation is a major obstacle to realizing the TB elimination target by 2030. The TB cure rate is still sub-optimal at 82 percent, below the global target for a treatment success rate of 90 percent (Laycock, Enane, & Steenhoff, 2021). TB patients who have not been found can be a source of TB transmission in the community, so this is a big

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challenge for TB control programs in Indonesia, including because there is still no early detection of latent TB cases (National TB Program, 2022).

Latent TB is a condition of persistent immune response to antigen stimulants without clinical evidence of active TB, radiographic and bacteriological abnormalities. Latent infection by Mycobacterium tuberculosis can occur when TB bacteria remain in the body in an inactive state. Memory T cells play an important role in controlling latent TB infection.

CD8 T cells can recognize and kill TB-infected cells, thereby helping control TB infection. Several cytokines produced by CD8 T cells, such as interferon and secretin IP-10, can influence TB1 cell function and strengthen the immune response against TB. A study shows that increased immune responses such as the production of interferon and secretin IP-10 can result in the expression of TB1 mRNA and the deletion of smooth muscle in TB cells (Dewi, 2020). In addition, TB1 can express other cytokines such as IL-12p, IL-15, IL-18, MIP-1α, and MIP-1β, all of which affect TB1 function (Setyawan, 2015). However, the immune response to TB can be influenced by other factors such as stress and smoking. Long-term stress can cause a decrease in the amount of the immune system which is influenced by the sympathetic nervous system and bone marrow, one of which is T lymphocyte cells (CD4 and CD8), thus making the body susceptible to pathogenic infections (Dewi, 2020; Wibowo et al., 2017; Infante et al., 2014). Exposure to cigarette smoke can also reduce the body's IFN-γ levels through inhibiting the function of macrophages and T cells (Syafaah, 2016). In another study, increased plasma IFN-γ concentrations were observed in 46.5% of multidrug-resistant tuberculosis (MDR-TB) patients. The immune response to TB infection is highly dependent on CD4+ T cells through the production of interferon-γ (IFN-γ). Individuals with MDR-TB are reported to have reduced IFN-γ production capacity by T cells (Arthamin et al., 2012). The number of memory T cells specific for Mycobacterium tuberculosis can increase in individuals who are latently infected with TB bacteria. This can happen because memory T cells can develop from T cells that are activated during the initial infection and remain in the body to provide protection in the future (An et al. 2022). Memory T cells can produce cytokines such as interferon-gamma (IFN-γ) and interleukin-2 (IL-2) which can strengthen the immune response against Mycobacterium tuberculosis. IFN-γ can stimulate macrophages to kill TB bacteria, while IL-2 can strengthen the activity of CD8+ T cells and CD4+ T cells (Syafaah, 2016). Memory T cells can express homing receptors that allow them to migrate to tissues infected by Mycobacterium tuberculosis. This allows memory T cells to provide local protection to infected tissue (Nabilah, 2020).

Next Generation Sequencing (NGS) technologies such as RNA-seq, have shown great potential in clinical research and diagnostic development, including discovery, characterization and detection. Due to its cost and time effectiveness, NGS methods can serve as an important diagnostic for understanding the spread of a disease (Satam et al, 2023). Research in RNA-seq involves examining pathways that are altered during infection or disease and analyzing changes in gene expression through differential expression analysis (Ewald et al, 2023). The focus of this research is to study the differential outcomes of transcription under different experimental conditions. Therefore, RNA-seq studies can be categorized into Differential Gene Expression studies, Differential Transcript Expression studies, and Differential Transcript/Exon Usage studies, where comparisons are made based on gene transcription, individual transcript size, and transcript/exon usage, respectively. Each (Liu et al., 2023; Park & Kim, 2016).

In this study, we analyzed differences in gene expression in latent tuberculosis samples using an RNA sequencing approach. The significant consensus identified from differentially expressed transcripts can be candidate genes that have key roles in significant biological processes and functional pathways associated with latent TB disease, so this research can contribute to the search for latent TB biomarker candidates that can be applied in diagnostics, detection, vaccine and specific treatment for latent TB cases.

METHOD

In this pipeline, RNA-seq datasets of memory CD8 T cells isolated from individuals suffering from latent tuberculosis and from controls not infected with tuberculosis were retrieved from NCBI-GEO. The datasets used include the NCBI-GEO accession GSM2643205 for memory CD8 T cells from latent tuberculosis patients and GSM2643198 for controls. Each group comprises eight sample files,

totaling sixteen sample files for the study. The data quality was initially assessed using FastQC, followed by preprocessing with Trimmomatic to ensure high-quality reads. Subsequently, the reads were aligned to the reference human genome (hg38) using the HiSAT2 aligner. The aligned reads were then quantified to obtain expression counts using featureCounts. These counts served as input for normalization and differential expression analysis. Normalization of the expression counts was performed using edgeR and DESeq2, two commonly used tools in RNA-seq analysis for differential expression analysis. Significant differential expression was identified using these tools, focusing on genes showing high consensus across the differential expression transcript (DET) analyses. The complete pipeline and workflow are illustrated in Figure 1.

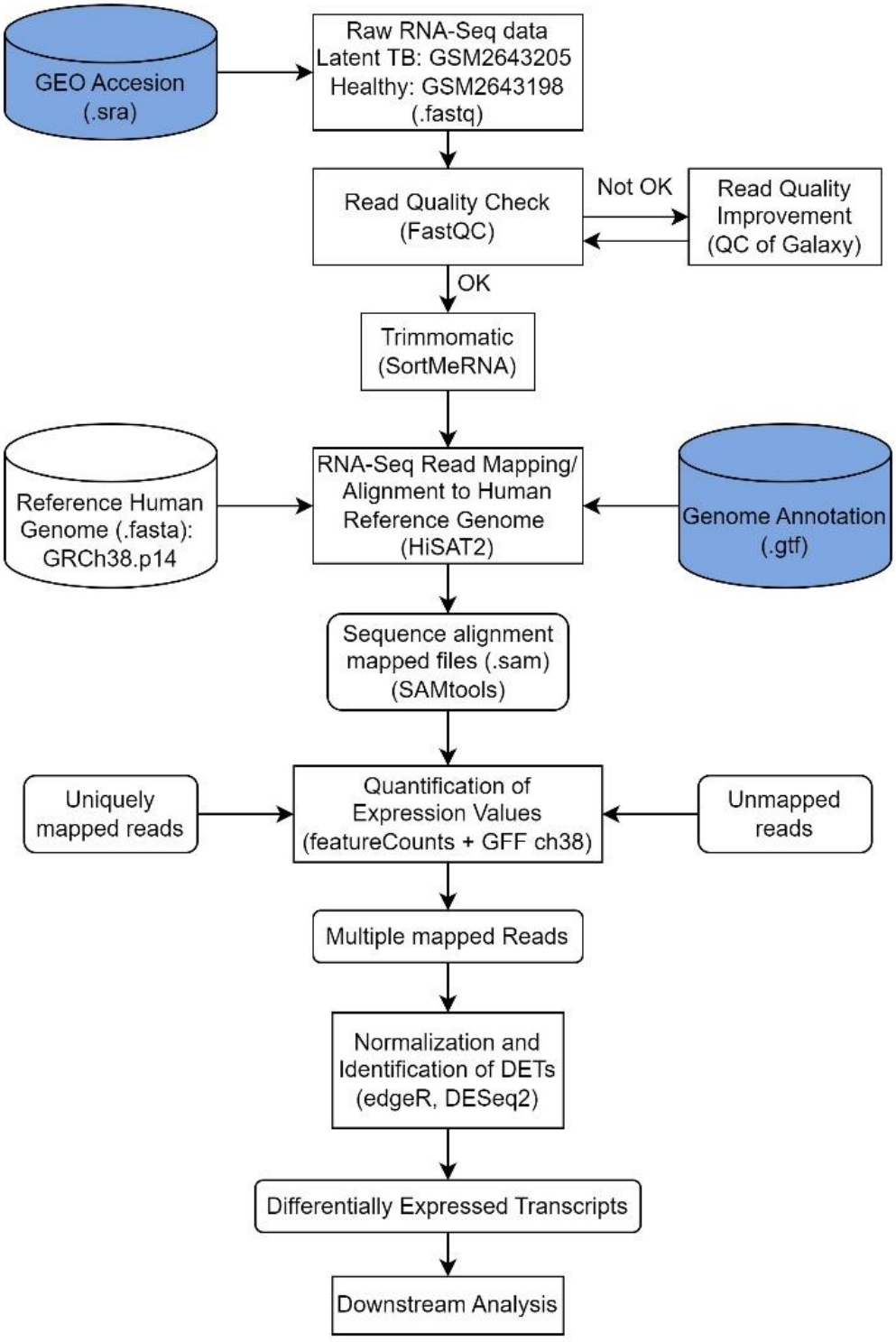

Figure 1. Workflow diagram for identification of Differential Expression Genes (DEGs)

Data Collection

RNA-seq datasets were retrieved from the NCBI Gene Expression Omnibus (GEO) repository under the accessions GSM2643205 and GSM2643198. These datasets pertain to memory CD8 T cells isolated from individuals with latent tuberculosis (GSM2643205) and controls without tuberculosis infection (GSM2643198).

1. **GSM2643205 (Latent Tuberculosis Group):** This dataset includes RNA-seq data from memory CD8 T cells isolated from individuals diagnosed with latent tuberculosis. It comprises eight sample files, each representing a different biological replicate or condition within the latent tuberculosis group.
2. **GSM2643198 (Control Group):** This dataset includes RNA-seq data from memory CD8 T cells isolated from controls who do not have tuberculosis infection. Similar to the latent tuberculosis group, it also consists of eight sample files, each representing distinct biological replicates or conditions within the control group. In total, the study utilizes sixteen sample files (eight from each group), facilitating comparative analysis between memory CD8 T cells from individuals with latent tuberculosis and those from healthy controls. These datasets serve as the foundational raw data for subsequent quality control, preprocessing, alignment to the human reference genome (hg38), and differential expression analysis using established RNA-seq analysis pipelines.

Preprocessing Data

All samples were first converted to fastq format and then checked for read quality using the FastQC tool. We used the Trimmomatic tool to trim the adapter to remove the adapter from the data. To distinguish rRNAs from their sequences, we used the SortMeRNA tool.

Read Mapping

We used the HiSAT2 alignment tool for preprocessed transmission against the human reference genome (hg38).

Read Counting

To count the number of reads deposited to the human reference genome, we used the featureCounts counting tool.

Differential Expression Analysis

Differential expression analysis begins with a normalization step, which is a method for adjusting the number of readings between samples in such a way that a normalized expression value is obtained that is even throughout the experiment. We implemented the following tool running on a benchmark value for a Bejamini-Hochberg controlled FDR of 0.05.

edgeR: edgeR is an analysis expression tool that models mapped read count data using a negative binomial model. This moderates the dispersion estimate calculated for each gene into a single generalized dispersion estimate, or local dispersion estimate, resulting from genes with similar expression weights calculated using the weighted conditional likelihood method. This is a measure to assess variation between libraries of these genes. For classical edgeR analysis, we took gene ID transcripts from sixteen sample libraries pooled in half. After dispersion estimation, we perform exact tests to determine differential expression. On the normalized expression values, we apply this tool.

DESeq2: The DESeq2 package uses negative binomial models to test differential expression. It calculates shrinkage based on distribution data, and then adjusts the logarithmic fold change to improve the stability of the results and their interpretability. For analysis via the DESeq2 package, we input the read counts of the sample library in matrix form, and also determine the condition of the sample, i.e. whether the sample is in a healthy or latent state. He first determined the size factor, and then calculated the distribution based on genes. This finally fits the model and tests for differential expression.

RESULTS AND DISCUSSION

Differential Expression Gene (DEG) analysis workflow (details in Figure 1) was performed on a workstation operating system.

Data Preprocessing

The quality of the raw RNA-seq data from both GSM2643205 (latent tuberculosis group) and GSM2643198 (control group) was initially assessed using FastQC. Analysis revealed that all samples exhibited a %GC content ranging from 47% to 50%, indicating typical nucleotide composition for RNA-seq data. Subsequently, the data underwent preprocessing using Trimmomatic to enhance data quality. Trimmomatic removed low-quality sequences, trimmed adapter sequences, and discarded reads that were too short, ensuring that only high-quality reads were retained for downstream analysis. Following Trimmomatic, the sorMeRNA tool was employed to efficiently filter out ribosomal RNA (rRNA) fragments from the metatranscriptomic data produced by Next Generation Sequencing. This step is crucial as rRNA can dominate sequencing reads and obscures the detection of mRNA transcripts of interest. sorMeRNA's high accuracy and specificity in matching RNA fragments against rRNA databases ensured effective removal of rRNA contaminants from the RNA-seq datasets. After preprocessing, the processed reads were aligned to the reference human genome (hg38) using the HiSAT2 aligner. Evaluation of the alignment results across all sixteen samples indicated an overall alignment rate exceeding 99%. This high alignment rate signifies robust mapping of sequencing reads to the human genome, reflecting the reliability and accuracy of the alignment process.

Read Counting and Normalization

As a standard assumption, read counting mapped to a gene/transcript is considered as a proxy for its expression. For data analysis, transcript feature-count results were further normalized by the total fragment count to create comparable counts across experiments. edgeR, and DESeq2 were used for this analysis, which first transforms the read count data into a continuous distribution by using the NB model to estimate the dispersion parameters for each transcript. This dispersion parameter provides a measure of the degree of inter-library variation of a particular transcript between samples. Generalized dispersion estimates provide an idea of the overall variability across a dataset.

Differential Expression Analysis

All samples were grouped into two groups, namely, eight latent TB samples and eight control samples followed by differential gene expression analysis using edgeR and Deseq2 tools. The principle of edgeR analysis is that the normalization factor calculated for each sample is close to 1, which indicates that the sixteen libraries have a similar composition using the Bayesian negative binomial objective model. The input estimates the common dispersion before estimating the tagwise dispersion to continue the differential expression analysis. First, the biological coefficient of variation (BCV) is applied to this input data. BCV is the mathematical square root of the generalized dispersion estimated using the NB model. With increasing read count, BCV remains unaffected, although a decrease in CV can technically be observed. Therefore, accurate BCV estimation is essential for differential expression analysis studies in RNA-seq experiments. The BCV calculated from the experiments was found to be 26%. Since, the higher the BCV size, the lower the number of differentially expressed genes/transcripts detected, it is therefore assumed that the assay detects a higher number of DETs. The results of edgeR analysis are logFC, logCPM, F, PValue and FDR. Meanwhile, the Deseq2 analysis principle uses a negative binomial model and uses the shrinkage estimator technique to handle genes with low expression. The results of the Deseq2 analysis are the normalized mean sum, average over all samples from both conditions, LogFC, standard error estimate for the log2FC estimate, Wald statistic, p-Value for the statistical significance of this change, p- Value adjusted for several testing with the Benjamini-Hochberg procedure which controls the false discovery rate (FDR).

Principal Component Analysis (PCA) is a statistical method used to reduce the dimensionality of complex datasets by projecting them into a lower-dimensional space. In this analysis, PCA was applied to RNA-seq data from memory CD8 T cells isolated from individuals with latent tuberculosis (latent sample group) and controls without tuberculosis infection (control sample group). PC1 (26% variance), PC1 represents the primary direction of maximum variance in the data. It explains 26% of the total variability observed across all samples. A higher percentage indicates that PC1 captures a substantial amount of the overall variation present in the dataset. In this context, PC1 likely reflects the major biological differences between the latent tuberculosis and control groups. PC2 (10% variance), PC2 represents the second principal component, explaining an additional 10% of the variance in the dataset. While PC2 has lower variance compared to PC1, it still contributes

significantly to the overall variability and captures additional distinct patterns or differences between the sample groups.

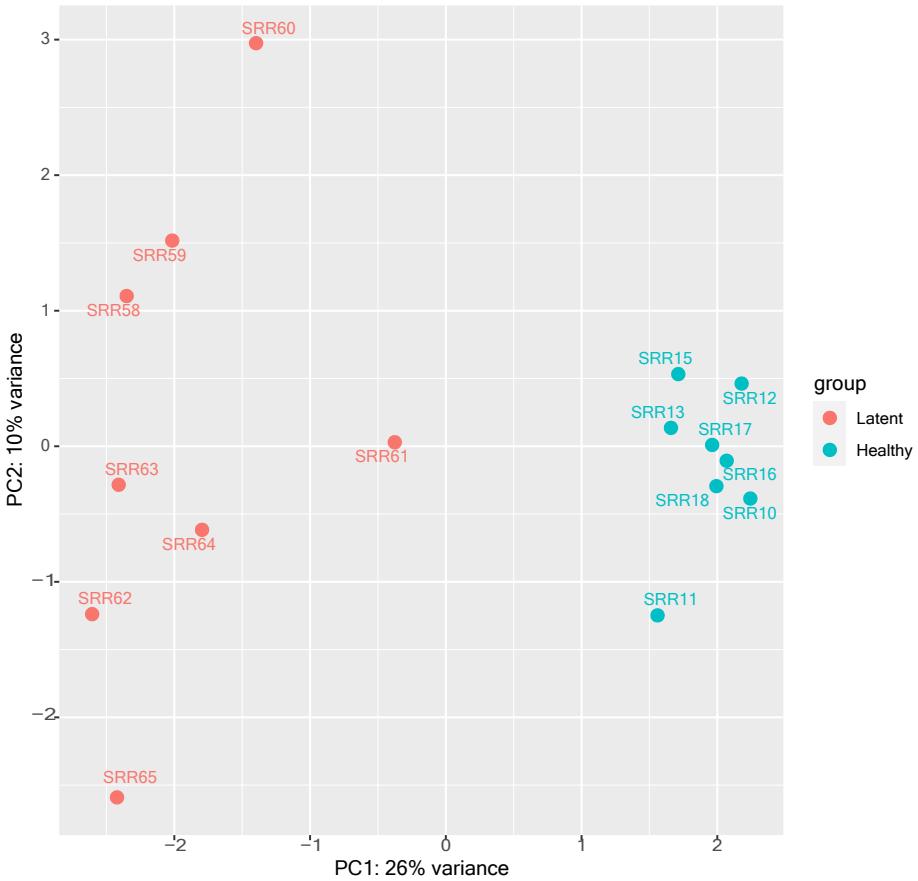

Figure 2. Principal Component Analysis (PCA) between PC1 (26%) and PC2 (10%)

The PCA plot (Figure 2) visually represents how samples are distributed in the reduced two-dimensional space defined by PC1 and PC2. The control sample group is clustered closely together in a distinct region of the plot, indicating similarities in gene expression profiles among control individuals. In contrast, the latent tuberculosis sample group exhibits greater dispersion across the plot, suggesting higher variability in gene expression profiles among individuals with latent tuberculosis. The separation between the two sample groups in the PCA plot indicates that there are distinct gene expression patterns distinguishing latent tuberculosis cases from healthy controls. This separation increases the potential to detect a higher number of Differentially Expressed Transcripts (DETs) between the two groups. DETs are genes whose expression levels significantly differ between experimental conditions (latent tuberculosis vs. control), and PCA helps visualize and interpret the underlying patterns driving these differences (See Figure 3).

The dispersion estimates graph on the x-axis which shows the normalized average of the data and the y-axis which shows the dispersion is a visual representation of the dispersion distribution of genes in RNA-seq data. Dispersion describes the extent to which gene expression variations vary between samples. Based on the graph, we can see that the red line shows that the data points that are closer to the red line indicate that the model can predict well for that gene.

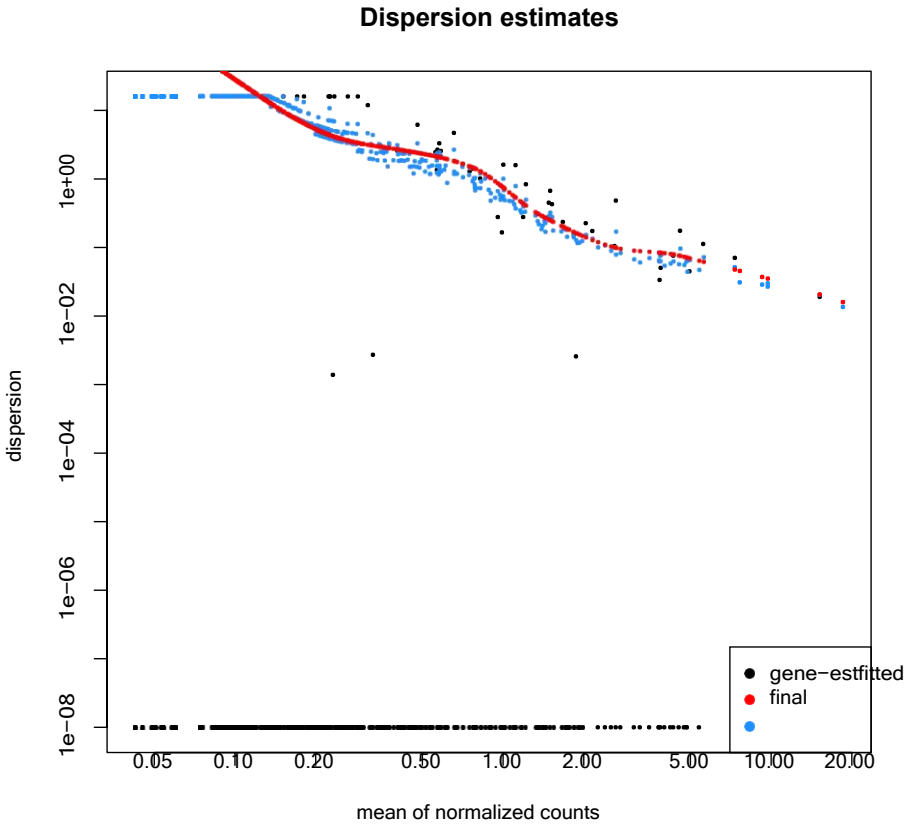

Figure 3. Distribution Prediction Graph (Dispersion estimates)

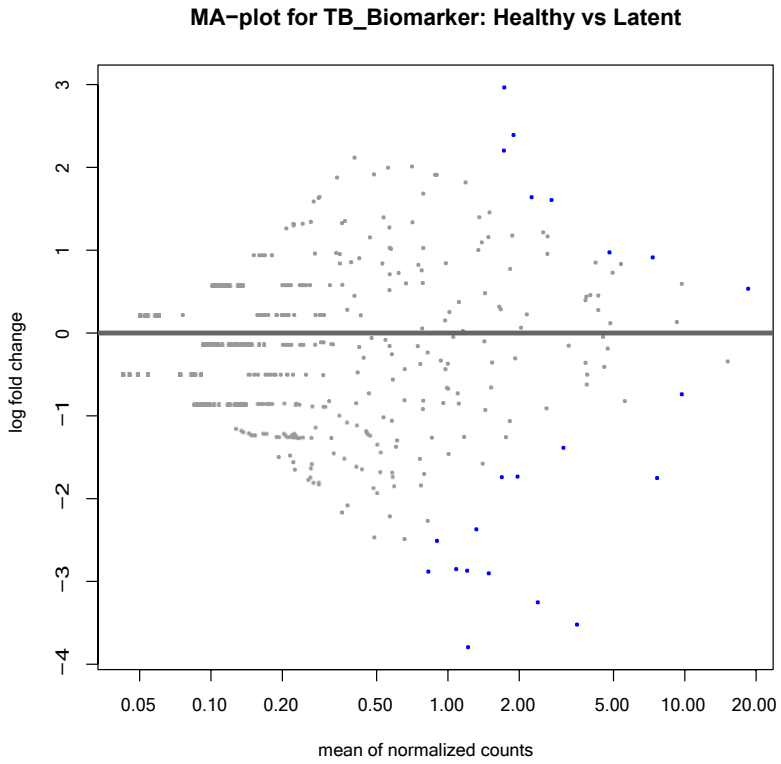

Figure 4. Mean-Abundance (MA) plot graph

In differential expression analysis of RNA-seq data, the MA-plot serves as a crucial visualization tool (see Figure 4). It plots the average of normalized counts (mean abundance) on the x-axis and the log fold change (log FC) between two conditions on the y-axis. Log FC indicates the magnitude of gene expression differences between groups; positive values denote increased expression in the first

group compared to the second, while negative values indicate decreased expression. Points outside the range of -2 to +2 on the y-axis highlight genes with significant expression changes, potentially indicating targets for further investigation. From the analysis of 12 differentially expressed genes (DEGs) with strong consensus, five were identified as Transcription Factors (TFs), crucial in gene regulation. Notably, LDB1, ZNF121, and STAT6 were among the identified genes, each known for roles in transcriptional regulation and immune response. Additionally, two novel genes were discovered, suggesting potential new players in tuberculosis-related pathways or immune responses. Ensembl (https://www.ensembl.org/) provides comprehensive genetic information, including gene IDs, symbols, descriptions, and expression data from various RNA-seq studies, aiding in the functional annotation of genes (Table.1).

Table 1. The Candidate genes as biomarkers for latent TB

RefSeq ID	Official Gene symbol	Gene name	Log2FC		Expression
			EdgeR	DESeq2	
ENSG00000198728	LDB1	(LIM domain binding 1)	3.25	3.52	Ubiquitous expression in skin (RPKM 42.1), ovary (RPKM 38.8) and 25 other tissues
ENSG00000197961	ZNF121	(zinc finger protein 121)	4.28	3.79	Ubiquitous expression in bone marrow (RPKM 3.6), spleen (RPKM 3.1) and 25 other tissues
ENSG00000166888	STAT6	(signal transducer and activator of transcription 6)	2.75	2.98	Ubiquitous expression in spleen (RPKM 48.4), skin (RPKM 47.3) and 25 other tissues

Metabolic Pathway Analysis

Figures 5, 6, 7, and 8 shows the metabolic pathway results using Panther.

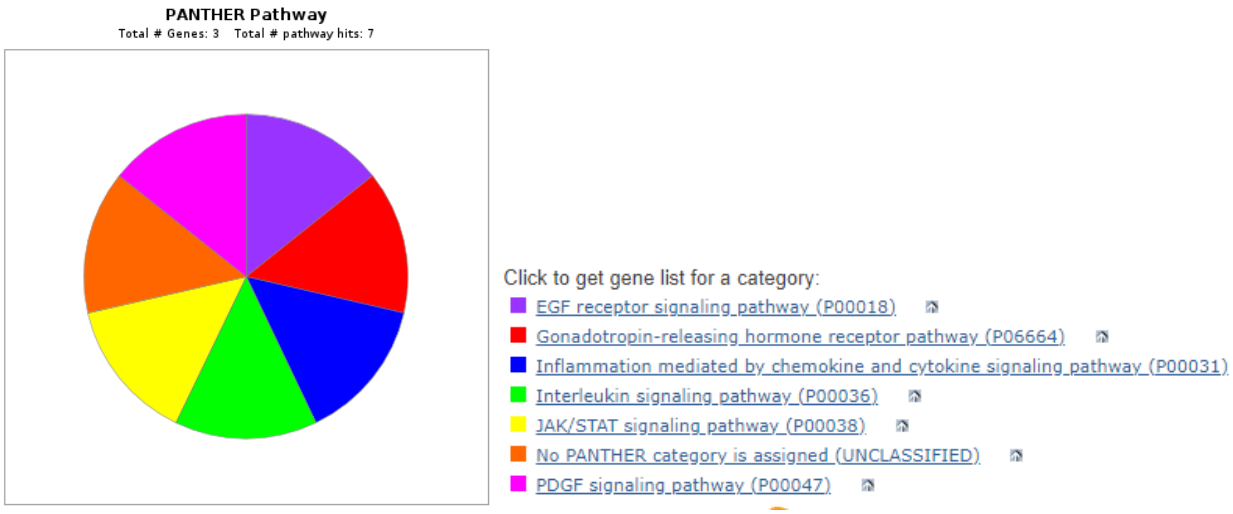

Figure 5. Metabolic pathway diagram resulting from advanced analysis by Panther

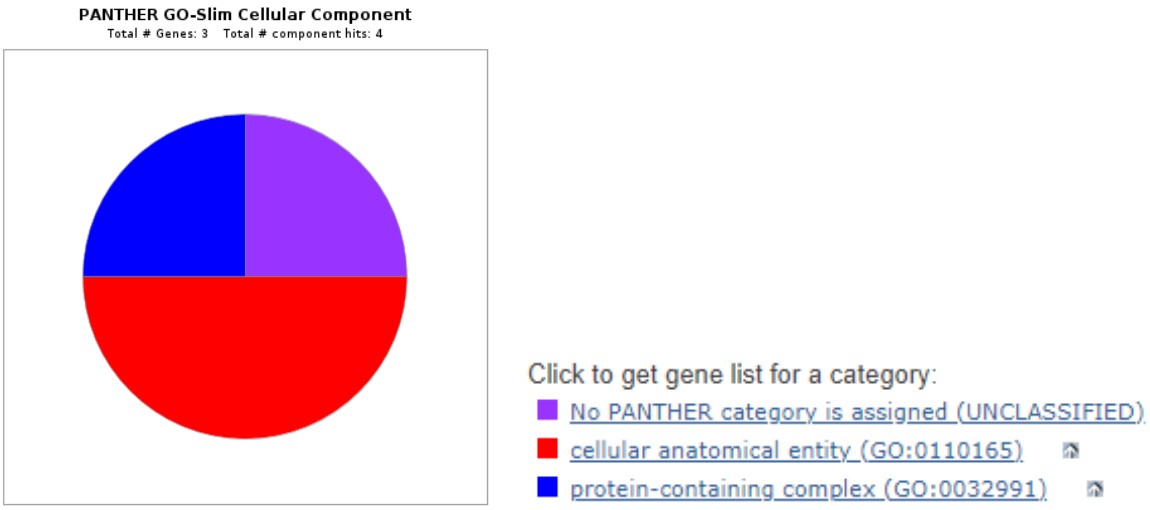

Figure 6. Diagram of the results of advanced *gene ontology (GO) analysis* of cell components by Panther

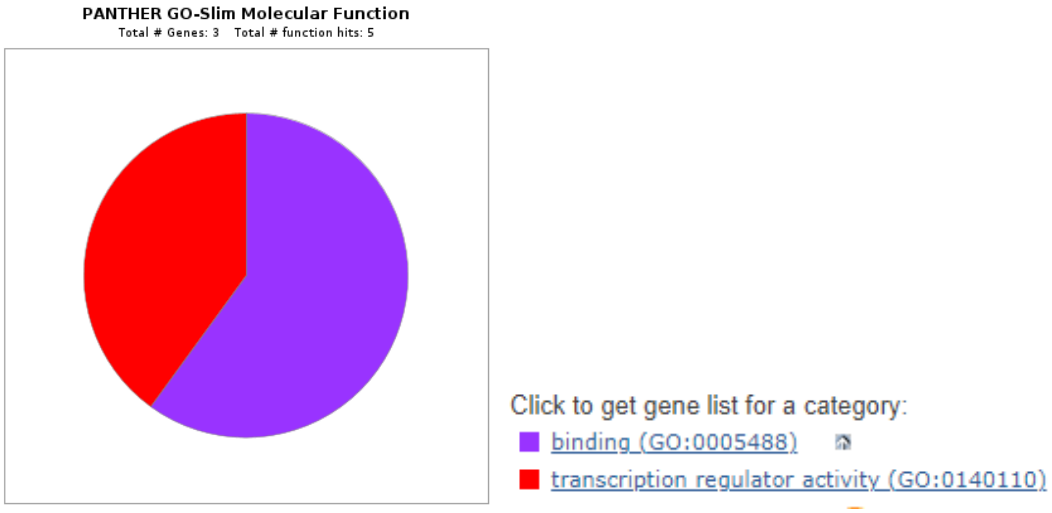

Figure 7. Diagram of the results of advanced *gene ontology (GO) analysis* of molecular function by Panther

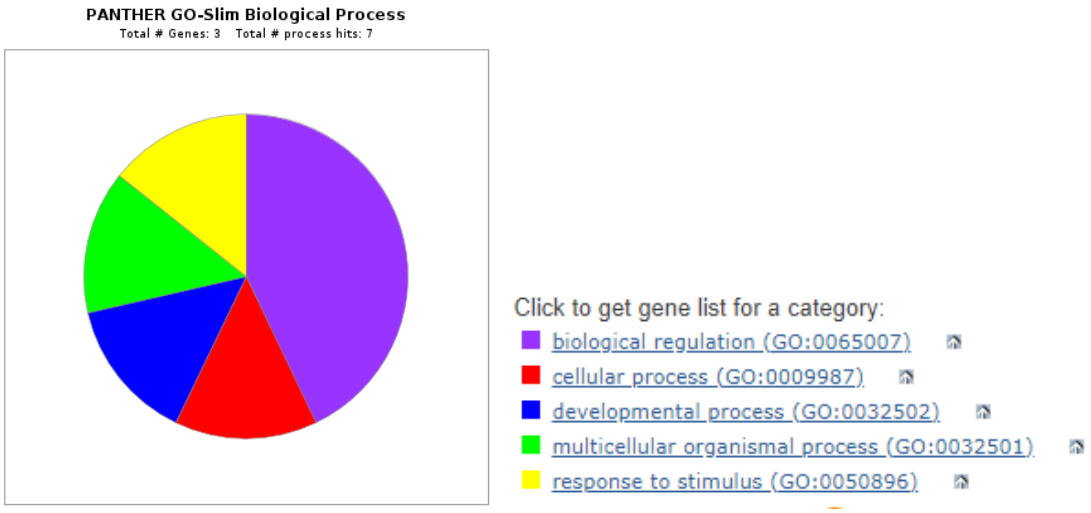

Figure 8. Diagram of the results of advanced *gene ontology (GO) analysis* of biological processes by Panther

Table 2. Metabolic pathway analysis of latent TB biomarker candidate genes

Pathway Accession	Mapped IDs	Pathway Name
P00031	HUMAN|HGNC=11.368|UniProtKB=P42226	Inflammation mediated by chemokine and cytokine signaling pathway.
P00018	HUMAN|HGNC=11.368|UniProtKB=P42226	EGF receptor signaling pathway.
P00036	HUMAN|HGNC=11.368|UniProtKB=P42226	Interleukin signaling pathway.
P06664	HUMAN|HGNC=6.532|UniProtKB=Q86U70	Gonadotropin releasing hormone receptor pathway.
P00047	HUMAN|HGNC=11.368|UniProtKB=P42226	PDGF signaling pathway.
P00038	HUMAN|HGNC=11.368|UniProtKB=P42226	JAK/STAT signaling pathway.

This pathway represents chemokine-induced adhesion and leukocyte migration that results in infiltration into the tissue and transcriptional activation that allows recruitment of more leukocytes. Thus, inhibiting certain chemokines and receptors may prevent excessive recruitment of leukocytes to sites of inflammation (based on Tabel 2). After binding to the G-protein family plus seven transmembrane receptors, chemokines (chemotactic cytokines) control and direct interaction and migration of immune cells. The development of tuberculosis biomarkers based on differential gene expression analysis has significant implications by identifying genes that are differentially expressed between healthy and sick individuals. This research provides the basis for the development of gene expression-based biomarkers that can detect TB disease. This has the potential to revolutionize TB diagnosis by enabling the identification of individuals at risk of active TB before they become infected with Mycobacterium tuberculosis. Early identification of susceptibility can lead to more targeted and timely interventions, including the use of antibiotics to treat latent TB in susceptible individuals, thereby reducing the risk of developing active disease (Nogueira et al., 2022; Wykowski et al., 2021). Additionally, research into these biomarkers may contribute to the development of more appropriate therapies for TB.

This study identifies genetic polymorphisms near differentially expressed genes associated with susceptibility to tuberculosis (TB), illuminating genetic factors influencing TB susceptibility. This knowledge could pave the way for personalized treatment strategies that consider an individual's genetic predisposition to TB (Blischak et al., 2017). Furthermore, the development of gene expression-based biomarkers holds promise in identifying TB-resistant individuals, potentially reducing unnecessary antibiotic use and mitigating the emergence of drug-resistant Mycobacterium tuberculosis (MTB) strains. Advancements in TB diagnosis are crucial to achieving the World Health Organization (WHO) End TB targets by 2030, aiming to reduce TB incidence by 80% and TB-related deaths by 90% from 2015 levels. Addressing current diagnostic challenges and limitations through innovative solutions is essential for enhancing TB diagnosis and treatment globally (Cho et al., 2020; McNerney et al., 2012).

CONCLUSION

Latent tuberculosis (TB) cases have prompted computational biologists and bioinformaticians to conduct differential expression analysis on patients infected with latent TB. This study computationally analyzed RNA-seq data to uncover transcriptomic changes, essential for developing improved diagnostic tools, therapies, and treatments. By employing edgeR and DESeq2 with stringent filter criteria (FDR < 0.05, p-value < 0.05, and |log2FC| > 2), the study identified LDB1, ZNF121, and STAT6 as promising biomarker candidates.

AUTHOR CONTRIBUTIONS

Each author of this article played an important role in the process of method conceptualization, simulation, and article writing

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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