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Original Article

A Novel Prognostic Biomarker with Potential of High Diagnostic Accuracy in Pulmonary Tuberculosis: An *in silico* Study

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ABSTRACT

Background: Due to the fact that pulmonary tuberculosis (PTB) is a highly infectious disease characterized by high herd susceptibility and hard to be treated. Cytokines have a crucial role in eliciting protective and pathologic consequences in infectious diseases, including tuberculosis. This study aimed to investigate the gene expression dataset of tuberculosis patients to identify the novel cytokine biomarkers in pulmonary tuberculosis. **Materials and Methods:** The expression dataset (GSE19435) having comparative study of drug treatment at 0-month (control), 2-months and 12 months was retrieved from National Center for Biotechnology Information (NCBI) geo datasets for bioinformatic analysis. Differential gene expression (DEGs) analysis of immune-related genes with treatment progression was performed which was further followed by Protein-Protein Interaction (PPI) network construction. Further, functional enrichment and KEGG pathway enrichment analysis were also performed. Finally, Receiver-operating characteristic curves (ROC) analysis was performed for selected biomarkers. **Results:** A total of 210 differentially expressed genes (DEGs) were identified, out of which 59 were upregulated, while 151 were downregulated. Gene ontology results revealed that the deregulated genes were enriched in immune response-regulation, cytokine pathways, and response to the bacterium. Also, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis revealed that deregulated genes are involved in the fork head box transcription factors (FOXO) signaling pathway, oxidative phosphorylation, and osteoclast differentiation pathways. Based on the combined score and degree of connectedness, novel genes like amyloid-beta precursor protein (APP), Transmembrane Immune Signaling Adaptor (TYROBP), Annexin A2 (ANXA2), Proteasome 20S Subunit Beta 2 (PSMB2), CD58 Molecule (CD58) (upregulated genes), and Thyroid Hormone Receptor Interactor 12 (TRIP12), RNA Polymerase III Subunit A (POLR3A), Nuclear Factor of Activated T Cells 5 (NFAT5), DEAD-Box Helicase 17 (DDX17), DNA Topoisomerase III (TOP3A) (downregulated genes) were identified as hub genes of which TRIP12 and POLR3A are cytokines (type1) biomarkers. Further analysis through ROC divulged TRIP12 as a potential diagnostic biomarker. **Conclusion:** Overall findings revealed that TRIP12 is a novel cytokine biomarker in *Mycobacterium tuberculosis*-infected patients with the highest diagnostic accuracy.

Keywords: Antibiotics, Biomarkers, Cytokines, Hub genes, *Mycobacterium tuberculosis*, Prognosis, Tuberculosis.

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INTRODUCTION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*MTB*), remains one of the deadliest infectious diseases worldwide.[1-2] According to WHO (World Health Organization, nearly 10 million became ill, and approximately 1.4 million died from TB (including HIV-positive people) across the globe.[3] As far as the highest mortality rate is concerned, the severely affected regions are reported in low- and middle-income countries.[4-5] According to a survey, India represents the country having the highest burden of TB cases.[6] Data archived from the health ministry department in India revealed that about 3 million new TB cases occur every year.[6] Besides, the development of TB involves various factors like virulence of *MTB* strain, host genetics, mechanism of evasion of the bacterium, and immune response of the host.[6,7] In addition, a balance between host immunity and bacterial evasion determines the severity of pathogenesis and progression of TB. Studies have shown that innate and adaptive immunity are involved in eradicating microorganisms through activating a cascade of immune responses.[8] Following the infection, components of innate immunity such as Toll-like receptors (TLRs), Nucleotide Oligomerization Domain (NOD)-like receptors recognizes the mycobacteria and elicits cytokine response followed by the phagocytosis of mycobacterium by macrophages and recruitment of T cells.[9] Activated macrophages, in turn, kill the bacteria through their cytotoxic activity.[8,10] The cytokines and chemokines play a critical role in mounting strong immunity against *MTB* .[11] *MTB* binding with pattern recognition receptors (PRRs) stimulates the production of chemokines and cytokines in the lungs, leading to the recruitment and activation of inflammatory cells.[12] Additionally, upon interaction with inflammatory cells, *MTB* migrates to the draining lymph nodes, effectively triggering the differentiation of the antigen-specific T-cells into cytokine-producing cells.[13-15] Thus, cytokines and chemokines have immense potential to be employed as critical biomarkers for early diagnosis and effective treatments of TB.

Considering the evidence mentioned above, the foremost aim of the present study is to investigate the effect of anti-mycobacterial treatment on the active TB signature and to decipher the network of novel cytokine biomarkers associated with tuberculosis during and after treatments. Since it is pertinent for pulmonary tuberculosis (PTB) treatment, the disease-related biomarkers are important to predict the treatment and follow-up of therapeutic protocols, the bacterial load and inflammatory response. In this dataset: PTB baseline, n = 7; PTB 2 months, n = 7; PTB 12 months, n = 7. A comparative transcriptomic

study on time-dependent drug treatment was performed at 0 months (control), 2 months, and 12 months. However, the availability of studies over a few genes prompted us to screen for more novel potential candidate genes associated with this disease and to disclose the associated mechanism behind tuberculosis infection at the molecular level through various bioinformatics applications.

In the present study, differential expression genes (DEGs) on the expression dataset (GSE19435) were retrieved. Then, with the help of combined scores Protein-Protein Interaction (PPI) network was formed. Also, a functional and gene enrichment study was performed. Our investigation led to the identification of a series of essential novel biomarkers, including cytokines like POLR3A and TRIP12, which are vital components of the immune system of vertebrates.

MATERIALS AND METHODS

Retrieval of Raw Expression Dataset

The raw expression dataset profile (GSE ID GSE19435) of TB patients at different time points of treatment was retrieved from the NCBI Gene Expression Omnibus datasets. TB Patients at 0 months of anti-tuberculosis drug treatment were considered as control and compared with TB patients at 2 months and at 12 months of treatment to reveal the effect of the drugs on samples in those periods. The array dataset contains one platform (GPL6947) Illumina Human HT-12 V3.0 expression bead chip and 33 samples. Multiple bioinformatics software were applied to reveal DEGs of TB samples.[16]

Availability of the Data

This dataset analyzed during the current study is available in the NCBI Gene Expression Omnibus (NCBI GEO) repository. The data set has the GEO accession number as (GSE19435) and can be publicly accessed through the following web link https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19435. NCBI GEO is an international public repository that archives and freely distributes microarray, next-generation sequencing, and other forms of high-throughput functional genomics data submitted by the research community.

Pre-processing of Retrieved Samples

Applying Benjamini and Hochberg's algorithm, pre-processing the retrieved raw dataset was performed. Adjusted p-value was determined, and log transformation was applied. Moreover, Limma precision weights and force normalization were applied to the dataset. The considerable level of cut-off was calibrated at 0.05.[17]

Extraction of DEGs

Extraction of DEGs from pre-processed dataset was performed depending on the variables with considerable *p*-values <0.05 and log fold change values >+1 for up-regulated and <-1 for down-regulated genes using GEO2R.[18] Extracted DEGs were visualized through the volcano and mean difference (MD) plot, highlighting significant differentially expressed genes at a default adjusted p-value cut-off of 0.05 (red=upregulated, blue=downregulated). Volcano plot and mean difference plot display log2fold change with statistical significance (-log10 p-value) and average log2 expression. Limma Package accessible in GEO2R was used to construct these plots.

Identification of Novel Biomarkers

To identify the novel biomarkers associated with tuberculosis, reported genes linked to TB were retrieved from Gene cards (https://www.genecards.org/)[19] and OMIM (https://www.omim.org/)[20] and compared with the extracted DEGs. Identified novel genes were further sorted based on the combined score and relatedness to other genes in the network. Furthermore, cytokine-related novel genes were sorted out. Venny2.1 was used to make comparisons between different groups and their visualization via a Venn diagram. Further, BioGRID v 4.4 https://thebiogrid.org/ was used to know the interactors of the selected biomarker.

Protein-Protein Interaction Analysis

STRING v 10.5 (https://www.string-db.org/),[21] an online server, was employed to screen the functional relationship among different proteins. For protein-protein interaction, STRING evaluates the combined scores between gene pairs. Thus, extracted DEG lists were analyzed by applying a combined score > 0.4 as one of the criteria for the evaluation. Further, Cytoscape v 3.2.1 (http://www.cytoscape.org),[22] a bioinformatics package for creating networks and sub-networks, was used to construct the network based on the combined score. The degree and edge betweenness option was implemented to develop networks and select hub nodes. Different colors and shapes were used in networks to differentiate between reported and novel DEGs.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway evaluation

The DEGs were further analyzed for gene ontology and pathway analysis. For this, DAVID (Database for Annotation, Visualization and Integrated Discovery) (https://david.abcc.ncifcrf.gov) server[23] includes merging functional annotation set using a comprehensive list of genes database, was used. DAVID builds a complete gene database with similar functions based on the hypergeometric distribution (Table 1).

Diagnostic accuracy of cytokine biomarkers- TRIP12 and POLR3A

To assess the diagnostic potential and precision of TRIP12 and POLR3A, we performed the Receiver-operating characteristic curves (ROC) analysis. An online tool for ROC Analysis (www.jrocfit.org.) was used.

RESULTS

Data Pre-processing

Normalization of DEGs of TB samples at different time points of anti-tuberculosis drug treatment (0 months, 2 months, and 12 months) through GEO2R was carried out. The box plot shows the distribution of the values of the selected samples (colored according to the groups). Values are median-centered in all samples, indicating normalized data (Figure 1A). Uniform Manifold Approximation and Projection (UMAP), a dimension reduction technique, shows the sample's distribution. The number of nearest neighbors (nbrs) considered for calculation is 8 (Figure 1B).

DEGs screening of TB

Screening of DEGs among three different groups of TB patients revealed significant enrichment of DEGs among TB at 0 months vs PTB at 2 months (Figure 2A, 2D), and TB at 0 months vs TB at 12 months (Figure 2B, 2E). However, no significant DEGs were enriched among PTB at 2 months vs TB at 12 months (Figure 2C, 2F). Further, DEGs screening among TB samples at a different time (0 months, 2 months, and 12 months) of treatment led to the identification of a total of 292 DEGs among PTB at 2 months and PTB at 12 months (Figure 3A) when compared with PTB at 0 months (control). Out of a total of 292 DEGs, only 210 DEGs with official gene symbols were found with 59 up-regulated and 151 down-regulated genes (Figure 3B). For extraction of DEGs, *p*-value < 0.05 and log2 FC > 1 were used as criteria.

Table 1: Gene Ontology Analysis for DEGs involved in PPI network.

Category	GO TERM	*p*- Value
GO_MF_FAT	GO:0047522~15-oxoprostaglandin 13-oxidase activity	1.512943
GO_MF_FAT	GO:0036132~13-prostaglandin reductase activity	1.512943
GO_MF_FAT	GO:0019899~enzyme binding	1.340952
GO_MF_FAT	GO:0005057~receptor signaling protein activity	1.178117
GO_MF_FAT	GO:0044822~poly(A) RNA binding	1.162663
GO_MF_FAT	GO:0051427~hormone receptor binding	1.152691
GO_MF_FAT	GO:0004702~receptor signaling protein serine/threonine kinase activity	1.143753
GO_MF_FAT	GO:0003676~nucleic acid binding	1.136598
GO_MF_FAT	GO:0004672~protein kinase activity	1.121377
GO_CC_FAT	GO:0005654~nucleoplasm	3.228188
GO_CC_FAT	GO:0005829~cytosol	1.84508
GO_CC_FAT	GO:0031967~organelle envelope	1.748839
GO_CC_FAT	GO:0031975~envelope	1.730477
GO_CC_FAT	GO:0034451~centriolar satellite	1.517698
GO_CC_FAT	GO:0033263~CORVET complex	1.287604
GO_CC_FAT	GO:0005798~Golgi-associated vesicle	1.230033
GO_BP_FAT	GO:0008219~cell death	3.410277
GO_BP_FAT	GO:0015031~protein transport	2.882265
GO_BP_FAT	GO:0006955~immune response	2.032978
GO_BP_FAT	GO:0045087~innate immune response	1.886771
GO_BP_FAT	GO:0001816~cytokine production	1.853086
GO_BP_FAT	GO:0006895~Golgi to endosome transport	1.708007
GO_BP_FAT	GO:0009617~response to bacterium	1.586507
GO_BP_FAT	GO:0050706~regulation of interleukin-1 beta secretion	1.444991
GO_BP_FAT	GO:0038093~Fc receptor signaling pathway	1.390727
GO_BP_FAT	GO:0007041~lysosomal transport	1.328386
GO_BP_FAT	GO:0002764~immune response-regulating signaling pathway	1.308027

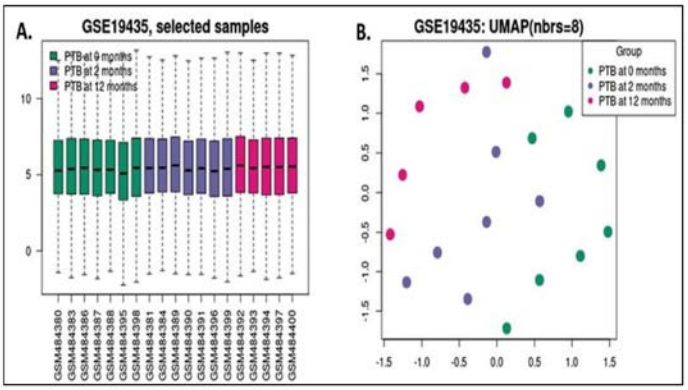

Figure 1: Microarray data normalization. 1A. The box plot shows that values are median-centred in all samples indicating that data are normalised for the selected samples. The lines in the box are coincident, indicating that these chips have been highly normalized. 1B. UMAP plot shows the pattern of data's distribution.

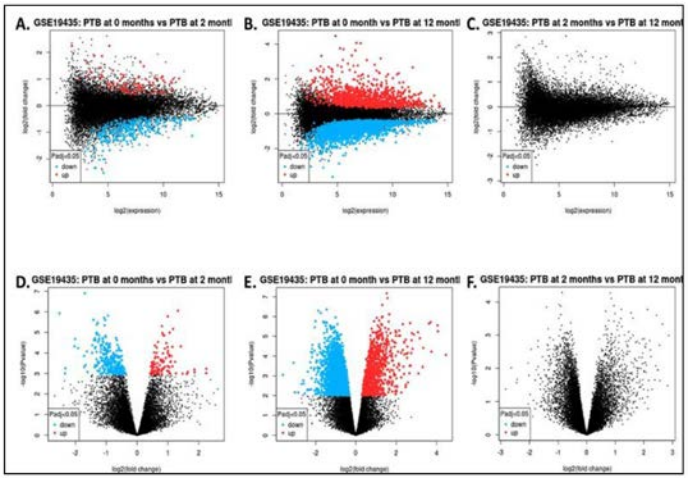

Figure 2: MD plot and Volcano for DEGs. MD plot and volcano plot was constructed for the DEGs in all three groups. 2A. MD plot for group PTB at 0 months and PTB at 2 months, 2B. MD plot for group PTB at 0 months and PTB at 12 months, 2C. MD plot for group PTB at 2 months and PTB at 12 months. Volcano plot is showing the significant DEGs between group 2D. PTB at 0 months and PTB at 2 months and 2E. PTB at 0 months and PTB at 12 months. Not any significant DEGs was enriched between groups PTB at 2 months and PTB at 12 months 2F. Blue dot represents significant down-regulated DEGs while red dots represent up-regulated DEGs.

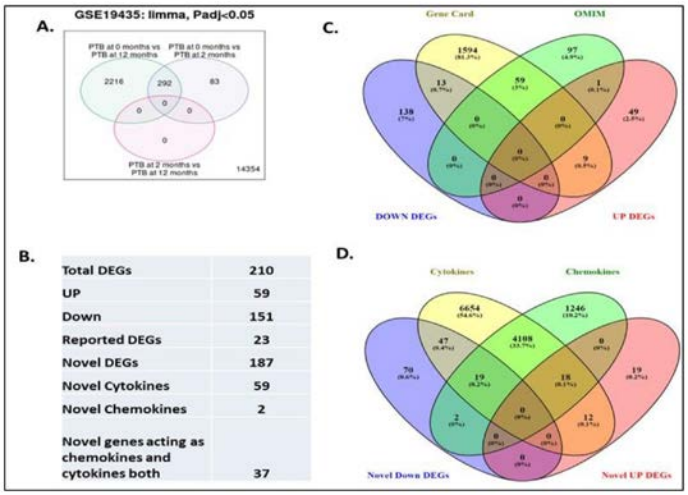

Figure 3: Venn diagram: Comparison of DEGs obtained at 2 months and 12 months resulted in a total of 292 common DEGs 3A. of which only 210 DEGs with official gene symbols were identified 3B. 3C. Further, comparison of DEGs with ALL related known genes obtained from Gene Cards and OMIM resulted in identification of 48 known DEGs while 185 novel genes.

Identification of Novel cytokine Biomarkers for Tuberculosis

Identifying novel cytokine biomarkers for different clinical pathologies and identification is of prime importance. To achieve this, comparisons were made between DEGs and the reported genes obtained from OMIM and Gene Cards database. Our results showed that out of 210 DEGs, around 23 DEGs were reported, while 187 DEGs were found to be novel DEGs related to TB. Of these 187 DEGs, 49 DEGs were upregulated, while 138 DEGs were down-regulated (Figure 3C). Furthermore, cytokines and chemokines were identified among these novel biomarkers (Figure 3D). These novel biomarkers were further enriched based on

networking which led to identifying some novel cytokine biomarkers forming hub nodes (following sub-section).

Principal Component and Hierarchical Clustering Analysis

Analysis of Principal Component (PC) for DEGs indicates the total variance of 80.6% (PC1) and 4.9% (PC2) between TB at 0 months vs TB at 2 months (Figure 4A) while 82.9% (PC1) and 3.3% (PC2) between TB at 0 months vs TB at 12 months (Figure 4B). Heat-map for DEGs was also constructed, which imparts a data matrix through different color patterns that disclose an outline of numerical variations (Figure 4C, 4D).

PPI Network Creation and Screening of TB Samples

Using the STRING database, a PPI network consisting of 526 nodes and 2708 edges was created for all DEGs (Figure 5A). A separate PPI for DEGs having a combined score >0.9 was constructed, which contains 121 nodes and 178 edges (Figure 5B). The red and blue color indicates the up-regulated and down-regulated genes of respective DEGs. Genes forming hub nodes were APP (Amyloid Beta Precursor Protein), TYROBP (Transmembrane Immune Signaling Adaptor), ANXA2 (Annexin A2), PSMB2 (Proteasome 20S Subunit Beta 2), and CD58 (CD58 Molecule) (up-regulated genes) and TRIP12 (Thyroid Hormone Receptor Interactor 12), POLR3A (RNA Polymerase III Subunit A), NFAT5 (Nuclear Factor of Activated T Cells 5), DDX17 (DEAD-Box Helicase 17), and TOP3A (DNA Topoisomerase III) (down-regulated genes). Since the direct relation of these hub nodes was found with known disease genes and being novel, they were considered as potential biomarker genes for TB. Based on the combined score and degree, 10 DEGs were retrieved as novel candidates. Further enrichment identifies POLR3A and TRIP12 as novel cytokine biomarkers for TB and MDR-TB. To create hub nodes, degree and edge betweenness criteria were applied. TRIP12 was found to interact with TP53. Although, TP53 is a tumor suppresser gene, recently it has been found to play a crucial role in immune responses and inflammatory diseases. On the other hand, POLR3A was found to interact with several others POLR genes like POLR2A/2C/2F/2G/2H/2L. All these POLR genes are involved in the positive regulation of interferon β and type I interferon (Supplementary

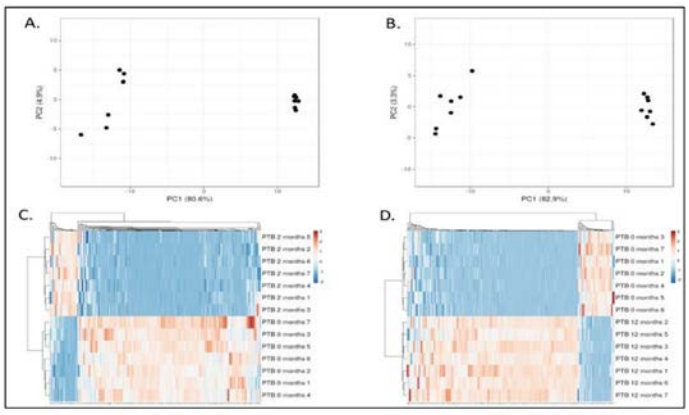

Figure 4: Principle Component Analysis (PCA) and heat map of differentially expressed genes. PCA for DEGs indicates total variance of 80.6% (PC1) and 4.9% (PC2) between TB at 0 months vs TB at 2 months (Figure 4A) while 82.9% (PC1) and 3.3% (PC2) between TB at 0 months vs TB at 12 months (Figure 4B). Heat-map for DEGs were also constructed which imparts a data matrix through different colour patterns that discloses an outline of numerical variations (Figure 4C, 4D). The blue to orange gradation represents the gene expression values change from small to large. ClustVis tool was used to draw heat map.

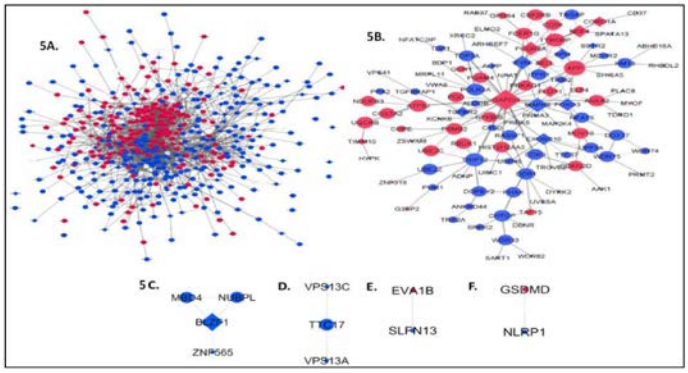

Figure 5: Protein-Protein interaction (PPI) of differentially expressed genes. PPI for all DEGs (5A) shows total 526 edges and 2708 nodes, a separate PPI network for DEGs having combined score >0.9 shows total 121 nodes and 178 edges (5B). Red Circle and Red Diamond represent up-regulated genes, while Blue Circle and Blue Diamond represent down-regulated genes. Lines are the correlation between genes. Thickness of lines (edges) is proportional to the combined score. Cytoscape v 3.2.1 was used to construct the network. Few DEGs were forming separate networks from main networks and were extracted separately (5C, 5D, 5E, 5F).

(Figure F1). Some of the DEGs formed four separate sub-networks extracted from the leading network. The first sub-network consists of 4 nodes and four edges (Figure 5C), the second sub-network consists of 3 nodes and two edges (Figure 5D), while the third and fourth sub-network consists of 2 nodes and one advantage (Figure 5E, 5F).

GO Enrichment and KEGG pathway construction

Functional enrichment for DEGs involved in the PPI network led to the identification of several major significant (p-value<0.05) biological processes, cellular components, and molecular functions. These DEGs were found to be regulating some major significant (p<0.05) biological processes like immune response, lysosomal transport, Fc receptor signaling pathway, regulation of interleukin-1β, response to the bacterium, Golgi endosomal transport, cytokine production, protein transport, and cell death (Figure 6A). Major significant functions carried out by these DEGs are protein kinase activity, hormone receptor binding, receptor signaling protein binding, prostaglandin reductase activity, and poly-A RNA binding. These DEGs were found to be localized in cytosol or nucleoplasm, organelle envelope, Golgi-associated vesicle, and centriolar satellite. FOXO Signaling pathway, oxidative phosphorylation, Epstein–Barr virus infection, Alzheimer's disease, osteoclast differentiation were the main pathways found enriched for these DEGs as identified through the KEGG pathway (Figure 6B).

TRIP12 has shown Better Diagnostic Accuracy Compared to POLR3A

In the ROC analysis, the "C index" (also called AUC-Area under the curve) for TRIP12 was highest, i.e., one, while lowest (0.5) for POLR3A. The biomarker with a larger AUC or "c index" has the best diagnostic accuracy. AUC for TRIP12 was larger than POLR3A and hence had better diagnostic accuracy than POLR3A (Figure 7).

DISCUSSION

Tuberculosis is a disease of poor prognosis, but with advancements in diagnosis and treatment, the condition is nowadays curable in maximum human populations. However, millions of individuals still suffer from TB, affecting people of any age group, but humans with weaker immune

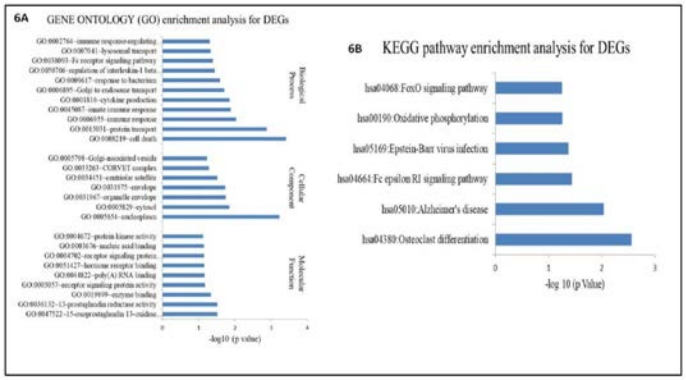

Figure 6: Gene Ontology and KEGG pathway analysis for DEGs in PPI network. 6A. Bar graph showing significant processes, function and cellular component enriched in TB patients for DEGs. DAVID v 6.7 was used for annotation. 6B. KEGG Pathway analysis for DEGs in TB Pathway enrichment for DEGs leads to identification of total 6 significant pathways. DAVID v 6.7 was used for annotation.

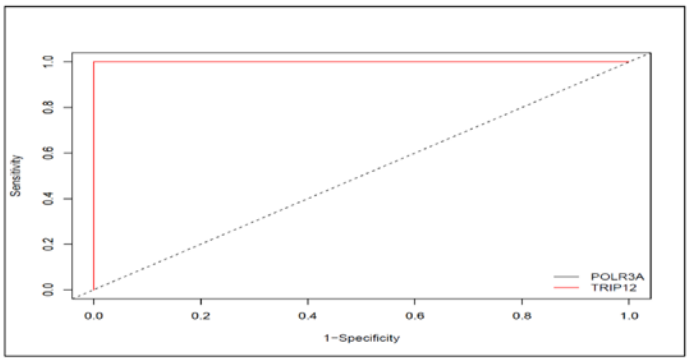

Figure 7: ROC Plot. ROC curves of TRIP12 and POLR3A for screening tuberculosis. Area under Curve (AUC) for TRIP12 is larger than AUC for POLR3A.

systems are more prone to risk.[24] Despite the significant progress in its treatment, TB is still a substantial challenge due to the emergence of new strains, specifically drug-resistant strains, that hinder tuberculosis management to a greater extent. Further, interplays between host immune response and *MTB* decide the disease's whole progression and severity. Several comprehensive studies showed that cytokines play a crucial role in mounting an immune response against mycobacterium.[25] Furthermore, initiation, progression, and combating TB is orchestrated by stimulation of a set of cytokines. Hence, the discovery of cytokine-based biomarkers is essential for the proper diagnosis and treatment of TB. Therefore, to find novel cytokine-based biomarkers related to tuberculosis, *in silico* studies were performed. The study revealed a total of 58 novel cytokines, of which 12 were up-regulated while 46 were downregulated. Based on networking, TRIP12 and POLR3A were considered novel cytokine biomarkers for tuberculosis. APP, TYROBP, ANXA2, PSMB2, CD58, NFAT5, DDX17, and TOP3A were other genes found to form hub nodes and can play an essential role in the pathophysiology of TB.

APP (Amyloid-beta precursor protein) are lipophilic metal chelators with metal-reducing activity. It encodes a neuronal protein that plays a vital role in the synaptogenesis process and axonogenesis. It has also been suggested to function as a receptor and growth factor and mediate cell-cell and cell-matrix interactions.[26] Further, APP is robustly expressed in monocytes and macrophages with increased expression or membrane localization during proinflammatory activation and a critical role in acquiring a reactive phenotype.[27-29] Additionally, APP and its proteolytic fragments have abilities to stimulate the macrophages. On the other hand, macrophages play an essential role in trapping, engulfing, and destroying the *MTB*.[30] Hence, all the above findings indicate a potentially important role of APP in tuberculosis. However, there is not any study regarding the role of APP in the context of TB.

TYROBP (TYRO protein tyrosine kinase-binding protein) is an adaptor protein that associates with the activating receptors found on various immune cells and thus mediates signaling and cell activation.[31] It encodes a transmembrane protein which leads to the activation of tyrosine kinases. TYROBP is required to activate neutrophils, monocytes [32], natural killer cells [31] and, myeloid cells.[33] Further, it associates with TREM2 on monocyte-derived dendritic cells and mediates the maturation and survival of dendritic cells.[34] All these immune cells have been shown to play a crucial role in controlling TB infection.[35-37] Thus, TYROBP is indirectly involved in maintaining the infection of TB via controlling the activation of various immune cells. Furthermore, several studies confirm the role of the TYROBP gene product in the pathophysiology of TB through network analysis.[38] Thus, our finding is consistent with the results of other reported works and establishes the role of the TYROBP gene in tuberculosis and thus making it a potential therapeutic target.

ANXA2 gene encodes annexin protein that plays an essential role in cellular growth, inflammatory responses, and different signaling pathways. It is one of the most studied members of the annexin superfamily and is produced by a wide variety of cells, including innate immune cells such as macrophages, monocytes and dendritic cells.[39] Several reports have suggested the protective role of ANXA2 during pathogenic infection.[39-41] Moreover, ANXA2 reduces the infection associated inflammation and promotes anti-inflammatory signals.[42] In an *in vivo* study, mice lacking AnxA2 showed a lower survival rate when infected with Cryptococcus, reflecting a dysregulated inflammatory response.[43]

On the contrary, ANXA2 has also been shown to facilitate productive infection.[44] In addition, ANXA2 facilitates the anchoring of P. aeruginosa (causes chronic pulmonary infection in patients with cystic fibrosis) on respiratory epithelial cells and its internalization followed by apoptotic cell death and release of pro-inflammatory cytokines.[42] Similarly, the EspL2 strain of *E. coli* manages to enter the host epithelial cells by reorganizing the cytoskeleton with the participation of ANXA2.[45] Thus, ANXA2 plays a dual role in inhibiting as well as promoting the infection. Although the role of the ANXA2 gene in tuberculosis is still unknown, its association with inflammation, as evident from the above report, may present it as a novel target candidate for tuberculosis.

PSMB2 (Proteasome subunit beta type-2) gene plays an essential role in proteasomal protein formation, which helps in processing MHC 1 peptides. One of the research works suggested through their meta-analysis studies that mutation in the PSMB2 gene may be considered one of the targets for TB.[46] Through their findings, F. Mrazek *et al*. disclosed that the PSMB2 gene is one of the targets for sarcoidosis.[47] As revealed through some reported work, the associative role of the PSMB2 gene in TB may provide a therapeutic target.

CD58 gene belongs to the immunoglobulin family that activates T-lymphocytes. Furthermore, it is reported that any specific mutation and deficiency in this gene leads to TB development,[48] thus validating its role in the onset of TB. W.A. Hanekom also found that dysfunctional CD58 affects dendritic cells in TB cases.[49] Hence, a mutation in the CD58 gene and its association with TB, as evident from various research works and our present study, reveals the CD58 gene as a potent marker for diagnosis and therapeutics of TB.

Another gene, TRIP12, encodes ubiquitin ligase protein, which plays a vital role in DNA damage response. Ubiquitylation and deubiquitylation have been shown to play an important role in the development and regulation of innate and adaptive immune responses. Several evidences support ubiquitin proteins' role in the orchestration of an immune response by ensuring the proper functioning of different cell types involved in the immune system. Following infection, the host innate immune system deploys a series of antimicrobial activities—such as autophagy, apoptosis, phagosomal maturation, and inflammatory signaling-all of which are fine-tuned by the ubiquitin system with the ultimate goal to destroy the invading pathogens and thereby protect the host.[50] TRIP12, being a ubiquitin ligase, can play a similar role in the development and regulation of the immune system. TRIP12 is an E3 enzyme of human ubiquitin fusion degradation (UFD) pathway having HECT (Homologous to the E6-AP Carboxyl Terminus) domain. This HECT domain of TRIP 12 acts as an E3 enzyme and catalyzes the ubiquitination of UFD substrates.

Interestingly increasing evidence has suggested the role of the HECT E3 domain in disease development.[51,52] Altered functioning of TRIP12 due to down-regulation or mutation may lead to disease development. Thus, it can be hypothesized that TRIP12 might be involved in the degradation of *MTB*, and its downregulation may cause the development of tuberculosis (Figure 8).

In the present study, TRIP12 expression is down-regulated, so *MTB* clinical features become more severe. On the other hand, the gene-gene interaction network (Figure F1) revealed the direct genetic and physical link of TRIP12 with p53 which has been shown to play an important role in chronic inflammation, viral infection, and innate immunity immune response. Thus, we hypothesized that TRIP12 coupled with TP53 might be involved in the regulation of immune signaling. Further, the involvement of the TRIP12 gene in acute myeloid leukemia (AML) patients has been experienced by RNA sequencing analysis.[53] In another study, it has been revealed that the frameshift mutation of the TRIP12 gene leads to colorectal and gastric carcinomas.[54] Our studies validate the link of TRIP12 with the onset of TB disease, and we further believe that our work will lead the way to devise effective treatments for TB possibly.

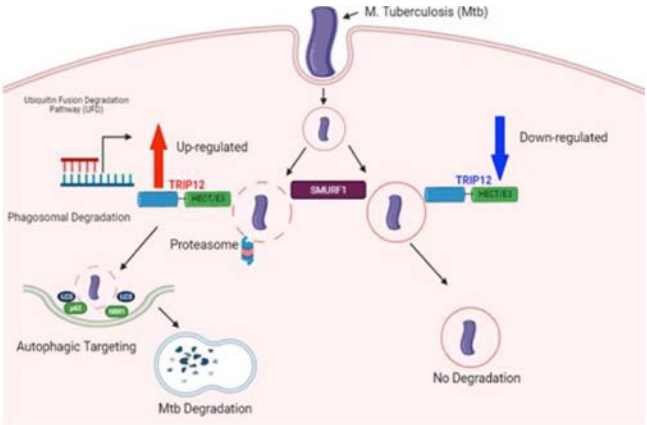

Figure 8: Proposed mechanism of TRIP12 action. TRIP12 belongs to E3 ubiqitine ligase family. SMURF1 is also a family member of E3 ubiqitine ligase family and has been found to directly involved in *MTB* degradation. TRIP12 contain a HECT domain which is E3 ubiquitine ligase, its function is largely unknown in mammals. Since TRIP12 and SMURF1 belongs to same family and have similar function hence it can be hypothesised that TRIP12 will also be involved in degradation of *MTB* via recruiting proteasome and several other downstream protein like LC3, p62 and NBR1.

The POLR3A is another class of genes that stimulates innate immune response and helps form RNA pol III; RNA pol III, in turn, serves as a viral DNA sensor in the natural immune system and induces interferon-1. Furthermore, a homozygous mutation in the POLR3 gene (which encodes a subunit of POL III) in children causes recurrent and systemic viral infections justifying its role in immune system.[55] This implies the non-canonical role of POLR3A gene as a cytokine and additionally it might be playing role in preventing the *MTB* infection. Interestingly this gene is downregulated in TB patient further reinforcing our idea of POLR3A as a cytokine. One study reports that a novel mutation in the POLR3A gene is responsible for leukodystrophy (degeneration of white matter in the brain).[56] In addition, another set of studies revealed the mutational role of POLR3A in leukodystrophy.[57] However, its role behind TB disease is still unclear. Still, as found in some studies and our findings, its response in immunity gains interest as becoming one of the targets for TB therapeutics.

NFAT5 (Nuclear factor of activated T-cells 5) is another critical gene that encodes a protein that plays an essential role in evoking immune response and plays a role by participating in transcription. S. Ranjbar *et al.* revealed the vital role of NFAT5 in toll-like receptor signaling, and its silencing leads to TB disease.[58] Moreover, N. Lee *et al.* also suggested the critical role of NFAT5 in evoking immune system response.[59] In addition, several studies provided the clue that NFAT5 gene mutation and TB disease are interlinked, which may represent a key target for diagnosis and treatment.

DDX17 (Probable ATP-dependent RNA helicase DDX17) gene is involved in cellular growth, embryogenesis, and cell division. Y. Xue *et al.* observed the mutational role of DDX17 in hepatocellular carcinoma.[60] Besides, S. Germann *et al.* kept an essential part of DDX17 in regulating NFAT5 gene function.[61] Since its role remains unclear in TB cases but its influential role with NFAT5 and its dysfunctioning suggested a direct link with TB infection, thus may provide a novel biomarker for TB.

TOP3A (DNA topoisomerase 3-alpha) gene encodes proteins involved in the DNA repair mechanism. Through their work, A. Seddak *et al.* reported the TOP3A gene duplication in TB infection.[62] K. Wu *et al.*, through their bioinformatics studies, suggested the involvement of the TOP3A gene in myocardial infarction.[63] Since some studies confirmed the role of the TOP3A gene in TB and hence it might serve as a marker for TB.

CONCLUSION

The daunting challenges posed by *MTB* due to its negating interactions with the immune system and its evasion mechanism needs clinical breakthroughs in its primary mechanisms of infection and pathogenesis to save the mortality of humans globally. In this context, scientists have relied mainly on reporting critical biomarkers to allow precise identification, quantitation, and pathogenesis of *MTB*. Our studies focused on the detailed investigation of biomarkers associated with the *MTB*. We concluded through our findings that APP, ANXA2, POLR3A, TRIP12, and DDX17 genes may act as novel candidates related to TB infection as they were found to play a vital role in immune response regulation, proteasomal degradation, and cytokine regulation. We identified TRIP12 as novel cytokine biomarkers for the prognosis of tuberculosis using a gene network approach. The present work discloses the ability of TRIP12 to directly target *MTB* leading to its degradation, a new outlook to find a full key behind the cause of TB infection occurrence, which leads to the progression of the disease. So, to validate the result, *in vitro* and *in vivo* studies will be carried out further.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

Author Contributions

Concept and Design of the study: MAM. Data curation: BAS, and BAB, Analysis of data: BAS, and BAB, Preparation of the manuscript: BAS, and BAB, Revision for important intellectual content: MAM, BAS, BAB and RAM, Supervision: MAM.

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ABBREVIATIONS

TB: Tuberculosis; ***MTB:*** *Mycobacterium tuberculosis;* **DEGs:** Differentially expressed genes; **KEGG:** Kyoto Encyclopedia of Genes and Genomes; **PTB:** Pulmonary tuberculosis**; TRIP12:** Thyroid Hormone Receptor Interactor 12; **POLR3A:** RNA Polymerase III Subunit A; **NFAT5:** Nuclear Factor of Activated T Cells 5; **DDX17**: DEAD-Box Helicase 17; **TOP3A**: DNA Topoisomerase III; **UMAP:** Uniform Manifold Approximation and Projection; **TLRs:** Toll-like receptors; **NOD:** Nucleotide Oligomerization Domain; **PRRs:** Pattern recognition receptors.

Availability of data and materials

The dataset analyzed in the present study is available in the Gene Expression Omnibus repository, https://www.ncbi.nlm.nih.gov/gds/?term=GSE19435, with accession number GSE19435.

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