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System biology study on MtrAB protein-protein interaction network of *Mycobacterium tuberculosis*

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ABSTRACT

Tuberculosis (TB) is the second largest infectious airborne disease that mostly affects the lungs and leads to damage to the respiratory system. There are mainly four factors susceptibility, exposure, infectiousness, and environment which determine the probability of transmission through *Mycobacterium tuberculosis* (MTB), a TB-causing pathogenic *bacteria*. To develop the antibiotic for the treatment of TB, two-component systems (TCSs) were mostly considered important targets. The mostly known TCS for MTB is MtrAB, which is present in all *mycobacterial* species. In this study, we applied network theory-based methods, using MtrAB protein-protein interactions, and identified potential drug targets for MTB. The constructed network showed hierarchical behavior having modules organization that performed a particular function. During our analysis, we found Rv1364c, regX3, gltB, dosT, and devS as five key regulators. The regX3 was found to be an important key regulator and showed interaction with other hubs. In addition, we also identified regX3, MtrA, MtrB, and Rv1364c formed four nodes motif in which regX3 regulates MtrA protein through MtrB and Rv1364c, which can be crucial for the network. The hub removal analysis showed that gltB is a key regulator mainly in communicating the signals. Furthermore, MtrA was not present in any of the identified modules, which suggested it is indirectly related to the modules function and also, possesses the potential to cross-talk between the modules through hubs. In the future, the development of a common drug target against regX3 and Rv1364c proteins could be a potential therapeutic cure for TB.

1. INTRODUCTION

The *Mycobacterium tuberculosis* (MTB) bacterium causes TB. Recently in 2020, 1.3 million people died from Tuberculosis (TB) disease. The effects of TB are observed in different age groups worldwide, mostly in young, adults, and people living in developing countries [1]. In past years, various antibiotics drugs utilized to treat, and fight against TB, but some strains have become resistant to drugs [2]. TB is challenging to diagnose using clinical, radiologic, bacteriological, and histological methods [3]. At present, the treatment of TB patients relies on clinical and laboratory evaluations, which have a number of limitations; the standardized methods do not account the individual variability in pathogenesis of MTB [4]. TB is treatable and curable but the fatality rate can be reduced only if we identify the potential drug targets where a bacterium is failed to develop resistance to the novel drug. The future treatment of TB patients may be improved using many systems biology domains. Next-generation sequencing and whole-genome sequencing methods provides a diagnostic role for treating patients with the disease [5,6]. Pharmacokinetic and pharmacodynamic techniques combined with systems biology have the potential to highlight the issues. Our understanding of the route of action of antibiotics is incorporated into systems pharmacology-based pharmacodynamic models to predict antibiotic effects on *bacteria,* for example, drug-target interactions [7]. High-throughput gene expression data made it possible to create large-scale gene regulatory networks. Gene regulatory networks provide as closer look to the clinical and medical application and can be seen as a bottleneck between the genotype and the phenotypes. Hence, it is important to understand the communication that occurs within MTB itself and with the host through molecular interaction. Two-component systems (TCSs) play an important role in *bacteria* which is ubiquitous and also important for cell signaling processes such as cell communications and cell adaptations [8]. The absence of TCS proteins in humans and other mammals' cellular systems makes them important drug targets potential. MTB has 11 pairs of TCS to perform its regular function and they are potentially attractive drug targets [9]. Understanding the pathways affected by these systems would unfurl effective measures for preventing MTB multiplications that ultimately

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result in pathogenesis. The MtrAB system is one of the two essential TCS in MTB, wherein MtrB is the sensor kinase, and MtrA is the cognate response regulator [10]. The MtrAB system is associated with replication, cell division, and cell wall formation. The MtrAB system is available in all *mycobacterial* species but is essential in MTB only for viability [10,11]. The virulence and infection mechanisms of MTB require signal transduction and one of the key biological processes is protein-protein interaction (PPI), which is a tool to analyze signal transduction.

The system biology approach using PPIs provided the key to understanding complex biological systems and helps to understand the importance of key regulators [12]. The network theory-based analysis helps to understand the topological properties of complex systems [13]. The modules in the hierarchical network are of particular interest because modules many correspond to independent functions [14,15]. The hub protein controls the stability of the network as well as modules [16-18]. Hence, the interaction between the hub and modules is crucial for network stability and communication. It was pertinent, therefore, to understand the communication within MTB and with its host based on MtrAB using a network biology approach.

2. METHODS

2.1. Construction and Statistical Analysis of MtrAB PPI Network

The PPIN of MtrA and MtrB genes was constructed using the STRING (Szklarczyk *et al*., 2019) database. The PPI information of MtrAB proteins in MTB was retrieved with the minimum required interaction score of 0.4. Here, the interaction contains both known and predicted PPIs, which are physical and functional interactions with confidence scores. These interactions are the bases to construct the network, so to obtain reliability; we used a medium confidence score. The duplicate interactions were removed from the interaction table and imported to the Cytoscape [19] to visualize the network.

2.2. Analysis of Statistical Parameters of MtrAB-PPI Network

To understand the topological behavior and importance of the key nodes, we used different statistical parameters such as degree distribution *(P(k))*, clustering coefficient C(*k*), neighborhood connectivity $C_N(k)$, and closeness centrality (*Cc*) using Network Analyzer a Cytoscape plugin [20].

Degree (K) and *(P(k))*.

Degree (K) denotes the number of interactions of a particular node with other nodes present in a network. The higher degree of nodes helps to identify the central nodes of the network which were further considered as hubs [21]. It can be determined by (Eq. 1).

$$P(k) = \frac{N_k}{N} \quad (1)$$

Where N_k denotes the total number of nodes having k degree whereas *N* denotes a node. The behavior of the networks can be categorized as random, small-world, scale-free, and hierarchical. In the small-world and random networks, the maximum number of nodes has a similar degree followed the poison distribution law, whereas, in scale-free networks, fewer nodes have a larger degree and followed power-law distribution $P(k) \sim k^{\gamma}$. The γ values define the network nature and mode of organization of nodes. (i) if γ is $2 \geq \gamma \leq 3$, larger degree nodes hold lesser degree nodes and the larger number of separated distributed nodes together, (ii) if $\gamma \leq 2$, showed the importance of consisted functional modules, a hierarchical nature, and (iii) if $\gamma > 3$ no relationship between the nodes losing scale-free topology [22].

Neighborhood connectivity $C_N(k)$.

Using the topological property $C_N(k)$, we can identify the relative mode of connectivity between interacting neighboring nodes and it can be determined (Eq. 2)

$$C_N(k) = \sum_q qP(q \mid k) \quad (2)$$

where $P(q \mid k)$ denotes the conditional probability to generate and create connections with nodes consisting of degree (k) to another node with degree q. [23]. For scale-free network topology, the value of $C_N(k)$ is constant, whereas $C_N(k)$ followed power law distribution, that is, $C_N(k) \sim k^{\beta}$, where, $\beta \sim 0.5$ in hierarchical behavior.

Clustering coefficient C(*k*).

It indicates the extent to which internal connections between a node's neighborhood can be formed, the strength of connections, and cluster organization. For any single node, it can be determined using the equation $C(k_i) = 2m_i/k_i(k_i\text{-}1)$ where m_i represent all of the links to its nearest neighbors. For scale-free and random networks, the value of C(*k*) is constant [24] means it is independent of k but in the case of a hierarchical network, it is dependent on a value of $C(k) \sim k^{-\alpha}$ where $\alpha \sim 1$ [25].

Closeness centrality (C_c).

It can be determined by calculating the "shortest path lengths" between nodes presented in a network. It is the reciprocal of farness and can be calculated by (Eq.3)

$$C_C(k) = \frac{N}{\sum_j d_{ij}} \quad (3)$$

where, d_{ij} is the sum of all the shortest distance between pair of nodes *i* and *j*, whereas N is the nodes present in the network. A node's (C_C) the value indicates how well it can transmit information throughout the network; a low value indicates its capacity to receive information faster.

2.3. Identification of the Nodes Highly Regulating mtrAB Network

According to the hypothesis, proteins with a large number of interacting partners in their network generally serve crucial roles in cells and showed their essentiality. In the constructed network, the proteins with high degrees interact with many other proteins and represented a key regulator of the network. In this study, we used a network analyzer [20] to identify the hub proteins' communication with many other significant proteins. Removal of hubs (higher degree proteins) may change the structural properties or stability in the network called the centrality-lethality rule [21]. In our analysis, we observed the network organization in the absence of higher degree nodes having large modules interactions one at a time, we considered the betweenness centrality (BC) parameter to re-analyze the rearranged network to measure regulating capacities of key nodes.

2.4. Module Construction and its Association with Hubs

To identify the number of clusters/modules (highly connected nodes or clusters of interactions) in the Mtrab PPIN, we used MCODE [26], a module prediction tool that identified the clusters consist of larger

interconnections between nodes. The algorithm uses a three-stage process: (i) Weighting: The nodes with the most linked neighbors receive a higher score. (ii) Molecular complex prediction: Recursively add nodes to the complex that are over a specified threshold, starting with the highest-weighted node (seed). (iii) Post-processing: Filters are applied to increase cluster quality (haircut and fluff). We used default parameter values of MCODE, node score cutoff (0.2), haircut, node density cutoff (0.1), K-score (2), maximum depth (100). To construct high-scoring modules in the network, we used the following default parameters of MCODE Cytoscape plugin. Here, we considered the top five modules that consist highest M-score. The identified hubs showed different interactions with a module, which further considered crucial nodes of the network.

2.5. Gene Ontology Enrichment Analysis of Modules

To understand the biological importance of functional modules, we annotated modules functions using the PANTHER tool. It combines gene-related information, such as gene function and cellular location to identify the gene annotation [27]. This tool provides a comprehensive set of efficient and concise annotations to the various genes and can categorize the terms as molecular functions, biological process (BP), and cellular components (CC).

3. RESULTS AND DISCUSSION

3.1. MtrA and MtrB Proteins in *M. tuberculosis* Strongly Regulated by Rv1364c

After the removal of duplicity in the genes, we constructed the two key proteins (*MtrA* and *MtrB*) in MTB, a based PPI network. Constructed MtrAB-PPIN consisted 909 edges (connections) in between 94 nodes (proteins) [Figure 1]. We found five sparsely distributed nodes namely, Rv1364c, regX3, devS, dosT, and gltB showed a high strength to attract a large number of nodes consists a lesser degree. These five proteins showed a high perturbation degree. Since the network is based on MtrA and MtrB interacting proteins, we found a degree of 73 and 51 for MtrB and MtrA, respectively. The key hub protein apart from these two (MtrA and MtrB) proteins has a degree of 48, 38, 37, 37, and 37 for Rv1364c, regX3, devS, dosT, and gltB, respectively. All five hub proteins were interacting with MtrB, but four of the hub proteins interact with MtrA except regX3.

3.2. Statistical and Topological Behavior of the Network

Based on a statistical analysis of the MtrAB-PPIN, we found network followed a power law distribution with degree means fewer nodes having a higher degree but a larger number of nodes having a lesser degree. The negative exponential value in *(P(k))* ([] = −0.35, correlation coefficient (r) = 0.37) provides a hierarchical scale-free (presence of modules and sparsely distributed hubs) nature to the network [Figure 2a] [28]. Both C_N (k) and C(k) resulted a negative exponent value ($\beta = 53.86 \pm 0.23$) and ($\alpha = 0.96 \pm 0.26$); with their corresponding r values (r = 0.76) and (r = 0.65) and followed power law distribution [Figure 2b and c]. The exponential values depicted that MtrAB-PPIN consisted of a hierarchical scale-free nature. The MtrAB-PPIN consisted of a disassortative behavior means that hubs play important role in network stability. To, recognize the signal processing strength of the hubs we included closeness centrality (Cc) topological parameter which showed that positive exponents value ($\eta = +0.28$) with corresponding (r = 0.90) indicated the leading hubs controlling the network stability with a higher strength [Figure 2d]. The topological and centrality parameters resulted from MtrAB-PPIN following a scale-free nature with a system level of organization (hierarchical scale-free).

3.3. Nodes Highly Regulating MtrAB Network

To identify the important genes in the network, we performed an independent hub-removal (gene knockout) experiment. Removal

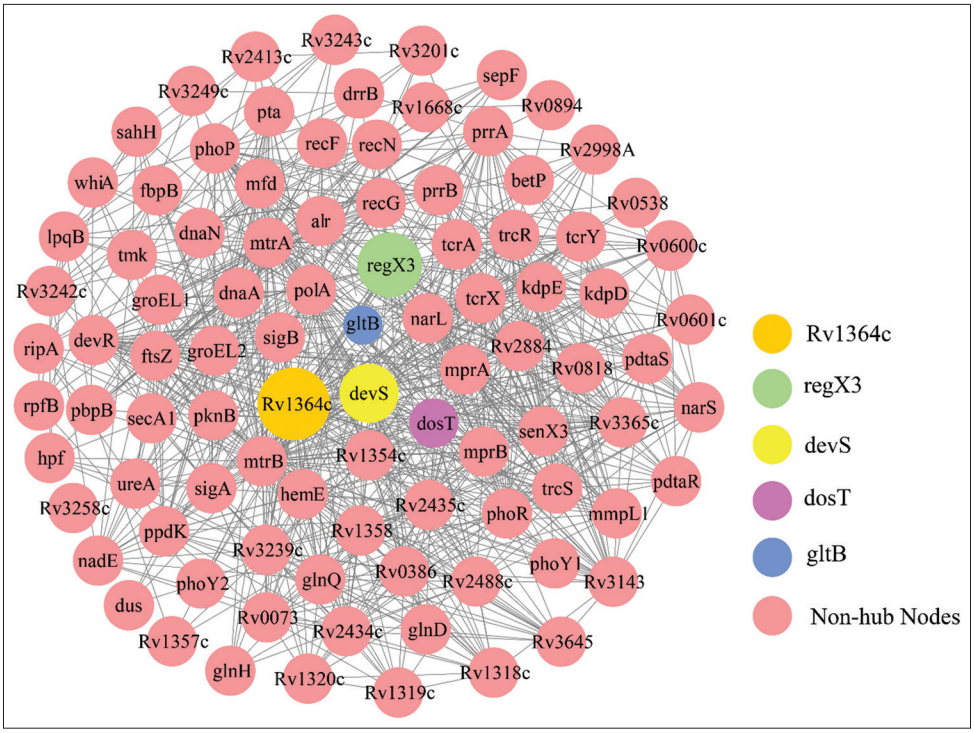

Figure 1: The hub in the protein-protein interaction of the MtrAB network. Expanded view of the network imported from Cytoscape, where nodes represent proteins and edge the physical interaction. The hub nodes circle size showed according to the degree (larger to smaller).

of crucial hubs may change the network properties referred to as the centrality-lethality rule [21]. In a hierarchical network, the hubs protein is less important as compared to the functional modules of the network. When it comes to network control, hubs are not as crucial as modules but may involve in the regulatory mechanisms of the network. In general, to understand the importance of a hub node remove the node from the network and then re-analyze the network's topological properties [29]. Here, we considered the BC parameter which may show a significant change. We removed all 5 hubs once at a time and re-analyzed them by deleting them from the complete PPI network. We found one hub gltB showed significant changes in BC that could increase signal propagation capacity in regulating mtrAB [Table 1]. The removal of the other four hubs (Rv1364c, regX3, dosT, and devS showed a same pattern of the regulation [Figure 3]. Due to the presence of modules that provided hierarchical nature to the network and on hub removal the network stability is still maintained. Most likely, the network's crosstalk between these hub proteins and functional modules aims to preserve the network's structural characteristics.

3.4. The Modular Structure of the MtrAB Network

The presence of modules in the network provides statistical and functional significance to interacting clusters of nodes, which leads to the formation of a functional community in the network. We identified five such significant modules in the MtrAB network. Here, we identified the top five modules based on the MCODE score (M-score). The module-1 consisted 16 nodes, 62 edges with a M-scoring of 8.26 [Figure 4a]. The module-2, 3, 4, and 5 had 23 nodes (scoring value 7.90) [Figure 4b], eight nodes (scoring value 4.28) [Figure 4c], five nodes (scoring value 3) [Figure 4d], ten nodes (scoring value 2.88) [Figure 4e] with the corresponding edges of 87, 15, 6, and 13, respectively. Among five significant hubs, three hubs present in module-3 and two hubs present in module-2 showed that the majority of the notably large hubs not only controlled their module but also influenced other modules to control network regulation. MtrB protein is present in module-1, and MtrA was not observed in all five modules, which suggested it is indirectly related to modular functionality; with a chance to cross-talk between the module through this protein. We also found modules were linked with sparsely distributed nodes, which provides the possibility of module cross-talk. We identified that these modules communicated with one another through the five most important hubs in the network.

Table 1: Average betweenness centrality values of complete MtrAB-PPIN and after the removal of hubs.

Name of hub-removed	Average betweenness centrality (BC)
Complete network	0.009258154
devS	0.009429794
dosT	0.009419519
gltb*	0.87840418
reg×3	0.009468325
Rv1364c	0.009465756

"*" hub showed significant changes in betweenness centrality.

3.5. Hubs and Modules Interaction in MtrAB Network Proven Rv1364c and regX3 are Crucial Proteins

The hub protein can directly cross-talk with the nodes present in modules to control the functionality of modules. Rv1364c hub protein showed the maximum interacting strength with five modules, followed by gltB, dosT, devS, and regX3 suggested that hubs were the key mediators of modules [Table 2 and Figure 5]. However, Rv1364c protein is not connected with any of the proteins in module-4. The Rv1364c hub protein interacted with most of the proteins in module-1, 2, and 3. It was identified that increased transcription of a potential sigma factor regulatory gene, Rv1364c, occurs, in *Mycobacterium bovis* (BCG) consequent to phagocytosis by macrophage [30]. The regX3 showed the fewer interactions with five modules compared to other hubs in the network, but they have a high number of interactions with module-5 compared to other hub proteins. Activated RegX3 limits persister

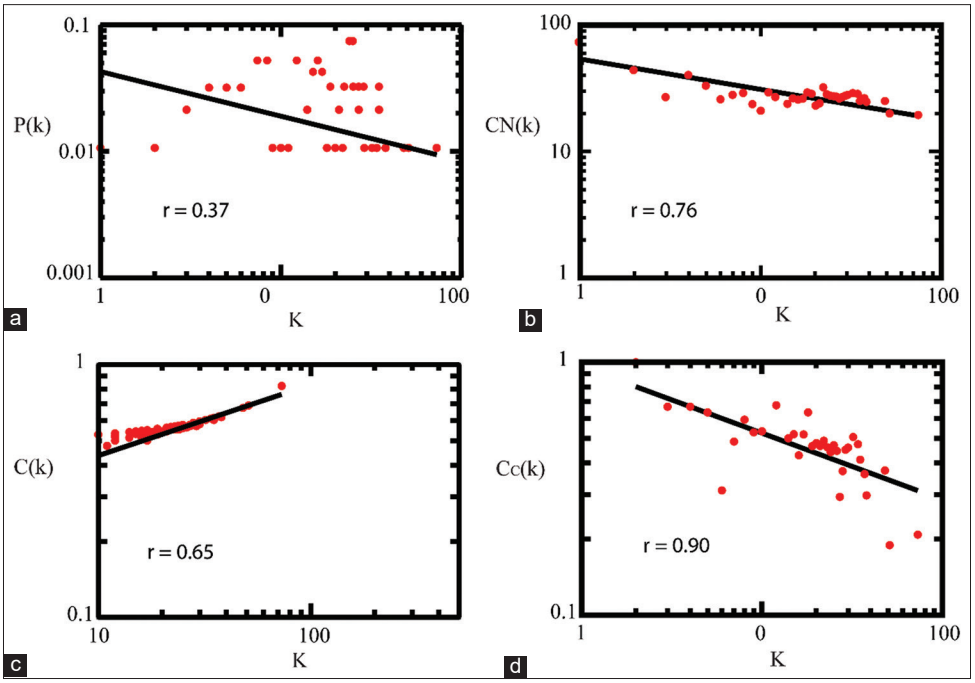

Figure 2: The topological and centrality properties of the network represented with correlation coefficient values (r). (a) probability of degree distribution *P(k),* (b) average neighborhood connectivity (C_N(k)), (c) average clustering coefficient C(*k*), (d) closeness centrality (C_C). All these properties follow the power law scale.

formation during growth under phosphate-limiting conditions [31]. Out of five hubs, two hub proteins did not connect with module-4 and the other three have only one connection each, here MtrB protein interacts with all the proteins in module-4. MtrA is not present in any of the modules, they interact with all the modules with a total connection of 31 [Figure 5]. The increased binding of acetylated MtrA to MtrB is also consistent with MtrA's repressor action, which also keeps the protein together and available for prompt switching if necessary [32]. We found that Rv1364c regulates one cluster of nodes (module1, 2, and 3), and regX3 hub along with MtrA and MtrB protein regulated module-4 and modules-5. The relationship between hubs and modules suggested that hubs also have capabilities to regulate the functionality of modules, stability, and signal processing in the network. The identified five modules observed in the network provided the hierarchical nature and hub interacting proteins were highlighted. It was clear that these five modules served as the MtrAB network's primary controller and regulator. The important two hubs (Rv1364c and regX3) in MtrAB network play significant roles through their interaction with two clusters of modules and these two hubs could preserve the stability of the network, information processing. All the five hubs, including MtrB protein present in module-1, 2, and 3 were identified as the most influencing nodes having stronger cross-talk in between the modules that interact with it. MtrA protein, absent in all five modules, but provide a link to cross-talk among the modules and their functions.

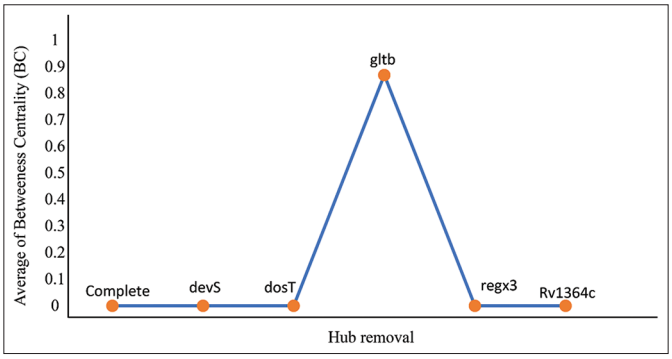

Figure 3: The representation of the network property comparison of average betweenness centrality (BC) of complete MtrAB-PPIN and after the removal of hubs.

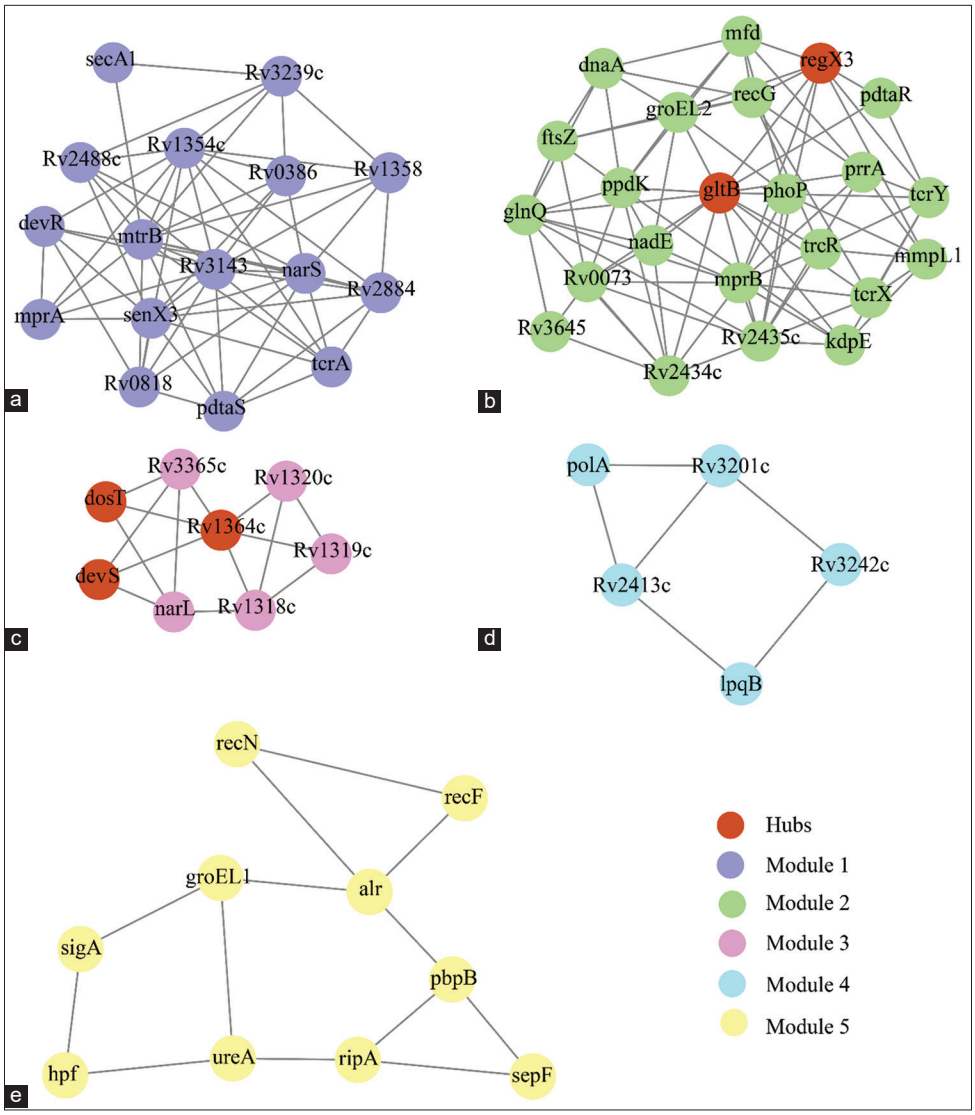

Figure 4: Structure of the modules in the MtrAB network. MCODE tool used to construct the modules. In the modules 1-5 (a-e) all the nodes are in filled circles with the corresponding edges in lines.

Table 2: The total number of nodes and the number of interactions in modules with five hubs.

Hub interaction networks	Module-1	Module-2	Module-3	Module-4	Module-5	Total
devS	12	11	3	1	1	28
dosT	13	11	3	1	1	29
gltB	11	15	1	0	3	30
regX3	8	9	2	1	4	24
Rv1364c	12	17	7	0	2	38
Number of nodes	16	23	8	5	10	62

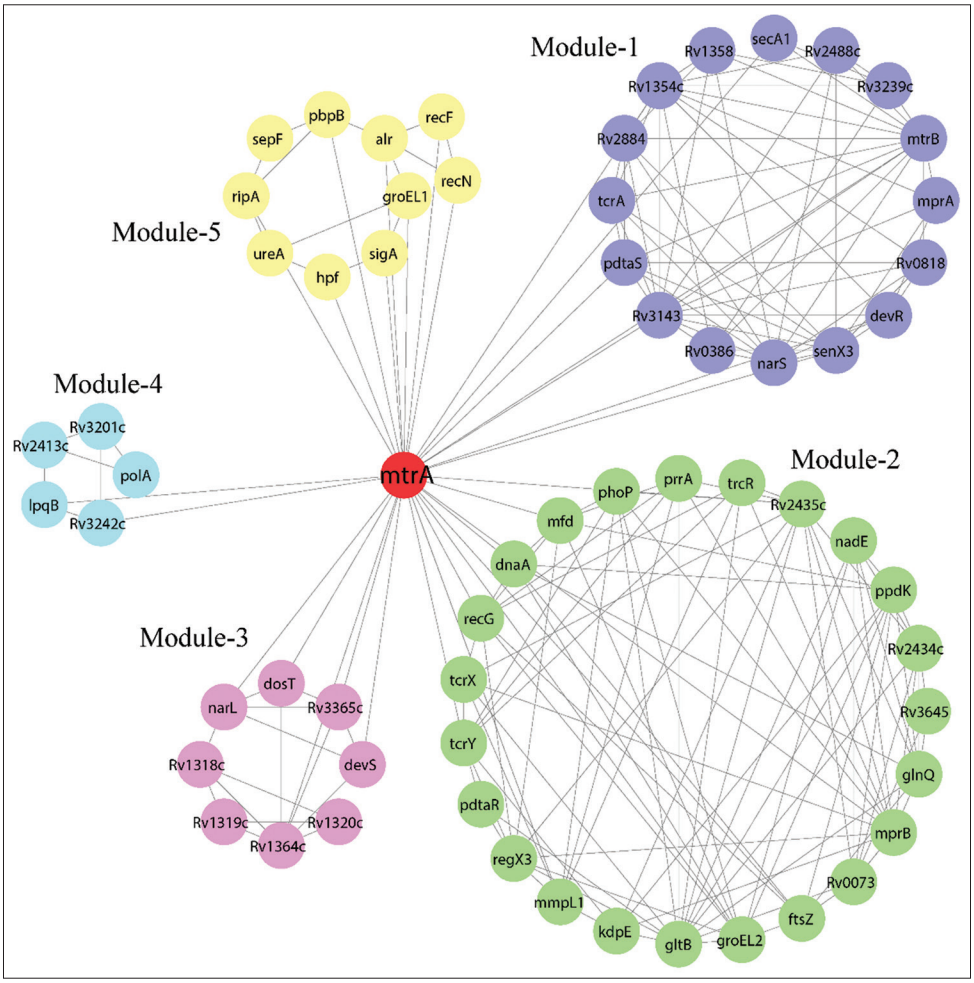

Figure 5: Cross-talk among the five modules and MtrA hub in the network. MtrA protein interacts with all the modules and hub-interacting nodes were connected via its edges.

3.6. Modules Functional Enrichment

We identified that module-1, 2, 3, 4, and 5 were functionally associated with catalytic activity [Figure 6]. Module-4 proteins showed 100% of functional enrichment with catalytic activity. Module-1, 2, and 3 also showed enrichment with molecular transducer activity. Apart from those modules 1 and 2 were functionally related to binding activities. Module-5 showed maximum enrichment of binding function as compared to other modules. All five modules are associated with biological cellular and metabolic processes but mostly with cellular processes. These proteins are mostly found to be localized in a protein complex, cellular anatomical entity, and intracellular. The proteins of module-4 showed 100% ofenrichment as cellular anatomical entities. Module-1 and module-3 proteins showed 50% of enrichment as cellular and another 50% as metabolic processes. Module-2, 4, and 5 also showed an association with response to stimuli BP 6.3%, 4.3%, and 18.2%, respectively [Figure 6].

4. DISCUSSION

Network system biology approach helps us to understand the disease biology, such disease pathogenesis, identification of potential biomarkers, drug targets, drug dosages, designing of therapeutic interventions, and synergism discovery [33]. A rapidly expanding area of computational and quantitative research, systems biology integrates a number of disciplines, including microbiome profiling,

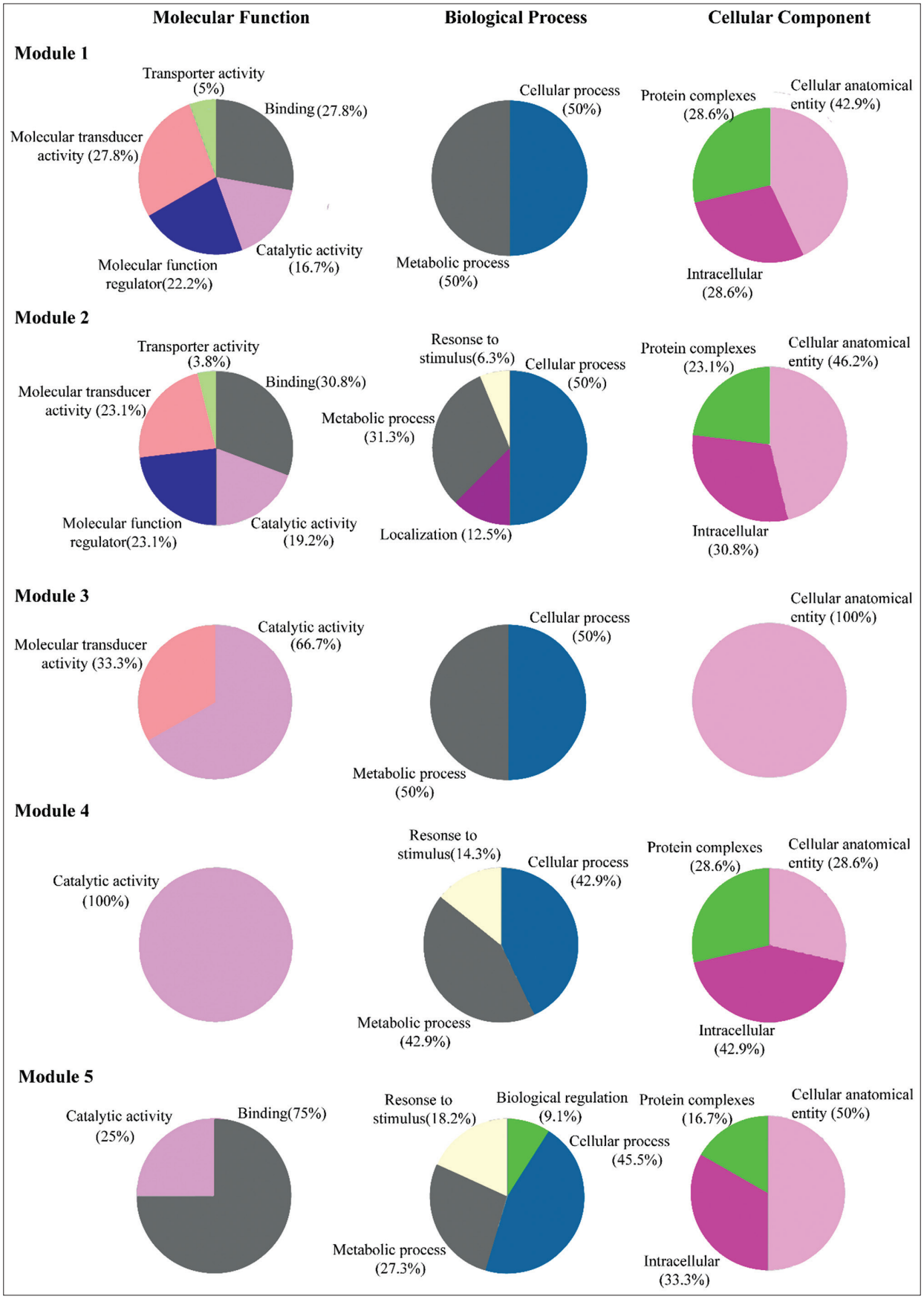

Figure 6: Functional annotation of five modules. The enrichment analysis in terms of molecular function, biological process, and cellular component shown with percentage represent the function hits of genes.

transcriptomics, proteomics, metabolomics, and genomics. In recent years, systems biology studies have been increasingly implemented in TB research to identify biomarkers and key targets that can showed changes in pathogen viability or its susceptibility to antituberculosis drugs, elucidate host signaling in response to the pathogen, and predict treatment response [4]. Network based systems biology study revealed diseasome and comorbidity associations of systemic sclerosis with different type of cancers [34]. Recent study on congenital heart diseases (CHDs) network regulating heart development and observed that a sub-network also regulates fetal brain development, thereby providing mechanistic insights into the clinical comorbidities between CHDs and neurodevelopmental conditions [35].

In this study, to unravel the signaling challenges associated with MTB, we applied a systems biology approach. Here, we analyzed the constructed PPI network of 94 proteins based on two key proteins (MtrA and MtrB) in MTB. The network analysis results suggested that Rv1364c and regX3 functions as key regulators of the MtrAB network, and both were regulating two modules observed in the network. Out of five key hubs of the network; Rv1364c was found as a major hub. The hub-module interactions showed Rv1364c and regX3 as key proteins, which regulated the network. Among the five hubs, regX3 is the only protein that interacts with other hubs. The connection among Rv1364c, regX3, and MtrB could be a critical three-node motif of the network.

5. CONCLUSION

The modeling and analysis of molecular interaction networks (MINs) could represent valuable resources in defining associated pathways and discovering novel therapeutic targets. Within living organisms, a single protein or other biomolecules will rarely act alone to effect a given function. Instead, there is a complex series of interactions between multiple biomolecules that contributes to a BP. Studying the structure and topology of MINs can help to identify biomolecules that are involved in biological processes and elucidate which biomolecules or processes are dysfunctional in disease.

PPINs have been developed for many different organisms, such as bacteriophages, yeast, *bacteria*, plants, animals, and human [36]. Recent developments in biomedical research have benefited greatly from PPIN and its uses because it is a useful index to measure their centrality and offer a benchmark for proteins as potential therapeutic targets. Therefore, PPINs become an effective method to understand the complex disease systems like cancer [37], multiple sclerosis [38], and Alzheimer's disease [39]. A basic aspect of network biology is that proteins implicated in the same disease have a propensity to interact with one another and form disease modules.

To comprehend the etiology of diseases and explain the penetrance and expressivity, it is helpful to study disease modules and provide an idea to the drug target of gene therapy and determines or specifies new disease and drug-protein targets.

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7. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

All the data is available with the authors and shall be provided upon request.

12. PUBLISHER'S NOTE

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