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Gene Expression Profiling of Tuberculous Meningitis

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Abstract

Tuberculous meningitis (TBM) is a form of extra pulmonary tuberculosis that is associated with severe neurological deficits and a high mortality. Early diagnosis of TBM is a major challenge despite the availability of several diagnostic methods. Existing diagnostic methods and markers are inadequate for early diagnosis of TBM owing to poor specificity and sensitivity. DNA microarray technology permits high-throughput identification of differentially expressed genes. In order to identify molecules as candidate biomarkers for early diagnosis or as therapeutic targets in TBM, we carried out transcriptomic analysis of brain tissue using whole human genome oligonucleotide arrays. From this gene expression analysis, we identified 2,434 genes that were differentially expressed at least two-fold in TBM cases as compared to controls. The large majority of the differentially expressed genes encoded proteins that are involved in metabolism, cell growth, transport, immune response, cell communication and signal transduction. We confirmed the upregulation of two molecules, serpin peptidase inhibitor, clade A member 3 (*SERPINA3)* and glial fibrillary acidic protein (*GFAP*), at the protein level by immunohistochemical analysis. The findings from our study should help us understand the molecular mechanisms underlying TBM and to develop better diagnostic and therapeutic strategies against this deadly disease.

Keywords: DNA microarrays; Biomarkers; Early diagnosis; Therapeutic target

Introduction

Although the causative organism of tuberculosis was discovered over a hundred years ago, this disease still remains a major public health problem worldwide. Tuberculosis primarily affects the lungs but can spread hematogenously to extra pulmonary sites such as lymph nodes, bones, meninges and genito-urinary-tract [1]. One of the most frequent sites of extra pulmonary disease is tuberculous meningitis (TBM), which is a common form of central nervous system tuberculosis with high morbidity and mortality [2-4]. The incidence of TBM is on the rise with the increase in immunodeficient states such as HIV/AIDS [5]. Concomitant with an increase in the incidence of TBM, development of multi-drug resistance in AIDS patients is a major obstacle associated with its treatment [6].

The diagnosis of TBM continues to be a challenge because the gold standard for diagnosis requires that *Mycobacterium tuberculosis* (*M.tb*) be demonstrated by culture of the cerebrospinal fluid (CSF) of suspected patients. This is a time consuming process which takes approximately 8 weeks [7]. Over the past several years, different molecular and biochemical assays have been developed for rapid diagnosis of *M.tb*. PCR based assays for detection of the pathogen, ELISA to detect *M.tb* protein antigens or host antibodies directed against *M.tb* are the most widely used assays for detection of *M.tb* [8]. A combined approach of detecting IFN-gamma levels by radioimmunoassay and use of IS6110 primer to detect *M.tb* by PCR shows a reasonably high sensitivity (80%) and specificity (92.6%), in the CSF of TBM patients [9]. Detection of anti-mycobacterial antibodies and mycobacterial immune complexes (IgG) have also been employed for diagnosis with variable sensitivity and specificity [10]. Other alternatives include demonstration of high lactate levels [11,12] and adenosine deaminase (*ADA*) in CSF for quick diagnosis and management of TBM [13]. However, the above described methods are still limited in their sensitivity and specificity in the clinical setting. As the mortality rate of TBM remains high [14,15], there is a critical need for identification of appropriate biomarkers for early diagnosis of TBM.

Gene expression profiling studies have previously been performed on CSF or blood of patients with TBM [16,17]. In the present study, we used whole genome DNA microarrays for investigating changes at the transcriptome level in infected brain tissues from TBM cases that were confirmed by autopsy as compared to uninfected brain tissues from controls. Further, we performed immunohistochemical validation of some of the differentially expressed genes identified from

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our microarray studies. Our results confirm that proteins encoded by *SERPINA3* and *GFAP* genes are indeed upregulated in TBM patients. Further studies to detect proteins encoded by these genes in the CSF as potential biomarkers in CSF could potentially lead to improved methods for diagnosing TBM.

Materials and Methods

Tissue samples

Human brain tissue samples from five cases of TBM (confirmed by detection of mycobacterial antibody/immune complexes in cerebrospinal fluid, and/or demonstration of acid fast bacilli by Ziehl Neilson's stain in the smear from basal exudates in meninges and histopathological features of granulomatous or chronic meningitis) and four control brains that were archived as frozen and formalin fixed specimens at Human Brain Tissue Repository (Human Brain Bank) in the Department of Neuropathology at National Institute of Mental Health and Neuro Sciences (NIMHANS), Bangalore, India, were used for the microarray experiments. The details of samples used are provided in the Supplementary Table 1.

The brains were collected at autopsy with written informed consent from close relatives to utilize them for research purposes. The study was approved by the Institutional Ethics Committee of NIMHANS. The dead bodies were shifted to 4°C within one hour of death. Tissues from frontal cortex (2x2 cm) were frozen at -86°C and preserved until analysis. The rest of the brain was fixed in 10% buffered formalin for 12–18 weeks. Representative tissue blocks were processed for histological evaluation. The postmortem interval (interval from the time of death to time the tissue was transferred to -86°C) varied from 6 hrs-13½ hrs for control cases and 1 hr 15 minutes–18 hrs for TBM cases. Frozen brain tissue samples with overlying meninges from the five cases and four controls were excised as small pieces, transferred to RNA Later (Ambion Inc Austin Tx) and incubated at 4°C for 12–16 hours to facilitate proper penetration into the tissues and stored at -86°C until further use.

RNA isolation

Approximately 100 mg of tissue from normal and infected brains was used for RNA isolation. The tissues were pulverized in QIAzol lysis reagent (Qiagen, Valencia, CA) using a homogenizer. Total RNA extraction and purification was carried out using RNeasy lipid tissue mini kit (QIAGEN, Valencia, CA) as per the manufacturer's protocol. The yield and quality of isolated total RNA was checked using the NanoDrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). Integrity of the isolated RNA was assessed by RNA gel and/or 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The samples were further processed based on RNA integrity.

cDNA synthesis, labeling and hybridization

Total RNA (600 ng) from each sample was reverse transcribed and linear amplified using Quick Amp Kit, One-color (Agilent Technologies, Palo Alto, CA) that employ OligodT-T7 promoter primers. The cDNA generated was used as template for *in vitro* transcription reaction with Cy3-CTP and RNA polymerase; thus cRNA was simultaneously synthesized and labeled. The labeled cRNA was purified using RNeasy spin columns (Qiagen, Valencia, CA). The samples with specific activity >9 pmol Cy3 per µg and yield >1600µg were selected for hybridization. Cy-3 labeled cRNA was fragmented and hybridized onto oligonucleotide-based whole human genome DNA microarrays (G4112F, 4x44 K, Agilent Technologies, Palo Alto, CA) for 16 hours at 65°C. The arrays were subsequently washed with gene expression wash buffers according to the manufacturer's hybridization protocol (Agilent Oligo Microarray Kit, Agilent Technologies).

Scanning and data analysis

The slides were scanned with Agilent microarray scanner (G2505B) using one color scan setting for 4x44 K array slides (scan resolution 5µm, dye channel was set to green, green PMT was set to 100%) and the images processed with Agilent's feature extraction software (9.5.3.1) to obtain the raw data files for further analysis. The raw data from microarray experiments were submitted to the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo accession#GSE23074). GeneSpring GX v11.0.2 (Agilent Technologies, Santa Clara) software was used to analyze the gene expression profiles. Raw data was imported into the GeneSpring GX software. The recommended quantile normalization without baseline transformation and t-test were applied. To determine the differentially expressed genes that were statistically significant, a p-value of <0.05 and a fold-change cut-off threshold of ≥2 were used. GO analysis was carried out for the differentially expressed genes using GeneSpring Gx software. A p-value threshold of 0.1 was used to filter out the significantly overrepresented GO categories.

Biological network analysis

Pathway analysis was carried out using Genespring GX v.11.0.2. Differentially expressed genes obtained after filtering based on fold-change cut off (≥4.0) was taken as input and biological networks were generated by comparing the input list to a reference list containing >1.4 million reactions generated by natural language processing algorithm and from different interaction databases. To obtain high confidence networks, analysis was carried out using filters that included binding, expression, metabolism, transport, promoter binding and regulation of the molecules. The number of molecules per network was restricted to 50. The entities which do not have connections were removed. The constructed network was overlaid with final input list to visualize differentially expressed genes.

Immunohistochemical analysis

For validation of the upregulated molecules in cases of TBM, immunohistochemical labeling was carried out using commercially available monoclonal antibodies directed against *GFAP* (Biogenex, Houston, Texas, USA) at 1:200 dilution and against *SERPINA3* (Sigma Aldrich, St. Louis, MO) at 1:100 dilution. HRP tagged secondary antibody provided with Envision kit (DAKO-K4011 and K4007, DAKO, Carpinteria, CA) was used and the immune reaction visualized with DAB/H_2O_2 as the chromogen. The immunohistochemical validation was carried out on an independent subset of 15 formalin fixed human brains from confirmed cases of TBM (Age range 1–55 years, postmortem interval 1 hour–18 hours) including the five cases used for gene expression profile and four controls. These cases were processed for paraffin embedding and histological evaluation to establish presence of TBM.

Briefly, 4µm thick serial sections from controls and TBM cases were collected on silane coated slides. The paraffin sections were deparaffinized and dehydrated. After stabilizing in PBS at room temperature, the endogenous peroxidase activity was quenched by blocking solution for 20 min at room temperature. Antigen retrieval was carried out in citrate buffer (pH 6.0) by microwaving the sections for 30 min. After blocking the non-specific binding sites in 3% non-fat dry milk powder for 15 min at room temperature, the serial sections were incubated with primary antibodies against *GFAP* and *SERPINA3*

overnight at 4°C. The sections were washed thrice in PBS and incubated with the appropriate secondary antibodies conjugated with HRP for 30 min at room temperature. The signal was developed with DAKO substrate buffer and chromogen. The sections were counterstained with hematoxylin and mounted. The immunolabeled sections were examined independently by two experienced neuropathologists (AM and SKS). The staining pattern, intensity and subcellular localization were visually scored.

Results and Discussion

Gene expression profiling using DNA microarrays was carried out to identify differential gene expression in TBM samples. The workflow adopted in this study is illustrated in Figure 1. We identified 2,434 genes that were differentially expressed with the p-value <0.05 and ≥2 fold change. A complete list of these genes is provided in Supplementary Table 2. A partial list of differentially expressed genes is shown in Table 1 along with protein associated information including protein domains, subcellular localization, biological processes and molecular function. For hierarchical clustering, we applied euclidean distance metric and centroid linkage as parameters. The heat map for differentially expressed genes is shown in Figure 2. Gene Ontology based classification of differentially expressed genes were carried out by using GeneSpring Gx gene expression analysis software. Significantly overrepresented GO categories are provided in Supplementary Table 3. Genes corresponding to immune response, integral to membrane, immune system process, intrinsic to membrane, antigen processing and presentation of peptide or polysaccharide antigen via MHC class II were

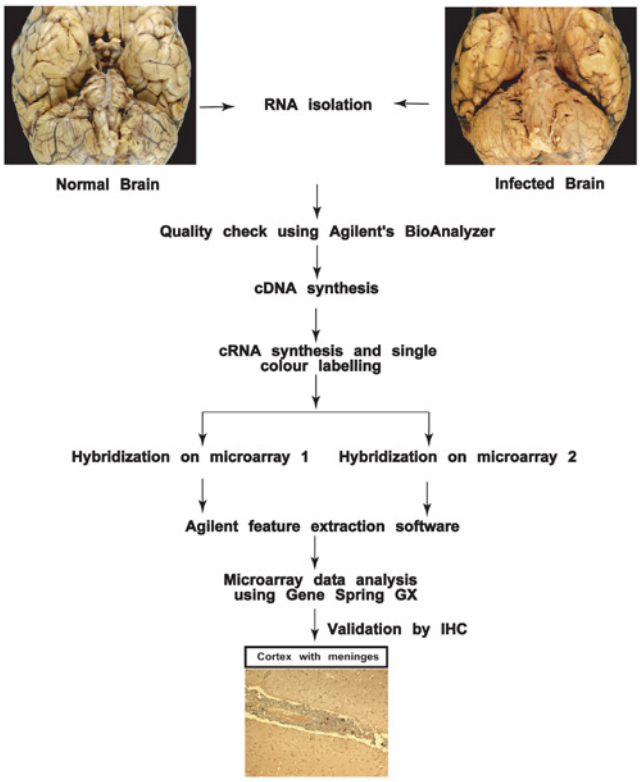

Figure 1: Work flow for transcriptomic studies in TBM. RNA isolation was carried out from brain tissue (frontal cortex) samples from confirmed TBM cases and from control cases. Based on the RNA integrity, selected samples were further processed for microarray experiments and analyzed. Selected upregulated molecules were validated by IHC.

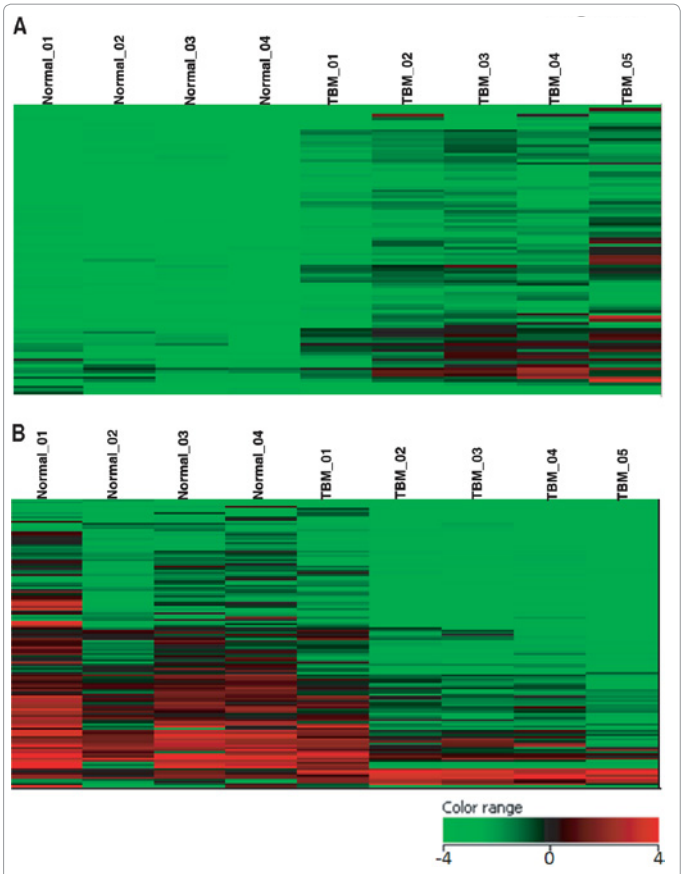

Figure 2: Heat map of differentially expressed genes in TBM. Unsupervised hierarchical clustering of differentially expressed genes with a p value <0.05 and fold-change ≥4 is shown in the figure. Cluster A represents higher expression levels in TBM cases while cluster B represents a lower expression levels in TBM cases as compared to controls.

significantly overrepresented in TBM as compared to normal. Since, TBM is an inflammatory disease, the genes related to host defense are likely to be altered. Thus the overrepresentation of immune response genes in TBM by GO analysis was found as anticipated.

Differentially expressed genes that have previously been reported in TBM studies

Earlier studies on gene expressional profiling of TBM have identified several genes [16,17]. We found a number of genes in this study which showed a similar differential expression as described previously. A subset of genes or gene products that have been reported to be differentially expressed either in TBM or in the context of tuberculosis-associated studies are discussed below:

Upregulated genes

Metallothionein 1F (*MT1F*) was shown to be upregulated in mice infected with laboratory strains of *M.tb* such as H37Rv and H37Ra [18]. We observed 2.5-fold upregulation of *MT1F* in the present study. MT1F belongs to cysteine-rich metal binding proteins family that is known to play a key role in regeneration of tissue after damage. Metallothioneins also have a functional role as antiapoptotic antioxidants in neurological abnormalities [19]. Changes in the expression levels of these metallothioneins mediates immune response under inflammatory conditions [20]. Polymorphisms of major histocompatibility complex

S.no	GeneSymbol	Architecture/Domain	Primary ocalization	Alternate localization	Biological process	Molecular function	Molecule class	+/-	Fold change TBM/Normal
1	*CHI3L2*	SP	Nucleus	N/A	Bone remodeling	Molecular function unknown	Unclassified	+	58
2	*UBD*	UBQ	Nucleus	Cytoplasm	Protein metabolism	Ubiquitin-specific protease activity	Ubiquitin proteasome system protein	+	56
3	*IGL@*	IGC; IGV; SP	N/A	N/A	Immune response	Antigen binding	Immunoglobulin	+	42.5
4	*IGHA2*	Ig_LIKE; IGV; SP	N/A	N/A	Immune response	Antigen binding	Immunoglobulin	+	40
5	*CXCL9*	SCY; CC; CXC; SP	Extracellular	N/A	Immune response	Chemokine activity	Chemokine	+	36
6	*IGHG1*	Ig_LIKE; IGV	N/A	N/A	Immune response	Antigen binding	Immunoglobulin	+	32
7	*SLAMF8*	TM; SP	Plasma membrane	N/A	Immune response	Receptor activity	Cell surface receptor	+	24
8	*GBP5*	CC	N/A	N/A	Cell communication; Signal transduction	GTPase activity	GTPase	+	22
9	*CARTPT*	SP	Extracellular	N/A	Biological_ process unknown	Molecular function unknown	Unclassified	+	20
10	*CIITA*	LRR	Nucleus	N/A	Immune response	Transcription regulator activity	Transcription regulatory protein	+	20
11	*LOC100131733*	N/A	N/A	N/A	N/A	N/A	N/A	+	19
12	*APOL6*	TM; CC	Cytoplasm	N/A	Metabolism; Energy pathways	Transporter activity	Transport/cargo protein	+	18.5
13	*CHI3L1*	SP	Extracellular	N/A	Cell growth and/or maintenance	Extracellular matrix structural constituent	Extracellular matrix protein	+	16
14	*IL4I1*	SP	N/A	N/A	Apoptosis; Immune response	Catalytic activity	Enzyme: Oxidase	+	15
15	*SERPINA3*	SERPIN; SP	Extracellular	Cytoplasm; Nucleus	Protein metabolism	Protease inhibitor activity	Protease inhibitor	+	14
16	*MYBPH*	FN3; IGC2	Cytoplasm	N/A	Cell growth and/or maintenance	Structural molecule activity	Structural protein	+	14
17	*SLAMF7*	IG; TM; SP	Plasma membrane	N/A	Immune response	Receptor activity	Cell surface receptor	+	14
18	*IL21R*	FN3; TM; SP; WSXWS	Plasma membrane	N/A	Immune response	Transmembrane receptor activity	Cytokine receptor	+	13
19	*IDO1*	N/A	Cytoplasm	N/A	Metabolism; Energy pathways	Catalytic activity	Enzyme: Oxygenase	+	13
20	*SLC14A1*	TM	Plasma membrane	N/A	Transport	Transporter activity	Transport/cargo protein	+	13
21	*SCIN*	GEL	Cytoplasm	Plasma membrane	Cell growth and/or maintenance	Cytoskeletal protein binding	Cytoskeletal associated protein	+	11
22	*PKD2L1*	EF; TM; CC	Plasma membrane	N/A	Transport	Ion channel activity	Ion channel	+	11
23	*CTAG1A*	N/A	N/A	N/A	Biological_ process unknown	Molecular function unknown	Unclassified	+	11
24	*TYMP*	N/A	Extracellular	Cytoplasm; Nucleus	Cell communication; Signal transduction	Growth factor activity	Growth factor	+	11
25	*LAX1*	TM	Integral to membrane	N/A	Cell communication; Signal transduction	Receptor signaling complex scaffold activity	Adapter molecule	+	10
26	*SPOCD1*	TFS2M	N/A	N/A	Biological_ process unknown	Molecular function unknown	Unclassified	+	10
27	*CD2*	TM; SP	Plasma membrane	N/A	Immune response	Receptor activity	Cell surface receptor	+	10
28	*TTR*	TRANSTHYR; SP	Extracellular	N/A	Transport	Transporter activity	Transport/cargo protein	-	23
29	*RELN*	EGF; SP	Extracellular	N/A	Protein metabolism	Serine-type peptidase activity	Serine protease	-	19
30	*LYVE1*	LINK; TM; SP	Plasma membrane	N/A	Cell communication; Signal transduction	Receptor activity	Cell surface receptor	-	19
31	*DEFA3*	SP	Extracellular	N/A	Immune response	Defense/immunity protein activity	Defensin	-	15
32	*HIAT1*	TM	N/A	N/A	Transport	Transporter activity	Transport/cargo protein	-	13
33	*EXPH5*	N/A	Nucleus	N/A	Biological_ process unknown	Molecular function unknown	Unclassified	-	12
34	*GLIPR1L2*	SCP	N/A	N/A	Biological_ process unknown	Molecular function unknown	Unclassified	-	12
35	*TMED7-TICAM2*	N/A	N/A	N/A	NA	NA	NA	-	12
36	*ABHD3*	SP	N/A	N/A	Biological_ process unknown	Molecular function unknown	Unclassified	-	11
37	*CNTN6*	FN3; Ig_LIKE; IGC2; SP	N/A	N/A	Cell communication; Signal transduction	Cell adhesion molecule activity	Adhesion molecule	-	11
38	*C9orf5*	TM	N/A	N/A	Biological_ process unknown	Molecular function unknown	Integral membrane protein	-	11
39	*EDNRB*	TM; SP	Plasma membrane	Lysosome; Endoplasmic reticulum	Cell communication; Signal transduction	G-protein coupled receptor activity	G protein coupled receptor	-	11
40	*TMCO3*	TM; CC; SP	Integral to membrane	N/A	Biological_ process unknown	Molecular function unknown	Integral membrane protein	-	10
41	*APPBP2*	CC	Cytoplasm	Cytoplasmic vesicle; Golgi apparatus	Regulation of gene expression, epigenetic	Molecular function unknown	Adapter molecule	-	10
42	*S100A12*	N/A	Cytoplasm	N/A	Cell communication; Signal transduction	Calcium ion binding	Calcium binding protein	-	10

Note: Positive sign (**+**) indicates upregulation, Negative sign (-) indicates downregulation and N/A: Not available

Table 1: A partial list of differentially regulated genes expressed in TBM.

class II, DR beta 1 (*HLA-DRB1*) gene have been found to be associated with susceptibility to TB [21]. HLA-DRB1 belongs to an important class of molecules which play a crucial role in the process of antigen presentation [22]. *HLA-DRB1* was shown to be upregulated 3-fold in the current study. *IL12RB1* encoded protein is a type-1 transmembrane protein, which belongs to the hemopoietin receptor superfamily [23]. Upregulation of *IL12RB1* has been reported earlier in *M.tb* infected mice [18]. Further, nucleotide polymorphisms in *IL12RB1* have also been reported to be associated with susceptibility to TB [21]. In this study, we found 3-fold upregulation of *IL12RB1* transcript in TBM. The protein encoded by solute carrier family 11, member 1 (*SLC11A1*) belongs to the solute carrier family and is also known as natural resistance-associated macrophage protein 1. Polymorphisms in *SLC11A1* have been associated with susceptibility to TB [21]. We observed a 4-fold upregulation of *SLC11A1* transcript in TBM cases. Tumor necrosis factor receptor superfamily, member 4 (*TNFRSF4*) is known to be involved in the pathogenesis of various immunological abnormalities including infectious, autoimmune, inflammatory related diseases. It has been shown to be upregulated in mice infected with *M.tb* strains H37Rv and H37Ra [18,24]. In this study, *TNFRSF4* transcript was 2-fold upregulated in TBM as compared to uninfected controls. Chemokine (C-X-C motif) ligand 9 (*CXCL9*) is a T cell trafficking chemokine [25], which is known to be upregulated in mice infected with laboratory strains of *M.tb* and high concentrations of *CXCL9* have been reported in the CSF of TBM patients [18,26]. We observed a 36-fold upregulation of *CXCL9* transcript in our study. Met proto-oncogene (*MET*), also known as hepatocyte growth factor receptor, encodes a receptor tyrosine kinase, which has shown to be overexpressed in *M.tb* stimulated monocyte-derived macrophages (MDMs) [17]. We found a 9-fold upregulation of this transcript in our transcriptomic study. CHI3L1 (Chitinase 3-like 1) is involved in the process of inflammation and tissue remodeling. *CHI3L1* was also shown to be upregulated in peripheral blood mononuclear cells (PBMCs) of recovered extra pulmonary tuberculosis patients upon incubating their PBMCs with whole lysates of *M.tb* [16]. *CHI3L1* transcript was 16-fold upregulated in the present study.

Downregulated genes

SLC15A2 (Solute carrier family 15, member 2) is known to be involved in the induction of proton-dependent transport for transporting small peptides [27]. In an *ex vivo* experiment, *M.tb*-stimulated MDMs showed downregulation of *SLC15A2* [17]. In this study, we found 5-fold downregulation of *SLC15A2* transcript in TBM. ITGB1 (Integrin beta 1) belongs to the membrane receptor family, which is involved in cell adhesion and recognition. ITGB1 is also involved in several cellular processes including immune response [28]. *ITGB1* transcript was observed to be 3-fold downregulated in this study. It has been reported that *ITGB1* was downregulated in fetal lung cell line in the presence of *M.tb* recombinant CFP-10/ESAT-6 protein (rCFES) [29]. Cathepsin L1 (*CTSL1*) encoded protein is a lysosomal cysteine proteinase, which is involved in intracellular protein catabolism. *CTSL1* transcript was shown to be 2-fold downregulated in this study and the encoded protein is localized to endosomes. CTSL1 activity and maturation was affected by *Mycobacterium avium* and *M.tb* infected macrophages [30].

Differentially expressed genes that were not reported earlier

A large number of differentially expressed genes identified in our study have not been previously reported in the literature to be associated with TBM. These genes are involved in various biological functions including cell-cell communication, enzymatic activity and signal transduction.

Novel upregulated genes

Over the past several years, various studies have proven the overexpression of GFAP (Glial fibrillary acidic protein) in CNS tuberculosis, toxocariasis and pneumococcal meningitis [31-33]. It has been shown to be overexpressed in astrocytes when there is an astrogliosis during CNS inflammation. In the present study, *GFAP* transcript showed 5-fold upregulation. Interleukin 4 induced 1 (IL4I1) expression has been shown to be induced by IL-4 in B-cells and this secreted protein is known to be involved in the regulation of T lymphocytes [34]. *IL4I1* transcript was found to be 15-fold upregulation in our study. Interleukin 21 receptor (IL21R) belongs to the type 1 cytokine receptor family and is involved in the maturation of natural killer cell and regulation of T lymphocytes [35]. IL21R binds to its ligand and activates the downstream signaling molecules like JAK 1, JAK3, STAT1 and STAT3 [36]. *IL21R* transcript showed 13-fold upregulation in the current study. Serpin peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 3 (SERPINA3) is a plasma protease inhibitor and is a member of the serine protease inhibitor class. *SERPINA3* transcript was found to be 14-fold upregulated in the current study. The overexpression of this protein has been reported in several neurological disorders like schizophrenia [37,38]. *GFAP* and *SERPINA3* were chosen for further validation by immunohistochemical analysis which is explained in the later section.

Novel downregulated genes

RELN mRNA was observed to be 19-fold downregulated in the

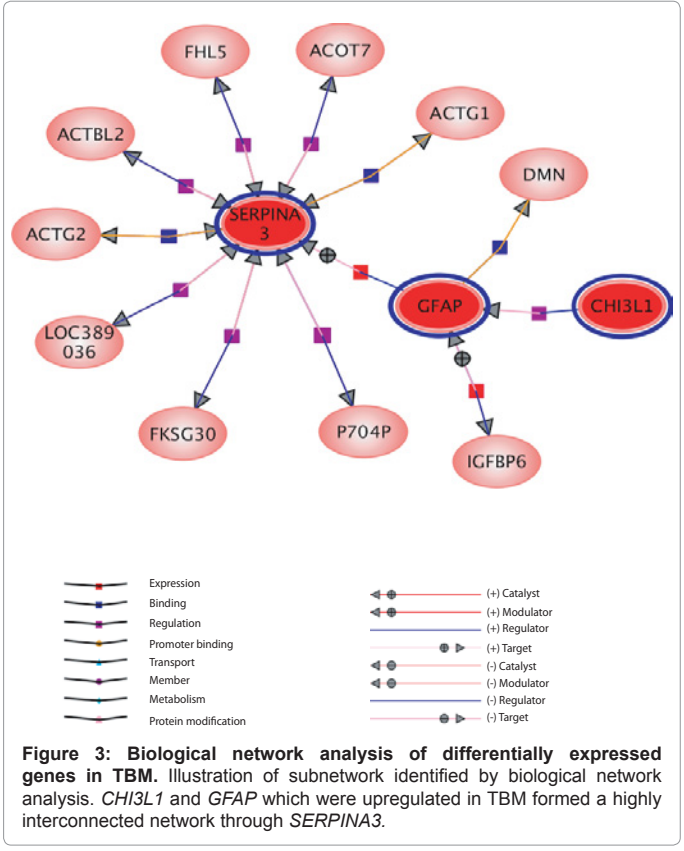

Figure 3: Biological network analysis of differentially expressed genes in TBM. Illustration of subnetwork identified by biological network analysis. *CHI3L1* and *GFAP* which were upregulated in TBM formed a highly interconnected network through *SERPINA3*.

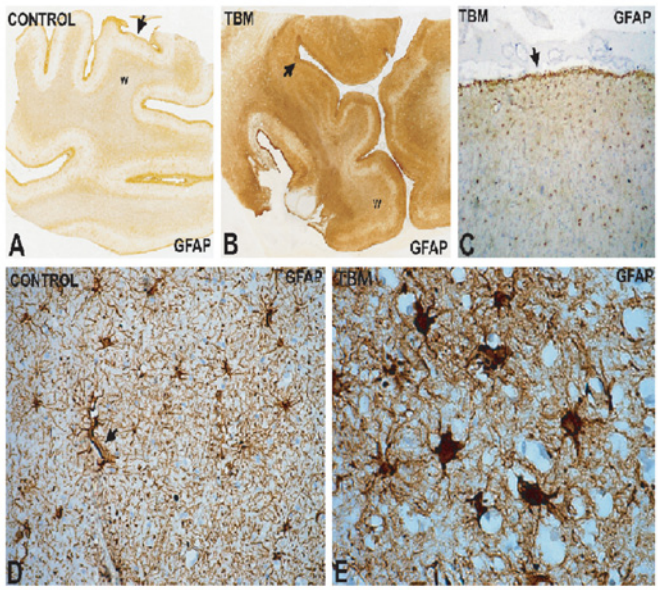

Figure 4: Immunohistochemical labeling of *GFAP*. A: Whole mount preparation of frontal cortex from a control shows thin subpial band of gliosis (arrow) and very light labeling of protoplasmic astrocytes. The white matter (w) shows diffuse, low intensity staining. (*GFAP*, Obj x5). **B:** Whole mount preparation of frontal cortex from a case of TBM showing thick subpial carpet (arrow) extending into the cortical grey matter. Note the reduced gradient of labeling of grey matter and diffuse dark staining of white matter (w). (*GFAP*, Obj x 5). **C:** Low power view of the frontal cortex from a case of TBM shows thick gliotic pial band (arrow) and hypertrophic glial cells in the superficial cortex. Above the pial band in the subarachnoid space, inflammatory exudates are observed. (*GFAP*, Obj x 10). **D:** The white matter at higher magnification from the control shows astrocytes with small body and thin long processes, some of them impinging on the vessel wall to form a fine lacy background. (*GFAP*, Obj x 20). **E:** Higher magnification of the subpial zone from the case of TBM showing large, hypertrophic reactive astrocytes with darkly labeled body and thick cell processes. The neuropil between the cells also has coarse fibres unlike the control. (*GFAP*, Obj x 40).

present study. Reelin (*RELN*) encodes a secreted extracellular matrix protein. RELN is involved in the molecular mechanism of cognitive functions. Defects in *RELN* expression have been associated with the abnormality of neuronal position and dendritic development in mouse models [39]. The protein encoded by Vacuolar protein sorting 26 (*VPS26*) is involved in retrograde transport of proteins. *VPS26* was found to be 2-fold downregulated in TBM cases. Sorting nexin 3 (SNX3), sorting nexin 12 (SNX12) and sorting nexin 18 (SNX18) belong to the sorting nexin family and are involved in intracellular trafficking. Inhibited expression of the SNX3 has been shown to affect membrane trafficking from early endosomes to recycling endosomes [40]. In this study, *SNX3* transcript was found to be 3-fold downregulated and protein encoded by this gene is localized to the endosomes. *SNX12* and *SNX18* were found to be downregulated 3-fold and 4-fold, respectively. Defensin alpha 3, neutrophil-specific (DEFA3) protein belongs to the family of microbicidal peptides which is involved in antimicrobial activity. *DEFA3* mRNA and protein expression levels have been reported to be decreased in transmigrated monocytes [41]. It showed 15-fold downregulation in our study.

Biological network data analysis

Several genes are involved in carrying out a specific biological process and they often interact with each other. We carried out biological network analysis using GeneSpring Gx software to identify such networks in relation to TBM. Natural language processing is used by GeneSpring to generate a database of interacting molecules. Genes that were differentially expressed in TBM were used as input which resulted in the generation of a complex network depending on the connectivity between the genes. It was comprised of several nodes forming distinct subnetworks. Expression values were overlaid onto the network. We identified a subnetwork with *SERPINA3* being the key molecule which is shown in Figure 3. *SERPINA3* is known to be associated with several neurological disorders including Parkinson's disease [42], Alzheimer's disease [43], schizophrenia and cerebrovascular disease [44]. Notably, we observed *GFAP* and *CHI3L1* which were upregulated in TBM to be part of this subnetwork. Earlier reports have shown that CHI3L1 and GFAP were co localized in the brain infarction and in other neurological diseases [45]. Other molecules that were part of the subnetwork include *P704P, FKSG30, ACTBL2*, *ACTG2*, *ACTO7* and *FHL5*. Although it is intriguing that we see the interaction of these molecules in the represented network, the exact functional role and significance with respect to TBM has to be investigated further.

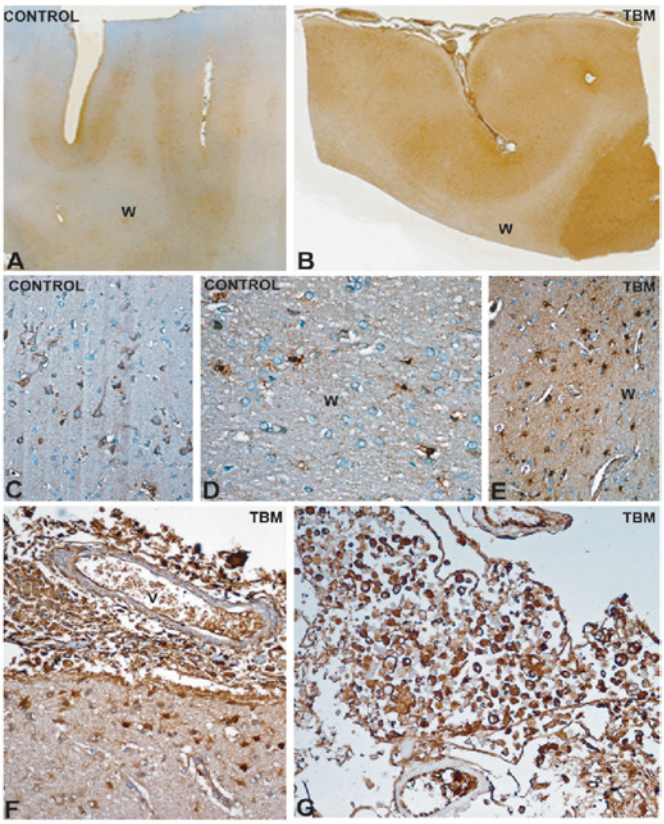

Figure 5: Immunohistochemical labeling of *SERPINA3*. A: Whole mount preparation of frontal cortex from a control showing low diffuse labeling of the cortical ribbon and low intensity staining of white matter (w). (*SERPINA3*, Obj x 5). **B:** Whole mount preparation of frontal cortex from a case of TBM showing diffuse intense staining of the cortical ribbon and exudates in the subarachnoid space (w - white matter). (*SERPINA3*, Obj x 5). **C:** The pyramidal neurons of the superficial cortex in controls have cytoplasmic labeling. (*SERPINA3*, Obj x 20). **D:** The astrocytes in the cortex of controls are stained well, though the density of labeled astrocytes is low. (*SERPINA3*, Obj x 20). **E:** The white matter shows a higher density of the *SERPINA3* labeled astrocytes in TBM. (*SERPINA3*, Obj x 10). **F:** Low power view of the frontal cortex showing dense labeling of subpial glial membrane, reactive astrocytes underneath and the granulomatous exudates in the subarachnoid space. (TBM case) (v: vessel) (*SERPINA3*, Obj x 20). **G:** Higher magnification of the subarachnoid exudates showing labeling of the round histocytes. (TBM case) (*SERPINA3*, Obj x 20).

Validation of candidate upregulated biomarkers by immunohistochemical labeling

In the present study, we chose to carry out IHC-based validation for two of the novel upregulated molecules identified in this study based on their biological significance and their fold expression. This was performed in 15 TBM cases, including the cases used for gene expression profiling.

GFAP: The staining pattern of GFAP in TBM cases as compared to control cases is shown in histological microphotographs in Figure 4. As shown in the figure, we observed thick subpial carpet staining of GFAP in the whole mount preparation of the frontal cortex of the TBM cases (Figure 4B) as compared to the control groups (Figure 4A). In the frontal cortex, astrocytes in the superficial cortex along with thick processes (Figure 4C), the white matter in the gyri and subcortical band were densely stained with GFAP in TBM cases. In controls (Figure 4D), GFAP labeling is conspicuous around the blood vessels with the foot processes (arrow) impinging on the vessel walls and forming a fine lacy background. In TBM cases, we found strong labeling of GFAP in the hypertrophic astrocytes (Figure 4E) with dark cell body and thick processes.

SERPINA3: Whole mount preparations of immuno stained sections from the frontal cortex in TBM (Figure 5B) showed marginally enhanced staining of the cortical ribbon in contrast to control (Figure 5A). On closer examination in controls, the neuronal cytoplasm in the superficial layers (Figure 5C) and astrocytes in the grey and white matter was labeled strongly with thick processes, but the density of astrocytes was conspicuously lower (Figure 5D). In the case of TBM, a thick subpial carpet was found in addition to numerous subpial astrocytes beneath the dense chronic granulomatous exudates (Figure 5F) and the cortex and white matter (Figure 5E). In the subarachnoid space the histiocytes in the exudates and around the vessels were densely labeled (Figure 5F and Figure 5G). The cortical neuronal labeling is essentially similar to controls, but marginally higher intensity of cell labeling involving all the layers of cortical ribbon unlike control. All these cellular elements contribute to the upregulated gene expression protein profile.

Conclusions

Though many clinical, morphological and biochemical studies have been carried out earlier on tuberculous meningitis, only a limited number of gene expression profiling studies are have been undertaken. In tropical developing countries, TBM is one of the commonest forms of chronic meningitis. A rise in HIV cases in these geographic regions further worsens the situation. Biomarkers that could facilitate early diagnosis of TBM provide a better opportunity for clinical management of this disease. By carrying out gene expression profiling of infected brain from TBM patients and control subjects, we have identified several differentially expressed genes. Systematic validation of some of the candidate molecules may provide biomarkers with potential clinical utility. Some of the differentially expressed genes encode proteins that are detectable in CSF and targeted studies to develop assay systems to monitor these molecules in CSF may prove useful.

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Conflict of Interest

Authors have declared no conflict of interest.

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