

Gene Expression Profiling of Tuberculous Meningitis Co-infected with HIV

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Abstract

Tuberculous meningitis (TBM) is a fatal form of *Mycobacterium tuberculosis* infection of the central nervous system (CNS). The similarities in the clinical and radiological findings in TBM cases with or without HIV make the diagnosis very challenging. Identification of genes, which are differentially expressed in brain tissues of HIV positive and HIV negative TBM patients, would enable better understanding of the molecular aspects of the infection and would also serve as an initial platform to evaluate potential biomarkers. Here, we report the identification of 796 differentially regulated genes in brain tissues of TBM patients co-infected with HIV using oligonucleotide DNA microarrays. We also performed immunohistochemical validation and confirmed the abundance of four gene products-glial fibrillary acidic protein (GFAP), serpin peptidase inhibitor, clade A member 3 (SERPINA3), thymidine phosphorylase (TYMP/ECGF1) and heat shock 70 kDa protein 8 (HSPA8). Our study paves the way for understanding the mechanism of TBM in HIV positive patients and for further validation of potential disease biomarkers.

Keywords: Single color labeling; GeneSpring; Cerebrospinal fluid; Neurological disorder; Early diagnosis

Introduction

Mycobacterium tuberculosis (MTB) is an acid-fast bacterium that primarily infects the lungs resulting in pulmonary tuberculosis. However, the infection can spread hematogenously to other sites including bone, lymph node, gastrointestinal tract, genitourinary tract and the central nervous system [1]. TBM is one of the most common forms of extrapulmonary tuberculosis. TBM is associated with high mortality and morbidity [2-4]. Diagnosis of TBM remains a major challenge even today. This is partly because the gold standard test used for the diagnosis of TBM requires culturing the bacterium from cerebrospinal fluid (CSF), which takes approximately 8 weeks [5]. Detection of MTB DNA using polymerase chain reaction (PCR), MTB protein or host antibodies against MTB antigens by Enzyme-Linked Immunosorbent Assay (ELISA) are the most common and extensively used assays for the detection of mycobacterial infection [6,7]. Some molecules are also being evaluated for their use as biological markers for the diagnosis of TBM. These include detection of high levels of lactate and adenosine deaminase (ADA) in CSF [8-10]. However, the specificity and sensitivity of these existing markers are still limited.

Approximately, 50 million people are infected globally with Human Immunodeficiency virus (HIV) and 20% cases of tuberculosis are associated with HIV in sub-Saharan Africa [11,12]. Cases of tuberculosis co-infected with HIV are frequent in Southeast Asia [13]. Occurrence of systemic TB in HIV positive patients is increasing in India as well [14]. The lifetime risk for a HIV negative individual to develop TB is 10-20% [15], whereas the lifetime risk of an HIV-infected individual to develop TB is >30% [16]. The risk of developing central

nervous system tuberculosis is higher in children [17] and HIV co-infected patients [18-20]. Occurrence of TBM in HIV-infected patients is more frequent (6.4%) as compared to people with no previous infection by HIV (1.2%) [21,22].

Diagnosis of TBM co-infected with HIV cases is more difficult because the AIDS (Acquired Immunodeficiency Syndrome) patients are susceptible to a wide range of opportunistic infections including virus, bacteria, protozoa and fungi mimicking tuberculous infections. As a consequence of the compromised immune system, any part of the CNS may be affected [23-25]. Confirmation at the clinical level of HIV mediated neurologic dysfunction is usually difficult until the advanced stage of disease is manifested by elevated viral load and compromised immune system with low CD4⁺ lymphocyte counts [26,27]. Global increase in incidence of AIDS and development of multidrug resistance

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adds to the lethality of various infections. Increasing numbers of multi-drug resistant tuberculous bacilli in TBM patients infected with HIV is a serious event, both clinically and epidemiologically [28].

The CSF mycobacterial count seems to be higher in patients with HIV infection compared to non-HIV infected individuals [29]. The clinical and radiological diagnostic features of TBM are similar in HIV positive and negative individuals [30]. Infections caused by *Toxoplasma gondii* or *Cryptococcus neoformans*, viral infections such as progressive multifocal leukoencephalopathy (PML) and malignancies including lymphoma may manifest as intra-cerebral mass lesions in patients with HIV, which makes it difficult to distinguish from TBM [31]. In this context, an algorithmic approach undertaken by investigators from South Africa was shown to be effective in identifying the etiology of lesions in 23/26 patients [32]. HIV changes the usual histological manifestations and tissue responses to tuberculosis as observed in lymphadenitis with the absence of granuloma and high bacterial load [33]. The rapid diagnosis and differentiation of TBM in HIV from other infections is crucial as the patients are immune compromised and the therapeutic regimens are different. Therefore, any delay in diagnosis may lead to a higher morbidity and mortality. This emphasizes the need for specific and sensitive biomarkers for diagnosis of TBM in HIV patients. Previously, we have identified and validated several candidate biomarkers for TBM based on transcriptome profiling studies of brain tissues from TBM infected and uninfected individuals [34].

In this study, we carried out gene expression profiling of brain tissues obtained from TBM patients co-infected with HIV and normal individuals. We identified 796 differentially expressed genes in tissues obtained from TBM co-infected with HIV patients as compared to controls. Further, we carried out immunohistochemical validation for a set of 4 proteins that were differentially expressed.

Materials and Methods

Tissue samples

Human brain tissue samples from the frontal cortex with overlying meninges from five autopsy confirmed cases of TBM + HIV-1 infection. Diagnosis was confirmed by (i) scrape smears from the basal meninges of the brain positive for acid fast bacilli (AFB); (ii) positive antibodies/immunocomplexes in CSF against MTB by ELISA; (iii) CSF culture positive for MTB; (iv) granulomatous meningitis on histological examination with or without demonstrable AFB in histological sections by Ziehl Neelsen's staining [35]. All cases were positive for HIV-1 on serological testing and confirmed to be HIV-1 clade C by clade specific PCR developed at Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore and validated [36]. HIV-1 viral load and CD4 counts were determined wherever possible.

The four control brain samples were from individuals who succumbed to road traffic accidents and the brains collected from 6 hrs-13½ hrs for postmortem analysis. These brain tissues were archived at the Human Brain Tissue Repository (National Research Facility), Department of Neuropathology, NIMHANS, Bangalore, India. The control tissues were confirmed to be negative for HIV-1 and tuberculosis infection by the above mentioned diagnostic tests. All tissues were stored at -86°C, after collecting tissues for routine histology in 10% neutral buffered formalin and preserved at room temperature. The tissues were collected following written informed consent provided by close relatives to archive the brain collected at autopsy and use for research purposes. A portion of the brain tissue samples were immediately transferred to RNA later (Ambion Inc.,

Austin, TX, USA) and stored at -86°C for RNA studies. Information pertaining to clinical samples used in the present study is provided in the Supplementary Table 1. The study was approved by an Institutional Ethics Committee.

RNA isolation

Approximately 100 mg of tissue was used as a starting amount from infected and normal brain tissue samples for RNA isolation. The tissues were homogenized in 1 ml of QIAzol lysis reagent (Qiagen, Valencia, CA). Total RNA was extracted and purified using Qiagen RNeasy Mini kit (QIAGEN, Valencia, CA) following manufacturer's instructions. The yield and quality of extracted total RNA was checked by the absorbance ratios A260/230 and A260/A280, using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Assessment of RNA integrity was checked using 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and/or RNA gel.

cDNA synthesis, labeling and hybridization

cDNA synthesis, labeling and hybridization were essentially carried out as previously described [34,37-39]. Briefly, we reverse transcribed 600 ng total RNA and linear amplified using quick Amp Kit, single color (Agilent Technologies, Palo Alto, CA) employing OligodT-T7 promoter primers. For *in-vitro* transcription, the cDNA generated was used as template with Cy3 labeled CTP and RNA polymerase. The labeled cRNA was synthesized and purified using RNeasy spin columns (Qiagen, Valencia, CA). The qualified samples according to the specific activity >9 pmol Cy3 per µg and yield >1600 µg were subjected to hybridization. The fragmented labeled cRNA was hybridized onto oligonucleotide-based whole human genome DNA microarrays (G4112F, 4 x 44 K, Agilent Technologies, Palo Alto, CA). The hybridization was continued for 16 hours at 65°C. Subsequently, the arrays were washed with gene expression wash buffers kit as per the manufacturer's hybridization protocol (Agilent Oligo Microarray Kit, Agilent Technologies).

Data analysis

An Agilent microarray scanner (G2505B) was used to scan the oligonucleotide-based whole human genome DNA microarrays with one color scan settings (scan resolution 5 µm, dye channel was set to green, green PMT was set to 100%). To obtain raw files for further analysis, the images were processed with Agilent's feature extraction software (9.5.3.1). The raw data was submitted to the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/> Accession GSE29507).

The gene expression data was analyzed using GeneSpring GX v11.0.2 (Agilent Technologies, Santa Clara) software. Quantile normalization without baseline transformation and t-test were applied to the data. To get a statistically significant data, we have used a p-value of <0.005 and a fold change with threshold cut-off of ≥ 2 for differentially expressed genes. The list was searched using Human Protein Reference Database (<http://www.hprd.org/>) [40,41] to list molecular functions and subcellular localizations of proteins. We also carried out an additional comparison of gene expression profiles of TBM patients co-infected with HIV to TBM patients without HIV. For this analysis, unpaired-t test was carried out and further filtered using p-value cut off of <0.005 and a fold change cut-off of ≥ 2 .

Biological network analysis

The biological network analysis was carried out using GeneSpring software. The list of differentially expressed genes with the p-value <0.005 and fold value of ≥ 5 were imported into GeneSpring as input list. The analysis was carried out as described earlier [34].

Immunohistochemical analysis

The transcriptomic data was validated by immunohistochemistry for four differentially expressed molecules, for which commercial antibodies were available. Immunohistochemical labeling was carried out with commercially available polyclonal antibodies directed against TYMP/ECGF1 (Catalog number HPA001072, Sigma Aldrich, St. Louis, MO) at 1:250 dilution and a monoclonal antibody directed against HSPA8/HSC70 (Catalog number ab51052, Abcam, PLC. 204 & 330 Cambridge Science Park, Cambridge) at 1:250 dilution. GFAP and SERPINA3 dilutions were carried out as previously described [34]. HRP conjugated with secondary antibody provided with Envision kit (DAKO-K4011 and K4007, DAKO, Carpinteria, CA) was used. The immune reaction was visualized with DAB/H₂O₂ as the chromogen. Immunohistochemical validation was carried out on an independent subset of 10 formalin fixed human brain tissues from confirmed cases of TBM co-infected with HIV in addition to the five cases and four controls used for gene expression profiling in this study.

Four micron thick serial paraffin sections from controls and TBM with HIV were collected on silane coated slides. The sections were deparaffinized and dehydrated by passing through graded alcohol and buffer. Sections were stabilized in tris buffer saline (TBS) at room temperature and endogenous peroxidase activity was quenched by blocking (3% H₂O₂ with methanol) solution for 20 min at room temperature. Citrate buffer (pH 6.0) was used for antigen retrieval by pressure cooking the sections for 30 min. Then sections were incubated with 3% skimmed milk powder at room temperature, followed by incubation at 4°C. After washing with TBS thrice, the reaction was visualized by incubating in HRP-tagged secondary antibody and DAB/H₂O₂ as chromogen. The sections were washed and mounted in DPX medium. The staining pattern, intensity and subcellular localization of molecules in the immuno labeled sections were independently scored and analyzed by two neuropathologists (AM and SKS).

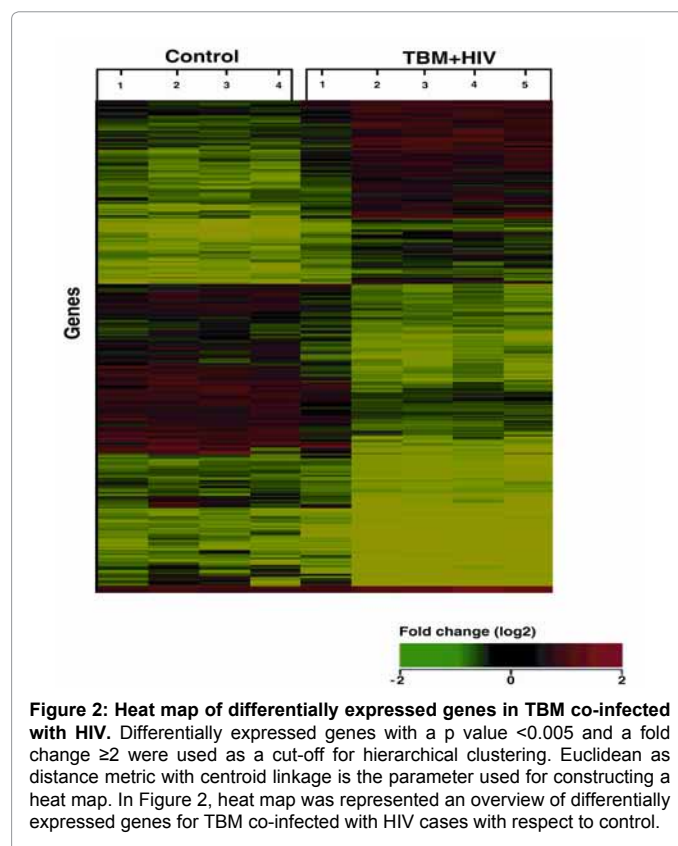
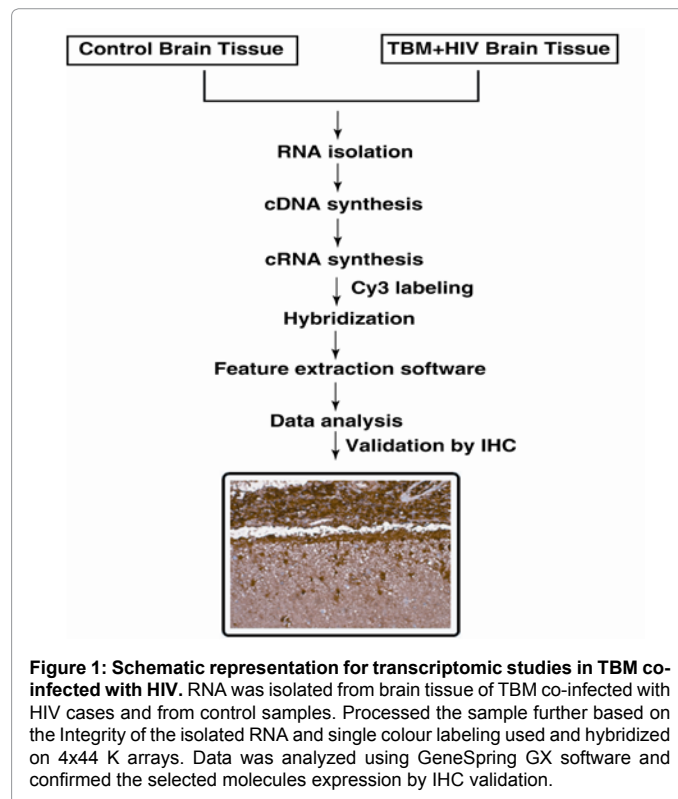
Results and Discussion

The current global gene expression profiling study was undertaken in brain samples from five TBM patients co-infected with HIV. The workflow of this study is represented in Figure 1. We have identified 796 genes, out of which 398 genes were upregulated and 398 genes were downregulated with a p-value cut off of 0.005 and a fold-change cut-off of ≥ 2 -fold. A complete list of differentially expressed genes in TBM patients co-infected with HIV is provided in Supplementary (Table 2) along with information on protein domains/motifs, subcellular localization, biological process, molecular function, molecular class and detection in tissues. Partial list of differentially expressed genes are provided in Table 1 and Table 2. The heat map of the differential expression is shown in Figure 2. Euclidean distance metric and centroid linkage were used as parameters for clustering the genes. We compared the cumulative list of differentially expressed genes with Human Protein Reference Database (HPRD, <http://www.hprd.org>) to compile molecular functions and subcellular localizations of corresponding proteins. The results of the analysis are depicted in Figure 3.

Upregulated genes

We identified 398 genes, which were upregulated by ≥ 2 -fold in TBM patients co-infected with HIV as compared to controls. A selected list of 10 highly upregulated genes are discussed below. We observed 143-fold upregulation of chitinase-3-like protein 2 (*CHI3L2*) mRNA. Colton *et al.*, have observed that *CHI3L2* mRNA was expressed at approximately 3-fold higher in the brains of Alzheimer's disease

compared to age matched controls [42]. mRNA of another member of this family, chitinase 3-like 1 (*CHI3L1*), whose encoded protein product



S.No	Gene Symbol	Protein Name	Description	Fold change
1	<i>TYMP</i>	Thymidine phosphorylase precursor	It promotes <i>in vivo</i> angiogenesis. It also stimulates the growth and migration of endothelial cells.	33.9
2	<i>SLAMF8</i>	SLAM family member 8 precursor	It is involved in lymphocyte activation.	33.6
3	<i>SERPINA3</i>	Alpha-1-antichymotrypsin precursor	It functions as an acute phase protein, increasing in plasma concentration in response to trauma and infection.	29.8
4	<i>CIITA</i>	MHC class II transactivator	Its functions as a positive regulator of class II major histocompatibility complex gene transcription.	28.7
5	<i>CHI3L1</i>	Chitinase-3-like protein 1 precursor	It is involved in inflammatory processes and tissue remodeling.	27.9
6	<i>IL4I1</i>	Interleukin 4 induced 1	It shares some similarity with L-amino acid oxidase. It is said to be involved in the catalysis and binding of the flavin adenine dinucleotide cofactor.	25.1
7	<i>C4B</i>	Complement C4-B preproprotein	It is involved in the classical activation pathway. Following cleavage and trimerization, it provides a surface for interaction between the antigen-antibody complex and other complement factors.	14.9
8	<i>IL21R</i>	Interleukin-21 receptor precursor	It acts as receptor for the cytokine Interleukin-21. It is involved in lymphocyte differentiation and proliferation.	13.8
9	<i>TCIRG1</i>	V-type proton ATPase 116 kDa subunit a isoform 3	It is involved in the process of intracellular organelle acidification in eukaryotes.	12.7
10	<i>IFI30</i>	gGamma-interferon-inducible lysosomal thiol reductase preproprotein	This enzyme is involved in MHC class II-restricted antigen processing.	12.0

Table 1: A partial list of upregulated genes in Tuberculous Meningitis co-infected with HIV.

S.No	Gene Symbol	Protein Name	Description	Fold change
1	<i>PCMT1</i>	Protein-L-isoaspartate (D-aspartate) O-methyltransferase	It is a protein repair enzyme involved in the conversion of abnormal D-aspartyl and L-isoaspartyl residues to the normal L-aspartyl form	20.7
2	<i>FBXW7</i>	F-box and WD repeat domain containing 7	It is one of the four subunits of SCF ubiquitin protein ligase complex and is involved in phosphorylation-dependent ubiquitination	18.7
3	<i>S1PR1</i>	Sphingosine-1-phosphate receptor 1	It plays a role in the differentiation of endothelial cells and cell-cell adhesion.	15.6
4	<i>HSPA8</i>	Heat shock 70kDa protein 8	It belongs to the HSP70 family and is implicated in protein folding. It is also regulates the transport of membrane components through the cell by acting as an ATPase.	13.9
5	<i>USP3</i>	Ubiquitin specific peptidase 3	It is a deubiquinating enzyme which deubiquitinates H2A/H2B and is implicated in maintaining genome integrity. Knocking out this gene results in a delay in the S-phase progression of the cell cycle and accumulation of DNA breaks.	13.0
6	<i>SCG3</i>	Secretogranin III	It belongs to a member of the chromogranin/secretogranin family of neuroendocrine secretory proteins. The function of this protein is unknown. However, some granins may play a role in protein sorting and proteolytic processing of prohormones.	12.2
7	<i>CLK4</i>	DC-like kinase 4	It is thought to regulate alternative splicing by interacting and phosphorylating serine and arginine rich proteins which are known to play an important role in the formation of spliceosomes.	12.5
8	<i>RAB1A</i>	RAB1A, member RAS oncogene family	It belongs to the Ras family of GTPases and is implicated in the regulation of vesicular traffic from the endoplasmic reticulum to the golgi apparatus.	11.0
9	<i>SRP68</i>	Signal recognition particle 68kDa	It is a component of the signal recognition peptide complex, which transport secreted and membrane proteins to the endoplasmic reticulum for processing.	9.8
10	<i>EIF4EBP2</i>	Eukaryotic translation initiation factor 4E binding protein 2	It is a member of the eukaryotic translation initiation factor 4E binding protein family which can bind eIF4E and inhibit translation initiation.	8

Table 2: A partial list of downregulated genes in Tuberculous Meningitis co-infected with HIV.

is involved in inflammation and tissue remodeling, was also found to be upregulated by 28-fold in the present study. *CHI3L1* was reported to be overexpressed in peripheral blood mononuclear cells (PBMCs) from patients recovered from extrapulmonary tuberculosis incubated with whole lysate of MTB [43]. It is considered to be a marker for later stages of macrophage differentiation [44]. SLAM family member 8 (*SLAMF8*) which encodes a member of the CD2 family of cell surface proteins was found to be upregulated 33-fold. *SLAMF8* is reported to play a role in B-cell differentiation and is also known as 'B lymphocyte activator macrophage expressed' (BLAME) [45]. *SLAMF8* mRNA is reported to be upregulated by 5-fold in pulmonary CD8 T-cells in BALB/c mice inoculated with MTB antigen 85A (Ad85A) intra-nasally as compared to intra-dermal route [46]. A progressive upregulation of *SLAMF8* mRNA was reported in mouse lungs inoculated with MTB at 20, 40 and 100 days of the infection [47]. Class II, major histocompatibility

complex, transactivator (*CIITA*) encodes a nuclear protein, which regulates the expression of class II major histocompatibility complex genes [48,49], was identified to be upregulated by 28-fold in the present study. Interleukin-4 induced 1 (*IL4I1*) mRNA was identified to be upregulated by 25-fold in the present study. Aerogenic infection with MTB in the mouse strains (DBA/2 and CBA/J) has also shown *IL4I1* to be highly upregulated [50]. It has also been found to be significantly upregulated by IL4 in macrophages [51]. Interleukin 21 receptor (*IL21R*) is a member of type I cytokine receptor family. This receptor binds to IL21 and IL21/IL-21R system is established to play a significant role in the control of viral infections. HIV-specific CD8+ T cells are shown to express high levels of *IL21R* in acute and chronic infection [52]. In the present study, we identified *IL21R* to be upregulated 13-fold in TBM patients co-infected with HIV. Guanylate binding protein 5 (*GBP5*) mRNA codes for an intracellular guanylate binding protein

and was found to upregulate 20-fold. GBP5 has also been shown to be upregulated in macrophages activated by heat-killed Group B streptococcus. Group B streptococcus is a main etiological agent of septicemia and meningitis in compromised immune system of adults and newborn infants [53,54]. T-cell, immune regulator 1, ATPase, H⁺ transporting, lysosomal V0 subunit A3 (*TCIRG1*) is reported to be downregulated in late TB granulomas in Rhesus macaque [55]. It has also been shown to be regulated by MTB H37Rv strain [56]. Our present study identified *TCIRG1* to be upregulated 12-fold in TBM patients co-infected with HIV. Apolipoprotein L1 (*APOLI*) is a high density lipoprotein involved in the transport of lipids. SNPs and haplotypes in *APOLI* have been associated with schizophrenia [57]. *APOLI* was found to be overexpressed by 7.9-fold in TBM+HIV cases as compared to control cases. *GFAP* is upregulated in astrocytes of several neurological diseases [34]. It is overexpressed in astrogliosis of CNS inflammation. In TBM cases co-infected with HIV showed 4.7-fold upregulation. *SERPINA3*, which codes for a plasma protease inhibitor, is identified to be a component of the neuritic plaques [58]. *SERPINA3* protein levels are reported to be upregulated in CSF of Alzheimer's disease patients [59,60] with elevated levels being associated with severity of the disease [59]. It has also been shown to be upregulated upon infection with MTB H37Rv strain [56] and 14-fold upregulated in our previous gene expression study of TBM infected human brain tissue [34]. The present study has identified *SERPINA3* to be overexpressed 29-fold in TBM co-infected with HIV patients as compared to control. TYMP is a protein, play a role in the process of angiogenesis [61]. TYMP also known as ECGF1, which encodes a cytosolic enzyme, was found to be upregulated by 3-fold in TBM co-infected with HIV patients as compared to TBM patients. Sequence variations in this gene are associated with the mitochondrial-neuro-gastro-intestinal encephalomyopathy (MNGIE) [62]. It is reported to inhibit the growth of glial cells and promote the growth of endothelial cells [63].

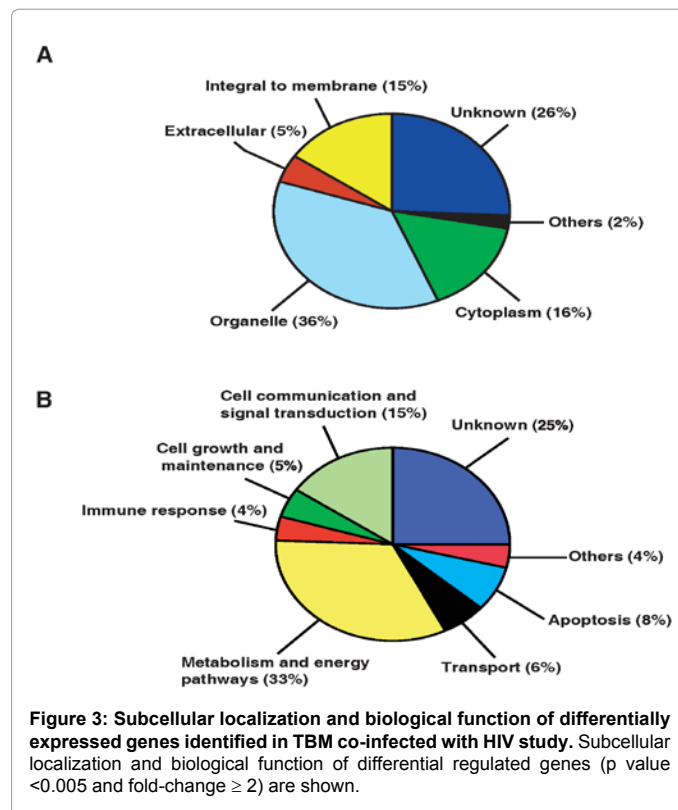
Downregulated genes

There were 398 genes, which were found to be downregulated by ≥ 2 -fold. Among these, regulator of G-protein signaling 5 (*RGS5*) transcripts were found to be downregulated about 23-fold. *RGS5* belongs to a family of signal transduction molecules involved in the regulation of heterotrimeric G-proteins. Sequence variations in *RGS5* have been associated with schizophrenia [64] and bipolar disorder [65]. Rap guanine nucleotide exchange factor 4 (*RAPGEF4*) encodes for the cytoplasmic rap guanine nucleotide exchange factor 4 protein. Non-synonymous variants of this gene have been found among patients with autism [66]. We found the *RAPGEF4* mRNA to be downregulated by 21-fold. N-acylsphingosine amidohydrolase (*ASAHI*) is a heterodimeric protein. It plays a role in ceramide metabolism [67] and hence is important in neuronal development. Gene variants of *ASAHI* have been observed in patients with Farber disease, a lysosomal storage disorder [68]. *ASAHI* mRNA was found to be downregulated by 21-fold in this study. The protein encoded by *CEP170* is localized in the centrosome and regulates microtubule organization and cell morphology [69,70]. This gene was found to be downregulated 20-fold in TBM patients co-infected with HIV. Sphingosine-1-phosphate receptor 1 (*S1PR1*), which binds to ligand sphingosine-1-phosphate is similar in structure to G protein coupled receptors [71] and has been associated with the regulation of endothelial cell differentiation [72]. *S1PR1* activation is known to induce cell-cell adhesion. In the current study, *S1PR1* was found to be downregulated by 15-fold. Ribophorin 2 (*RPN2*) is a membrane protein found only in the endoplasmic reticulum. It has been found to be associated with a risk for sporadic

Alzheimer's disease [73]. We found *RPN2* mRNA to be underexpressed by 15-fold in TBM co-infected with HIV as compared to normal. *CDK8* encodes a protein belonging to the family of cyclin dependent protein kinases. This protein is known to be an important regulator of cell cycle progression [74]. *CDK8* is also known to regulate transcription by acting on the subunits of the general transcription initiation factor IIIH. We found *CDK8* to be downregulated 15-fold in the current study. Non-imprinted in Prader-Willi/Angelman syndrome 1 (*NIPA1*) transcript was found to be downregulated by 14.5-fold in the current study. *NIPA1* protein is a magnesium transporter known to be associated with early endosomes in neuronal and epithelial cells [75]. Kinesin light chain 1 (*KLC1*) codes for a protein member of the kinesin light chain family. It functions as an adaptor molecule that binds to the Golgi complex, mitochondria and vesicles. A *KLC1* variant was found to extend a protective effect against multiple sclerosis [76]. It has been hypothesized that this gene plays an important role in the disease pathogenesis of Alzheimer's disease during early stages [77] and acts as a susceptibility gene to the development of age-related cataract [78]. *KLC1* was found to downregulate by 3.9-fold in TBM co-infected with HIV patients as compared to controls. *HSPA8* mRNA, which encodes for a protein belonging to the heat shock protein 70 family, was found to be 14-fold downregulation in TBM cases co-infected with HIV showed. *HSPA8* had been reported to be differentially expressed in patients with schizophrenia [79,80]. Sequence variations in this gene had also been implicated in the development of schizophrenia [81]. It has also been as observed to be expressed in stressed neurons during acute phase of epilepsy [82].

Differentially expressed genes in TBM co-infected with HIV as compared to TBM cases

We used the gene expression data from TBM patients co-infected



with HIV and compared to our earlier study on gene expression of patients with TBM but without HIV infection [34]. Unpaired t-test was performed to determine differential expression of genes in patients with TBM alone as compared to TBM patients co-infected with HIV. We found 49 genes to be differentially expressed with p-value cut-off of 0.005 and a fold-change cut-off of ≥ 2 . The list of the differentially expressed genes between two groups is provided in Supplementary Table 3. We discuss below a subset of genes/genes products which are differentially expressed in TBM patients co-infected with HIV as compared to TBM patients.

Solute carrier family 1, member 2 (*SLC1A2*), otherwise known as excitatory amino acid transporter 2 (EAAT2), is a membrane bound protein expressed by neurons and glial cells is known to be involved in extracellular glutamate clearance in the CNS [83]. Glutamate transported dysfunction caused by viral infections (HIV and human herpesvirus 6 (HHV-6)) has been directly correlated with the reduced expression of EAAT2 [83]. We also observed reduced expression of *SLC1A2* in TBM patients co-infected with HIV as compared to TBM patients without HIV infection. Leucine rich repeat and fibronectin type III domain containing 4 (*LRFN4*) is a neuronal transmembrane protein that has been shown to interact with NMDA receptors which are ionotropic glutamate transporters involved in multiple neuronal functions [84]. We found *LRFN4* to be 2-fold overexpressed in patients with TBM co-infected with HIV as compared to TBM. Lysophosphatidic acid receptor 3 (*LPAR3*) is a member of the G protein-coupled receptor and the EDG family of proteins. *LPAR3* receptor has been shown to play a role in the infiltration of leukocytes during inflammation [85]. In the present study, we observed a 4-fold downregulation of *LPAR3* transcript in TBMHIV as compared to TBM alone. Aggrecan (*ACAN*) is a chondroitin sulfate proteoglycan well known for its function in cartilage as a structural component [86]. However, studies have shown the expression of this protein in the CNS, specifically in the cerebral cortex, the precise function of this protein in neuronal cells is not clear [86]. We observed this gene to be 4-fold upregulated in TBM patients co-infected with HIV as compared to patients with TBM. Other notable genes that were differentially expressed include BCL2-like 15 (*BCL2L15*), THAP domain containing 9 (*THAP9*), Ferric-chelate reductase 1 (*FRRS1*) and *XAF1* (XIAP associated factor 1) which were upregulated 5-fold, 5-fold, 4.8-fold and 3.6-fold, respectively.

Biological network analysis

We have used GeneSpring GX v11.0.2 (Agilent Technologies, Santa Clara) software for pathway analysis. Differentially expressed genes with a fold value cut off of ≥ 5.0 were chosen as an input file. The differentially expressed genes derived from the microarray data were employed to construct a gene network with the organism-specific interaction databases that include targets and regulators of the molecules as well as biological processes associated with the molecules. To achieve a greater confidence of the networks expression values were overlaid on the networks and applied biological filters such as metabolism, transport, binding, expression, regulation, promoter binding of the molecules. Our analysis identified networks that could be indicative of various biological contexts. We identified a subnetwork involving the transcription factor signal transducer and activator of transcription 1 (*STAT1*), shown in (Figure 4A), which is involved in the transcription of various cytokine-induced genes. *STAT1* plays a role in HIV induced BBB disruption. The consequences of this may have a role in HIV neuropathogenesis [87]. *STAT1* interacting molecules include interleukin 27 (*IL27*), interferon, epsilon (*IFNE1*),

interleukin 27 receptor alpha (*IL27RA*), chromosome 19 open reading frame 10 (*C19orf10*), interleukin 17D (*IL17D*), signal transducer and

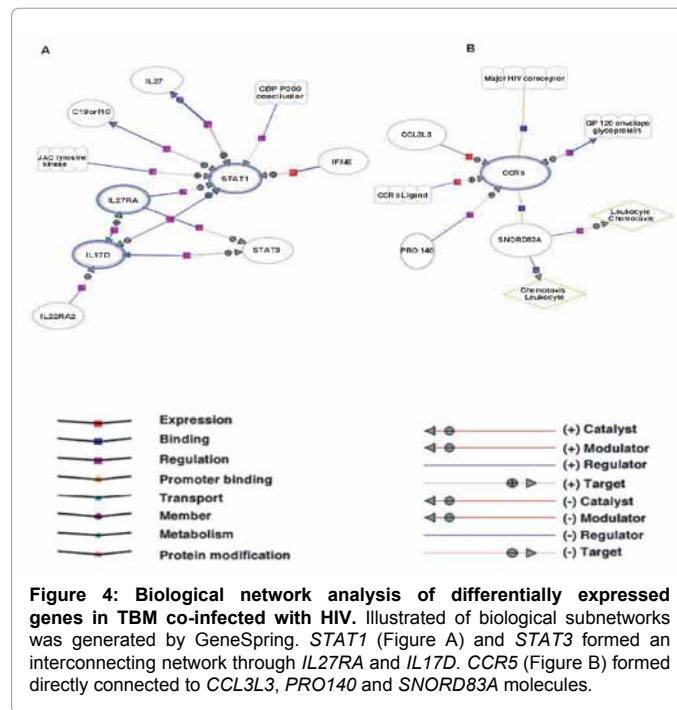


Figure 4: Biological network analysis of differentially expressed genes in TBM co-infected with HIV. Illustrated of biological subnetworks was generated by GeneSpring. *STAT1* (Figure A) and *STAT3* formed an interconnecting network through *IL27RA* and *IL17D*. *CCR5* (Figure B) formed directly connected to *CCL3L3*, *PRO140* and *SNORD83A* molecules.



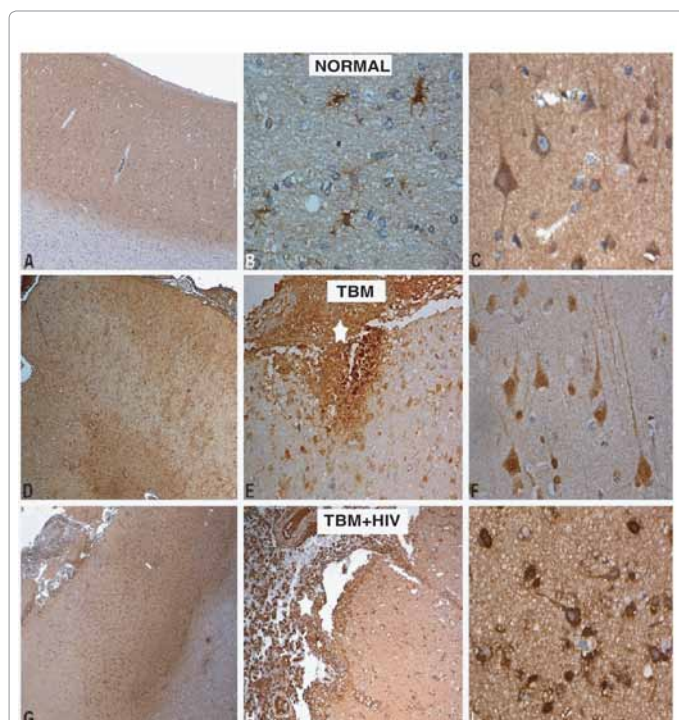
[A, C, F: xObj.1.6, B: Obj.x5, D, F: xObj.20]

Figure 5: Immunohistochemical labeling of GFAP. A-B: GFAP expression in normal cortex is seen confined to subpial zone (B), with relatively low labeling of cortical ribbon and diffuse labeling of white matter (A,B). C-D: TBM showing upregulation of GFAP diffusely and in patchy manner in cortical ribbon (A), subcortical U fibers and white matter (A). The reactive astrocytes with thick fibers are seen surrounding epithelioid granuloma (asterix, D) E-F: In case of TBM with HIV, subpial gliosis and dense gliosis in subcortical U fibers and white matter extending into the cortical ribbon as triangular zones is seen (E). Higher magnification of subpial zone shows dense band of labeling streaming thick glial fibers into underlying cortex are noted (F).

activator of transcription 3 (*STAT3*), interleukin 22 receptor alpha 2 (*IL22RA2*). Their roles have to be further evaluated in the context of TBM co-infected with HIV cases. Another subnetwork identified in our analysis is chemokine (C-C motif) receptor 5 (*CCR5*), which is shown in Figure 4B. *CCR5* is expressed by macrophages, T-cells and dendritic cells. *CCR5* used as a co-receptor for HIV to enter in to the target cells and *CCR5* plays a role in HIV pathogenesis [88]. *CCR5* binding molecules include chemokine (C-C motif) ligand 3-like 3 (*CCL3L3*), small nucleolar RNA, C/D box 83A (*SNORD83A*) and PRO 140. PRO 140 is a monoclonal antibody found on T-lymphocytes of immune system to inhibit *CCR5* and Progenics Pharmaceuticals group has been investigating this molecule as a potential therapy in the treatment of HIV infection [89]. The role of *CCR5* and its interacting partners have to be elucidated further in TBM co-infected with HIV cases.

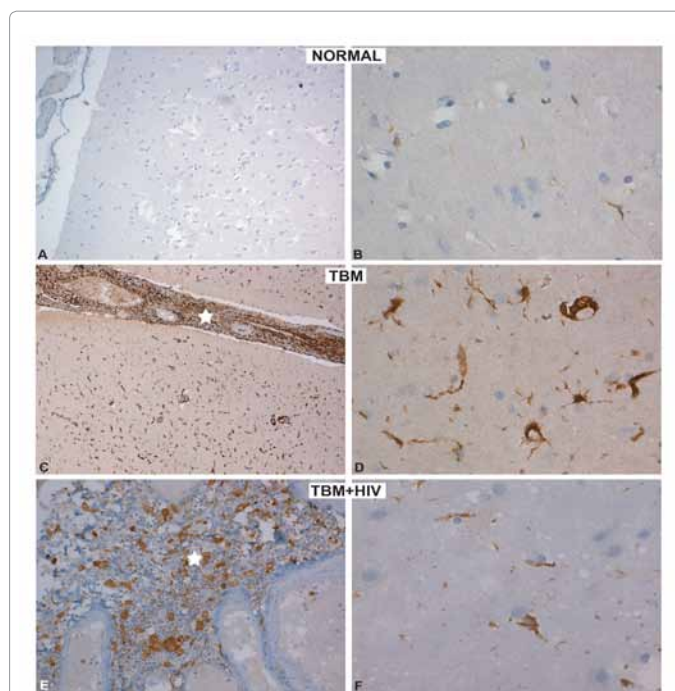
Validation of potential candidates by immunohistochemical labeling

We chose IHC based validation for three upregulated and one downregulated molecule from the differentially expressed genes identified in this study. These molecules were selected based on their biological significance and fold-change. IHC was performed on tissues from 10 cases of TBM+HIV (including the cases used in microarray experiments), and 15 TBM only cases, in addition to 10 control cases.



[A,D,G: Obj.x5, B,E,H: Obj.x 10, C,F,I: Obj.x20]

Figure 6: Immunohistochemical labeling of SERPINA3. A-C: Normal cortex reveals labeling of cortical ribbon in synaptic pattern (A). The neurons with dendritic processes in grey matter (C) and branching fibrous astrocytes in white matter are labeled (B). D-F: In cases of TBM, intense labeling of cortical ribbon is seen (A), concentrating around exudates in meninges (E, asterix) and in neurons with dendritic arborization (F). Note dense labeling of inflammatory cells in exudates (E, asterix) G-I: In cases of TBM with HIV, the labeling of cortical ribbon (G), neurons (I) and glial cells close to inflammatory exudates in subarachnoid space (H, asterix) is more intense than in TBM alone. In addition, a few glial shrubs intensely labeled are found in the cortical ribbon (I) unlike in cases of TBM without co-infection with HIV.



[A, C; Obj.x5, E: xObj.10, B, D, F: Obj.x20]

Figure 7: Immunohistochemical labeling: TYMP. A-B: Normal cortex shows minimal expression in cortex restricted to scattered microglial cells in grey matter (B). C-D: In cases of TBM, intense expression was seen in meningeal exudates (C, asterix) and microglial cells in cortical ribbon (C, D) and white matter was strongly labeled highlighting branching pattern of activated microglia. E-F: In cases of TBM with HIV, labeling of meningeal exudates (E, asterix) was more intense compared to cases of TBM, while activated microglia in grey matter attained amoeboid morphology (F).

GFAP

GFAP protein expression in normal cortex was restricted to a narrow band in the subpial zone with very minimal expression in the underlying grey matter (Figure 5A and figure 5B). The white matter demonstrated uniform labeling of the glial fibers parenchyma in addition to highlighting the fibrous astrocytes (Figure 5A). In TBM in contrast, prominent reactive astrocytosis was evident in the subpial zone bordering meningeal exudates (Figure 5C and figure 5D). This extended into the underlying cortical ribbon to variable extent with perivascular collections of reactive astrocytosis and the white matter also revealed diffuse astrogliosis. The astrocytes were hyperplastic with accumulation of GFAP distending the cytoplasm and producing irregular thick processes surrounding foci of meningeal exudates (Figure 5D). In cases of TBM co-infected with HIV, similar astrocytosis was evident in upper layers of cortex and perivascularly in the grey in large patchy zones (Figure 5E and figure 5F). In the white matter, the astrogliosis was extensive compared to normal cortex and cases of TBM (Figure 5E).

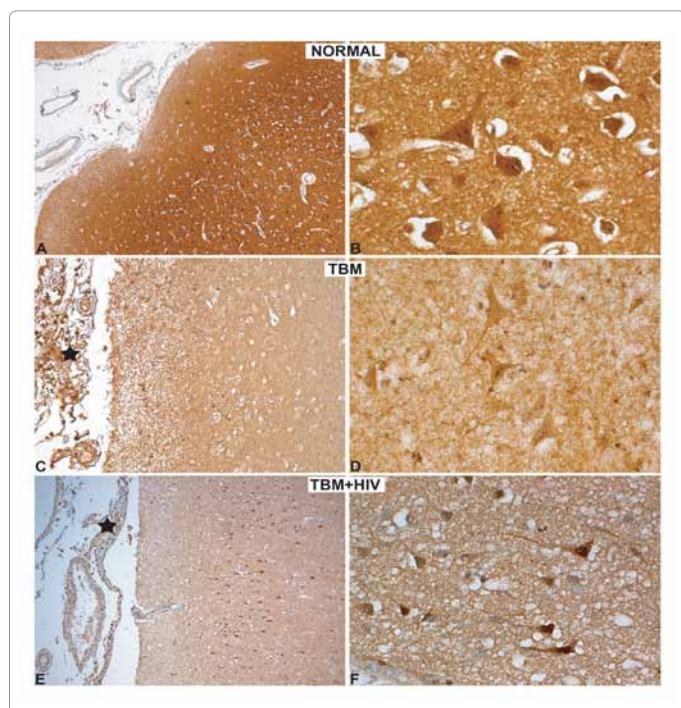
SERPINA3

In normal control cases, SERPINA3 protein was seen in the cortical ribbon with light to moderate labeling of neuropil and neuronal soma (Figure 6A and figure 6C). The white matter demonstrated labeling restricted to astrocytes, highlighting branching processes of fibrillary astrocytes (Figure 6B). In TBM cases, there was marked upregulation of SERPINA3 expression in grey and white matter (Figure 6D) with

strong labeling seen in reactive astrocytes in subpial molecular layer underneath the meningeal exudates (Figure 6E). The neurons also showed variably intense labeling of cytoplasm spreading along the apical dendrites and occasionally in the nuclei (Figure 6F). Several parenchymal vessels showed expression of SERPINA3 in the vascular lining endothelium and wall. Similarly inflammatory cells in the meningeal exudates revealed cytoplasmic labeling Figure 6E. In the white matter, the astrocytes and glial processes revealed accumulation of SERPINA3. In cases of TBM with HIV, in the grey matter, strong labeling was seen in neuronal cytoplasm and occasionally nuclei and to a lesser extent in the neuropil (Figure 6G and figure 6H) whereas in the white matter, several oligodendroglial cells in addition to astrocytes revealed nuclear labeling. Several glial cells with granular labeling of cytoplasm were highlighted in grey and white matter resembling glial plaques. (Figure 6I).

TYMP/ECGF1

TYMP protein was overexpressed in cases of TBM and TBM co-infected with HIV as compared to control (Figure 7F). TYMP in normal cerebral cortex showed very low expression (Figure 7A) mostly restricted to scattered microglia in grey and white matter (Figure 7B). In cases of TBM and TBM co-infected with HIV, significant upregulation of TYMP was evident in the macrophage rich meningeal exudates in subarachnoid space and in the inflamed grey matter, bordering the meningeal exudates (Figure 7C and figure 7E). Strong labeling of activated microglial with branching processes were highlighted in both TBM (Figure 6D) and TBM with HIV cases (Figure 7F).



[A, C, E: xObj, 5, B, D: Obj.x20, F: Obj.x10]

Figure 8: Immunohistochemical labeling: HSPA8. A-B: Normal cortex shows intense labeling of neuropil of cortical ribbon (A) and neuronal soma (B). C-D: In cases of TBM, the labeling of both the neuropil (C) and neurons in grey matter is low (D) reflecting downregulation. The histiocytes in the exudates (C, asterisk) are intensely labeled. E-F: In TBM with HIV, the neuropil labeling is very low (E) except for few large pyramidal neurons (F) that reveals nuclear labeling and pale dendritic arborization. Labeling is also seen of meningeal macrophages in the subarachnoid space (E, asterisk).

HSPA8/HSC70

HSPA8/HSC70 protein, in cerebral cortex of normal controls, revealed intense labeling in the grey matter highlighting neuronal soma as well as nucleus in addition to diffuse labeling of neuropil in the cortex and to a lesser extent the white matter (Figure 8A and figure 8B). In the white matter, astrocytes and oligodendroglial cells showed variable labeling of nucleus and cytoplasm (not shown). Meningeal blood vessels and few vessels in the parenchyma demonstrated positive expression in smooth muscle coat and endothelium. In cases of TBM (Figure 8C and figure 8D), decreased expression was evident in comparison with controls with light labeling of neuropil, scattered neuronal and glial cell labeling in grey and white matter. Inflammatory infiltrates and epitheloid granuloma in the subarachnoid space and vascular smooth muscle of meningeal vessels revealed moderately strong labeling. Similarly in cases of TBM co-infected with HIV, reduced expression in neuropil and neuronal soma was evident in comparison with controls (Figure 8E and figure 8F).

Conclusions

TBM is one of the most frequent forms of chronic meningitis. In tropical developing countries, a rise in HIV co-infections has worsened the scenario. The pathogenesis and tissue response of meninges inflamed because of *M. tuberculosis* infection in the presence of HIV is not well understood at the molecular level. To elucidate a candidate biomarker and for better understanding the molecular mechanisms involved in disease process, we undertook the present study on human brain tissue from cases of TBM co-infected with HIV. We identified many novel transcripts in the context of TBM co-infected with HIV. Differentially regulated expression levels of transcripts of *GFAP*, *SERPINA3*, *TYMP* and *HSPA8* were confirmed by IHC validation. We believe that further studies on these (*SERPINA3*, *TYMP* and *HSPA8*) candidates could lead to development of biomarkers.

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