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Combined gene expression analysis in HIV Associated Dementia, Alzheimer's disease and Parkinson's disease- An in-silico approach

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ABSTRACT

Human immunodeficiency virus (HIV) Type 1 infection predominantly affects the immune system. Nevertheless, scientific studies have proven its association with the Central Nervous system (CNS) causing several neurological complications leading to HIV Associated Dementia (HAD). HAD is characterized by a progressive, disabling decline in essential CNS functions such as cognition, motor control and behavior. These are the general characteristics of the most common Neuro degenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD). The genetics of AD and PD is widely studied and clinical studies have shown cohesion in the pathology of HAD, AD and PD. Analysing the concurrent expression patterns of large number of genes amongst these related diseases will aid in establishing correlations between the genes and their functions.

We have analysed the gene expression datasets of HAD, AD and PD from GEO database to determine the overlapping genes and transcription factors involved. The datasets were normalized using R-Bioconductor, and statistical analysis was performed to identify the significant genes using limma and related packages in R. Although substantial amount of common proteins among HAD and other Neuro degenerative diseases have been previously reported, our findings can help in expanding the pool of target genes and further enhancing the knowledge about the convergent pathways among HAD, AD and PD. These common markers identified will provide insights into parallel pathways of disease mechanisms and further assist in the understanding of progression of HAD pathogenesis.

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1. INTRODUCTION

The existence of HIV infection in patients suffering from AIDS induces HIV Associated Dementia (HAD), a neurological condition and a common complication of HIV disease. The pre-highly active antiretroviral therapy (HAART) era reported the prevalence of HAD at least 20% - 30% in advanced HIV infections [1]. With the advent of HAART, incidence of HAD has decreased; however, there is an increase in its prevalence due to the prolonged existence of HIV [2]. HAD patients generally suffer from HIV-associated Encephalitis (HIVE)

with viral replication limited to cells of monocyte origin [3]. The neuropathology of HIVE includes infiltration of central nervous system (CNS) by macrophages, activation of microglial cells, perivascular or parenchymal monocytes, multinucleated giant cells, and lymphocytes [4], [5]. Additionally, HIVE and HAD are associated with substantial neuronal loss, formation of abnormal dendrites, secretion of chemokines, pro-inflammatory cytokines, nitrous oxide, and other neurotoxic factors produced by infected and activated cells leading to damaging cellular responses. This is followed by the altered gene expressions of neuronal growth factors, cytokines and cell death genes leading to potential impairment of blood–brain barrier permeability, development of brain atrophy lastly culminating in brain injury [5 - 7]. Scrupulous investigations of HIV-1 infection carried out over the years have identified various factors and genes associated with the pathogenesis of HAD, constituting T-cell receptor-mediated signaling, subcellular trafficking, transcriptional regulation, and diverse cellular metabolic pathways [8]. Remarkable gene expression profiles have been demonstrated as a result of HIV-altered signal transduction of neuronal cells along with alteration in astrocyte function [9]. Diverse range of serological markers like tumour necrosis factor alpha, monocyte chemo-attractant protein 1, interleukin-6, and high-sensitivity C-reactive and soluble CD14 proteins are recognized to play a role in progression of HAD pathogenesis leading to rigorous metabolic alterations [10]. Nevertheless, despite the numerous advances in the revelation of the HIV-1 pathophysiology, the molecular mechanisms implicated in HIV associated dementia remains poorly understood.

Gene expression studies have been extensively used to investigate the various cellular mechanisms linked to HAD pathogenesis [11]. Ongoing studies dealing with genomic and transcriptomic factors underlying the disease have provided significant insights about neuro-pathogenesis associated with HAD and has positively driven identification of treatment targets. Gelman et al. in their study revealed two distinct transcriptome profiles linking two distinct pathways to HIV associated neurocognitive disorder (HAND). A complete variation in the expression was observed in HAND patients with and without HIVE which reflected the up-regulation and down-regulation of the inflammatory pathways [12]. Numerous studies have drawn parallels among diverse neurodegenerative diseases with respect to pathophysiology of unusual protein assemblies suggesting commonalities with HAD development [13]. Studies dealing with identification of amylodoigenic potential of HIV-1 proteins has revealed that they have a potential to contribute aggregation [14]. Thus, along with the physiologic features, these diseases do share common genetic factors leading to the diseased state. AD is a type of dementia which is ranked third by the experts and is a widely studied multifaceted, irreparable, progressive brain sickness, which reduces memory and thinking skills in a slow pace [15]. A study on the neurocognitive impairment in AD and HIV identified commonly associated genes between them and reported the dysregulation of shared mitochondrial gene networks and upregulation of various cancer-related genes [16]. A meta-analysis of transcriptomes derived from different regions of brain from individuals with HIVE and AD also presented shared up-regulation of several immune response genes and downregulation of synaptic transmission and cell signalling genes [17]. Neurodegenerative diseases with diverse etiologies possess a general characteristic feature of amyloid fibril deposition in brain tissues linked with neuronal apoptosis and cell death [18], [19]. Thus, existence of various commonly affected pathways in these diseases is definitely not astonishing.

In accordance with this fact, and in the view of expanding the pool of target genes we have analyzed the gene expression data from HAD, PD and AD datasets. PD is one of the most studied and the second most widespread neurodegenerative disorder of the central nervous system [20]. The analysis involves individual assessment of respective datasets as well as a combined analysis to put forth the convergent set of genes that are concurrently affected in these three diseases. Normalized gene expression data is subjected to statistical analysis for determining the most significantly affected genes and the possible role of few genes is discussed further. The prospect of the study is to provide insights into the identification of common disease mechanisms underlying HAD and other neurodegenerative diseases.

2. RESEARCH METHOD

2.1 Gene expression datasets

The raw gene expression profiling datasets by three individual studies of AD, PD and HAD were obtained from Gene Expression Omnibus database, an online and publicly available repository that archives microarray, next generation sequencing, and other forms of high- throughput functional genomic data [21]. The microarray engineering offers unique chances to obtain molecular signatures of the region of activity of diseased cells and patient samples. A microarray is a 2D array on a solid substrate that evaluates large amounts of biological samples using high-throughput screening. Using random sampling technique 39 non-demented

controls and 43 AD affected samples from the AD dataset series GSE528; 9 control and 16 disease replicate PD dataset from series GSE7621 and 18 controlled and 36 affected HAD dataset from series GSE35864 samples were used in the current study. All these experimental data is generated on GPL570 [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array, ensuring uniformity for combined integrated analysis.

Table 1. Count of differentially expressed genes determined in SAM and T-test analysis

Dataset	SAM	T-LIMMA (p value < 0.05)	Common
AD	9682	12806	7205
PD	426	188	141
HAD	1044	146	109
Combined	6087	10838	4796

2.2 Dataset Normalization

Normalization was done for resolving the systematic errors and bias introduced by the microarray experimental platform; thus, correcting hybridization intensity to make decisive analysis [22]. The Robust Multi array Average (RMA) method available in Bioconductor's affy package was applied in this study. RMA process includes 3 steps: 1) RMA Background Correction; To distinguish the probe-level signal from background signal (optical noise & non-specific binding), 2) RMA Normalization; makes all the arrays to have same quantile values, thus removing all the variations incorporated during sample preparation and hybridization and 3) RMA Summarization; a robust system for centering, using probe-level background-corrected and quantile normalized intensities thus giving summarized expression values [23]. Individual diseased datasets as well as combined data sets of our study have been normalized and converted to expression set objects for statistical analysis using R software package

R Version 3.4.1 (https://www.r-project.org/) is a language and environment for statistical computing and graphics. R provides a wide variety of statistical (linear and nonlinear modelling, classical statistical tests, time-series analysis, classification, clustering) and graphical techniques [24].

Bioconductor-3.5 (https://www.bioconductor.org/) is an open source and open development software project based on R used for the analysis and comprehension of high-throughput genomic data. Bioconductor is based on the R programming language. Major workflows in Bioconductor include pre-processing, quality assessment, differential expression, clustering and classification, and gene set enrichment analysis [25]. Our study uses affy and its dependent packages to read and normalize data and limma and its dependent packages to perform differential expression of genes.

2.3 Statistical Analysis of Differential Expression

Identification of differentially expressed genes in individual diseased datasets and combined dataset was carried out using limma package in Bioconductor [26]. Normalized data is submitted for statistical tests like t-test and SAM test. Limma uses moderated t-statistic with shrunken standard deviation for each gene to identify gene specific difference between means of expression levels of 2 groups (Control and Affected) [26]. The topTable( ) method returns a table ranking the genes according to evidence for differential expression. Genes with adjusted p value < 0.05 are identified to be differentially expressed. SAM (Significance Analysis of Microarrays) is performed using Web-enabled and Cross-platform SAM via Shiny (https://github.com/MikeJSeo/SAM) which is a statistical system for identifying significant genes in a set of microarray investigates. The input to SAM is gene expression measurements from the normalized microarray datasets, as well as a response variable from each dataset. The response variable may be a grouping like control and diseased (either unpaired or paired) samples. SAM computes a statistic for each gene, measuring the strength of the relationship between gene expression and the response variable. In SAM delta value of 1.01 is used to control the false positives. Web platform lists the significant genes in order of q value. Lesser the q value results in more statistically significant results. SAM test was performed for 100 permutations with mean difference as 10 and delta value as 1.

2.4 Metabolic engineering analysis

Metabolic engineering provides a framework for genome-wide differential gene expression data analysis. It combines with data on protein content that further correlates to the pathways involved, thus having biotechnological applications for screening candidate drugs or designing gene therapies [27]. Here in we have analyzed metabolic engineering pathways of the ITPKB gene in combined dataset using KEGG database [28].

3. RESULTS AND ANALYSIS

The normalized expression sets of individual diseases were compared to check for overlapping genes; however no overlapping genes were found among the three diseases. Hence, an integrated analysis was performed wherein the raw datasets of all three diseases were normalized together to form a combined normalized set and then subjected to statistical analysis.

3.1 Box plot analysis

Box plots are graphical tools to visualize key statistical measures, such as median, mean and quartiles [29]. Boxplots for microarray experiments shows distribution of overall gene expression values between arrays. Meaningful statistical analysis and inferences from the data can be performed only if the samples are comparable. Boxplots helps to visualize the expression across samples by comparing raw and normalized data. After normalization, probe intensity values are centred on the median and the range of distribution is reduced by eliminating the highly diverse values as outliers. The supplementary file (SF) figures 1 - 3 displays the box plots of raw and normalized AD, PD and HAD datasets respectively. SF figures 4 and 5 displays respective box plots of raw and normalized data of the combined (all three diseased states) dataset.

3.2 Histogram Analysis

A histogram is used to graphically summarize and display the distribution of a processed data set. A frequency distribution shows how often each different value in a set of data occurs. It is most commonly used to show frequency distributions of log2 intensities of perfect match probes for comparison of probe intensity behavior between different arrays. Histograms of all the individual datasets have been plotted for visualization while histogram for the combined dataset (raw and normalized) is displayed in SF figure 6.

3.3 Visualization of data

In heat maps, the data is represented in a grid where each row denotes a gene and each column denotes a sample. A heat map of normalized expression dataset gives a graphical representation of individual gene intensity values with respect to the samples under study in form of a color scheme. It also depicts hierarchical or k-means clustering of the samples (normal vs diseased) according to their expression values, so as samples expressing similar variations in gene intensities are clustered together forming a tree like structure (https://www.ebi.ac.uk/training/online/course/functional-genomics-ii-common-technologies-and-data-analysis-methods/biological-0). Figure 1 represents the heat map generated from the integrated data for AD, PD and HAD datasets reflecting gene expression values in several conditions.

3.4 Statistical Tests

T test and SAM test listed a significant amount of differentially expressed genes in individual datasets of AD, PD and HAD as well as in combined dataset as described in Table 1. The common genes identified from SAM and T-test were considered to form a consensus dataset. Table 2 and 3 describes the top 5 upregulated and 5 downregulated genes from the consensus set of 4796 genes identified after statistical tests. We have also derived set of overlapping genes between the consensus genes of Combined Dataset and AD. Total of 5171 genes were identified to overlap out of which top 5 upregulated and top 5 downregulated genes are described in SF table 1 and 2. A total of 72 overlapping genes were identified between the consensus set of combined and HAD genes. SF table 3 describes the top 5 genes of this set. No downregulated genes were obtained in this combination. A total of 63 overlapping genes were identified between consensus set of combined and PD. SF table 4 describes top 5 and SF table 5 describes the top 3 genes of this combination. About 55 overlapping genes were derived between the consensus set of HAD and AD as denoted in SF table 6, however no genes were identified to overlap between the HAD and PD dataset. SF table 7 describes top 5 genes found to be upregulated in HAD but downregulated in AD. The total genes set derived of each combination will be made available in excel sheet upon request to the author.

ITPKB gene identified to be upregulated in the top 5 differentially expressed genes set of combined dataset as shown in table 3 is reported to be involved in enhancing amyloid production by overexpressing in the neurons of APP mice. ITPKB is involved in Inositol phosphate metabolism and Calcium signaling pathway. The protein Inositol-trisphosphate 3-kinase B is responsible in regulating the levels of several inositol polyphosphates that are critical in cellular signaling [30]. Salta et al. in their study have also reported the upregulation of ITPKB in human AD patients [31]. Thus, its inclusion in this combined set explains that this gene is upregulated in all three conditions AD, PD and HAD. It is also evident from table 10 that it is one of the top five differentially expressed genes between HAD and AD. NFASC gene codes for neurofascin protein functions in neurite outgrowth, neurite fasciculation, organization of the axons and involved in neural development [32]. NFASC has been reported to be a predicted target of hsa-miR-5010-3p in AD [33]. HIPK2 found to be upregulated in the combined set is reported to be associated with ER-stress-mediated neurodegeneration on activation and is a potential biomarker [34]. CFLAR (C-FLIP) can suppress caspase 8 activation and mediate apoptosis and its activities are seen to be increased in AD [35]. Along with CFLAR, PDCD6 is a part of the functional group of genes that represent a cluster of B-cell lymphoma/leukaemia 11B (BCL11B) targets and reported to have a relative abundance in frontal cortex samples in the study performed on latent HIV infection [36]. BCL11B is an important transcriptional regulator in the brain [37]. PDCD6 was found to be overlapping in the top 5 upregulated genes of HAD and AD. One of the common gene seen to be upregulated in HAD and AD is LPP coding for lipoma preferred partner that has already been reported to show an increase in AD [35]. Most of the genes found in the integrated study have a higher fold change value for upregulation than downregulation. Our combined analysis of all three disease datasets has shown that HAD shares more differentially expressed genes with AD than with PD. Shapshak et al. in their work on determining relationship between AD and HAD with respect to structure, expression and function have reported genes like APBB1 APBB2, APBB3, APLP2 which are known biomarkers in AD to be sharing close chromosomal locations with genes viz. HSHIN1, LOC391810, HTATIP2 HTATIP that are expressed in HAD condition [38]. Few of the standard deviated genes identified in our study are discussed here, however the knowledge base of such genes is quite huge and thorough study on each and every gene may give possible prospects of it to serve as biomarker for these diseases.

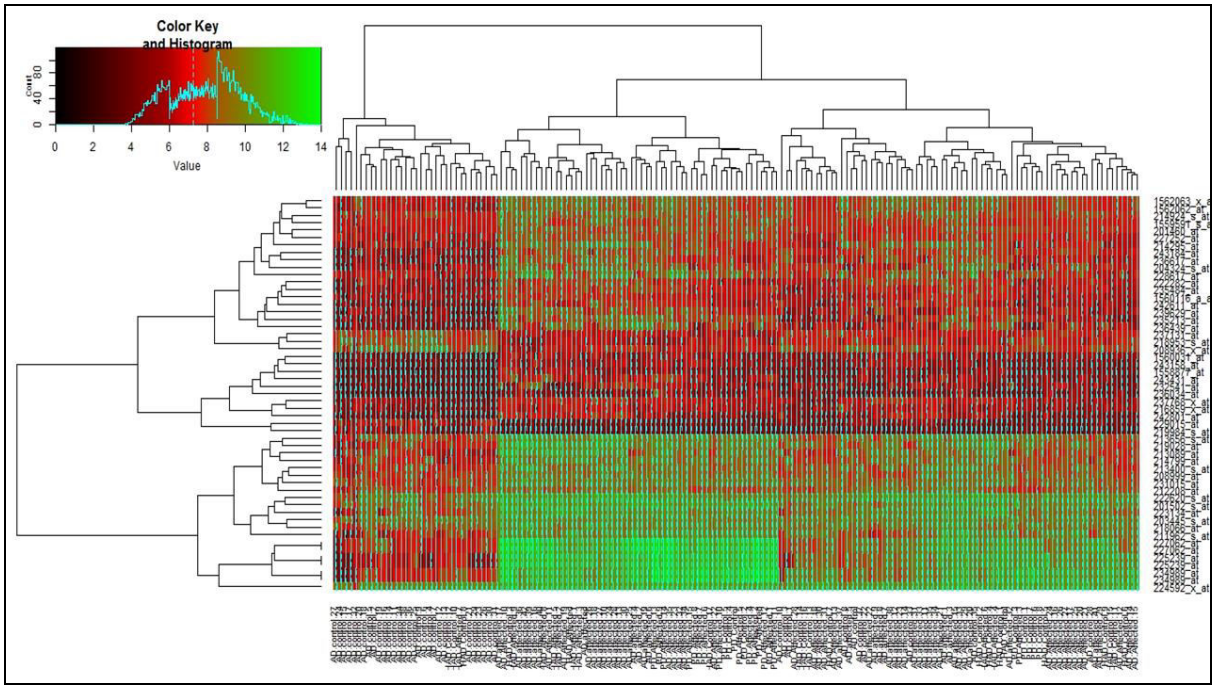

Figure 1: Heat map generated from the integrated data for AD, PD and HAD datasets reflecting gene expression values in several conditions.

Table 2. Top 5 upregulated common (SAM and T-test) genes from combined dataset

Gene Symbol	Description	T-Statistics	P-value	SAM Score	Log Fold Change
PTAR1	Protein prenyltransferase alpha subunit repeat containing 1 [*Homo sapiens*	8.55	1.52E-10	7.047	1.827

	(human)]				
ITPKB	Inositol-trisphosphate 3-kinase B [*Homo sapiens* (human)]	8.256	6.24E-10	7.209	2.541
NFASC	Neurofascin [*Homo sapiens* (human)]	8.231	6.24E-10	7.028	1.972
HIPK2	Homeodomain interacting protein kinase 2 [*Homo sapiens* (human)]	7.823	3.06E-09	6.88	2.252
CFLAR	CASP8 and FADD like apoptosis regulator [*Homo sapiens* (human)]	7.728074	4.73E-09	6.781	2.384

Table 3. Top 5 downregulated common (SAM and T-test) genes from combined dataset

Gene Symbol	Description	T-Statistics	P-value	SAM Score	Log Fold Change
PCYOX1L	Prenylcysteine oxidase 1 like [*Homo sapiens* (human)]	-7.96396	1.67E-09	-6.483	0.559
LGALS8	Galectin 8 [*Homo sapiens* (human)]	-7.43614	1.36E-08	-6.150	0.56
RIIAD1	Regulatory subunit of type II PKA R-subunit (RIIa) domain containing 1 [*Homo sapiens* (human)]	-7.41312	1.39E-08	-5.983	0.6514
FAR2	Fatty acyl-CoA reductase 2 [*Homo sapiens* (human)]	-6.85978	1.13E-07	-5.779	0.5311
ASAH2B	N-acylsphingosine amidohydrolase 2B [*Homo sapiens* (human)]	-6.74849	1.69E-07	-5.741	0.4979

4. CONCLUSION

Despite the differences in the mechanisms of AD, PD and HAD, the integrated analysis of these diseases has revealed the overlap of differentially expressed genes in this condition. Analysis of HAD and AD datasets have presented a potential gene pool of commonly affected genes, while genes shared by HAD and PD did not appear in the differentially expressed category. However, a combined integrated analysis did put forth few genes possibly affected in neurodegenerative conditions caused by viral as well as non-viral counterparts. Identification of biomarkers may assist researchers and clinicians in forecasting the onset of the disease progression and in determining the effects of new therapies. A meta- analysis approach allows us to have an overall assessment of the gene expressions of the genes involved in various diseases and integrated analysis may aid to present genes shared by similar diseases.

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SUPPLEMENTARY FILES

1. BOXPLOTS, HISTOGRAMS and TABLES

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