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Identification of molecular signatures and pathways common to blood cells and brain tissue of amyotrophic lateral sclerosis patients

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a progressive motor neuron disease that is characterized by the death of neurons controlling voluntary muscles. Early diagnosis of ALS is difficult and detection is limited in sensitivity and specificity as well as by cost. Therefore, detecting ALS from blood cell analysis could improve the early diagnosis and treatment of the disease. The present study aimed to identify blood cell transcripts that reflect brain expression levels of factors linked to ALS progression. We analyzed blood cell and brain transcriptomics gene expression datasets (RNA-seq and microarray) in blood and brain. We identified 13 differentially expressed genes (DEG; ALS versus controls) common to blood cells and brain (DNAH6, HLA-DMB, HLA-A, EHD2, CMKLR1, PROS1, GAPT, CCR1, THBS1, CDK2, RAB27A, ITGB3 and C1orf162) that were commonly dysregulated between ALS blood and brain tissues. These data revealed significant neurodegeneration-associated molecular pathways in the signaling systems. Integration of these different analyses revealed dysregulation of a number of transcription factors, namely SP1, MYC, TP3, CTCF and SRF. In addition, we identified microRNAs altered in ALS: miR-29c, miR-21, let-7a, miR-377, miR-103, miR-369-3p, miR-494, miR-204, miR-29a. Thus, we have identified possible new links between pathological processes in the brain and transcripts in blood cells in ALS subjects that may enable the use of blood samples to diagnose and monitor ALS onset and progression.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive fatal neuromuscular disease that predominantly affects upper and lower motor neurons [1,2]. While the great majority of cases are classed as sporadic ALS (sALS), i.e., without evidence of inherited susceptibility, a minority of cases (typically cited as around 10%, but depending on the stringency of heritability definitions) do show evidence of familial involvement (fALS) [3]. The underlying causes of ALS and the mechanisms that drive the associated neurodegeneration are unclear, even where genetic involvement is indicated. No cure for ALS exists, and there is a dearth of useful clinical biomarkers that might improve diagnosis or aid identification of clinical targets. Genetic and transcript levels in patient tissues can help identify possible new biomarkers to help define the strikingly similar phenotypes seen in ALS patients. Such studies have already identified a role for the disruption of RNA processing in ALS affected tissues with the discovery of fALS mutations in genes coding for RNA binding proteins (reviewed by Prasade et al. [4]), such as an RNA splicing and transportation factor TARDBP (TDP-43), which is important in neuronal development and in regulating expression of 601 genes [5] but which can also accumulate in cell inclusions. Mutations in TARDBP account for 5% of fALS cases. Other RNA-binding protein genes include FUS [6], TAF15, HNRNPA2B1, HNRNPA1, EWSR1 and ATXN2 [7]. Some of these are regulated by TARDBP, but over 15 other ALS-associated genes with a range of functions have been identified, many of which (e.g., SOD1) greatly affect the transcriptome, as do a number of micro-RNAs (miRNA) [4]). TARDBP protein has also been implicated in sALS, suggesting wider

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roles that those evident from heritable mutations. Thus, a strategy to identify features of RNA signatures in ALS for early diagnosis or mechanistic insights seems a promising one.

While the pathogenesis of ALS is likely to be multifactorial in nature, the application of molecular methods to improve diagnosis and assessment of ALS has yet to provide substantiated results and hence the quest for early ALS biomarkers in peripheral blood has received increased attention [8–11]. Successful identification of such blood molecular biomarkers could potentially have a high impact on ALS diagnosis, care and treatment [8]. Recently, there has been a number of studies that have profiled human tissues [12–19]. Several gene expression profiling studies have been performed in ALS to characterize its associated mRNA signatures [8–11,13,14,16,17], and a number of differentially expressed genes (DEGs) have been reported. However, these findings were limited at transcript level since functional interactions among the gene products were not considered. Since the biological molecules interact with each other to carry out functions in biological processes in cells and tissues, integrative analyses within network medicine context are essential to understand the molecular mechanisms behind diseases and to identify critical biomolecules.

We thus employed an integrative approach to identify molecular biomarker signatures for ALS that is expressed under similar genetic control in both peripheral blood cells and brain tissue using transcriptome analyses. Gene over-representation analysis was performed on mutually dysregulated DEGs between brain tissues and blood cells termed as core DEGs in ALS followed by gene ontology (GO) analysis. Pathway enrichment analysis was then used to identify the enriched pathways by the identified DEGs. These DEGs were further analyzed to identify regulatory factors, such as transcription factors (TFs) and microRNAs (miRNAs) that may influence levels of these DEGs in ALS-affected tissues. This study particularly focused on biomarker signatures at both transcriptional (mRNAs and miRNAs) and translational levels (hub proteins and TFs), as summarized in Fig. 1, in order to clarify pathogenic mechanisms in ALS and identify potential biomarkers for early diagnosis.

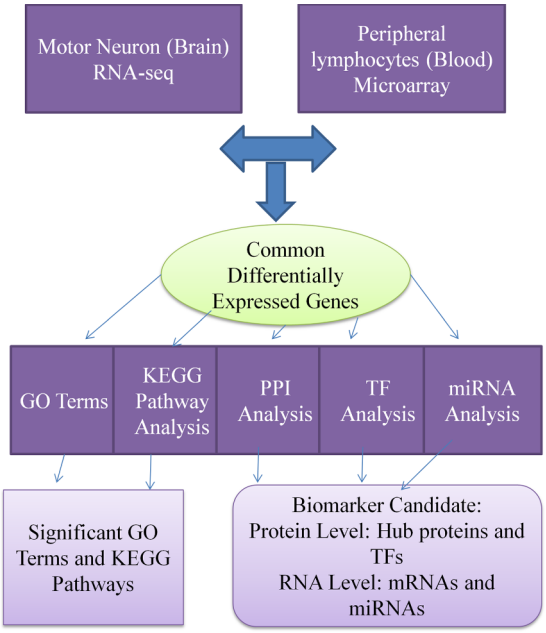

Fig. 1. The systems biology pipeline employed in this study. Gene expression datasets from Amyotrophic lateral sclerosis (ALS) matched control comparison studies of blood and brain tissue were obtained from the Gene Expression Omnibus (GEO) repository. The datasets were analyzed to identify common differentially expressed genes (DEGs) between brain and blood tissue. The significantly enriched pathways and Gene Ontology (GO) terms were identified through enrichment analyses. Protein-protein interaction network was analyzed to identify hub proteins. Transcription Factor (TF)-target gene interactions and microRNA-target gene interactions were also studied to identify regulatory biomolecules.

2. Materials and methods

2.1. Identification of differentially expressed genes from high-throughput RNA-Seq and microarray datasets

We obtained the gene expression Illumina microarray dataset GSE28253 (peripheral lymphocytes of blood tissues) and RNA-seq dataset GSE76220 (motor nerve) of ALS patients from NCBI-GEO database [20]. The RNA-seq data (GSE76220) was analyzed via GREIN [21] and identified DEGs with p-value < 0.05 and log2 absolute values for fold control FC ≥ 2. We applied linear models for microarrays (limma) through the GEO2R online tools in order to identify the DEGs from the peripheral lymphocytes dataset (GSE28253). The overlapping DEGs between the two datasets were considered for further analysis. The Benjamini-Hochberg (BH) method was used to adjust p-values.

2.2. Gene set enrichment analyses to identify gene ontology and molecular pathways

We performed gene set enrichment analysis via Enrichr [22] and NetworkAnalyst [23] to identify GO and pathways of the overlapping DEGs. The ontology comprised three categories: biological process, molecular function and cellular component. An adjusted p-value < 0.05 was considered as the cut-off criterion for all enrichment analyses.

2.3. Protein-protein interaction network analysis

We retrieved the PPI networks based on the physical interaction of the proteins of DEGs from the STRING database [24] through the NetworkAnalyst [23]. A medium confidence score of 400 was selected in the STRING Interactome. Network visualization and topological analyses were performed through NetworkAnalyst [23]. Using topological parameters, the degree (greater than 15°) was used to identify highly interacting hub proteins from PPI analysis.

2.4. In-silico cross-validation of the differentially expressed gene

In order to cross-validate the identified DEGs, we performed an in-silico cross-validation by comparing the DEGs with another microarray gene expression dataset with accession number GSE112680. This dataset is the whole blood gene expression profiling of ALS of 376 samples where 397 ALS patients and 645 control subjects. We analyzed the microarray dataset and identified the DEGs based on with adjusted p-value < 0.05 and absolute values of t-statistics ≥ 1.5 as statistically significant.

2.5. TF-miRNA coregulatory interactions analysis

We studied the TFs-miRNA coregulatory interactions obtained from the RegNetwork repository [25] to identify regulatory TFs and miRNAs that regulate DEGs of interest at the transcriptional and post-transcriptional level. The network was analyzed in NetworkAnalyst [23].

2.6. Identification of candidate drugs/small molecules

We performed drug-target enrichment or over-representation analysis using the DSigDB: drug signatures database gene sets [26].

Table 1
The functional enrichment analysis of differentially expressed genes to identify Gene Ontology terms in the ALS. Top 10 significant GO terms summarized.

Category	GO ID	GO Term	Adj p-value	Gene
Biological Process	GO:0010759	positive regulation of macrophage chemotaxis	0.0012	THBS1; CMKLR1
	GO:1905523	positive regulation of macrophage migration	0.0012	THBS1; CMKLR1
	GO:0002690	positive regulation of leukocyte chemotaxis	0.0012	CCR1; THBS1; CMKLR1
	GO:0071622	regulation of granulocyte chemotaxis	0.0020	THBS1; CMKLR1
	GO:0071675	regulation of mononuclear cell migration	0.0020	THBS1; CMKLR1
	GO:0032695	negative regulation of interleukin-12 production	0.0020	THBS1; CMKLR1
	GO:0010758	regulation of macrophage chemotaxis	0.0023	THBS1; CMKLR1
	GO:0002576	platelet degranulation	0.0028	ITGB3; PROS1; THBS1
	GO:0045055	regulated exocytosis	0.0042	ITGB3; PROS1; THBS1
	GO:0043277	apoptotic cell clearance	0.0046	ITGB3; THBS1
Molecular Function	GO:0001637	G-protein coupled chemoattractant receptor activity	0.0011	CCR1; CMKLR1
	GO:0019956	chemokine binding	0.0011	CCR1; ITGB3
	GO:0004950	chemokine receptor activity	0.0011	CCR1; CMKLR1
	GO:0017134	fibroblast growth factor binding	0.0011	ITGB3; THBS1
	GO:0004896	cytokine receptor activity	0.0096	CCR1; CMKLR1
	GO:0005178	integrin binding	0.0131	ITGB3; THBS1
	GO:0043184	vascular endothelial growth factor receptor 2 binding	0.0304	ITGB3
	GO:0019957	C–C chemokine binding	0.0304	CCR1
	GO:0035173	histone kinase activity	0.0304	CDK2
	GO:0005172	vascular endothelial growth factor receptor binding	0.0304	ITGB3
Cellular Component	GO:0042470	melanosome	0.0011	ITGB3; RAB27A
	GO:0042611	MHC protein complex	0.0011	HLA-DMB; HLA-A
	GO:0048770	pigment granule	0.0011	ITGB3; RAB27A
	GO:0031091	platelet alpha granule	0.0011	ITGB3; PROS1; THBS1
	GO:0055038	recycling endosome membrane	0.0044	EHD2; HLA-A
	GO:0031093	platelet alpha granule lumen	0.0075	PROS1; THBS1
	GO:0034774	secretory granule lumen	0.0075	PROS1; RAB27A; THBS1
	GO:0055037	recycling endosome	0.0173	EHD2; HLA-A
	GO:0032585	multivesicular body membrane	0.0275	RAB27A
	GO:0031528	microvillus membrane	0.0275	ITGB3

3. Results

3.1. Identification of common deferentially expressed genes between blood and brain tissues in ALS

We analyzed high-throughput RNA-seq and microarray gene expression datasets of motor neuron and peripheral blood lymphocytes of ALS. The transcriptomics datasets of motor neuron and blood showed similar expression of 13 common genes (DNAH6, HLA-DMB, HLA-A, EHD2, CMKLR1, PROS1, GAPT, CCR1, THBS1, CDK2, RAB27A, ITGB3, and C1orf162) in both tissues.

To clarify the biological significance of the identified DEGs, we performed a gene set enrichment analysis. The significant GO terms were enriched in biological processes, molecular functions and cellular components (Table 1). The pathways analysis revealed significant pathways involving graft-versus-host disease, allograft rejection, p53 signaling pathway, antigen processing and presentation, Epstein-Barr virus infection, cell adhesion molecules, Focal adhesion, ECM-receptor interaction and hypertrophic cardiomyopathy (Fig. 2).

3.2. Protein-protein interaction analysis to identify hub proteins

A protein-protein interaction network was constructed, encoded by the DEGs to reveal the central protein, the so-called hub proteins considering the degree measures (Fig. 3). CDK2, CCR1, DNAH6, HLA-A, EHD2, THBS1, and RAB27A were identified as the hub proteins. These are potential biomarkers and may lead to new ALS therapeutic targets.

3.3. In-silico cross-validation of the differentially expressed genes

The cross-validation of the differential expression of the identified DEGs revealed HLA-A, EHD2, CMKLR1, GAPT, THBS1, CDK2, RAB27A and C1orf162 were significantly differentially expressed (adj p-values < 0.05) in the blood cell based gene expression profiling dataset GSE112680 of ALS. CCR1 and ITGB3, initially found to be differentially expressed, but further analysis showed their expression levels were not statistically significant (adj. p-value > 0.05).

3.4. Identification of transcriptional and/or post-transcriptional regulator

We identified TFs and miRNAs and their targeted DEGs to reveal regulatory biomolecules that may regulate the expression of DEGs at transcriptional and post transcriptional levels (Fig. 4). The analysis revealed TFs (SP1, MYC, TP3, CTCF, SRF) and miRNAs (miR-29c, miR-21, let-7a, miR-377, miR-103, miR-369-3p, miR-494, miR-204, miR-29a) which play roles in the regulation of the DEGs.

3.5. Identification of candidate drugs/small molecules

The present study identified the candidate small molecule interactors from DSigDB database (Table 2). In this way, we identified candidate intereacting compounds enriched in the gene list; these might indicate useful drugs and drug targets, although it should be noted that these relate to gene expression in blood cells.

4. Discussion

The lack of peripheral blood biomarkers for ALS has led to efforts to identify much needed methods for the early diagnosis of this debilitating disease. The identification of peripheral biomarkers may also shed light on molecular mechanisms of ALS and enable the monitoring of treatment. Transcriptomics analysis (by RNA-seq and microarray) is extensively employed in identifying candidate biomarkers for many diseases [27–31]. Some studies aimed to encode the mRNAs expression signatures [8–14]; for instance, van Rheenen and coworkers identified 2943 DEGs in ALS from blood gene expression profiling [10]. Despite

Fig. 2. *The significant* KEGG pathways enriched by the identified differentially expressed genes shared by the brain tissues and blood cells in Amyotrophic Lateral Sclerosis (p-value < 0.05).

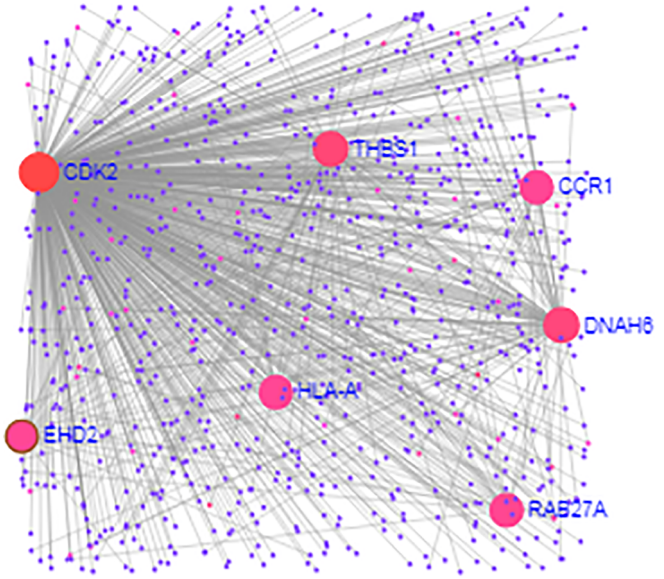

Fig. 3. Protein-protein interaction network of the common differentially expressed genes (DEGs) shared by brain tissues and blood cells of Amyotrophic Lateral Sclerosis. The nodes indicate the DEGs and the edges indicate the interactions between two genes.

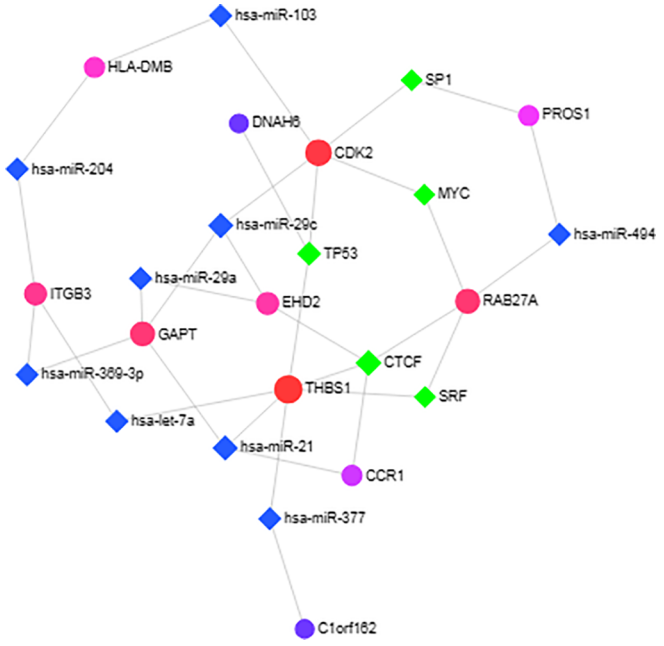

Fig. 4. Analysis of Transcription Factors-miRNAs coregulatory interaction networks based on differentially expressed genes identified shared by blood cells and brain tissues of ALS.

Table 2
Top 10 drugs identified in drug-target enrichment or over-representation analysis using the DSigDB gene sets.

Drugs/Compounds	p-value	Genes
Clopidogrelum	0.000116	ITGB3; THBS1
Beryllium sulfate	0.000285	ITGB3; HLA-A
Zebularine	0.000451	CDK2; THBS1
simvastatin	0.000897	CCR1; CDK2; THBS1
17-Ethynyl estradiol	0.000897	CCR1; PROS1; THBS1
genistein	0.006539	ITGB3; PROS1; CDK2; THBS1
dexamethasone	0.002279	HLA-DMB; CDK2; THBS1
estradiol	0.011076	EHD2; ITGB3; PROS1; CDK2; HLA-A; THBS1; CMKLR1
Medroxyprogesterone acetate	0.00182	CDK2; C1ORF162; THBS1
aspirin	0.005114	ITGB3; CDK2; THBS1

these studies providing some candidate biomarkers, there is no report of a study so far that attempted to identify candidate biomarkers signatures that are common in blood cells and brain tissues of ALS. We analyzed two gene expression datasets from peripheral blood and motor neuron of the brain of the ALS patients in an attempt to identify potential biomarker candidates that were regulated similarly in blood cells and brain tissues.

Our analysis revealed DEGs, as well as TFs and miRNAs that strongly influence gene expression at the transcriptional and post-transcriptional levels. Our analysis revealed 13 DEGs common to the two transcriptomic datasets of blood and brain tissues. Geneset enrichment analyses also revealed ALS-associated molecular signaling pathways that included the cell adhesion molecules, antigen processing and presentation. The p53 signaling pathways we identified is already implicated in apoptosis leading to the death of spinal motor neuron in ALS [32,33]. The pathways such as focal adhesion, ECM interactions and cell adhesion molecules (CAM) also identified in the present study were consistent with similar findings of Kotni et al. who studied the gene expression profiling in ALS [34]. Employing protein-protein interaction networks, we also identified dysregulated hub proteins that participate in many cellular processes. These hub proteins are considered key drivers in the mechanisms underlying a disease

development [35]. Therefore, we reconstructed the protein interaction network focusing on the DEGs in an attempt to identify related hub proteins. Such proteins have the potential to contribute to the formation and progression of ALS. Of the DEGs we identified, mRNA levels of CDK2 expression in the spinal cord of motor neurons in ALS patients showed a potential role in ALS [36,37]. Misra et al. discussed the role of HLA-A was implicated in neurodegenerative diseases [38]. Satoh et al. identified the down-regulation of various collagen gene including THBS1 in ALS [39].

Regulatory biomolecules are now increasingly being studied as potential biomarkers for important conditions such as neurodegenerative diseases [27,28,30,40–42]. With this in mind, we studied the role of TFs and miRNAs in the regulation of the DEGs through TF-miRNA coregulatory networks in the pathogenesis of MS. Among TFs we identified were SP1 which is involved in alteration of vascular endothelial growth factor [43] and is significantly involved in multiple sclerosis, another neurodegenerative disease [44]. TP53 encodes p53 which is central to DNA repair and oncogenesis, but is also associated with TP53 include Li-Fraumeni syndrome and osteogenic sarcoma. Among its related pathways are Apoptosis Modulation and Signaling and Glioma. Gene Ontology (GO) annotations related to this gene include DNA-binding transcription factor activity and protein heterodimerization activity. CTCF as a multifunctional protein in genome regulation and gene expression [43].

miRNAs play important roles in gene regulation and there is emerging evidence demonstrating their potential for use as biomarkers for ALS and other diseases and it is very likely that many miRNAs play significant roles in the pathogenic process underlying ALS [45–47]. Di Pietro et al. showed that miR-29c was significantly upregulated in the ALS slow group [48]. miR-369-3p was upregulated in the spinal cord of ALS [49]. The let-7a involved in the inhibition of migration and invasion of nasopharyngeal carcinoma [50]. The mir-494-3p might play a protective role in the survival of motor nerve cells, a discovery that could lead to the development of new therapies for ALS. miR-29a decreased in Alzheimer disease [51].

Finally, we identified drugs/compounds due to the possibility that identified biomarkers (i.e., hub proteins and TFs) may be drug targets to generate drug repurposing hypothesis in ALS. Thus, associations between the identified ALS markers and drugs were discovered that suggest that they may influence important pathways in disease progression, but further investigations required to evaluate the consequences of proposed biomarkers blockade.

5. Conclusion

In the present study, we analyzed the transcriptomics of blood cells and brain tissue to identify common DEGs between these two tissue types in ALS. We integrated these common DEGs into pathway analysis for protein-protein interactions, TFs and miRNAs. 13 DEGs were identified from RNA-seq and microarray data of blood cells and motor neurons in ALS. Neurodegeneration associated molecular signaling pathways were identified; several TFs and miRNAs were identified as putative transcriptional and post-transcriptional regulators of the DEGs we identified. Thus, we have identified potential biomarker transcripts that are commonly dysregulated in both blood cells and brain tissues in ALS. Furthermore, we identified candidate drugs targeting the identified biomarkers. We propose that these biomarkers may enable the rapid and cost effective assessment of blood sample analysis for the diagnosis of ALS. This novel approach to identify markers can be employed in easily accessible tissue (blood) to assess its expression in an inaccessible tissue (brain), and is one that could be applied to other related clinical problems. We now propose a more detailed validation of this approach and of the putative biomarker transcripts we have identified with clinical-based investigations.

Conflict of interest statement

The authors declare no conflict of interest.

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Author contributions

Conceptualization: M.R.R., and M.A.M.; Formal analysis: M.R.R., T.I..; Methodology: M.R.R., T.I. and M.A.M.; Supervision: F.H.., J.M.W.Q., and M.A.M.; Writing-original draft: M.R.R..; Writing-review & editing: M.R.R., J.M.W.Q., and M.A.M.

Ethical statement

No ethical clearance is not required from our institution for this study.

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