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Expression analysis of protein homeostasis pathways in the peripheral blood mononuclear cells of sporadic amyotrophic lateral sclerosis patients

Abhishek Vats[a,b], Mandaville Gourie-Devi[c,d], Kavita Ahuja[e], Ankkita Sharma[c], Saima Wajid[b], Nirmal Kumar Ganguly[a], Vibha Taneja[a,*]

[a] Department of Research, Sir Ganga Ram Hospital, Rajinder Nagar, Delhi 110060, India
[b] Department of Biotechnology, Jamia Hamdard, Hamdard Nagar, New Delhi, Delhi 110062, India
[c] Department of Neurophysiology, Sir Ganga Ram Hospital, Rajinder Nagar, Delhi 110060, India
[d] Department of Neurology, Institute of Human Behaviour and Allied Sciences, New Delhi 110095, India
[e] Department of Bio and Nano Technology, Guru Jambheshwar University of Science and Technology, Hisar, Haryana 125001, India

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ABSTRACT

Misfolded protein aggregates are the hallmark of Amyotrophic Lateral Sclerosis (ALS) which suggests involvement of protein homeostasis pathways in etiology of ALS. However, status of protein homeostasis in peripheral blood of ALS is not well established. We analyzed expression levels of key genes of proteostasis pathways in peripheral blood mononuclear cells (PBMCs) of sporadic ALS (sALS) patients and healthy controls. Increased protein carbonylation was observed in patients reflecting oxidative damage in PBMCs. We observed increased transcript and protein levels of GRP78 suggesting Endoplasmic reticulum (ER) insult to cells. Further, significant upregulation of spliced *XBP1* and two stress sensors: *IRE1α/ERN1* and *ATF6* indicated induction of unfolded protein response (UPR). Genes involved in autophagosome initiation (*ULK1, ULK2, ATG13*); nucleation and elongation (*BECLIN1, ATG7, ATG16L1, ATG5, ATG10*) and vesicular trafficking genes were significantly increased in patients. Increased lipidation of LC3 validated induction of autophagy. Accumulation of low molecular weight ubiquitinated proteins in patients suggested deregulation of proteasome (UPS) pathway. In addition, cytosolic chaperones (*HSP70* and *HSP27*) and HSF1 were elevated in patients. Increased TDP43 indicated role of TDP43 in disease pathology. Our findings suggest that there is oxidative insult and upregulation of UPR, vesicular trafficking and autophagy in PBMCs of sALS patients.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is characterized by loss of upper and lower motor neurons in cerebral cortex, brain stem and spinal cord resulting in motor dysfunction, progressive muscle weakness, atrophy, paralysis, difficulty in speaking, swallowing, respiratory failure and death in 3 to 5 years with exceptions of longer period of survival. Neuroepidemiological studies showed prevalence rate of ALS 4 per 100,000 in India [1] which is comparable to the global rate. Majority of the ALS cases (> 90%) occur sporadically (sALS) and < 10% are familial (fALS) but in India fALS is found to be < 1% [2,3]. Mutations in ~13 genes have been shown to account for ~70% of fALS cases.

The precise etiology of ALS remains unresolved and probably interplays of environmental, toxins, infections, nutritional and genetic influences contribute to the development as well as progression of the disease. Similar to other neurodegenerative disorders, protein misfolding/aggregation is one of the major pathological features of ALS. Histopathological accumulation of TDP43- and FUS-positive ubiquitinated inclusions have been documented in ALS [4,5]. In addition, aggregates of mutant SOD1 have also been implicated in the pathogenesis of ALS [6,7].

Adequate evidence suggests that the pathological accumulation of aberrantly folded proteins in neurodegenerative disorders may be due to deregulation of proteostasis pathways [8–10]. Evidences suggest altered expression of chaperones, genes involved in unfolded protein response (UPR) [11], vesicular trafficking and autophagy lysosomal pathway [10,12,13] and ubiquitin proteasome system (UPS) [14] in brain, spinal cord and cerebrospinal fluid (CSF) of ALS patients. Moreover, several ALS-associated mutations are present in proteins involved in proteostasis pathways [15–19].

Peripheral blood transcriptome dynamically reflects system-wide biology and ~80% of the genes expressed in central nervous system (CNS) are detectable in circulating blood cells. Recent studies have focused on scanning peripheral blood for transcriptional alterations to

* Corresponding author at: Department of Research, Sir Ganga Ram Hospital, Rajinder Nagar, Delhi 110060, India.
E-mail address: vibha.taneja@sgrh.com (V. Taneja).

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assess molecular pathogenesis in neurodegenerative disorders including ALS. SOD1 transcript was found to be increased in the lymphocytes and brain of ALS patients [20]. Recently proteome analysis of peripheral blood mononuclear cells (PBMCs) of ALS patients showed differential expression of cytoplasmic and ER chaperones [21] as well as genes involved in redox regulation. Differential expression of inflammatory genes in circulating monocytes of ALS patients also showed involvement of peripheral blood cells in the disease [22]. A very recent study showed altered subcellular distribution of TDP43 in the monocytes of ALS patients having mutation in *TDP43* and *VCP* [23]. Thus, it is likely that proteostasis pathways are also altered in the peripheral blood cells of ALS patients. Hence, we analyzed the key factors involved in protein folding and degradation of misfolded/aggregated proteins in PBMCs of sALS patients.

2. Material & methods

2.1. Subjects

Forty-seven subjects from North India were diagnosed to have ALS based on the Revised El Escorial criteria [24,25]. The age at onset, duration of illness at presentation, site of onset and activities of daily living were assessed using the Revised ALS Functional Rating Scale (ALSFRS-R). Age and gender matched healthy volunteers (n = 47; males n = 26, females n = 21) without any neurological disorder from North India were also included in the study. The study was approved by the Institute Ethics Committee of Sir Ganga Ram Hospital, New Delhi. Informed consent was taken before collection of blood sample. The study was conducted according to the principles of the Helsinki Declaration of 1975, as revised in 1983.

2.2. Isolation of PBMCs

Blood sample (5-6 ml) was collected from sALS patient and healthy individual in EDTA coated vial. Peripheral blood mononuclear cells were separated by density gradient centrifugation on Histopaque-1077 (sigma#10771). Briefly, blood was diluted with Phosphate buffer saline (PBS) in 1:1 ratio and layered over histopaque (Sigma Aldrich, USA) in a ratio of 2:1 (diluted blood:histopaque) followed by centrifugation at 1200 rpm for 35 min at 18–20 °C. The white buffy coat was aspirated out and washed twice with DPBS by centrifuging at 2000 rpm and second wash with DPBS at 1000 rpm for 10 min respectively.

2.3. Isolation of protein from PBMCs

Total protein was isolated by resuspending PBMCs in a non-denaturing cell lysis buffer (137 mM NaCl, 1% Triton X-100, 10% glycerol, 20 mM Tris pH 8.0, 2 mM EDTA, 2 mM PMSF) containing protease inhibitor cocktail. Cells were incubated in ice for 1 h and centrifuged at 11000 × *g* for 10 min at 4 °C. Supernatant was collected and quantified by Pierce BCA protein assay kit (Thermo#23225).

2.4. Protein carbonylation assay

The carbonylation content of total protein was measured using simplified 2,4-dinitrophenylhydrazine (DNPH) spectrophotometric assay in which addition of alkaline sodium hydroxide (NaOH) shifts the maximum absorbance of the derivatized protein from 370 to 450 nm [26]. Briefly, 100 µl of 10 mM DNPH in 0.5 M phosphoric acid was mixed with 100µg of normalized protein lysate in 100 µl from PBMCs and incubated at room temperature for 15 min. 50 µl of 6 N NaOH was added and incubated for another 10 min and absorbance was taken at 450 nM. Optical density (OD) of the test samples was normalized by subtracting the OD of buffer. The protein carbonylation amount was calculated by using molar absorptivity of 22,308 $M^{-1} cm^{-1}$ for the hydrazone derivatives of carbonyl groups [26].

2.5. Relative expression analysis of transcript by manual RTPCR and qRTPCR array

RNA isolation was done using RNAzol (sigma#R4533) as described by manufacturer protocols.

High capacity cDNA Reverse Transcription Kit (appliedbiosystem#4368814) was used to synthesize cDNA for manual RTPCR. Briefly, cDNA reaction was set up using 1.5 µg of RNA with the random primers and reverse transcriptase enzyme in a 20 µl total volume. For qRTPCR array cDNA was synthesized by RT^2 first strand kit (Qiagen#330401) using 1µg of RNA.

Relative expression profiling of *GRP78*, unspliced *XBP1* (*uXBP1*) and spliced *XBP1* (*sXBP1*) (Patients, n = 40; controls, n = 43) was carried out by real time PCR (RTPCR) using SYBR Green chemistry (appliedbiosystem#43855612) with the 10 ng cDNA/reaction. β-actin (*ACTB*) was used as an endogenous control. The primers used are: GRP78-F - GGTGCCTACCAAGAAGTCTC and GRP78-R- TTGTTCCCTGTACCCTTGTC; sXBP1-F – TGCTGAGTCCGCAGCAGGTGC and sXBP1 R – GCTGGCAGGCTCTGGGGAAG; uXBP1-F- TGCTGAGTC CGCAGCACTCAGA and uXBP1-R- GCTGGCAGGCTCTGGGGAAG; β-ACTIN-F- GGACTTCGAGCAAGAGATGG and β- ACTIN-R – AGCACTG TGTTGGCGTACAG. Further, RTPCR of 41 genes was carried out using commercially customized qRTPCR array from Qiagen. The reactions were setup using 8.5 ng cDNA/reaction (patients, n = 14; controls n = 15) and *GAPDH* was used as an endogenous control. Fold change was calculated by dividing the $2^{-\Delta Ct}$ of patients by average $2^{-\Delta Ct}$ of Controls.

2.6. Immunoblotting

Total protein was isolated as described above; normalized and resolved on SDS-PAGE followed by probing with antibodies against anti-GRP78 (sigma#G9043, dilution 1:4000), anti-LC3 (sigma#L8918, dilution 1:2000), anti-Ubiquitin (sigma#U5379, dilution 1:100) and anti-actin (sigma#A2066, dilution 1:500). Alkaline Phospahatase conjugated anti-rabbit IgG antibody (sigma#A9919, dilution 1:30,000) was used as secondary antibody and the blots were developed using Novex® AP Chemiluminescent Substrate (Invitrogen#WP20002) and visualized on ChemiDoc system (Syngene Genegnome 5 75,000). Images were scanned with Genesys software and quantification of proteins was done by densitometry using ImageJ.

2.7. Statistical analysis

Statistical analysis was performed by the SPSS software for Windows, version 17.0 (SPSS, Chicago, Illinois). Data was checked for normality and continuous variables were compared using Mann-Whitney *U* test between patients and healthy individuals. The possible correlation of altered expression between two genes in ALS patients was evaluated by Spearman's rho coefficient. For all statistical tests, a p value < 0.05 was taken to indicate a significant difference.

3. Results

3.1. Demographic and clinical details of studied patients

There were 25 males and 22 females, with the mean age at onset of 54.91 ± 9.52 years (range 35 to 74 years) (Table 1). It is noteworthy that in 36% the age of onset was < 50 years. The mean duration of illness at presentation was 20.14 ± 14.90 months (range 3 to 60 months). The duration was two years or less in 78% of the patients. The bulbar onset was observed in 47% and limb onset in 53%. Mean ALSFRS-R score was 37.19 ± 5.26 with a range of 25–44.