Biomedical & Pharmacology Journal, June 2023. Vol. **16**(2), p. 797-807

Comprehensive Assessment of Human Cerebrospinal Fluid for Protein Bio-Marker Identification Following Japanese Encephalitis Viral Infection

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https://dx.doi.org/10.13005/bpj/2662

(Received: 06 October 2022; accepted: 23 December 2022)

Identifying potential biomarkers, which can be used for diagnostic and therapeutic purposes, is urgently needed for successful Japanese encephalitis (JE) viral infection disease management. In the present study, we identified key CSF protein biomarkers of Japanese encephalitis virus (JEV) infected patients. We compared them to those from non-JE acute encephalitis syndrome and other neurological non-infectious patients to determine their discriminatory potential to detect JEV infection. Demographic and clinical information including fever, headache, vomiting, altered sensorium, behavioral abnormalities, neck stiffness, and Glasgow coma scale (GCS) score were recorded for all patients. CSF protein biomarkers were analyzed using 2D gel electrophoresis and liquid chromatography-tandem mass spectrometry (LC-MS/MS). Total 22 CSF based protein biomarkers were identified and a out of them three protein spots were further processed for biomarkers identification on the basis of size and density. Functional enrichment analyses of Gene Ontology (GO) were performed using Cytoscape software to explore the biological functions and relevant pathways. GO enrichment analysis showed that the G.O. terms were mainly enriched in immune responses, inflammatory and apoptotic cell death pathways, autophagy regulation, cellular organization, cellular protein modification, lipid transportation, fatty acid metabolism and iron regulation specifically associated with JE disease. Taken together, it showed that a combination of multiple CSF protein biomarkers constitutes a founding set for the discrimination of JEV infection individuals, which can be used for diagnosis and therapeutic targets; however, it demands further extensive independent cohorts study.

Keywords: Cerebrospinal fluid; 2D-gel electrophoresis; Japanese encephalitis disease; Liquid chromatography-tandem mass spectrometry; Proteomics; Virus.

Acute encephalitis syndrome (AES) is characterized by an acute onset of fever and clinical neurological manifestation that includes mental confusion, disorientation, delirium, or coma. It is triggered by many infectious viruses for eg; Dengue, West Nile Virus, Epstein-Barr virus, including the Japanese encephalitis virus (JEV) or other infectious agents such as fungus, bacterial

and even non-infectious when the immune system responds to a previous infection or, it mistakenly attacks brain tissue.[1, 2] Japanese encephalitis (J.E.) virus infection emerged as Asia's most common viral encephalitis. Globally, JEV is responsible for ~68,000 clinical cases each year. The mortality associated with JE disease is as high as 25-30% in India, and even after treatment, 50% of survivors, especially children; suffer from lifelong disabilities due to late intervention and treatment.[3, 4] This emphasizes the need for detection of JEV infection with a comprehensive understanding of the biology of the disease so that the pathogenesis could be controlled in a better way.

JE virus is a single-stranded, positive-sense RNA virus that belongs to the family *Flaviviridae*.[5] The virion is composed of three structural proteins– nucleocapsid or core protein (C), non-glycosylated membrane protein (M) and glycosylated envelope protein (E), andseven non-structural (NS) proteins – NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5.[6] JE viral infection has a complex molecular strategy to evade the host immune cell response. NS proteins play a crucial role in generating neurovirulence in host neuronal cells by assisting the virus in the neuronal invasion, eliciting host immune response, and protecting immunity via generation of host antibodies.[7, 8] Furthermore, it is evident that immune cells (e.g., microglia) act as a plausible reservoir of the virus particles in JEV infection, thus mediating the neuroinflammatory response by producing chemokines and cytokines, which further leads to neuronal cell death in bystander fashion.[9, 10]

During JE viral infection, systemic protein analysis of host and viral proteins can provide dynamic insights on virus-host interaction. These include immunity components, viral-host protein interaction products, and proteins targeted for degradation by viruses. Cerebrospinal fluid (CSF) proteomic analysis is a well-accepted powerful technique to examine protein changes in the brain.[11] This analysis will allow us to see how host cells change after being infected with the J.E. virus and help identify critical targets for JEV disease. In the present study, we compared the CSF protein profiles of JE-AES patients with non-JE AES and other neurological disorders using 2-dimensional gel electrophoresis (2-DGE) and LC/MS-MS.

MATERIALS AND METHODS

Human samples

Patients with acute encephalitis syndrome admitted to the Department of Medicine and Department of Pediatrics, King George Medical University, Lucknow, India, were investigated for JEV infection and divided into JE positive and non-JE AES patients. Non-JE AES and Other non-infectious neurological disease patients were included as a control in the study. This study has been approved by the Institutional ethics committee (Ref: 92nd ECM II A/P3). Demographic and clinical data of patients were recorded at the time of enrolment with the help of a questionnaire, as provided in table 1. The groups of this study as follows:

A: JE patients (n=25)

B: Non-JE AES patients (n=25).

Inclusion criteria for JE and non-JE AES patients were acute onset of fever with change in mental status (including symptoms such as confusion, disorientation, coma, or inability to talk) and new onset of seizures (excluding simple febrile seizures)[1].

*C: Non infectious other neurological diseases (n=2*0). Note: this group consists non-infectious other neurological diseases like seizures patients (n=7), stroke patients (n=11) and glioblastoma (n=2).

CSF sample collection

CSF samples were collected from all individuals by lumbar puncture followed by centrifugation for 15 min at 2,000 rpm. The supernatant was transferred into new vials and protease inhibitor cocktails were added in each sample and were stored at -80°C for further analysis.

Detection of JE Virus

Enzyme-Linked Immunosorbent Assay (ELISA) was performed for all CSF samples using JE virus IgM ELISA kit (National Institute of Virology, Pune, India). JEV-IgM positive samples were further tested for IgM antibodies against Dengue by using Dengue IgM ELISA kit (National Institute of Virology, Pune, India) to avoid the possibility of cross-reaction with Dengue virus infection.

CSF protein sample preparation

40µl CSF samples from each patient per

group were pooled and precipitated using acetone procedure. Briefly, CSF and acetone were mixed uniformly at a ratio of 1:6 and kept overnight at -20°C and were centrifuged at 10,000 × g for 30 min at 4°C. The supernatant was discarded, and the protein sample pellets were air-dried for further proteomic analysis.[12]

Protein quantification

The protein concentration of all CSF samples was determined by using Bradford's method according to the manufacturer's instructions (Bradford Protein Assay Kit, Bio-Rad).

2D Gel Electrophoresis and mass spectrometry analysis

2D Gel Electrophoresis

The first part of the 2 D gel electrophoresis was performed using the SCI-PLAS Isoelectric focusing System (IEF-SYS), Germany. Total 200 ìg protein samples were dissolved in 125 µl of strip rehydration solution (8M urea, 2%CHAPS, 0.2M DTT, 0.2% bromophenol blue, and 0.4% resolytes). The prepared samples were loaded onto IPG Stip (3-10 pH, 7 cm, Biorad ReadyStrip™ IPG Strips) by in-gel sample rehydration process for overnight at room temperature. IEF strips were run in IEF unit at 500 V for 30 min, followed by 1,000 V for 30 min, 3,000 V for 6 hr, and 300V for 2 hr. The IPG strips were equilibrated in reducing buffer (6 M Urea, 20% glycerol, 2% SDS, 375 mM Tris-HCl pH 8.8 and 2% DTT) for 10 min, and in alkylating buffer containing 6 M Urea, 20% glycerol, 2% SDS, 375 mM Tris-HCl pH 8.8 and 2.5% iodoacetamide for additional 10 min. The IPG strips and molecular weight standard were placed on top of 13% SDS-PAGE gel and were sealed with 0.5% agarose. The second dimension electrophoresis protein separation was carried out under 60V for 10 min and then under 90V until the front of dye reached the bottom of the gel. After electrophoresis, gels were stained by silver nitrate.

Sample Preparation for mass spectrometry

Selected protein spots were excised from the stained gel and were transferred into microcentrifuge tubes for in-gel digestion. Destaining solution [40 mM ammonium bicarbonate (NH_4CO_3) and 40% acetonitrile (ACN)] was added to cover the gel pieces and was agitated until the gel pieces completely destained. 100% ACN was added to each tube and was incubated for 10-15 min followed by addition of sufficient reduction solution (5 mM DTT in 40 mM NH_4CO_3). The mixture was incubated at 60°C for 30 min. After cooling down to room temperature, sufficient alkylation solution (20 mM iodoacetamide (IAA) in 40 mM NH_4CO_3) was added. The mixture was kept in the dark at room temperature for 10 min. The solution was removed, and gel pieces were dehydrated with 100% ACN. Trypsin solution was prepared with a concentration of 10ng/µl in 40 mM NH_4CO_3 and enough trypsin solution was added to each microcentrifuge tube. The tubes were placed on ice for 45 minutes until the gel pieces were completely rehydrated. Subsequently, 40 mM NH_4HCO_3 was added to each tube. The tubes were incubated at 37°C for overnight. The next day, 100 µl of 5% formic acid was added to each tube and incubated for 10 min at 37°C for peptide extraction, and the supernatant was collected into a fresh microcentrifuge tube. After that, 100 µl extraction buffer (5% Formic acid; 40% ACN) was added to each tube and was incubated for 10 min. For final extraction, 100% ACN was further added and was incubated for 10 min, and was dried down by vacuum drying procedure.

Mass spectrometry analysis

Digested peptides samples were analyzed using 120 minutes runtime on Orbitrap QE+ mass spectrometer interfaced with Vanquish3000 LC system (Thermo Scientific, Bremen, Germany). Each fraction was reconstituted in 0.1% formic acid and then loaded onto a 2 cm long pre-column (50 µ x 2 cm, 3 µ particle, and 100 Å pore size). The peptides were resolved on an analytical column (50 µ x 20 cm, 3 µ particle, and 100 Å pore size) using a linear gradient of 5% to 30% of solvent B (0.1% formic acid in 95% Acetonitrile) over 100 minutes and flow rate of 300 nl/min. Both MS and MS/MS were acquired using Orbitrap mass analyzer. MS scans were acquired in 350-1800 m/z range with an ion injection time of 60 ms, AGC target of 500,000, and resolution of 120,000 at 400 m/z. Most abundant peptides with 2-5 charges were acquired in data-dependent mode, and exclusion duration was set to 30 seconds. Higher-energy collisional dissociation (HCD) was used for fragmentation and set at 37%. Fragment ions were detected in Orbitrap with a mass resolution of 30,000 at 400 m/z and AGC target value was set to 50,000. MS3 scans were acquired on Orbitrap for accurate relative quantitation. Synchronous precursor selection was

enabled, and HCD collision energy of 65% was used with AGC target set to 2,000 and maximum ion injection time of 200 ms. Internal calibration was carried out using the lock mass option (m/z 445.1200025) from ambient air.

Biological pathways analysis

The identified proteins were then used to fetch Gene Ontology terms using Cluego plugging.[13-15] Only p-Value significant Gene Ontology terms associated processes and pathways were considered.

RESULTS

Demography and clinical characteristics

The demographic and clinical characteristics of patients are detailed in table 1.

2-DGE and mass spectrometry analysis

The final protein concentration of CSF samples were found 1223.81µg/ml, 1392.21µg/ml, and 1561.91µg/ml of JEV (group A), non-JE AES (group B), and non-infectious other neurological diseases (group C), respectively. We observed 22 protein spots in JE patients CSF, 32 protein spots in non-JE AES patients, and 49 protein spots in non-infectious other neurological diseases patients after silver staining (Figure 1). Inter gel comparison of these three groups was analyzed using SameSpots v 5.1.012 online software. Total 16 protein spots were differentially expressed in JE CSF. Out of these sixteen differentially expressed protein spots, three spots were selected on the based on size and density were further processed for biomarker identification using liquid chromatography mass spectrometry LC-MS/MS. The proteins identified in these three spots are illustrated in table 2.

Proteins biological function

Biological functions of JE CSF proteins were analyzed by using Cytoscape system for direct and indirect relationships. P-value of proteins for eg,serum albumin, keratin, type II cytoskeletal 1, immunoglobulin lambda-like polypeptide 5 isoform 1, immunoglobulin heavy variable 4-38-2-like, metalloproteinase inhibitor 1, hemoglobin subunit beta, immunoglobulin J chain, serotransferrin, alpha 2 microglobulin found significant in Gene Ontology terms associated biological pathways. Serotransferin and immunoglobulin J chain were found related to antimicrobial humoral response while immunoglobulin lambda five, A2M were found related to complement activation and apoptotic pathway. Keratin 1 protein was found involved in the establishment of skin barriers as a cell organization process. Tissue metalloproteinase inhibitor 1 (TIMP-1) was found associated with endopeptidase inhibitor activity and apoptotic processes. Serum albumin and hemoglobin subunit beta were found associated with blood plasma lipoprotein particle remodeling and platelet aggregation. Similarly, other CSF proteins for eg; tumor-associated calcium signal transducer 2, Ubiquitin-60S ribosomal protein L40 isoform X1, Cystatin-C, Chitinase-3-like protein 1 (YKL40), Tumor necrosis factor receptor superfamily member

Table 1. Demographic and clinical characteristics of all group patients

S. No.	Characteristics	Non JE AES patients (N=25)	JE patients (N=25)	Other neurological (N=20)
Demographic characteristic				
1	Mean age (±SD)	14.80 ± 2.572	20.42 ± 3.000	21.58 ± 4.118
2	Male	17	14	13
3	Female	08	11	07
Clinical features				
1	Fever	21	24	0
2	Headache	23	24	03
3	Vomiting	17	16	07
4	Altered sensorium	16	21	0
5	Behavioral abnormalities	17	18	02
6	Neck Stiffness	05	03	0
7	GCS≤8	09	11	11
8	GCS≤8	16	14	09

6B, Fatty acid-binding protein, heart isoform 1, Fatty acid-binding protein, heart isoform 1, Apolipoprotein D, Eukaryotic translation initiation factor 5A-1 isoform X1, Alpha-crystallin A chain isoform 1, Dermcidin isoform 2 preproprotein, Beta-2-microglobulin isoform X1, Compliment C3 and their biological functions are summarized in table 3A and B.

DISCUSSION

In our study, we found that all three differential protein spots from CSF of JEV patients contained multiple immunological proteins such as α2M (Alpha 2 microglobulin), beta-2microglobulins (β2M), immunoglobulin lambda-like polypeptide 5, immunoglobulin heavy variable 4-38-2-like, immunoglobulin J chain, indicating host immune cell response against JEV infection and blood-brain barrier (BBB) impairment. α2M is a member of alpha macroglobulin, which acts as a marker for BBB impairment and has protein transport function and anti-protease activity.[16-18] Conversely, α2M has also been shown to bind and internalize viral proteins, regulate immune responses, and increase virus infectivity.[16-19] Similarly, we also observed another key protein, beta 2 microglobulins (β2M) which is a consistent marker in different inflammatory diseases, neuroinfectious disorders such as HIV and other neurodegenerative disorders such as Alzheimer's and Parkinson's disease.[20-26] Previous study investigated that Beta-2-microglobulin (B2M) protein upregulated after JE viral infection.[27]

Table 2. Protein Identified by 2D-GE LC-MS/MS

Spot No.	Protein ID	Accession No.	Peptides	Unique Peptides
1	Serum albumin	gi|4502027	11	11
	Keratin, type II cytoskeletal 1	gi119395750	6	5
	Tumor-associated calcium signal transducer 2	gi|166795236	2	2
	Immunoglobulin lambda-like polypeptide 5 isoform 1	gi|295986608	2	2
	Cystatin-C	gi|4503107	1	1
	Immunoglobulin heavy variable 4-38-2-like	gi|768041374	1	1
	Ubiquitin-60S ribosomal protein L40 isoform X1	gi|1034609204	1	1
	Metalloproteinase inhibitor 1	gi|4507509	1	1
	Chitinase-3-like protein 1	gi|144226251	1	1
	Tumor necrosis factor receptor superfamily member 6B	gi|4507585	1	1
2	Serum albumin	gi|4502027	21	21
	Immunoglobulin lambda-like polypeptide 5 isoform 1	gi|295986608	2	2
	Keratin, type II cytoskeletal 1	gi|119395750	4	4
	Hemoglobin subunit beta	gi|4504349	1	1
	Immunoglobulin J chain	gi|1004426878	1	1
	Fatty acid-binding protein, heart isoform 1	gi|1004426878	1	1
	Immunoglobulin heavy variable 4-38-2-like	gi|21489959	1	1
	Apolyprotein E Isoform a	gi|705044002	5	5
	Cystatin C	gi|4503107	5	5
	Serum albumin	gi|4502027	10	10
	Serotransferrin isoform 1	gi|4557871	3	3
3	Hemoglobin subunit beta	gi|4504349	2	2
	Apolipoprotein D	gi|4502163	2	2
	Alpha-2-macroglobulin isoform X1	gi|578822814	1	1
	Eukaryotic translation initiation factor 5A-1 isoform X1	gi|1370470360	1	1
	Alpha-crystallin A chain isoform 1	gi|4503055	1	1
	Dermcidin isoform 2 preproprotein	gi|665505971	1	1
	Beta-2-microglobulin isoform X1	gi|530406197	1	1
	Compliment C3	gi|115298678	1	1

Table 3. Proteins biological functions

S. No.	Protein	Biological functions
A. p-value significant Gene Ontology terms associated biological pathways of proteins		
1	Serum albumin	Blood coagulation
2	Keratin, type II cytoskeletal 1	Cell organization
3	Immunoglobulin lambda-like polypeptide 5 isoform 1	Immune responses
4	Immunoglobulin heavy variable 4-38-2-like	*Immune response*
5	Metalloproteinase inhibitor 1	Apoptotic pathway
6	Hemoglobin subunit beta	Blood coagulation
7	Immunoglobulin J chain	Immune responses
8	Serotransferrin	Immune response and iron transport
9	Alpha 2 microglobulin	Immune response & apoptotic pathway
B. Other proteins biological pathways		
1	Tumor-associated calcium signal transducer 2	Sensory transduction
2	Ubiquitin-60S ribosomal protein L40 isoform X1	Cellular protein modification,
3	Cystatin-C	Inflammation
4	Chitinase-3-like protein 1	Immune responses (Inflammation)
5	Tumor necrosis factor receptor superfamily member 6B	Immune responses(Inflammation, apoptosis)
6	Fatty acid-binding protein, heart isoform 1	Fatty acid metabolism
7	Apolyprotein E Isoform a	Lipid Transport
8	Apolipoprotein D	Lipid Transport, Immune response
9	Eukaryotic translation initiation factor 5A-1 isoform X1	Cellular response to virus, Protein transport
10	Alpha-crystallin A chain isoform 1	Apoptotic process
11	Dermcidin isoform 2 preproprotein	Antimicrobial humoral response
12	Beta-2-microglobulin isoform X1	Immune response
13	Compliment C3	Immune response

Sero-transferrin (Tf) was found in the CSF of J.E. patients. Tf is an important beta-globin that helps in iron transportation, energy balance, and metabolism. The presence of Tf in CSF represents a case of BBB damage and disease pathology following JE viral infection.[28] Along with this, we found tissue inhibitor metalloproteinases (TIMP1) in JE patients, which is consistent with our previous findings, that highlights that matrix metalloproteinase (MMPs) and TIMP play key roles in the pathogenesis of JE viral infection by modulating BBB integrity and infiltration of peripheral immune cells in CNS[29] and found significantly higher TIMP-1 concentration in CSF of JEV infected children compared to control group.[30]

Complement protein C3 that complement system activation may play a role in JEV infection pathogenesis. Privious study shows that in flavivirus infections, the complement system plays an antagonistic role, either by restricting viral replication and protecting the host, or by exacerbating the inflammatory response, increasing illness severity.[31] Jongen PJH et al. (2000) demonstrated that the CSF level of C3 protein represents intrathecal C3 production and significantly affects immunopathogenesis or effector mechanism in immune-mediated inflammatory neurodegeneration disorders. In continuation of these findings, it has also been observed that the CSF level of complement protein (C1q/C3) correlates with patients' neuronal injury level.[32]

Along with this, we also noted ubiquitin-60S ribosomal protein L40 isoform X1 and cystatin C protein in CSF of JE patients, which are found to be an essential components of cellular pathways for viral processing, protein transport, protein degradation, and cell-matrix interactions.[33]

In addition, we found YKL-40, which belongs to the conservative Chitinase family in mammals. This protein has been investigated previously in the role of the inflammatory process like multiple sclerosis, inflammatory bowel

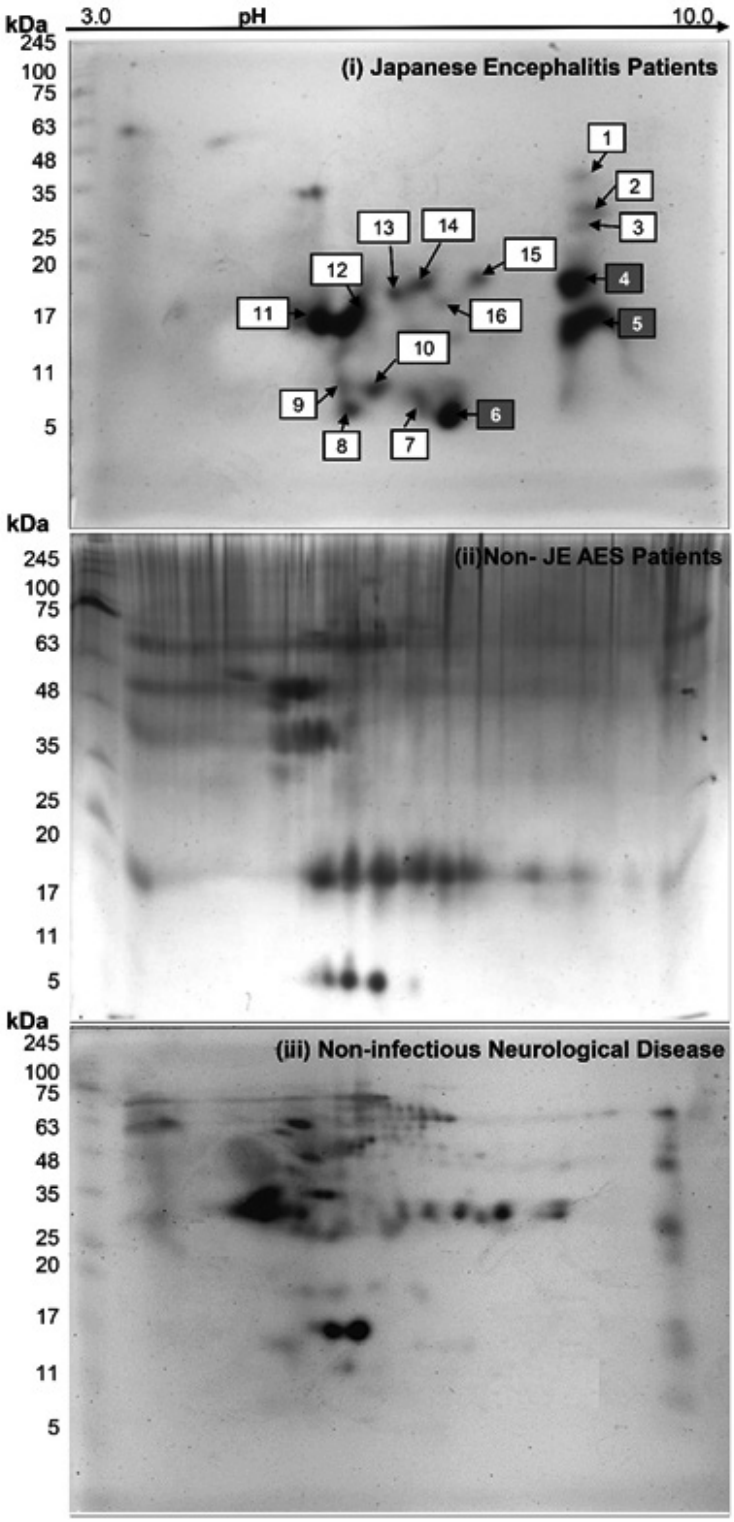

Fig. 1. CSF 2DGE of JE patients (i), non-JE AES patients (ii), and non-infectious other neurological disease patients (iii). 16 protein spots differentially expressed compared to Non-JE AES and other neurological disease patients

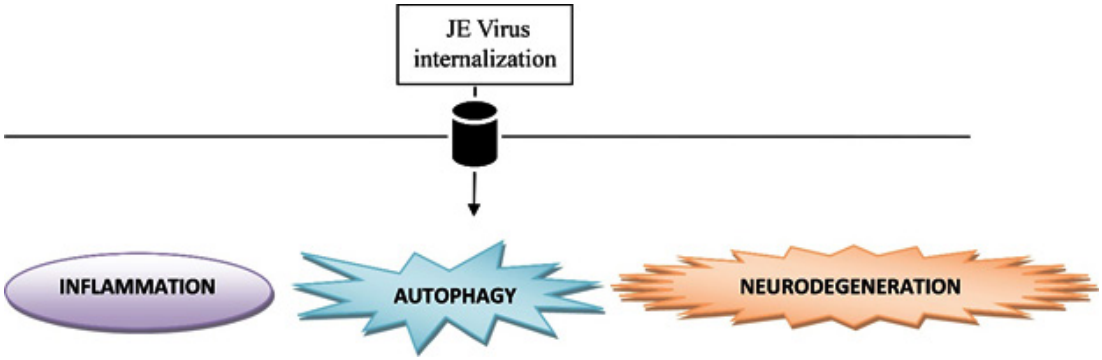

Fig. 2. Proteins which were found in Japanese encephalitis patients CSF, mainly related to autophagy, inflammation and neurodegeneration

diseases, rheumatoid arthritis, inflammatory lung diseases, osteoarthritis, cardiovascular disease, psoriasis, and viral hepatitis.[34-36] In previous observations of other viral diseases,the level of YKL-40 is observed to be increased in CSF and found to be correlated with the increased level of axonal injury in HIV.[37, 38] Our results agree with the hypothesis that JEV infection in the brain results in activation of glial cells, for instance microglia and astrocytes and leads to neuronal injury, suggesting the importance of using YKL-40 as a prognostic marker in neuroinflammation mediated neurodegenerative diseases.

Tumor necrosis factor receptor (TNFR) superfamily member 6b (TNFRSF6B) is known as Decoy receptor 3 (DcR3) and expressed after inflammatory stimuli by T cells, monocytes, and epithelial cells.[39] DcR3 was shown to block the signaling of three TNF ligand members, FasL, LIGHT, TL1A, and disease pathogenesis.[40-42] In a previous studies, detecting DcR3 in cerebrospinal fluid is helpful as a diagnostic and prognostic marker of different neurological diseases and other infectious disorders such as bacterial meningitis acute respiratory diseases syndrome (ARDS).[43, 44] However, the presence of DcR3 in CSF of J.E. patients needs further investigation.

Interestingly, Apolipoprotein E (ApoE) and Apolipoprotein D (ApoD) are found in CSF of J.E. patients. In one study, ApoE level is found increased in CSF from patients with Alzheimer's disease (A.D.) and other neurological and psychiatric disorders, which indicates that CSF ApoE tests can generally represent neuronal damage and inflammatory brain reactions.[45] However, their role in viral infectious diseases is controversial as it is involved in both pro and antiviral activity of HIV.[46-48] Similarly, we also noted ApoD in CSF of JEV patients; likeApoE, ApoD is also expressed in glial cells in the brain and associated with the development and repair of the nervous system.[49, 50] In another study Apolipoprotein D (ApoD) expression was found to be elevated in the central nervous system (CNS) of mice after Japanese encephalitis virus (JEV) infection.[51]

Along with this, the biological functions of proteins were identified by Cytoscape in gene ontology (G.O.) term and found many proteins related to immune responses, inflammatory and apoptotic cell death pathways, and other key pathways e.g., autophagy regulation, cellular organization, cellular protein modification, lipid transportation, fatty acid metabolism, iron regulation, are explicitly involved in JEV pathologies.

CONCLUSION

In summary, we found protein biomarkers by CSF proteome studies in JE disease, which are related to autophagy, inflammation and neurodegeneration that may serve as reference for future studies (Figure 2). Extensive prospective studies would be required to evaluate the efficacy of these biomarkers in JE-specific biomarkers for global application.

ACKNOWLEDGMENT

The authors, duly acknowledges, late Dr T. N. Dhole and Dr. Gourav Dey for providing technical as well as methodology assistance during the work.

Ethical and funding source information

This study was approved by the Institutional ethics committee (Ref: 92nd ECM II A/P3) and was supported by the Ramalingaswami fellowship (BT/RLF/Re-entry/13/2014) from the Department of Biotechnology, Ministry of Science and Technology, Govt. of India to A.K.

Credit authorship contribution statement

A.K., S.G., DHR, Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Visualization. **A.K., S.G.:** Writing - original draft, **DHR, MB:** Resources for patient samples and Supervision. All authors reviewed and edited the manuscript

Conflict of interest

None

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