Subarachnoid hemorrhage enhances the expression of TDP-43 in the brain of experimental rats and human subjects

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Abstract. The transactive response DNA-binding protein of 43 (TDP-43) may be involved in neurodegenerative disease and in the response to brain injury; however, alterations in the expression of TDP-43 following subarachnoid hemorrhage (SAH) require further investigation. The present study reported a notable elevation in the expression of TDP-43 within the cerebrospinal fluid (CSF) of patients with aneurysmal SAH and increased brain expression of TDP-43 in a rat model of SAH. The TDP-43 protein and a derivative migrated at 43 and 24 kDa, respectively, as observed via the immunoblotting of concentrated CSF samples obtained from patients with SAH; no signal was detected in the CSF from healthy controls. SAH in rats was induced by intravascular suture puncture. The expression levels of TDP-43 in rat cortical lysates following SAH were increased at 0.5 h, peaked at 48 h and remained significantly elevated at 72 h post-injury, compared with sham controls. TDP-43 immunolabeling indication localization within neurons, astrocytes and microglia in the experimental rats. Collectively, the findings of the present study indicated the early involvement of TDP-43 in the brain in response to SAH, and that expression levels of TDP-43 in the CSF may serve as a prognostic biomarker among patients with this condition.

Introduction

Aneurysmal subarachnoid hemorrhage (SAH) is a neurological disease associated with high rates of mortality; only ~50% patients survive this clinical condition (1). A large proportion of the survivors may also suffer from neurological disorders, including cognitive impairment and dementia later in life (2). Extensive investigations have focused on managing early post-morbidity and improving long-term care following this type of cerebral stroke. For example, brain imaging studies have suggested that SAH causes white matter damage, and monitoring the extent of this lesion may be of prognostic value (3,4). The factors and mechanism underlying SAH-induced neurological deficits remain to be fully elucidated; mechanical and ischemic stress, and neuroinflammation are expected to occur during and following subarachnoid bleeding (5).

The transactive response DNA-binding protein of 43 (TDP-43) has an important physiological role in the nervous system. TDP-43 regulates mRNA transport and stability, and is involved in microRNA biosynthesis, apoptosis, cell division and the regulation of neuronal plasticity. TDP-43 is mainly localized in the nucleus under normal conditions. By interacting with RNAs, TDP-43 may regulate the alternative splicing of genes associated with the development of neurodegenerative diseases (6). Alterations in the expression of TDP-43 have been associated with Alzheimer's disease, Lewy body-related diseases, Pick's disease and Huntington's disease (7,8). TDP-43 may exhibit neurotoxicity via phosphorylation, ubiquitination, insolubility and the formation of ectopic cytoplasmic inclusions, which has been defined as TDP-43 proteinopathy (6). Aggregated TDP-43 may also occur in axons, causing neuronal pathology in amyotrophic lateral sclerosis and other conditions (9-11). Similarly, the accumulation of TDP-43 in neurons may inhibit dendritic growth, and impair dendritic complexity and neurocircuitry communication (12).

The present study aimed to investigate whether alterations in the protein expression of TDP-43 occur in human subjects suffering from SAH via western blot analysis of cerebrospinal fluid (CSF). Marked elevations in the expression levels of TDP-43 were observed within CSF samples from patients with SAH relative to healthy controls in the present study. To determine the potential cellular origin of the SAH-induced upregulation of TDP-43, a rat model of SAH was established, which was also used to investigate SAH-induced behavioral deficits Additionally, alterations in the expression of TDP-43 within the brains of experimental animals were examined relative to sham controls.

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Materials and methods

Ethics statement. The present study was performed in compliance with Chinese legislations and guidelines of the National Institutes of Health involving human subjects and for the use and care for experimental animals. All animal experiments were approved by the Ethics Committee of Central South University Xiangya School of Medicine (Changsha, China). Clinical information and CSF samples from patients were obtained with written informed consent prior to enrollment in the study. The experiments involving human subjects were approved by the Ethical Committee of The Third Xiangya Hospital of Central South University (Changsha, China).

CSF samples from patients with SAH and controls. All SAH subjects (4 males, each with aneurysmal SAH; age range, 57-69 years) and controls (3 males, each with migraines; age range, 30-43 years) were inpatients at the Department of Neurology of The Third Xiangya Hospital of Central South University. Patients with ruptured aneurysms did not have any underlying disease prior to the incident and were treated with interventional therapy. CSF was obtained from March 2017 to April 2017 by lumbar puncture at 48 h post-SAH; a 10-ml sample was collected from each patient (n=4). Control CSF samples (n=3) were obtained from patients clinically diagnosed with a migraine. CSF samples were centrifuged at 3,200 x g for 20 min at 4˚C. Following this, 8 ml supernatant was placed in an ultrafiltration tube (Amicon® Ultra-15 10K Centrifugal Filter Devices; Amicon; EMD Millipore, Billerica, MA, USA) and centrifuged at a speed of 4,000 x g for 20 min at 4˚C, leading to a 4-fold concentration increase.

Animals and grouping. A total of 72 Sprague-Dawley (SD) male rats (8 weeks old) weighing 220-250 g were used in the present study. The animals were obtained from and housed in the Animal Center of Central South University, with free access to food and water during the entire period of experiment. Rats were housed in a temperature (25˚C) and humidity (40-50%) controlled room with a 12 h light/dark cycle. The rats were randomly divided into the sham (n=8) and SAH groups, and were decollated 0.5, 3, 10, 24, 48 and 72 h (n=8/group), respectively following surgery. A total of three rats surviving 48 h post-surgery were used for immunohistochemical analysis; 10 rats succumbed to mortality following SAH and three rats were excluded from analysis due to having an SAH grade ≤7.

Surgery and SAH grading. The endovascular perforation model of SAH was established according to a previously described method (13). Briefly, under anesthesia via inhalation of 3% isoflurane, a 4-0 monofilament nylon suture was introduced from the stump of the external carotid artery to the right internal carotid artery. By further extension, perforation via the suture was performed at the bifurcation of the anterior cerebral artery and middle cerebral artery. The sham group rats underwent the same procedure but without arterial perforation. Following recovery from anesthesia, the animals were housed for the aforementioned durations; prior to sacrifice, the behavior of animals was assessed by an experimenter in a blinded manner using the SAH Grand scoring system (14).

Neurobehavioral assessment. The behaviors of the animals were assessed at 10, 24, 48 and 72 h post-surgery, respectively, but not at the earlier time points due to the effects of anesthesia (15). The modified Garcia score test constitutes 18-point sensory assessments comprising spontaneous activity, side impact, vibrational touch, limb symmetry, climbing and forelimb walking. A beam balance test was performed to measure the ability of an animal to walk on a narrow wooden beam (22.5 mm diameter) within 60 sec.

Western blot analysis. Protein extraction and western blot analysis were performed as previously described (16). Left hemispheres were collected and extracted using radioimmunoprecipitation buffer (Beyotime Institute of Biotchenology, Haimen, China) and protein concentrations were measured using a BCA assay kit (Beyotime Institute of Biotechnology). Lysates (10 μl) from the left cerebral hemisphere (perforation side) were separated using 10% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. Blocking of nonspecific binding was performed using 5% skimmed milk for 2 h at room temperature. Immunoblotting was performed overnight at 4˚C by incubation with the following primary antibodies: Rabbit polyclonal antibodies against TDP-43 cat. no. BM5082; Wuhan Sanying Biotechnology, Wuhan, China, 1:1,000) and mouse anti-GADPH (cat. no. 60004-1-Ig; Wuhan Sanying Biotechnology; 1:5,000), followed by incubation with fluorescent secondary antibodies (cat. no. SA00001-9; Wuhan Sanying Biotechnology; 1:10,000) for 90 min at room temperature. The immunoblotting signals were detected using the Odyssey® CLx Infrared Imaging System (LI-COR Biosciences, Lincoln, Nebraska USA). To assess the protein expression in human CSF, 20 μl of concentrated CSF sample from patients with SAH and controls were used according to the aforementioned protocol.

Immunocytochemistry. Histology and immunohistochemistry were performed as previously described (17). The brain tissues of the SAH and sham groups were perfused with 4% paraformaldehyde at 48 h post-surgery, post-fixed for 24 h in the same fixative and cryoprotected via 15 and 30% sucrose prior to sectioning in a cryostat at 35-μm thickness. The sections were rinsed in 0.01 M phosphate-buffered saline (PBS; pH 7.4) three times, and treated with 3% hydrogen peroxide for 30 min to inhibit endogenous peroxidase activity and then 5% normal horse serum (cat. no. S-2000; Vector Laboratories, Inc., Burlingame, CA, USA) for 2 h at room temperature. The sections were then incubated with rabbit polyclonal antibodies against TDP-43 (1:1,000) overnight at 4˚C, and biotinylated horse anti-rabbit IgG (cat. no. BA-1100; Vector Laboratories, Inc.; 1:400) for 2 h at 37˚C. The immunolabeling was visualized with ABC reagents (Vector Laboratories, Inc., 1:400), 0.05% DAB and 0.003% H_2O_2. The immunolabeled sections were mounted on micro-glass slides, dehydrated and cover-slipped prior to light microscopic examination by ordinary light microscope (Olympus BX51; Olympus Corporation, Tokyo, Japan).

Double immunofluorescence. Double immunofluorescence labeling of rat brain sections was performed as previously described (18). The sections were washed with PBS and 0.01% Triton X-100, and then blocked with 5% donkey serum

(cat. no. 017-000-121; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 2 h at room temperature. Subsequently, the sections were incubated overnight at 4˚C with rabbit anti-TDP-43 (1:500) in combination with mouse anti-neuron-specific nuclear antigen (NeuN; cat. no. MAB377; EMD Millipore; 1:1,000), mouse anti-glial fibrillary acidic protein (GFAP; cat. no. ab10062; Abcam, Cambridge, UK; 1:1,000) or mouse anti-ionized calcium-binding adaptor molecule 1 (Iba-1; cat. no. ab15690; Abcam; 1:500) antibodies. The sections were then incubated with Alexia 488 and 594-conjugated donkey anti-rabbit and anti-mouse IgG antibodies (cat. nos. 711-545-152 and 715-585-15, respectively; Jackson ImmunoResearch Laboratories, Inc.; 1:400) for 2 h at room temperature. As a negative control, the primary antibodies were omitted and the same staining procedures were performed. All sections were incubated with 4,6-diamidino-2-phenylindole for 10 min to visualize cell nuclei via fluorescence microscopy.

Data acquisition, quantification and statistical analysis. Densitometric data of western blot analysis were obtained using Odyssey® CLx Infrared Imaging System (LI-COR Biosciences). The expression levels of TDP-43 were normalized to the signal of the internal standard (GADPH). Data are expressed as the mean ± standard deviation. Mean values were statistically analyzed with GraphPad Prism software (Version 7.0; GraphPad Software, Inc., La Jolla, CA, USA) using analysis of variance followed by a Turkey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Protein expression levels of TDP-43 are elevated in the CSF of patients with SAH. Initially, the present study analyzed the protein expression of TDP-43 in the blank CSF of patients with SAH by western blot analysis, which revealed an elevation of this protein, however, the blotted bands were not readily detected. Therefore, a freeze enrichment method was applied to concentrate the CSF samples ~4-fold. Subsequent immunoblotting using the concentrated samples resulted in detectable bands of TDP-43 proteins in the CSF of patients with SAH. By contrast, the protein expression of TDP-43 in the concentrated CSF from all control human subjects appeared to be low (Fig. 1). As the alterations in the protein expression of TDP-43 of patients with SAH relative to controls were qualitative, due to distinct bands of the SAH group compared with in the control, quantification was not performed.

Induction of SAH induces behavioral changes. In the present study, a total of 61 male rats were subjected to SAH surgery, among them, 10 animals succumbed to mortality, accounting for a mortality rate of 16.3%. No rats succumbed to mortality in the sham group. Postmortem examination indicated subarachnoid blood clots around the Circle of Willis and ventral surface of the brainstem within the brains from the SAH group (Fig. 2A); blood infiltration was not observed in the sham-operated group (Fig. 2B). There were significant differences of the SAH scores between the sham group and each SAH group (Fig. 2C). The modified Garcia score and the balance beam score were decreased 10 h following the induction of SAH, but subsequently improved (Fig. 2D and E).

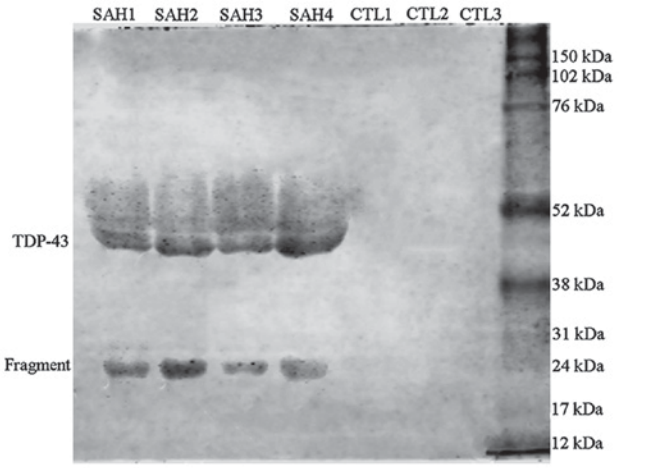

Figure 1. Western blot analysis of TDP-43 protein in concentrated CSF samples from patients who suffered from acute SAH for 72 h compared with in controls. TDP-43 protein migrated at 43 kDa; a fragment at ~25 kDa was observed in samples from four patients with SAH (SAH1-4), which were absent in samples from three CTL subjects (CTL1-3). CTL, control; SAH, subarachnoid hemorrhage; TDP-43, transactive response DNA-binding protein of 43.

Protein levels of TDP-43 in rat brain following the induction of SAH. The present study comparatively assayed the protein expression levels of TDP-43 in the lysates of the ipsilateral cortex to vascular perforation in the SAH and sham groups. Within the rats of the SAH group surviving 0.5-75 h post-surgery, the expression levels of TDP-43 appeared to increase with longer durations of survival, relative to the sham control ($P<0.05$; Fig. 3A). Increases in the expression levels of TDP-43 peaked at 48 h and remained elevated at 72 h post-surgery (Fig. 3A and B). No significant differences were observed in the expression levels of TDP-43 between the ipsilateral and contralateral cortices of the SAH group surviving 48 h following surgery (Fig. 3C and D).

TDP-43 is expressed in neurons and glial cells within SAH rat brains. The immunoreactivity of TDP-43 in coronal sections across the entire rostrocaudal extension of the brain was assessed using the ABC-DAB method in the present study. Sections from the sham-operated and SAH animals were processed collectively under identical conditions. The immunolabeling intensity of the cortical and subcortical areas, predominantly nucleus-like, appeared to be more marked in the SAH group compared with that in the sham group of animals. The difference in labeling intensities between the two groups were markedly apparent in the hippocampal formation (Fig. 4A) and the ventral cortical regions around the sites of accumulated blood clotting (Fig. 4B).

To characterize the types of cells that exhibited TDP-43 immunoreactivity, sections from rats surviving 48 h following the induction of SAH were used for confocal double immunofluorescence analysis. The immunofluorescence suggested that TDP-43 was co-localized with NeuN, a marker of mature neurons, GFAP, a marker of astrocytes and Iba-1, a marker of

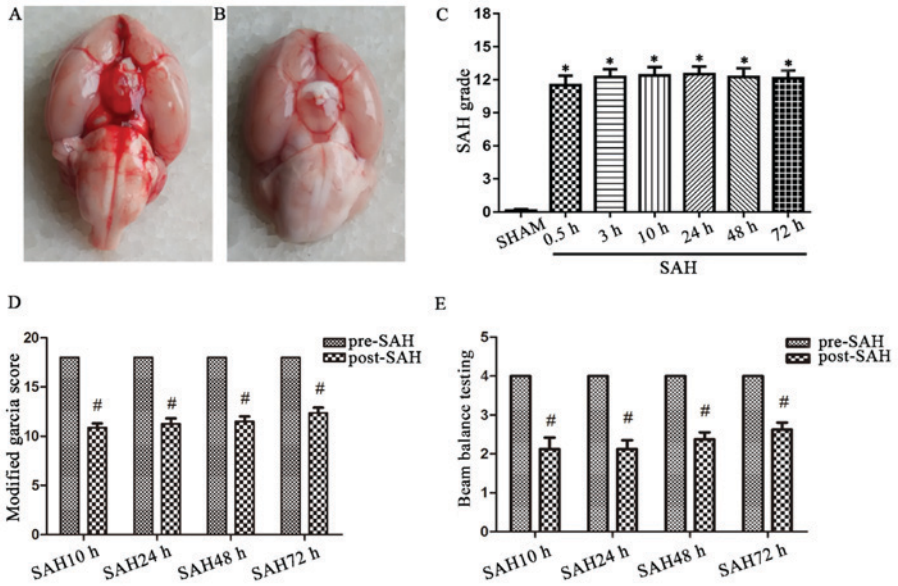

Figure 2. Gross cranial appearance and neurological scores of rats with surgically induced SAH relative to sham-operated controls. Blood clotting was observed on the ventral surface of the brain around the Circle of Willis from (A) an SAH-induced rat, but not in that from (B) a sham-operated rat. (C) No significant differences in SAH grade scores were found among groups surviving for different durations. (D) Modified Garcia scores and (E) beam balance testing scores were significantly decreased following induction of SAH (n=8). *P<0.001 vs. the sham group $^{\#}$P<0.05. SAH, subarachnoid hemorrhage.

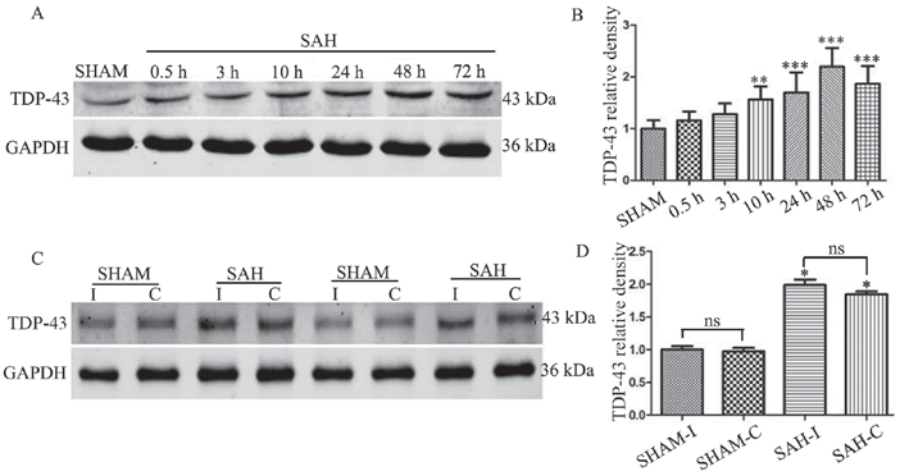

Figure 3. Western blot analysis of the expression of TDP-43 in rat brain tissue. (A) Blotted TDP-43 bands of the sham and SAH rat groups surviving for different durations (0.5, 3, 10, 24, 48 and 72 h). (B) Relative to the control, expression levels of TDP-43 gradually increased, peaked at 48 h, and remained elevated in the experimental groups (**P<0.01 and ***P<0.001 vs. the sham group). (C) Expression levels of TDP-43 of the I and C hemispheric cortex at 48 h following SAH. (D) No significant difference were observed between the two sides (n=8). ns, not significant. *P<0.05 vs. the sham group. C, contralateral; I, ipsilateral; SAH, subarachnoid hemorrhage; TDP-43, transactive response DNA-binding protein of 43; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

microglia, in the cerebral cortex, hippocampal formation and subcortical areas (Fig. 4C).

Discussion

As one type of acute cerebral stroke, SAH has a high early mortality rate and can cause devastating long-term neurological outcomes. Therefore, it is important to identify potential prognostic biomarkers, and understand the neurobiological and pathological mechanism induced by subarachnoid bleeding. TDP-43 has critical biological roles in the central nervous system (CNS), and its dysregulation has been associated with numerous chronic neurodegenerative diseases (6-12). The present study aimed to investigate whether TDP-43 may be used as clinical biomarker in patients with SAH, and whether it may be associated with neurological and cognitive deficits among survivors of SAH.

As a pilot study, performed assessing TDP-43 in the CSF for predictive prognosis in patients suffering acute SAH attack, the present study reported an elevation of this protein in concentrated CSF samples from patients 72 h following disease onset. Initially, a classical method of centrifugation of CSF was used prior to ELISA and western blot analyses, which failed to reliably detect the protein. It has been reported that TDP-43 protein and a derivative may be detected by immunoblotting in the CSF from patients with SAH only, but not in that from control subjects, according to a previously described

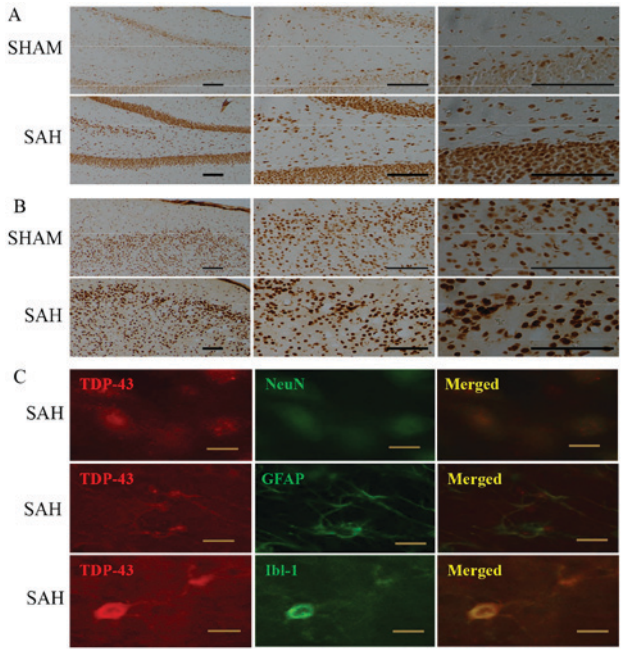

Figure 4. TDP-43 immunolabeling at 48 h following surgical induction of SAH. Images of the (A) hippocampal dentate gyrus and (B) ventral region of the cerebral cortex from sham-operated and SAH-induced rats as indicated. The labeling intensity was higher in the SAH group than the sham group. Scale bar=100. (C) Double immunofluorescence images revealed colocalization of TDP-43 labeling with neuronal, astrocytic, microglial markers, respectively in sections from SAH animals. Scale bar=10 μm. SAH, subarachnoid hemorrhage; TDP-43, transactive response DNA-binding protein of 43; NeuN, neuron-specific nuclear; GFAP, glial fibrillary acidic protein; Ibl-1, ionized calcium-binding adaptor molecule 1.

protocol (19). It appears that this approach may allow the investigation and follow-up of a large sample size to evaluate the prognostic value of CSF-associated TDP-43 regarding mortality risk and long-term neurological outcomes following SAH. Using the same method, a previous study reported that, within 24 h of disease onset, TDP-43 may be detected within the CSF of patients following traumatic brain injury (19). In addition, increased expression levels of TDP-43 in biological fluids, including CSF and plasma, have been detected in patients with amyotrophic lateral sclerosis (ALS) and fronto-temporal lobar degeneration (20). Extracellular TDP-43 may be the result of cell death induced by TDP-43 inclusion bodies in the cytoplasm (21). In addition, TDP-43 can be released from cells by secretory vesicles, known as exosomes (22,23).

Using a rat experimental model, the present study demonstrated that, following the induction of SAH, the expression levels of TDP-43 in brain tissues were rapidly increased and this elevation persisted for up to 72 h post-injury. Immunohistochemistry also indicated increased TDP-43 immunolabeling in the nuclei and cytoplasm of the cortical, hippocampal and subcortical neurons. Double immunofluorescence suggested that the expression of TDP-43 was notable in neurons, and may co-localize with additional proteins in astrocytes and microglia. Of note, the present study used phosphorylated-TDP-43 antibodies for the immunoblotting and immunohistochemical analysis of human CSF samples and rat brain tissues, which failed to obtain a clear signal. The results of the present study suggested that the acute upregulation of TDP-43 may largely involve the regular form of TDP-43 protein. Future investigations aim to investigate the mRNA expression of TDP-43 in the SAH model.

Inflammation is considered to be an important factor that mediates the upregulation and translocation of TDP-43 into the cytoplasm under pathological conditions. Inflammatory factors, including tumor necrosis factor-α and lipopolysaccharide, may promote the cytoplasmic accumulation of TDP-43 in neurons and glial cells, as suggested by the proteinopathy of TDP-43 in CNS-associated inflammatory diseases, including ALS (24,25). It has been reported that extracellular TDP-43 activates glial cells and induces the microglial caspase-3 and interleukin-18 signaling pathway, resulting in the formation of TDP-43 cytoplasmic inclusion bodies via reverse translocation of endogenous TDP-43 from the nucleus to the cytoplasm (21). Therefore, inflammation induced by SAH may account for the observed elevation in expression levels of TDP-43 within the CSF and brains of patients with SAH and experimental rats, respectively.

Future investigations are required to determine whether, and if so, how the early increases in expression levels of TDP-43 may impact recovery or may be associated with certain long-term neuronal dysfunction in patients and experimental animals with SAH (26,27). TDP-43 has important physiological effects in neuronal production and axon maintenance, however, it can also cause abnormal axonal truncation and axonal transport defects (10,11). Persistent upregulation induced by injury and the accumulation of TDP-43 may be potentially harmful by inhibiting dendritic growth and the recovery of neurocircuitry (12).

Taken together, the clinical analyses of the present study revealed that CSF-associated levels of TDP-43 were markedly elevated in patients with SAH soon following the onset of disease. The animal experiments performed in the present study revealed an early upregulation of TDP-43 in the rat brain following the surgical induction of SAH.

The present study analyzed alterations in the expression of TDP-43 within the CSF and brain tissue following SAH, which is considered to be a pathological marker protein of neurodegenerative diseases. Of note, the aggregation and phosphorylation of TDP-43 may lead to cytotoxicity and long-term cognitive dysfunction (22). Therefore, future investigations are required to examine the long-term effects of the aggregated upregulation of TDP-43 relative to neuronal damage and recovery in an experimental rat model of SAH, and then to reduce aggregated TDP-43 via activation of the heat shock factor 1/heat shock protein pathways (28). Reductions in aggregated TDP-43 in the early stage following SAH may improve or reverse long-term behavioral changes. Future investigations also aim to evaluate the potential of using the CSF-associated expression of TDP-43 as a prognostic biomarker for SAH clinically.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

FL and TBH designed the present study, analyzed the data and wrote the manuscript; TBH and YCZ established the podocyte injury model; JL and HXZ collected the CSF samples. KA and XXY assisted with technical performance and contributed to writing the manuscript. The final version of the manuscript has been read and approved by all authors and each author considers the manuscript to represents honest work.

Ethics approval and consent to participate

The present study was performed in compliance with Chinese legislations and guidelines of the National Institutes of Health involving human subjects and for the use and care for experimental animals. Written informed consent was obtained from all patients. Experiments involving animals and humans were approved by the Ethics Committees of Central South University Xiangya School of Medicine and The Third Xiangya Hospital of Central South University, respectively.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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