

Apoptosis-antagonizing transcription factor is involved in rat post-traumatic epilepsy pathogenesis

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Abstract. The present study aimed to explore the pathogenesis behind post-traumatic epilepsy (PTE). In the present study, a chloride ferric injection-induced rat PTE model was established. The expression levels of apoptosis-antagonizing transcription factor (AATF), cleaved caspase-3, p53, Bcl-2 and Bax were measured by western blotting or immunofluorescence staining (IF). The expression of AATF *in vivo* was downregulated by microinjection of lentiviral-mediated short-hairpin RNA. Compared with control and sham groups, at day 5 after PTE, neuron apoptosis was significantly increased and the expression levels of AATF, p53, cleaved caspase-3 and Bax were significantly upregulated. In addition, IF revealed co-localization of AATF and cleaved caspase-3 in the cortex. Additionally, AATF was expressed mainly in neurons and astrocytes. Following AATF inhibition, the expression levels of p53 and cleaved caspase-3 were significantly reduced as compared with the control group. Taken together, these findings suggested that following PTE, AATF is involved in neuronal apoptosis and may serve as a potential target for its alleviation.

Introduction

Post-traumatic epilepsy (PTE) is characterized by the development of delayed unprovoked seizures after traumatic brain

injury (TBI) (1). It has been estimated that approximately 20% of symptomatic epilepsies are caused as a result of TBI, indicating that PTE is one of the most common focal epilepsy types (2). The incidence rate of PTE was found to range from 4.4-53% worldwide, which accounts for 10-20% of symptomatic epilepsy cases and for 5% of all epilepsy cases (3-5). Despite significant efforts made to understand the pathogenesis and improve the efficacy of PTE treatment, no effective treatment option for PTE has been introduced (6). Thus, it is a crucial medical need to further explore the pathogenesis of PTE to provide effective therapeutic options.

Neuronal apoptosis has been previously reported to occur during seizures (7,8). Moreover, repeated PTE seizures damage neurons and cause neuronal death and secondary neuron loss (9). Evidence has revealed the typical apoptotic features in damaged regions and elevated expression levels of caspase 3 and Bax after PTE (10). A recent study observed that cleaved caspase-3 exerted an important role in chronic TBI pathologies and neurodegenerative diseases (11). All these findings suggest that neuronal apoptosis is involved in PTE pathogenesis.

Apoptosis-antagonizing transcription factor (AATF) is an RNA polymerase II-binding protein that was identified as an interaction partner of the death-associated protein-like kinase (12). Previous studies observed that AATF inactivation or inhibition results in DNA damage accumulation (13,14) and induces apoptosis (12,15-17). Furthermore, AATF protects neurons against amyloid β -peptide-induced oxidative damage (18), indicating that AATF may serve as a novel cytoprotective factor against apoptotic insult in neurological diseases. However, the role of AATF in PTE is still poorly understood. Therefore, the present study aimed to explore the role of AATF in the pathogenesis of PTE.

Materials and methods

Animals. A total of 54 male Sprague-Dawley rats (220-275 g, 6-8 weeks), obtained from the Experimental Animal Center of Nantong University (Jiangsu, China), were maintained under a 12/12-h light/dark cycle at $23\pm 1^\circ\text{C}$ and received food and water *ad libitum*. All animal care and surgical treatments and

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Abbreviations: AATF, apoptosis antagonizing transcription factor; PTE, post-traumatic epilepsy; TBI, traumatic brain injury; GFAP, glial fibrillary acidic protein; Iba-1, ionized Ca^{2+} -binding adaptor molecule

Key words: PTE, AATF, neuronal apoptosis

procedures were carried out in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and the study protocol was approved by the Ethics Committee of Nantong University. A PTE rat model was established as previously described (19). In brief, after anesthetization with 10% chloral hydrate (350 mg/kg, intraperitoneal), the overlying bone of the rat was drilled with a stereotaxic atlas (2.0 mm behind the left coronal suture and 2.0 mm beside the sagittal suture). Then, using a microinjection pump, 5 μ l of FeCl₃ (0.1 mol/l) or sterile saline was injected into the motor cortex via a trephine hole over a period of 5 min. Afterwards, the trephine hole was covered by medical bone wax. Suturing of the skin and the muscle were performed separately. During the experimental procedure, no signs of peritonitis were observed.

The behavioral changes of the mice were recorded 1 h before and after modeling at 8-10 am every day, and assessed from 12 h to 28 days after the FeCl₃ injection using the Racine scale (20). The Racine stages employed were as follows: Grade 0, no behaviors of epileptic seizure; grade 1, face clonus, chewing, yawning; grade 2, nodding, face clonus, neck muscle convulsion, in addition to the behaviors of grade 1; grade 3, forelimb or hindlimb convulsion, in addition to the behaviors of grade 2; grade 4, hindlimb standing, sudden standing, in addition to the behaviors of grade 3; grade 5, rhythmic convulsion of four limbs, hindlimb stiffness, and dorsal flexure or convulsion, in addition to the behaviors of grade 4. Rats at grade 4 or higher were selected as PTE models.

Rats injected with saline were classified as the sham group. The rats in the experimental group (n=3/time point) were sacrificed at 12 h or 1, 3, 5, 7, 14 and 28 days to extract protein for western blot analysis. Rats in the control (n=3) and sham groups (n=3) were sacrificed on day 5 according to the preliminary western blot results (Fig. 1). Additional experimental rats were sacrificed at each time point for further immunohistochemical analysis (n=3). The rats were anesthetized by chloral hydrate (400 mg/kg), transcardially perfused with 0.9% sterile saline, then the cortex tissues of the injection area were collected and stored at -70°C before analysis.

Western blotting. Hypotonic lysis buffer (0.05% NP-40; 50 mmol/l Tris-HCl; 5 mmol/l EDTA; 10 mM NaCl; pH 7.5) was used to separate the nuclear and cytoplasmic fractions of the cortex tissues. The protein concentration was detected by BCA protein assay (cat. no. P0006; Beyotime Institute of Biotechnology). Next, a total of 20 μ g protein was separated by 10-12% SDS-Polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes (EMD Millipore). After blocking with 5% skim milk for 1 h at room temperature, the membranes were incubated with the following primary antibodies: Anti-AATF (1:2,000; cat. no. WH0026574M4; Sigma-Aldrich; Merck KGaA), anti-cleaved caspase-3 (1:5,000; cat. no. 9664; Santa Cruz Biotechnology, Inc.), anti-p53 (1:5,000; cat. no. 2527; Santa Cruz Biotechnology, Inc.), anti-Bcl2 (1:5,000; cat. no. 15071; Santa Cruz Biotechnology, Inc.), anti-Bax (1:1,000; cat. no. 14-6999-37; Zymed; Thermo Fisher Scientific, Inc.) and anti-GAPDH (1:5,000; cat. no. sc-365062; Santa Cruz Biotechnology, Inc.), overnight at 4°C. Then, the membranes were further incubated with secondary antibodies (1:10,000; goat anti-mouse and

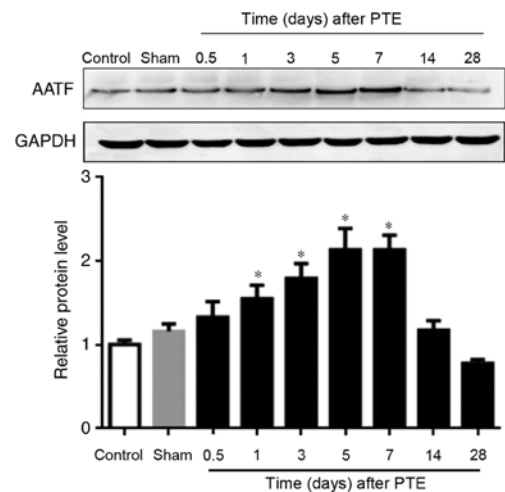


Figure 1. Expression profile of AATF following PTE. The expression of AATF at the protein level in the ipsilateral cortex following PTE. * $P < 0.05$ vs. sham. AATF, apoptosis antagonizing transcription factor; PTE, post-traumatic epilepsy.

goat anti-rabbit; cat. nos. F0257 and F0382; Sigma-Aldrich; Merck KGaA). The bands were detected using an enhanced chemiluminescence system (Amersham; Cytiva). GAPDH was used as an internal control. For quantification of the protein bands, ImageJ software (v1.8.0; National Institutes of Health) was utilized for densitometric value evaluation.

Immunofluorescence staining. The cortex was fixed overnight using 4% paraformaldehyde at 4°C and dehydrated in 30% sucrose at 4°C for 2-3 days. After embedding in optimal cutting temperature compound, 7- μ m coronal sections were cut within 1-3 mm posterior to the bregma and placed on gelatin-coated microscope slides. All sections were frozen at -20°C before use.

For immunofluorescence analysis, all sections were blocked using 10% serum-blocking buffer (0.1% Triton X-100, 3% (w/v) bovine serum albumin (BSA) and 0.05% Tween-20) for 2 h at room temperature. Then, the sections were incubated with primary antibodies against AATF (1:100; cat. no. WH0026574M4; Sigma-Aldrich; Merck KGaA), NeuN (1:100; cat. no. SAB4300883; Sigma-Aldrich; Merck KGaA), glial fibrillary acidic protein (GFAP; 1:100; cat. no. sc-33673; Santa Cruz Biotechnology, Inc.), ionized Ca²⁺-binding adaptor molecule 1 (Iba-1; 1:100; cat. no. sc-32725; Santa Cruz Biotechnology, Inc.) and cleaved-caspase-3 (1:100; cat. no. sc-3073; Santa Cruz Biotechnology, Inc.) overnight at 4°C. The sections were next incubated with FITC- and Tetramethylrhodamine-conjugated secondary antibodies (1:1,000; cat. no. F0257; Sigma-Aldrich; Merck KGaA) for 2 h at room temperature. The sections were dehydrated and covered with coverslips, and the slides were photographed under a 20x objective using a Leica DM4000B fluorescence microscope (Leica Microsystems GmbH).

Terminal deoxynucleotidyl transferase-mediated biotinylated-dUTP nick-end labeling (TUNEL). The animals were sacrificed by perfusion-fixation. Dissected rat brains were postfixed in 4% paraformaldehyde solution for another 24 h

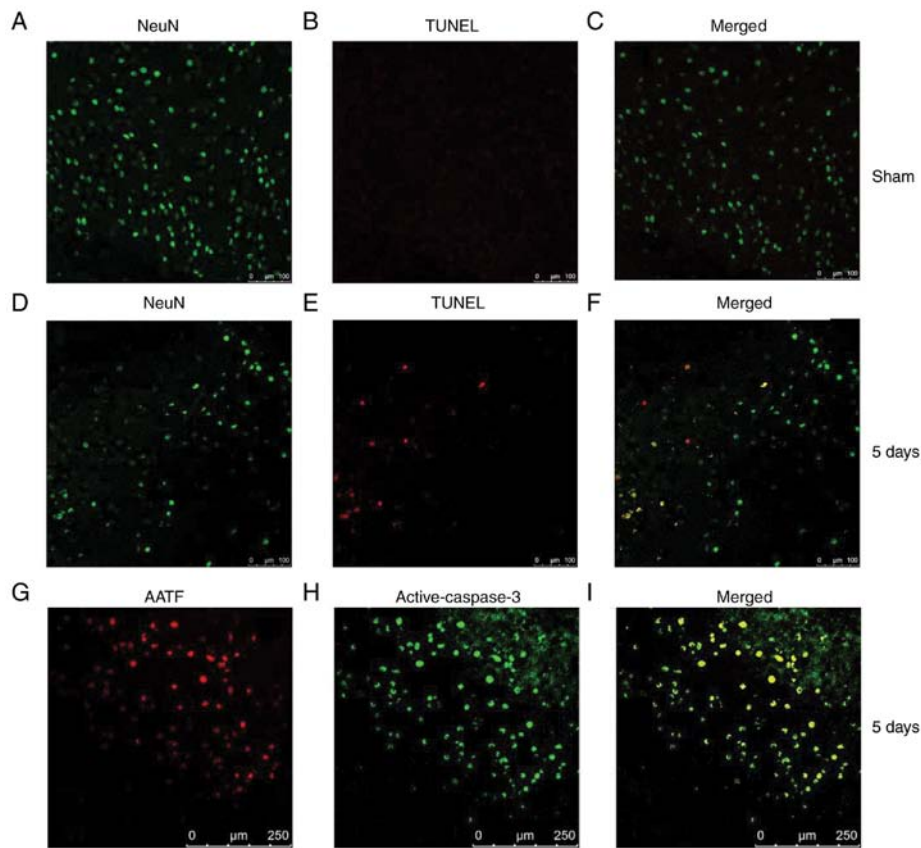


Figure 2. Apoptosis of neurons was significantly increased on the 5th day after PTE and AATF was co-localized with cleaved caspase-3. (A) NeuN (green) and (B) TUNEL (red) staining, as well as (C) double staining in the sham group; (D) NeuN (green) and (E) TUNEL (red) staining, as well as (F) double staining in the PTE group. Immunofluorescence staining of (G) AATF (red) and (H) cleaved caspase-3 (green) revealed (I) co-localization at 5 days following PTE. AATF, apoptosis antagonizing transcription factor; PTE, post-traumatic epilepsy.

at 4°C and dehydrated for 2 days at 4°C. The brains were then immersed in 4% paraformaldehyde for 48 h, embedded in optimal cutting temperature compound (OCT) for 10 min in a freezing microtome (Leica Model CM 1900; Leica Microsystems GmbH) at -25°C. The OCT-embedded brain tissues were then sliced into 30 μm sections. The sections were rinsed with PBS and then were blocked with 3% goat serum (cat. no. 005-000-001; Jackson ImmunoResearch Laboratories, Inc.) for 60 min at room temperature and incubated with rabbit anti-NeuN antibody (1:100; cat. no. SAB4300883; Sigma-Aldrich; Merck KGaA) overnight at 4°C. The slides were then probed using the *in-situ* Cell Death Detection kit (cat. no. 11684795910; Roche Diagnostics) according to the manufacturer's instructions. In brief, frozen tissue sections were rinsed with PBS and then treated with 1% Triton X-100 for 2 min on ice. The slides were next washed in PBS and incubated with 50 μl of TUNEL reaction mixture for 60 min at 4°C. Subsequently, the sections were counterstained with DAPI (cat. no. C1006; Beyotime Institute of Biotechnology) for 10 min at room temperature. Images were acquired under a 20x objective using a Leica DM4000B fluorescence microscope (Leica Microsystems GmbH). The negative control sections were incubated for 60 min with a labeling solution.

Lentiviral transfection of shRNA targeting AATF. After FeCl₃ induction, polybrene (5 μg/ml) was used to dilute the lentivirus (Invitrogen; Thermo Fisher Scientific, Inc.) to 3x10⁶ TU/μl. Rat

brain cortex was microinjected with lentivirus by the brain stereotaxic method, as previously described (21). The complementary oligonucleotides targeting AATF were obtained from Shanghai GeneChem Co., Ltd. The targeted sequence for AATF was 5'-GGAGGAGATCTCAGAAGAT-3' and 5'-ATC TTCTGAGATCTCCACC-3'. The sequence for negative control was 5'-TTCTCCGAACGTGTCACGT-3' and 5'-ACG TGACACGTTCCGAGAA-3'. These complementary oligonucleotides was annealed and ligated to GV118 vector (Clontech Laboratories, Inc.). The shRNA-carrying lentiviruses were produced in 293T cells and subjected for microinjection, as were the negative controls. Five days after microinjection, the rats in both the negative control and AATF groups were sacrificed, then brain cortex was collected and subjected for further analysis.

Statistical analysis. Data were expressed as mean ± standard error of the mean (SEM). The statistical significance between groups was determined by one-way ANOVA, followed by Tukey's post hoc multiple comparison tests. All data were analyzed using SPSS 17.0 (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of AATF is enhanced at the early stage of PTE. Western blotting was employed to investigate the expression

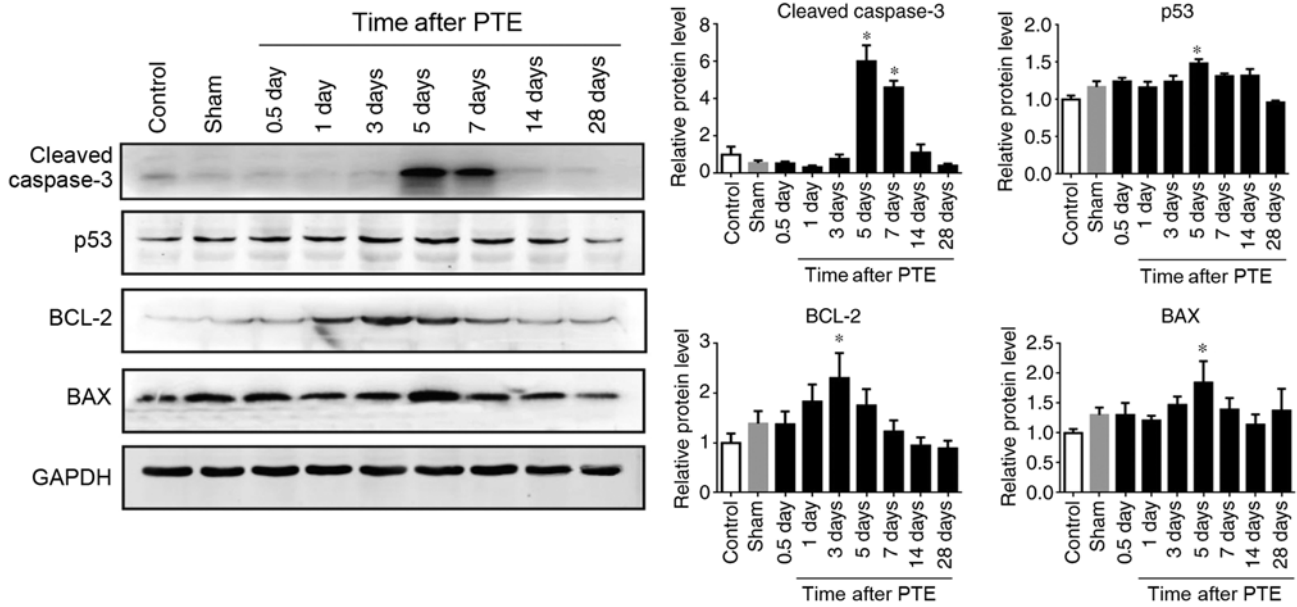


Figure 3. Temporal change in protein expression levels of cleaved caspase-3, p53, Bcl-2 and Bax after PTE. Expression of cleaved caspase-3, p53, Bcl-2 and Bax in the ipsilateral cortex following PTE at the protein level. * $P < 0.05$ vs. sham. PTE, post-traumatic epilepsy.

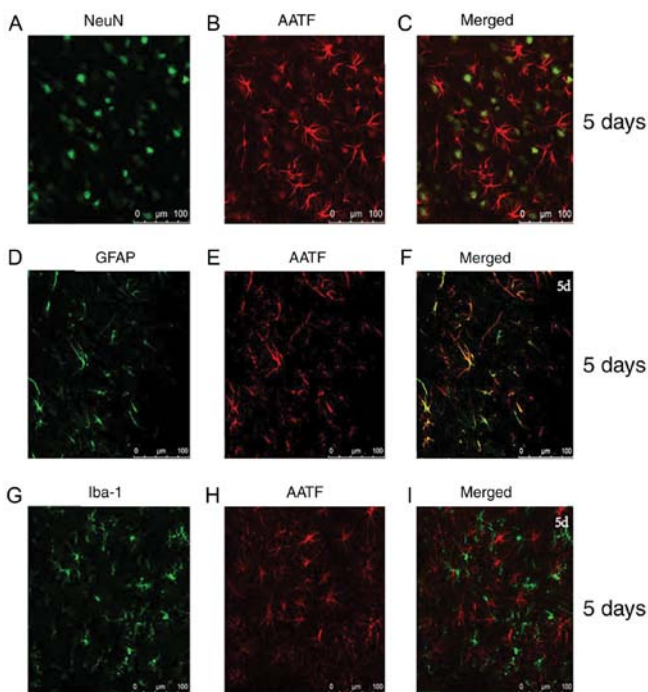


Figure 4. Localization of AATF in brain cortex. The expression of AATF in different cell types in the adult rat brain cortex within 5 mm of the lesion site on the 5th day after PTE. (A) NeuN, green staining and (B) AATF, red staining, as well as (C) co-localization of NeuN and AATF. (D) GFAP green staining, and (E) AATF red staining, as well as (F) co-localization of GFAP and AATF. (G) Iba-1 green staining and (H) AATF red staining, as well as (I) co-localization of Iba-1 and AATF. AATF, apoptosis antagonizing transcription factor; GFAP, glial fibrillary acidic protein; Iba-1, ionized Ca^{2+} -binding adaptor molecule; PTE, post-traumatic epilepsy.

pattern of AATF following PTE. The results demonstrated that the expression levels of AATF in the sham and control groups were low, but gradually elevated and peaked at day 5, and declined thereafter (Fig. 1). These results indicated that

the expression levels of AATF was increased at the early stage of PTE.

Apoptosis-related proteins have an expression pattern similar to that of AATF after PTE. Since the expression levels of AATF peaked on day 5, neural apoptosis 5 days post PTE was detected. TUNEL staining demonstrated that the apoptosis of the neurons was increased 5 days post PTE (Fig. 2A-F). Then, the expression levels of apoptosis-related proteins were examined. The expression levels of cleaved caspase-3 significantly increased after PTE and peaked at day 5 ($P < 0.05$; Fig. 3), which is consistent with the expression profile of AATF (Fig. 1). Moreover, immunofluorescence staining demonstrated that AATF and cleaved caspase-3 were co-localized (Fig. 2G-I). Additionally, the expression levels of apoptosis-related proteins, such as p53 and Bax and Bcl-2, increased gradually in the PTE group, with p53 and Bax reaching their peak 5 days after PTE and Bcl-2 reaching its peak 3 days after PTE ($P < 0.05$, Fig. 3). These results suggested that AATF is associated with neuronal apoptosis after PTE.

AATF is expressed primarily in the neurons and astrocytes.

To determine whether AATF was specifically expressed in the neurons after PTE, double-immunofluorescence labeling was employed to detect the co-localization of AATF (red) and NeuN (Fig. 4A-C), GFAP (Fig. 4D-F) and Iba-1 (Fig. 4G-I). The results demonstrated that AATF was expressed mainly in the neurons and astrocytes (Fig. 4), indicating that neural apoptosis after PTE may be associated with AATF. However, no colocalization between AATF and IBA-1 was detected (Fig. 4G-I).

AATF inhibition attenuates the expression of apoptosis-related proteins in the cortical neurons. To elucidate the role of AATF in PTE, the expression levels of AATF was significantly decreased following knockdown using shRNA targeting

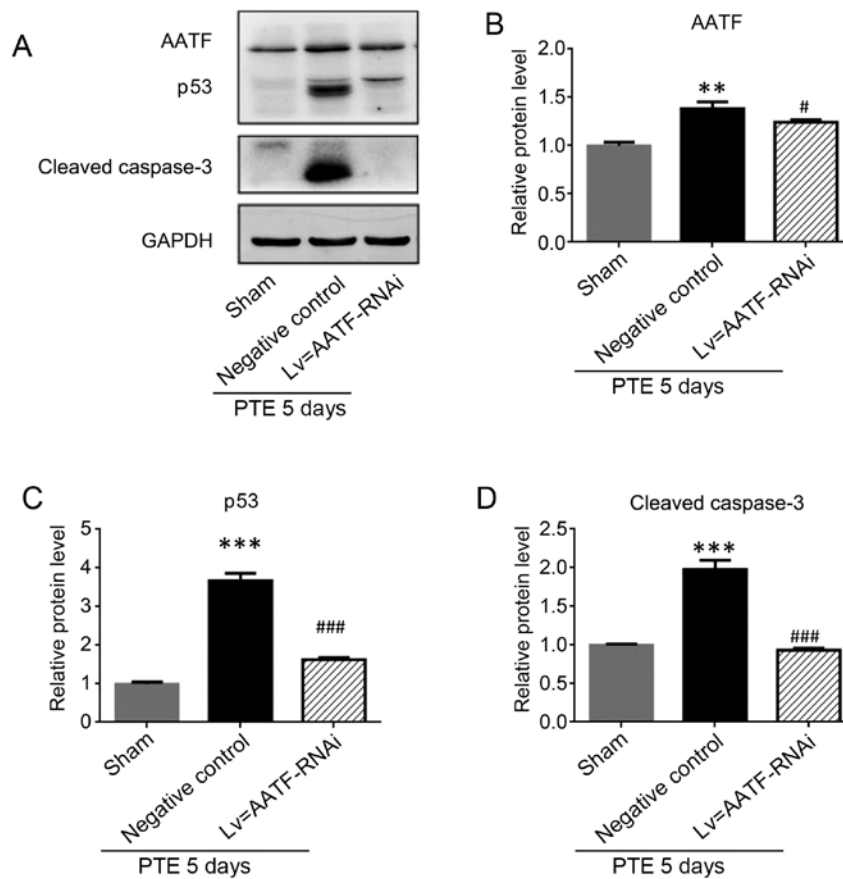


Figure 5. Expression of AATF and apoptosis-related proteins post-AATF silencing in vivo. The expression levels of AATF (A and B), p53 (A and C) and cleaved caspase-3 (A and D) after AATF knockdown. ** $P < 0.01$ vs. sham; *** $P < 0.001$ vs. sham; # $P < 0.05$ vs. negative control; ### $P < 0.01$ vs. negative control. AATF, apoptosis antagonizing transcription factor, LV, lentivirus; PTE, post-traumatic epilepsy; RNAi, RNA interference.

AATF (Fig. 5A and B). The results demonstrated that the expression levels of p53 and cleaved caspase-3 were significantly decreased in the AATF-knockdown group compared with those of the sham group ($P < 0.05$; Fig. 5A, C and D), which supported the notion that the intervention of AATF may have influenced neuronal apoptosis after PTE.

Discussion

The results of the present study demonstrated that the expression levels of AATF was gradually elevated and peaked at the early phase of PTE, which was similar to the expression pattern of apoptosis-related proteins, such as cleaved caspase-3, p53, Bcl-2 and Bax. In addition, AATF was expressed primarily in the neurons and was co-localized with cleaved caspase-3. These findings suggested that AATF is involved in neural apoptosis after PTE and may be a therapeutic target for PTE treatment.

Neuronal death following a seizure may be triggered by apoptosis (22), and neuronal apoptosis may also cause repeated antiepileptic events (23). Thus, a comprehensive understanding of the underlying mechanism of neuronal apoptosis after epileptic seizures is needed to improve the efficacy of epilepsy treatment. Neuronal apoptosis is reportedly accompanied by a change in the expression pattern of apoptosis-related proteins, such as p53, Bcl-2 and Bax (24,25). Morrison *et al* (26) knocked out p53 in the PTE rat model and

reported decreased apoptosis in the hippocampus of epileptic rats compared with the control group. Bruno *et al* (27) observed a significant increase in apoptosis-related protein expression levels in patients with refractory temporal lobe epilepsy compared with control patients. The results of the present study demonstrated significantly increased expression levels of apoptosis-related proteins, which is consistent with the aforementioned earlier findings, suggesting that altering the neuronal apoptosis pattern may be a potential therapeutic strategy for PTE treatment.

AATF is associated with gene transcription regulation, cell proliferation, DNA damage response and apoptosis (27). However, the association between AATF and central nervous system function following epilepsy is still not clearly understood. The results of the present study demonstrated that the expression levels of AATF were increased 5 days after PTE, and the apoptosis of the neurons was also significantly increased. Moreover, AATF and cleaved caspase-3 were co-localized in the cortex. Additionally, the expression levels of p53, cleaved caspase-3 and Bax exhibited a trend similar to that of AATF after traumatic brain injury. In addition, immunofluorescence staining evidenced that AATF was expressed predominantly in the neurons and astrocytes. Notably, this study demonstrated that AATF was highly expressed in the astrocytes after PTE and that astrocytes also served an important role in PTE. These results strongly suggested the involvement of AATF in the neuronal apoptosis of PTE.

Recent studies have reported that certain molecules or drugs can alleviate epilepsy by reducing neuronal apoptosis (28-30). For example, it has been found that ferulic acid ameliorates pentylentetrazol-induced seizures through reducing neuron cell death (28). Furthermore, AATF was previously demonstrated to be involved in Alzheimer's disease, and could be considered a novel cytoprotective factor against apoptosis (18,31). In the present study, AAT expression was inhibited and the results demonstrated that AATF silencing significantly reduced the expression levels of p53 and cleaved caspase-3 compared with those of the control group. These findings suggested that AATF may represent a potential target for therapeutic application.

The present study is not without limitations. Though cleaved caspase-3 could reflect the apoptosis state of cells, this study failed to detect the expression levels of caspase-3, but since the expression levels of p53 were also altered post AATF silencing it could be inferred that the conclusion was robust.

In conclusion, neuronal apoptosis serves a crucial role in epilepsy pathogenesis and may be involved in AATF expression changes. The inhibition of AATF may represent a potential therapeutic strategy for PTE treatment.

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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

WW conceived and coordinated the study, designed, performed and analyzed the experiments and wrote the study. YMM, ZLJ, ZWG and WGC performed the data collection and data analysis and revised the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Nantong University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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