Treatment with melatonin ameliorates febrile convulsion via modulating the MEG3/miR-223/PTEN/AKT signaling pathway

GEFEI WU*, JIASHENG HU*, HONGMIN ZHU, SHUHUA WU, SHENG HUANG and ZHISHENG LIU

Neurology Department, Wuhan Children's Hospital (Wuhan Maternal and Child Healthcare Hospital), Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei 430016, P.R. China

Received May 15, 2019; Accepted January 29, 2021

DOI: 10.3892/ijmm.2021.4987

Abstract. The PTEN/AKT signaling pathway is involved in the pathogenesis of febrile convulsion (FC), a convulsion caused by abnormal electrical activity in the brain. The objective of the present study was to evaluate the therapeutic effect of melatonin (MT) on FC and the according underlying molecular mechanisms. Reverse transcription-quantitative PCR and western blot analysis were used to explore the effects of MT on the expression levels of MEG3, microRNA (miRNA/miR)-223, phosphatase and tensin homolog (PTEN) and protein kinase B (AKT). Luciferase assay was performed to verify the downstream targets of MEG3 and miR-223. An animal model was established to evaluate the effects of MT on the MEG3/miR-223/PTEN/AKT pathway. TUNEL staining was carried out to assess the effect of MT on neuronal apoptosis. Finally, the duration of seizure/convulsion was recorded to determine the effect of MT on FC. In both cell and animal models, mRNA levels of MEG3 and PTEN increased in the apoptosis group, while treatment with MT decreased the expression levels of MEG3 and PTEN. miR-223 expression was decreased in the apoptosis group, whereas treatment with MT increased the expression level of miR-223. Protein levels of PTEN and cleaved caspase-3 increased in the apoptosis group, whereas treatment with MT decreased the protein level of PTEN. Phosphorylated (p)-AKT expression was decreased in the apoptosis group and treatment with MT reversed this effect. miR-223 could directly bind to MEG3, and PTEN was a direct target of miR-223. MT could decrease the duration of seizure/convulsion. In all experimental groups, treatment with MT could decrease the ratio of β waves, while increasing the ratios of α, θ and δ waves. Therefore, the results from the present study collectively suggested that treatment with MT alleviated FC via the MEG3/miR-223/PTEN/AKT pathway, which also indicated that MT could be considered as a novel strategy for the treatment of FC disease.

Correspondence to: Professor Gefei Wu, Neurology Department, Wuhan Children's Hospital (Wuhan Maternal and Child Healthcare Hospital), Tongji Medical College, Huazhong University of Science and Technology, 100 Xianggang Road, Wuhan, Hubei 430016, P.R. China
E-mail: fseizures@yeah.net

*Contributed equally

Key words: melatonin, MEG3, microRNA-223, phosphatase and tensin homolog, protein kinase B, febrile convulsion

Introduction

As a type of seizure often diagnosed in childhood or infancy, febrile seizure (FS) is one of the most commonly encountered acute neurologic conditions in children (1). In the 1990s, the International League Against Epilepsy defined FS as a seizure observed in children of >1 month that is not induced by infection to the central nervous system (CNS) and does not meet the standard for other acute seizures (2).

A separate study carried out by Molina-Carballo *et al* (3) in 54 children with febrile and epileptic convulsive crisis showed that serum melatonin (MT) expression was enhanced during seizure attacks and returned to normal values within 1 h. The authors further concluded that excitation of MT generation by a convulsive crisis may help to achieve homeostasis in the body (3). Similar results were observed in other studies (4).

Long non-coding RNAs (lncRNAs) are long RNA transcripts that do not encode proteins (5). The expression of lncRNAs is usually specific to cell and tissue types (6,7). Thus far, lncRNAs have been reported to participate in the pathogenesis of numerous malignancies by regulating gene modification and transcription (8,9). Previous results showed that lncRNA MEG3 is enriched in the endothelium and acts as a competing endogenous RNA of microRNA (miRNA/miR)-223, while MEG3 was also suspected to participate in MT-induced prevention against atherosclerosis (10). Moreover, MEG3 can inhibit the activity of miR-223, while increasing the expression of cryopyrin (NLRP3) and the pyroptosis of endothelial cells (10).

It has been demonstrated that miR-223 harvested from macrophages can enhance the chemoresistance of EOC cells via the phosphatase and tensin homolog (PTEN)-PI3K/protein kinase B (AKT) signaling (11). PTEN was illustrated to be inhibited by miR-223 to enhance the level of PI3K/AKT activation, indicating that AKT is a key mediator in the miR-223/PTEN signaling. Additionally, miR-223 is negatively

correlated with PTEN expression in various types of tumor cells, indicating the important role of PTEN in tumorigenesis (11).

PTEN has the properties of lipid phosphatase. Moreover, the substrate of PTEN is PIP3, which is hydrolyzed by PTEN to generate phosphatidylinositol 4,5-bisphosphate (PIP2) (12-15). PTEN inhibits PI3K/AKT signaling by dephosphorylating PIP3 (14). On the other hand, the inhibition of PTEN activates PI3K/AKT signaling and promotes cell survival (15). PTEN was reported to impair AKT activation by suppressing its membrane recruitment, thus suppressing the proliferation, growth and survival of cells. Therefore, PTEN plays an important role in suppressing the malignant transformation of normal cells (16).

It is commonly known that AKT is important in the regulation of numerous cellular processes, including protein translation, gene transcription, cell survival and cell proliferation (17). For example, accumulating evidence has implied that the inhibition of the kinase activity of AKT promotes FS-mediated neuronal apoptosis in the hippocampus, while FS and febrile convulsion (FC) did not affect the level of total AKT in rats, indicating that AKT/MTOR signaling is involved in FS-induced neuronal injury, and the initialization of endoplasmic reticulum stress promoted hippocampal cell apoptosis by affecting the phosphorylation of AKT (18). Furthermore, γ-aminobutyric acid (GABA), which acts as the main inhibitory signal in the peripheral nervous system and CNS, has been associated with severe neurological and neuromuscular disorders (19). There is increasing evidence suggesting that the low expression of GABA is an important trigger of seizures (20). For instance, Mackenzie and Maguire (21) reported that chronic stress-induced GABA downregulation in the mouse hippocampus can increase the susceptibility to seizure.

It has been reported that treatment with MT can downregulate the expression of lncRNA-MEG3 and thereby upregulate the expression of miR-223, a competing endogenous RNA of MEG3 (10). Furthermore, PTEN has been identified as a direct target of miR-223, while the PTEN/AKT signaling pathway has been shown to be involved in the pathogenesis of FC (18,22). In the present study, the aim was to evaluate the therapeutic effect of MT on FC and the according underlying molecular mechanisms. Accordingly, it was demonstrated that MT alleviated FC via the MEG3/miR-223/PTEN/AKT pathway, which further indicated MT as a potential novel treatment strategy for FC.

Materials and methods

Animals. In the present study, a total of 24 Wistar rats (age, 5-10 days; weight, 6-8 g) were used to mimic newborn infants delivered after full-term pregnancy in humans. In addition, brain tissues isolated from rats aged 15 to 20 days were used to mimic the brain of human infants with an age of ≤1 years old. Specifically, male Wistar rats aged 17 days were utilized to establish the animal model. During the experiments, these male Wistar rats were placed under anesthesia using a mixture of 3 mg/kg xylazine and 80 mg/kg ketamine administrated intraperitoneally. After the anesthesia of rats was successfully established, the head of each rat was opened via a small incision to remove the brain tissue. In the next step, a pair of electrodes made of stainless steel were inserted into the frontal region of the dura mater of each rat. In addition, a third electrode made of stainless steel was inserted into the occipital region of the dura mater of each rat. Subsequently, the electrodes were fixed to the skull of each rat using dental acrylic. After the operation, all rats were placed in an SPF animal facility with temperature control (the room temperature was set to 23±1˚C). In addition, the light/dark cycle was set to 12/12 h, and all rats had free access to a standard chow diet and drinking water throughout the entire experiment. By the end of the experiment, the animals were killed by a lethal intravenous dose of sodium pentobarbital (100 mg/kg). Death was confirmed by various combined factors, including lack of pulse, breathing, corneal reflex, and response to toe pinch; inability to hear respiratory sounds and heartbeat. No secondary procedure was used. Moreover, percutaneous cardiac puncture was performed after the rats were unconscious, and failure of movement of the needle and attached syringe after insertion into the heart (aspiration of blood provides evidence of correct location) also indicates lack of cardiac muscle movement and death. All animal procedures were approved by Wuhan Children's Hospital (approval no. WHET20170731V1; Wuhan, China) and were in strict compliance with the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health (23).

Establishment of an animal model of FC. An animal model of FC was established. During the animal model establishment, all rats were exposed to hyperthermia by being placed in a water tank holding water with a water level (5 cm depth of water at 45˚C). In addition, the rats were also exposed to hyperthermia by keeping the water tank temperature at 45˚C for ~5 min until the symptoms of seizure were observed. A total of 10 such experimental operations were repeated every 2 days. For the rats of the NC group, they were placed in water of 37˚C during similar experimental operations. In addition, after the rats were exposed to hyperthermia, their core temperature was immediately measured using a temperature probe via the rectum. Furthermore, the duration of seizure in each rat was determined based on the time interval when the rat was put into the water tank until the initial signs of seizure were first observed. For comparison, 10-time intervals of seizure (also named seizure latencies) were recorded in each rat. The duration of seizure in each rat was calculated as the time interval between the onset of initial seizure signs to the moment when the rat first gained consciousness, which was evaluated based on the responsiveness of each rat to a range of multiple stimuli, including cage tapping, loud clapping sound, touch and trace of object movement in front of the eyes of the rat. By the end of each experimental operation, all rats were dried with towels first and further dried underneath a heating lamp before they were placed back in their cages. In this study, the seizure intensity in each rat was evaluated. In brief, the scoring of seizure intensity was as follows: i) 0 points indicated no sign of convulsion; ii) 1 point indicated facial clonus; iii) 2 points indicated head nodding; iv) 3 points indicated forelimb clonus; v) 4 points indicated rearing; and vi) 5 points indicated rearing and back falling. In this study, animals were divided into three groups with 8 rats in each group: i) NC group (sham-operated rats treated with PBS); ii) FC group (rats suffering from FC

induced by a high temperature); and iii) FC + MT group (rats were given 80 mg/kg MT via intraperitoneal injection at 15 min before the induction of seizure).

RNA isolation and reverse transcription-quantitative (RT-q) PCR. Total RNA was isolated from tissue and cell specimens using a miRNeasy Mini kit (Qiagen GmbH) according to the manufacturer's protocols. RNA concentration and purity were confirmed using an UV spectrophotometer (Eppendorf). After purified RNA was made into cDNA templates via RT reactions using a TaqMan™ RNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) on a 9700 PCR Machine (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the conditions described as follows: 30 min at 16˚C, 30 min at 42˚C, 5 min at 85˚C, and then kept at 4˚C. The cDNA templates were then subjected to qPCR carried out on a 7900HT Fast Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) using a TaqMan Universal PCR Master Mix II (Applied Biosystems; Thermo Fisher Scientific, Inc.) per the kit instructions. The thermocycling conditions were as follows: 10 min at 95˚C; 40 cycles for 15 sec at 95˚C; and finally 60 sec at 60˚C. The relative expression of MEG3, miR-223 and PTEN mRNA was calculated using the $2^{-\Delta\Delta Cq}$ method (24). The primer sequences were as follows: MEG3 forward, 5'-GCCAAGCTTCTTGAA AGGCC-3' and reverse, 5'-TTCCACGGAGTAGAGCGA GTC-3'; miR-223 forward, 5'-GTCGTATCCATGGCAGGG TCCGAGGTATTCGCCATGGATACGACTGGGGT-3' and reverse, 5'-TGTCAGTTTGTCAAATACCCCA-3'; and PTEN forward, 5'-CTGGGTACCATGACAGCCATCATCAAAG-3' and reverse, 5'-GGCCTCGAGTCAGACTTTTGTAATTTG TG-3'. U6 (forward, 5'-GTGCTCGCTTCGGCAGCA-3' and reverse, 5'-CAAAATATGGAACGCTTC-3') was used as an internal reference gene for miR-223 and GAPDH (forward, 5'-CGACTTCAACAGCGACACTCAC-3' and reverse, 5'-CCC TGTTGCTGTAGCCAAATTC-3') was used as an internal reference gene for MEG3 and PTEN.

Cell culture and transfection. SH-SY5Y (cat. no. ATCC® CRL-2266™; American Type Culture Collection) and U251 MG cells (cat. no. 09063001; Sigma-Aldrich; Merck KGaA) were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS (Sigma-Aldrich; Merck KGaA). The culture was carried out at 37˚C and 5% CO_2. To determine the effect of miR-233, SH-SY5Y and U251 cells were seeded into 24-well plates at a density of $5x10^5$ cells/well and transfected with miR-233 mimics (5'-CCGAGGTATTCGCCATGGATACGACTG GGGT-3'; Thermo Fisher Scientific, Inc.) and scramble miRNA (5'-UGGGCGUAUAGACGUGUUACAC-3'; Thermo Fisher Scientific, Inc.), negative control (NC) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) per the recommendation of the manufacturer. SH-SY5Y cells were in the following three groups: i) NC group, cells were treated with PBS at 37˚C for 24 h; ii) H_2O_2 group, cells were treated with H_2O_2 to model apoptosis at 37˚C for 24 h; and iii) H_2O_2 + MT group, cells were treated with 1 μM MT at 37˚C for 24 h.

Vector construction, mutagenesis and luciferase assay. The 3'untranslated region (UTR) of PTEN containing the miR-233 binding site was inserted into a pcDNA3.1 vector (Promega Corporation) to generate the wild-type (WT) PTEN 3'UTR vector. Then, the miR-233 binding site in the 3'UTR of PTEN was subjected to site-directed mutagenesis to generate the mutant (MUT) PTEN 3'UTR vector using a site-directed mutagenesis kit (Agilent Technologies, Inc.). Similarly, the WT and MUT vectors of MEG3 promoter containing the WT and MUT miR-233 binding sites were also generated. Subsequently, SH-SY5Y and U251 cells seeded in 24-well plates at a density of $5x10^5$ cells/well were co-transfected with WT/MUT PTEN/MEG3 vectors in conjunction with miR-233 mimics (5'-CCGAGGTATTCG CCATGGATACGACTGGGGT-3'; Thermo Fisher Scientific, Inc.) and NC (5'-UGGGCGUAUAGACGUGUUACAC-3'; Thermo Fisher Scientific, Inc.) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), per the recommendation of the manufacturer for 48 h before the luciferase activity of transfected cells was assayed using a Bright-Glo™ Luciferase Assay System (Promega Corporation). *Renilla* luciferase activity was used for the normalization of the relative luciferase activity.

Western blot analysis. Protein was extracted from the SH-SY5Y and U251 MG cells using RIPA (Sigma-Aldrich; Merck KGaA) and protein concentration was determined with a BCA assay kit (Bio-Rad Laboratories, Inc.). Then, 50 μg/lane protein was resolved on a 10% NuPAGE™ Novel Bis-Tris gel (Invitrogen; Thermo Fisher Scientific, Inc.), and subsequently separated proteins were transferred to a nitrocellulose membrane (Amersham; Cytiva). The membrane was then blocked with 5% non-fat milk for 60 min at room temperature, and incubated overnight at 4˚C with anti-PTEN (1:1,000; cat. no. ab267787; Abcam), anti-AKT (1:500; cat. no. ab8805; Abcam), anti-phosphorylated (p)-AKT (1:1,000; cat. no. ab38449; Abcam), anti-cleaved-caspase-3 primary antibodies (1:500; cat. no. ab32042; Abcam) and anti-β-actin (1 μg/ml; cat. no. ab8226; Abcam). β-actin was used as the loading control. Following which, membranes were further incubated for 2 h at 37˚C with the appropriate secondary antibody (1:2,000; cat. no. ab6721; Abcam). After the protein bands were visualized using a SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.), the relative expression of PTEN, AKT, p-AKT and cleaved caspase-3 was calculated using Quantity One software (version 4.6.7; Bio-Rad Laboratories, Inc.).

TUNEL staining. The apoptotic status of treated samples was measured using a TUNEL staining assay kit (Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. The samples were immersed in a 3% H_2O_2 solution at room temperature for 10 min, and then hydrolyzed in 20 μg/ml proteinase K solution for 20 min, washed with PBS, and treated with a citrate buffer (0.01 M) for 30 min of high-pressure antigen retrieval. The samples were then processed with 50 μl TdT enzyme solution at 37˚C for 1 h. Subsequently, the samples were washed with PBS and incubated with 50 μl peroxidase-labeled anti-digoxigenin at 37˚C for 30 min in the dark. Neutral resin was used as the mounting medium, and a fluorescence microscope (magnification, x400) was used to observe the samples, 10 visual fields of view were selected randomly.

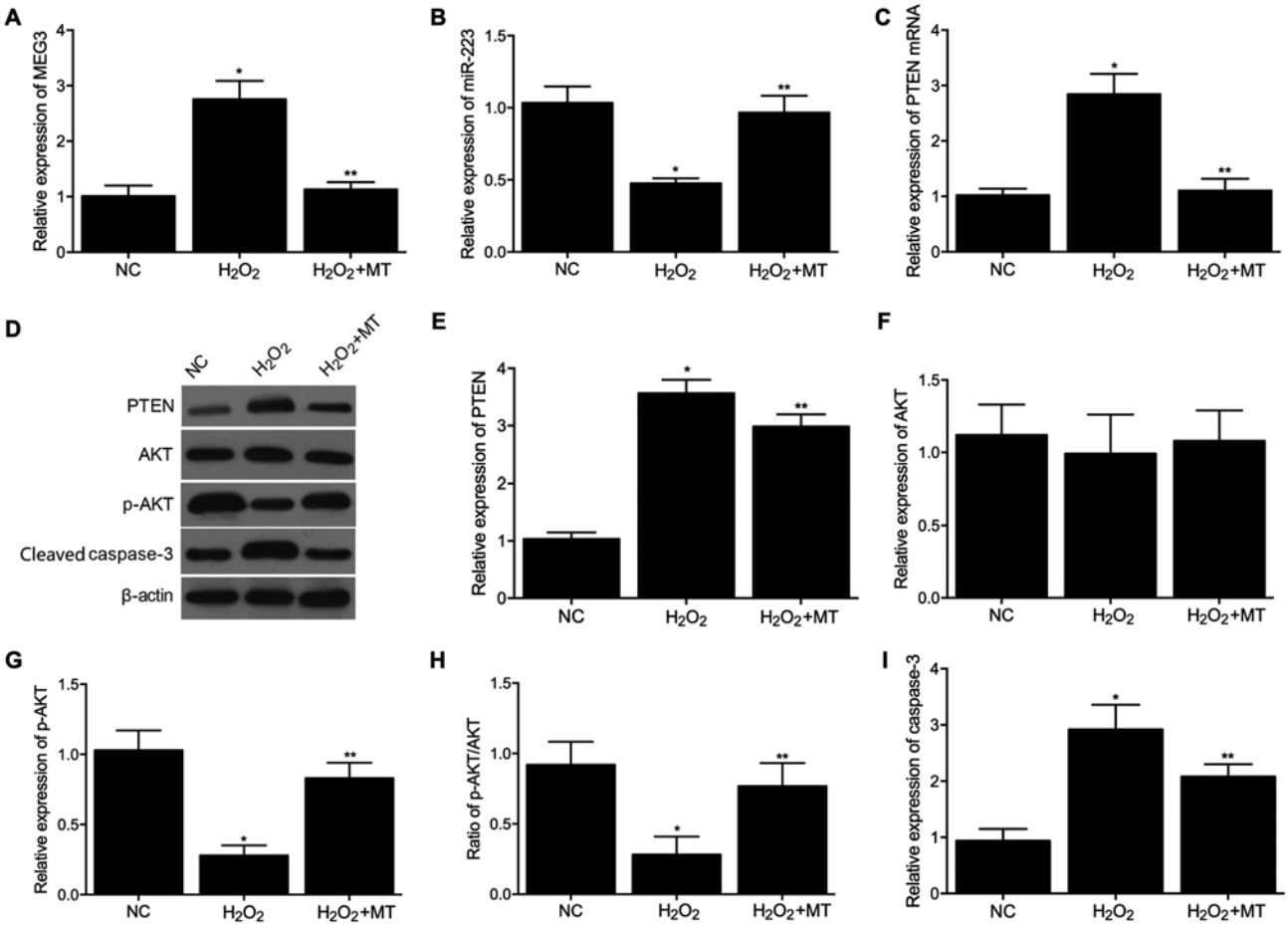

Figure 1. Expression levels of MEG3, miR-223, PTEN, AKT, p-AKT and cleaved caspase-3 among control, H_2O_2 and H_2O_2 + MT groups in SH-SY5Y cells. (A) mRNA expression levels of MEG3 in the three groups. (B) mRNA expression levels of miR-223 in the three groups. (C) mRNA expression levels of PTEN in the three groups. (D) Protein expression levels of PTEN, AKT, p-AKT and cleaved caspase-3 in the three groups. (E) Densitometry analysis of PTEN in the three groups. (F) Densitometry analysis of AKT in the three groups. (G) Densitometry analysis of p-AKT in the three groups. (H) Expression of p-AKT normalized to the expression of AKT in the three groups. (I) Densitometry analysis of cleaved caspase-3 in the three groups. *P<0.05 vs. NC group; **P<0.05 vs. H_2O_2 group. miR, microRNA; PTEN, phosphatase and tensin homolog; AKT, protein kinase B; p-, phosphorylated; MT, melatonin; NC, negative control.

Detection of seizure and duration of convulsion with ECG recordings of β, θ, α and δ waves. Electroencephalography (EEG) signals of β, θ, α and δ waves were recorded using a NicoletOne™ Neurodiagnostic system (Natus Medical Incorporated). In brief, the EEG signal recording was evaluated in two steps: In the first step, the first 30 min of the recorded EEG signals were immediately evaluated to detect any seizure activity. In the second step, the recording was continued for ≥18 h and all recorded EEG signals were evaluated the next day. Only the seizure activity that lasted for ≥10 sec was included for the evaluation. At the end of evaluation, the entire recording of EEG signals was assessed again to carry out the trend analysis using Nicolet One software (version 32; Natus Medical Incorporated).

Statistical analysis. Statistical analysis was carried out using GraphPad Prism (v5.0; GraphPad Software, Inc.) and SPSS for Windows (16.0; SPSS, Inc.). Data is expressed as the mean ± SD. The differences among different groups were analyzed using an unpaired Student's t-test. Comparisons between multiple groups were performed via one way ANOVA, with Bonferroni's test being utilized as the post hoc test. TargetScan (http://www.targetscan.org) and PicTar-Vert (https://pictar.mdc-berlin.de) were employed to predict the potential targets of MEG3 and PTEN. Each experiment was repeated in triplicate. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression levels of MEG3, miR-223 and PTEN among the three groups. RT-qPCR was performed to detect the mRNA levels of MEG3, miR-223 and PTEN in different SH-SY5Y cell groups. As shown in Fig. 1, mRNA expression of MEG3 (Fig. 1A) was increased in the H_2O_2 group, while treatment with MT decreased the expression of MEG3. The expression of miR-223 (Fig. 1B) was decreased in the H_2O_2 group, while treatment with MT increased the expression levels of miR-223 (Fig. 1B). The mRNA expression of PTEN (Fig. 1C) showed the same tendency as MEG3.

Additionally, western blotting was used to compare PTEN, AKT, p-AKT and cleaved caspase-3 expression among the three groups. The protein level of PTEN (Fig. 1D and E) was increased in the H_2O_2 group, while treatment with MT decreased PTEN protein expression. The protein expression of AKT (Fig. 1D and F) was comparable among the

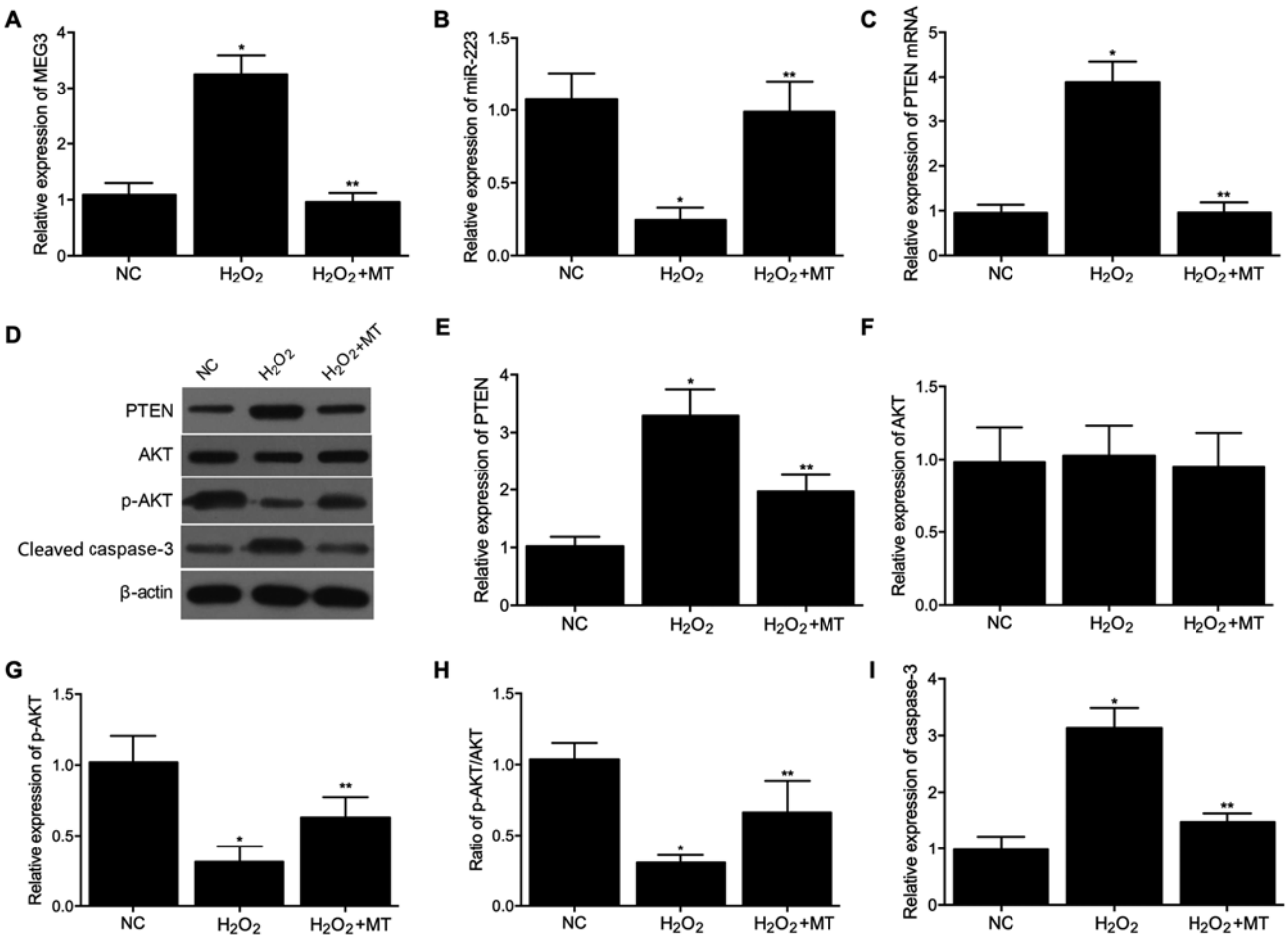

Figure 2. Expression levels of MEG3, miR-223, PTEN, AKT, p-AKT and cleaved caspase-3 among the control, H_2O_2 and H_2O_2 + MT groups in U251 cells. (A) mRNA expression levels of MEG3 in the three groups. (B) mRNA expression levels of miR-223 in the three groups. (C) mRNA expression levels of PTEN in the three groups. (D) Protein expression levels of PTEN, AKT, p-AKT and cleaved caspase-3 in the three groups. (E) Densitometry analysis of PTEN in the three groups. (F) Densitometry analysis of AKT in the three groups. (G) Densitometry analysis of p-AKT in the three groups. (H) Expression of p-AKT normalized to the expression of AKT in the three groups. (I) Densitometry analysis of cleaved caspase-3 in the three groups. $^{*}P<0.05$ vs. NC group; $^{**}P<0.05$ vs. H_2O_2 group. miR, microRNA; PTEN, phosphatase and tensin homolog; AKT, protein kinase B; p-, phosphorylated; MT, melatonin; NC, negative control.

three groups. In addition, the protein expression of p-AKT (Fig. 1D, G and H) was decreased in the H_2O_2 group, while treatment with MT reversed this effect. The protein level of cleaved caspase-3 (Fig. 1D and I) was increased in the H_2O_2 group, while treatment with MT decreased cleaved caspase-3 protein expression. The same experiment was then performed in U251 cells and similar results were observed (Fig. 2A-I).

Verification of the MEG3/miR-223/PTEN axis. TargetScan and PicTar-Vert were employed to predict the potential targets of MEG3 and PTEN. A potential binding site for miR-223 was found in MEG3 (Fig. 3A) and PTEN (Fig. 3B), indicating that MEG3 or PTEN may be direct targets of miR-223. To confirm the putative miR-223 binding site in MEG3 and PTEN, vectors containing WT or MUT MEG3/PTEN were constructed. Then, SH-SY5Y and U251 cells were co-transfected with miR-223 mimics or miR-223 NC. The successful transfection of miR-223 mimics was validated by the evidently upregulated miR-223 expression in SH-SY5Y (Fig. S1A) and U251 cells (Fig. S1B). Moreover, as shown in Fig. 3C, in SH-SY5Y cells, WT MEG3 significantly decreased the relative luciferase activity of miR-223, suggesting that miR-223 could directly bind to MEG3. Also, WT PTEN significantly decreased the relative luciferase activity of miR-223 in SH-SY5Y cells, suggesting that PTEN could directly bind to miR-223 (Fig. 3D). Additionally, the same results were observed in U251 cells (Fig. 3E and F). Taken together, these findings indicated that MEG3, miR-223 and PTEN formed a regulatory axis.

Expression levels of MEG3, miR-223 and PTEN among the three animal model groups. Animals were divided into three groups: NC, FC and the FC + MT. Then, RT-qPCR was performed to detect the mRNA expression levels of MEG3, miR-223 and PTEN. Western blotting was used to examine protein expression levels of PTEN, AKT, p-AKT and cleaved caspase-3. As shown in Fig. 4A-I, the results were consistent with those observed in cells.

To quantify neurological deficits, TUNEL staining was performed to visualize neuronal apoptosis. The results showed that the FC group had a notably higher number of apoptotic neurons than the control group. However, compared with the FC group, MT treatment could markedly decrease the number of apoptotic neurons (Fig. 5).

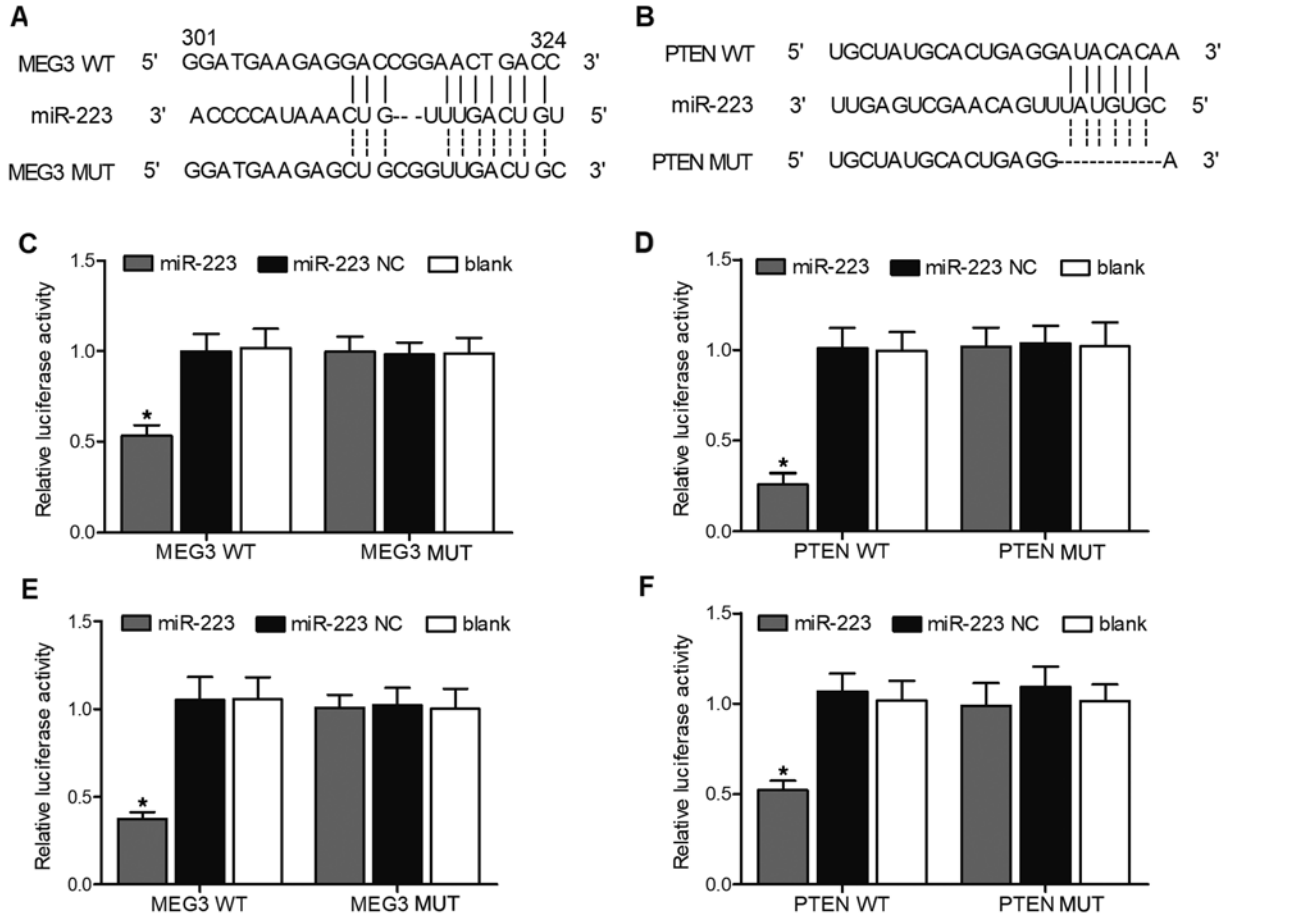

Figure 3. Verification of the targets of MEG3 and miR-233 using a luciferase assay. (A) Predicted binding sites of MEG3 and miR-223. (B) Predicted binding sites of PTEN and miR-223. (C) Luciferase activity of miR-223 in SH-SY5Y cells co-transfected with WT/MT of MEG3 and miR-223 or miR-223 NC. (D) Luciferase activity of miR-223 in SH-SY5Y cells co-transfected with WT/MT of PTEN and miR-223 or miR-223 NC. (E) Luciferase activity of miR-223 in U251 cells co-transfected with WT/MT of MEG3 and miR-223 or miR-223 NC. (F) Luciferase activity of miR-223 in U251 cells co-transfected with WT/MT of PTEN and miR-223 or miR-223 NC. *P<0.05 vs. cells transfected with miR-223 NC. miR, microRNA; PTEN, phosphatase and tensin homolog; NC, negative control; WT, wild-type; MT, mutant.

Detection of seizure/convulsion duration among the different animal groups. The seizure/convulsion duration in different groups on the same day were detected, as shown in Fig. 6. The duration of convulsion (except the first episode of convulsion) in the FC group was significantly higher than those in the FC + MT group.

ECG recordings were then taken for the three groups. As shown in Fig. 7A, there was no significant difference in the first b power ratios among all groups. However, the last β power ratios of FC group and FC + MT group were significantly decreased compared with the first β power ratios of these two groups. The last β power ratio of the FC group was significantly lower than the last β power ratio in the NC group, whereas treatment with MT further decreased the last β power ratio.

As shown in Fig. 7B, there was no significant difference in the first a power ratios among all groups. The last α power ratios of FC group and FC + MT group were significantly increased compared with the first α power ratios of these two groups. The last α power ratio of the FC group was significantly higher than the last α power ratio in the NC group, while treatment with MT further increased the last α power ratio. The same phenomenon was observed in the first θ power ratios, last θ power ratios (Fig. 7C), first δ power ratios and last δ power ratios (Fig. 7D).

Discussion

MT is a neuroendocrine factor produced by the pineal gland (25,26). MT is involved in a wide range of biological functions in the body, including the regulation of circadian rhythm, inflammation, sleep and immunity, as well as having roles in cancer development (27-29). Of note, it has been suggested that MT can alleviate different cardiovascular diseases, such as myocardial ischemia-reperfusion injury, atherosclerosis, hypertension and heart failure (30-32). Previous studies also reported that MT can alleviate atherosclerosis through the MEG3/miR-223/NLRP3 signaling pathway (10). In the present study, it was found that miR-223 could bind to MEG3 and was negatively regulated by MEG3. PTEN was a direct target of miR-223 and was negatively regulated by miR-223.

MEG3 is a lncRNA that carries several miRNAs. Furthermore, it has been demonstrated that miR-223 can directly bind to PTEN to induce chemoresistance in a wide range of tumor cells (33,34). PTEN has been illustrated to inhibit cell survival while promoting apoptosis (34,35). PTEN

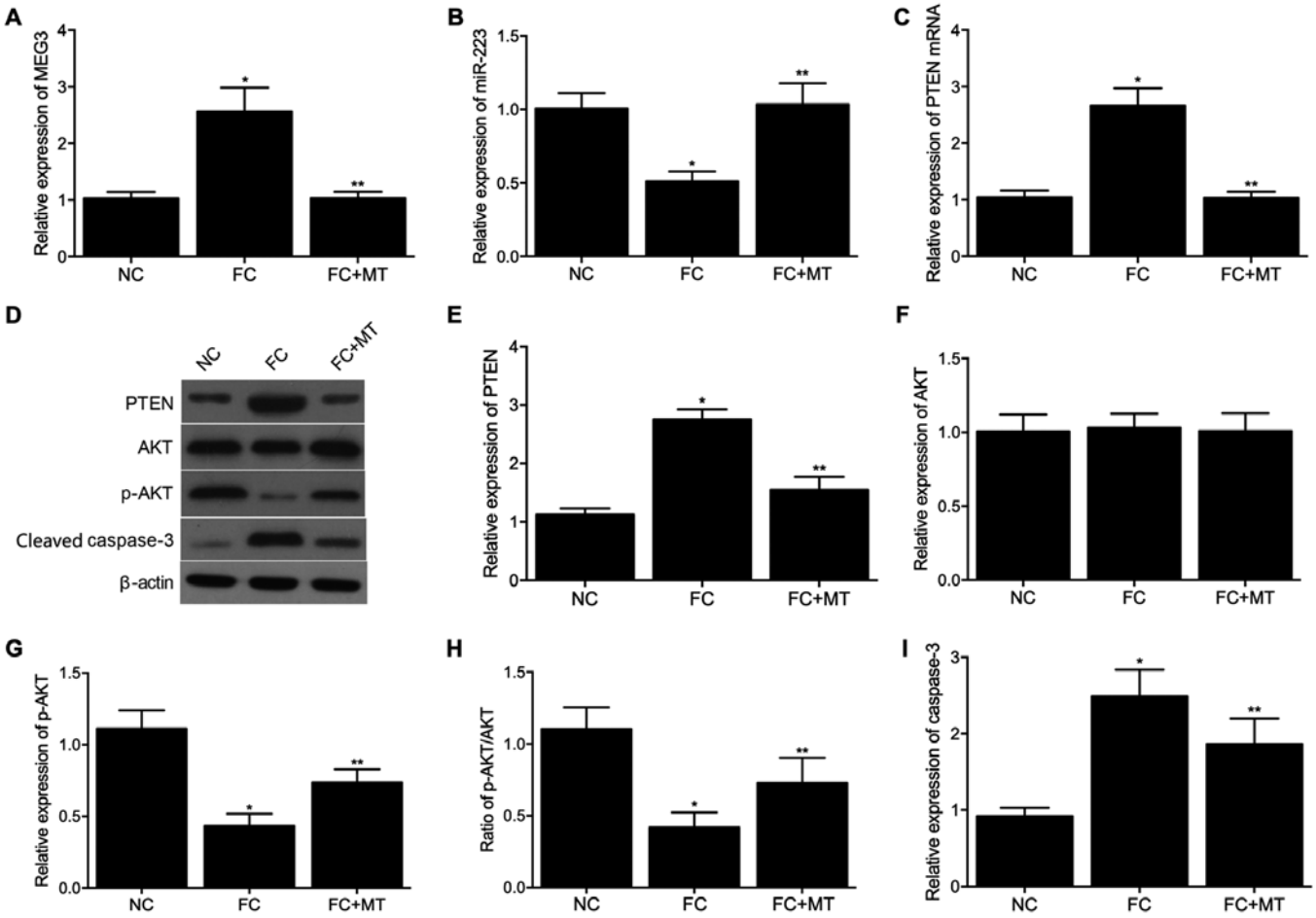

Figure 4. Expression levels of MEG3, miR-223, PTEN, AKT, p-AKT and cleaved caspase-3 among NC, FC and FC + MT groups. (A) mRNA expression levels of MEG3 in the three groups. (B) mRNA expression levels of miR-223 in the three groups. (C) mRNA expression levels of PTEN in the three groups. (D) Protein expression levels of PTEN, AKT, p-AKT and cleaved caspase-3 in the three groups. (E) Densitometry analysis of PTEN in the three groups. (F) Densitometry analysis of AKT in the three groups. (G) Densitometry analysis of p-AKT in the three groups. (H) Expression of p-AKT normalized to the expression of AKT in the three groups. (I) Densitometry analysis of cleaved caspase-3 in the three groups. *P<0.05 vs. NC group; **P<0.05 vs. FC group. miR, microRNA; PTEN, phosphatase and tensin homolog; AKT, protein kinase B; p-, phosphorylated; MT, melatonin; NC, negative control; FC, febrile convulsion.

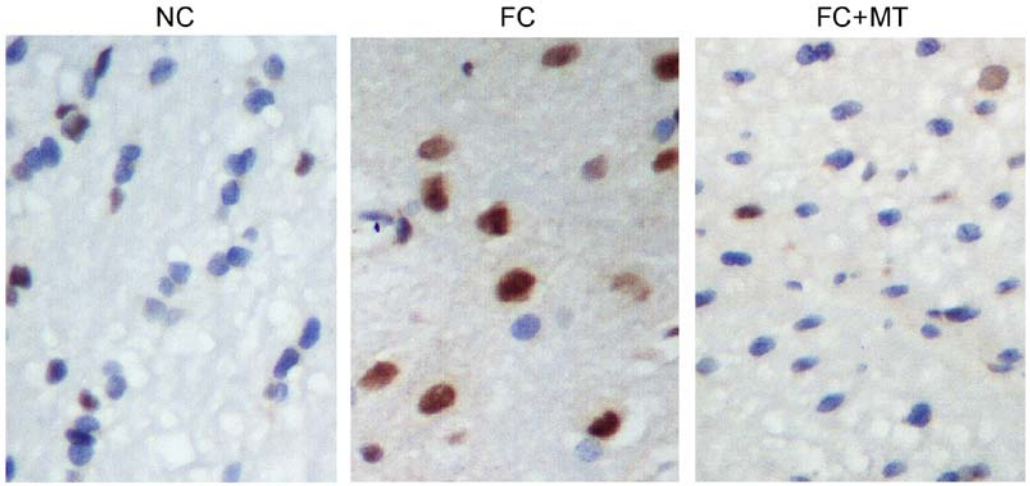

Figure 5. Neuronal apoptosis detected by TUNEL staining among control, FC and FC + MT groups. MT, melatonin; NC, negative control; FC, febrile convulsion.

is also a negative mediator of the PI3K/AKT signaling pathway and is implicated in a wide range of malignancies (36-38). In particular, PTEN expression is dysregulated in Alzheimer's disease (AD) (39). In addition, PTEN nitrosylation is enhanced in early AD (40). Moreover, PTEN dysfunction was found to cause abnormal social behavior in mice (41).

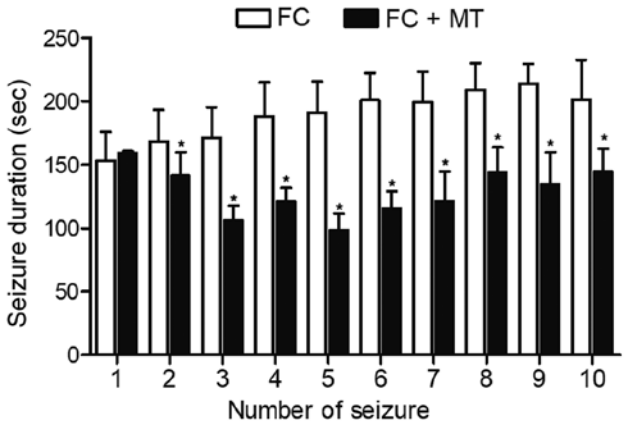

Figure 6. Comparison of seizure duration among all groups on the same day. *P<0.05 vs. FC group. MT, melatonin; FC, febrile convulsion.

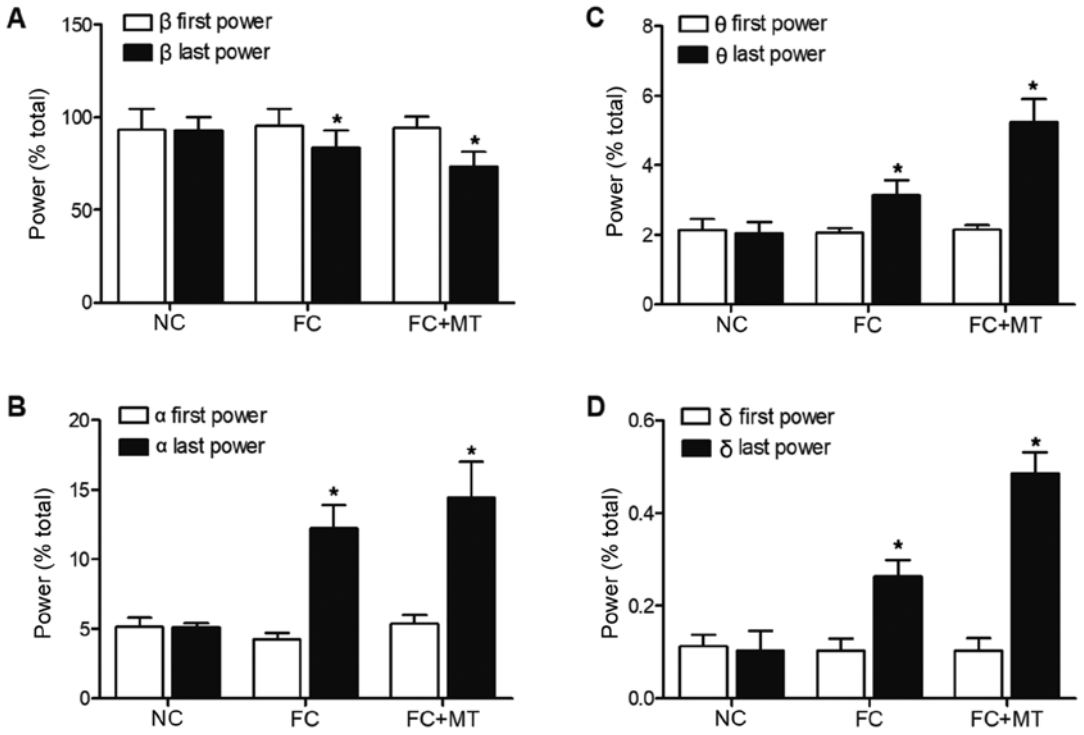

Figure 7. Comparison of EEG recordings among control, FC and FC + MT groups. Comparison of EEG recordings of first and last (A) β, (B) α, (C) θ and (D) δ power ratios among the groups. *P<0.05 vs. NC group. MT, melatonin; FC, febrile convulsion; EEG, electroencephalography; NC, negative control.

PTEN-α, which is one type of PTEN isoform, has been demonstrated to induce the production of ATP by mitochondria (42). Furthermore, inhibition of PTEN signaling was shown to reduce the permeability of mitochondria (43). Additionally, hydrogen sulfide (H2S) was reported to improve the dysfunction in cardiac contractility by inhibiting the function of AKT2, thus alleviating the apoptosis of mitochondrial cells (44). Similarly, it was observed that H2S could reduce the expression of PTEN in mitochondria, and knockdown of PTEN expression in mice has the ability to impair learning and memory, as well as inducing spontaneous seizure (45-47). Furthermore, mice with PTEN dysfunction also show defunct social behaviors (48). Thus, mice that lack PTEN may develop significant behavioral deficits and seizure (49). The results of the present study showed that MEG3, cleaved caspase-3 and PTEN were increased in FC, whereas miR-223 and p-AKT were increased. Treatment with MT could reverse the effects of FC.

The downregulation of PTEN has been demonstrated to activate PI3K/AKT signaling and promote cell survival (15,50). Furthermore, the function of PTEN is significantly affected by PI3K/AKT signaling (51,52). Moreover, the C-terminal hydrophobic motif of AGC kinases has been demonstrated to regulate the catalytic functions of AGC kinases (53,54). In particular, the activation of AKT kinase has been shown to be mediated by several receptor tyrosine kinases (RTKs), such as the RTKs related to epidermal growth factor, insulin, platelet-derived growth factor, fibroblast growth factor and vascular endothelial growth factor (55,56). Moreover, RTKs can directly activate PI3K or indirectly activate PI3K via certain adaptor proteins, including insulin receptor substrate 1/2. A previous

study evaluated AKT phosphorylation at position Ser473 and illustrated that AKT phosphorylation is reduced in the hippocampus of rats suffering from recurrent FS (18).

To conclude, taken together, the present results suggested that MT prevented FC via the MEG3/miR-223/PTEN/AKT signaling pathway. Therefore, MT treatment could be considered as a novel strategy for the treatment of FC disease.

Acknowledgements

Not applicable.

Funding

This study was sponsored by The Project of Wuhan Clinical Medical Research Center in Children's Neural Disease (Wuhan Bureau of Science and Technology, grant no. 2014-160) and Wuhan Yellow Crane Medical Talent Program (Wuhan Bureau of Talent Program, grant no. 2014-10).

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

GW designed the study. JH supervised the study. GW, JH, HZ, SW and ZL performed the experiments and drafted the manuscript. SH performed the experiments and analyzed the data. ZL performed the literature research, GW and JH drafted and reviewed the manuscript. GW and SW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal procedures were approved by the Wuhan Children's Hospital (Wuhan, China) and were in strict compliance with the 'Guide for the Care and Use of Laboratory Animals' published by the US National Institutes of Health.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Freeman JM: Febrile seizures: A consensus of their significance, evaluation, and treatment. Pediatrics 66: 1009, 1980.
2. Karaagac N, Yeni SN, Senocak M, Bozluolcay M, Savrun FK, Ozdemir H and Cagatay P: Prevalence of epilepsy in Silivri, a rural area of Turkey. Epilepsia 40: 637-642, 1999.
3. Molina-Carballo A, Muñoz-Hoyos A, Sanchez-Forte M, Uberos-Fernandez J, Moreno-Madrid F and Acuna-Castroviejo D: Melatonin increases following convulsive seizures may be related to its anticonvulsant properties at physiological concentrations. Neuropediatrics 38: 122-125, 2007.
4. Molina-Carballo A, Acuna-Castroviejo D, Rodriguez-Cabezas T and Muñoz-Hoyos A: Effects of febrile and epileptic convulsions on daily variations in plasma melatonin concentration in children. J Pineal Res 16: 1-9, 1994.
5. ENCODE Project Consortium: An integrated encyclopedia of DNA elements in the human genome. Nature 489: 57-74, 2012.
6. Gibb EA, Brown CJ and Lam WL: The functional role of long non-coding RNA in human carcinomas. Mol Cancer 10: 38, 2011.
7. Brunner AL, Beck AH, Edris B, Sweeney RT, Zhu SX, Li R, Montgomery K, Varma S, Gilks T, Guo X, *et al*: Transcriptional profiling of long non-coding RNAs and novel transcribed regions across a diverse panel of archived human cancers. Genome Biol 13: R75, 2012.
8. Ponting CP, Oliver PL and Reik W: Evolution and functions of long noncoding RNAs. Cell 136: 629-641, 2009.
9. Schmitt AM and Chang HY: Long noncoding RNAs in cancer pathways. Cancer Cell 29: 452-463, 2016.
10. Zhang Y, Liu X, Bai X, Lin Y, Li Z, Fu J, Li M, Zhao T, Yang H, Xu R, *et al*: Melatonin prevents endothelial cell pyroptosis via regulation of long noncoding RNA MEG3/miR-223/NLRP3 axis. J Pineal Res 64: 37-38, 2018.
11. Zhu X, Shen H, Yin X, Yang M, Wei H, Chen Q, Feng F, Liu Y, Xu W and Li Y: Macrophages derived exosomes deliver miR-223 to epithelial ovarian cancer cells to elicit a chemoresistant phenotype. J Exp Clin Cancer Res 38: 81, 2019.
12. Li DM and Sun H: TEP1, encoded by a candidate tumor suppressor locus, is a novel protein tyrosine phosphatase regulated by transforming growth factor beta. Cancer Res 57: 2124-2129, 1997.
13. Myers MP, Pass I, Batty IH, Van der Kaay J, Stolarov JP, Hemmings BA, Wigler MH, Downes CP and Tonks NK: The lipid phosphatase activity of PTEN is critical for its tumor supressor function. Proc Natl Acad Sci USA 95: 13513-13518, 1998.
14. Maehama T and Dixon JE: The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J Biol Chem 273: 13375-13378, 1998.
15. Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP and Mak TW: Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell 95: 29-39, 1998.
16. Hopkins BD, Hodakoski C, Barrows D, Mense SM and Parsons RE: PTEN function: The long and the short of it. Trends Biochem Sci 39: 183-190, 2014.
17. Zhu F, Kai J, Chen L, Wu M, Dong J, Wang Q and Zeng LH: Akt inhibitor perifosine prevents epileptogenesis in a rat model of temporal lobe epilepsy. Neurosci Bull 34: 283-290, 2018.
18. Zhao Y, Han Y, Bu DF, Zhang J, Li QR, Jin HF, Du JB and Qin J: Reduced AKT phosphorylation contributes to endoplasmic reticulum stress-mediated hippocampal neuronal apoptosis in rat recurrent febrile seizure. Life Sci 153: 153-162, 2016.
19. Freyd T, Warszycki D, Mordalski S, Bojarski AJ, Sylte I and Gabrielsen M: Ligand-guided homology modelling of the GABAB2 subunit of the GABAB receptor. PLoS One 12: e0173889, 2017.
20. Zhang B, McDaniel SS, Rensing NR and Wong M: Vigabatrin inhibits seizures and mTOR pathway activation in a mouse model of tuberous sclerosis complex. PLoS One 8: e57445, 2013.
21. MacKenzie G and Maguire J: Chronic stress shifts the GABA reversal potential in the hippocampus and increases seizure susceptibility. Epilepsy Res 109: 13-27, 2015.
22. Xu XK, Wang SY, Chen Y, Zhan L, Shao ZY, Lin L, Yan WC and Mei SF: Fangjing decoction relieves febrile seizures-induced hippocampal neuron apoptosis in rats via regulating the Akt/mTOR pathway. Biosci Rep 38: BSR20181206, 2018.
23. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals: Guide for the Care and Use of Laboratory Animals, 8th edition. National Academies Press (US), Washington, DC, 2011.
24. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
25. Stehle JH, Saade A, Rawashdeh O, Ackermann K, Jilg A, Sebesteny T and Maronde E: A survey of molecular details in the human pineal gland in the light of phylogeny, structure, function and chronobiological diseases. J Pineal Res 51: 17-43, 2011.

26. Acuña-Castroviejo D, Escames G, Venegas C, Díaz-Casado ME, Lima-Cabello E, López LC, Rosales-Corral S, Tan DX and Reiter RJ: Extrapineal melatonin: Sources, regulation, and potential functions. Cell Mol Life Sci 71: 2997-3025, 2014.
27. Manchester LC, Coto-Montes A, Boga JA, Andersen LP, Zhou Z, Galano A, Vriend J, Tan DX and Reiter RJ: Melatonin: An ancient molecule that makes oxygen metabolically tolerable. J Pineal Res 59: 403-419, 2015.
28. Reiter RJ, Mayo JC, Tan DX, Sainz RM, Alatorre-Jimenez M and Qin L: Melatonin as an antioxidant: Under promises but over delivers. J Pineal Res 61: 253-278, 2016.
29. Lax P, Otalora BB, Esquiva G, Rol Mde L, Madrid JA and Cuenca N: Circadian dysfunction in P23H rhodopsin transgenic rats: Effects of exogenous melatonin. J Pineal Res 50: 183-191, 2011.
30. Yu L, Liang H, Dong X, Zhao G, Jin Z, Zhai M, Yang Y, Chen W, Liu J, Yi W, *et al*: Reduced silent information regulator 1 signaling exacerbates myocardial ischemia-reperfusion injury in type 2 diabetic rats and the protective effect of melatonin. J Pineal Res 59: 376-390, 2015.
31. Yu L, Liang H, Lu Z, Zhao G, Zhai M, Yang Y, Yang J, Yi D, Chen W, Wang X, *et al*: Membrane receptor-dependent Notch1/Hes1 activation by melatonin protects against myocardial ischemia-reperfusion injury: In vivo and in vitro studies. J Pineal Res 59: 420-433, 2015.
32. Favero G, Rodella LF, Reiter RJ and Rezzani R: Melatonin and its atheroprotective effects: A review. Mol Cell Endocrinol 382: 926-937, 2014.
33. Chen Q, Qin R, Fang Y and Li H: Berberine sensitizes human ovarian cancer cells to cisplatin through miR-93/PTEN/Akt signaling pathway. Cell Physiol Biochem 36: 956-965, 2015.
34. Keniry M and Parsons R: The role of PTEN signaling perturbations in cancer and in targeted therapy. Oncogene 27: 5477-5485, 2008.
35. Cowell JK, Qin H, Hu T, Wu Q, Bhole A and Ren M: Mutation in the FGFR1 tyrosine kinase domain or inactivation of PTEN is associated with acquired resistance to FGFR inhibitors in FGFR1-driven leukemia/lymphomas. Int J Cancer 141: 1822-1829, 2017.
36. Lue H, Thiele M, Franz J, Dahl E, Speckgens S, Leng L, Fingerle-Rowson G, Bucala R, Luscher B and Bernhagen J: Macrophage migration inhibitory factor (MIF) promotes cell survival by activation of the Akt pathway and role for CSN5/JAB1 in the control of autocrine MIF activity. Oncogene 26: 5046-5059, 2007.
37. Han Z, Chen F, Ge X, Tan J, Lei P and Zhang J: miR-21 alleviated apoptosis of cortical neurons through promoting PTEN-Akt signaling pathway in vitro after experimental traumatic brain injury. Brain Res 1582: 12-20, 2014.
38. Reynolds C, Roderick JE, LaBelle JL, Bird G, Mathieu R, Bodaar K, Colon D, Pyati U, Stevenson KE, Qi J, *et al*: Repression of BIM mediates survival signaling by MYC and AKT in high-risk T-cell acute lymphoblastic leukemia. Leukemia 28: 1819-1827, 2014.
39. Sonoda Y, Mukai H, Matsuo K, Takahashi M, Ono Y, Maeda K, Akiyama H and Kawamata T: Accumulation of tumor-suppressor PTEN in Alzheimer neurofibrillary tangles. Neurosci Lett 471: 20-24, 2010.
40. Kwak YD, Ma T, Diao S, Zhang X, Chen Y, Hsu J, Lipton SA, Masliah E, Xu H and Liao FF: NO signaling and S-nitrosylation regulate PTEN inhibition in neurodegeneration. Mol Neurodegener 5: 49, 2010.
41. Napoli E, Ross-Inta C, Wong S, Hung C, Fujisawa Y, Sakaguchi D, Angelastro J, Omanska-Klusek A, Schoenfeld R and Giulivi C: Mitochondrial dysfunction in Pten haplo-insufficient mice with social deficits and repetitive behavior: Interplay between Pten and p53. PLoS One 7: e42504, 2012.
42. Liang H, He S, Yang J, Jia X, Wang P, Chen X, Zhang Z, Zou X, McNutt MA, Shen WH and Yin Y: PTENα, a PTEN isoform translated through alternative initiation, regulates mitochondrial function and energy metabolism. Cell Metab 19: 836-848, 2014.
43. Zheng X, Zu L, Becker L and Cai ZP: Ischemic preconditioning inhibits mitochondrial permeability transition pore opening through the PTEN/PDE4 signaling pathway. Cardiology 129: 163-173, 2014.
44. Hu N, Dong M and Ren J: Hydrogen sulfide alleviates cardiac contractile dysfunction in an Akt2-knockout murine model of insulin resistance: Role of mitochondrial injury and apoptosis. Am J Physiol Regul Integr Comp Physiol 306: R761-R771, 2014.
45. Kwon CH, Luikart BW, Powell CM, Zhou J, Matheny SA, Zhang W, Li Y, Baker SJ and Parada LF: Pten regulates neuronal arborization and social interaction in mice. Neuron 50: 377-388, 2006.
46. Lugo JN, Smith GD, Morrison JB and White J: Deletion of PTEN produces deficits in conditioned fear and increases fragile X mental retardation protein. Learn Mem 20: 670-673, 2013.
47. Backman SA, Stambolic V, Suzuki A, Haight J, Elia A, Pretorius J, Tsao MS, Shannon P, Bolon B, Ivy GO and Mak TW: Deletion of Pten in mouse brain causes seizures, ataxia and defects in soma size resembling Lhermitte-Duclos disease. Nat Genet 29: 396-403, 2001.
48. Clipperton-Allen AE and Page DT: Decreased aggression and increased repetitive behavior in Pten haploinsufficient mice. Genes Brain Behav 14: 145-157, 2015.
49. Smith GD, White J and Lugo JN: Superimposing status epilepticus on neuron subset-specific PTEN haploinsufficient and wild type mice results in long-term changes in behavior. Sci Rep 6: 36559, 2016.
50. Sun H, Lesche R, Li DM, Liliental J, Zhang H, Gao J, Gavrilova N, Mueller B, Liu X and Wu H: PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. Proc Natl Acad Sci USA 96: 6199-6204, 1999.
51. Liu JL, Sheng X, Hortobagyi ZK, Mao Z, Gallick GE and Yung WK: Nuclear PTEN-mediated growth suppression is independent of Akt down-regulation. Mol Cell Biol 25: 6211-6224, 2005.
52. Blanco-Aparicio C, Renner O, Leal JF and Carnero A: PTEN, more than the AKT pathway. Carcinogenesis 28: 1379-1386, 2007.
53. Arencibia JM, Pastor-Flores D, Bauer AF, Schulze JO and Biondi RM: AGC protein kinases: From structural mechanism of regulation to allosteric drug development for the treatment of human diseases. Biochim Biophys Acta 1834: 1302-1321, 2013.
54. Manning BD and Cantley LC: AKT/PKB signaling: Navigating downstream. Cell 129: 1261-1274, 2007.
55. Liao Y and Hung MC: Physiological regulation of Akt activity and stability. Am J Transl Res 2: 19-42, 2010.
56. Scheid MP and Woodgett JR: Unravelling the activation mechanisms of protein kinase B/Akt. FEBS Lett 546: 108-112, 2003.