Decreased expression of ubiquilin-1 following neonatal hypoxia-ischemic brain injury in mice

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Abstract. Ubiquilin-1 (Ubqln), a ubiquitin-like protein, regulates degradation of misfolded proteins and has been reported to have a crucial role in multiple pathologic and physiologic conditions. The current study was undertaken to investigate the expression of Ubqln in the brain of a neonatal hypoxia-ischemic (HI) brain injury model induced using the Rice method with some modifications. Mouse pups at postnatal day 7 day were used in this study. Pups underwent permanent ligation of the left common carotid artery and a consecutive hypoxic challenge (8% O_2 and 92% N_2 for 120 min). The expression of Ubqln in the brain of pups following HI was analyzed by immunofluorescence staining and western blot analysis. Immunofluorescence staining demonstrated that Ubqln was extensively distributed in the cerebral cortex and hippocampus, and Ubqln was expressed in neurons, astrocytes and microglia in the brains of the HI brain injury model mice. Western blot analyses revealed decreased expression of Ubqln in the HI penumbra of the mouse model compared with Ubqln in the sham control group. The results of this study revealed that HI alters the expression of Ubqln, thus may provide a novel understanding of role of Ubqln in neonatal hypoxic ischemic encephalopathy.

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Abbreviations: Ubqln1, ubiquilin-1; CCA, common carotid artery; HIE, hypoxic-ischemic encephalopathy; TTC, triphenyl tetrazolium chloride

Key words: ubiquilin-1, hypoxia-ischemic brain injury, neonatal mice

Introduction

Neonatal hypoxic-ischemic encephalopathy (HIE) is a neurological condition in newborns characterized by hypoxia and ischemia, and is a major cause of neonatal mortality, neurological behavior deficient and long-term disability (1). Not only are patients in distress, an enormous burden and pressure is placed upon parents, and the rest of society (2). Therefore, there is an urgent need to identify effective treatments for HIE in neonates. Furthermore, elucidating the mechanisms underlying HIE is required (3). Energy failure, intracellular calcium overload, glutamate-mediated excitotoxicity, oxidative stress and inflammation have all been reported to contribute to HIE (4-6). In the current study, the association between the expression of ubiquilin-1 (Ubqln) and the extent of neonatal HI brain injury was investigated.

Abnormal protein aggregation, and intracellular or extracellular accumulation of misfolded and aggregated proteins are major events in the pathogenesis of different neurodegenerative diseases. The ubiquitin-proteasome system has a key role in protecting neuronal homeostasis by removing misfolded/aggregating proteins (7). Ubqln1, also known as proepithelin, is a ubiquitin-like (UbL) protein, including a N-terminal UbL domain, which regulates the interaction with the proteasome and a C-terminal Ub-associated domain and preferentially binds poly-ubiquitinated proteins (8). Previous studies have demonstrated that Ubqln overexpression promotes the degradation of misfolded proteins, inhibits misfolded protein-induced

cytotoxicity, and protects neurons against ischemia and oxidative stress-induced brain injury; whereas knockdown of Ubqln aggravates cerebral ischemia-induced neuronal injury and delays nerve function recovery (7,8). Disruption of Ubqln function is involved in the pathologic process of a number of human neurodegenerative disorders, such as Alzheimer's disease (9,10) and Huntington's disease (11); however, the precise location and distribution of Ubqln in neonatal HIE remains largely unknown. In the current study, the distribution and co-localization of Ubqln in brain tissue was analyzed using immunohistochemical methods. The study determined the level of Ubqln during the development of neonatal hypoxic-ischemic (HI) brain injury.

Materials and methods

Animals. Timed-pregnant C57 mice (n=5; age, 2-3-months) were acquired from Sun Yat-sen University (Guangdong, China). The day of birth of pups was designated day 0 (P0); postnatal 7-day-old (P7) pups of either sex were used in the subsequent experiments. A total of 40 pups were used in this study. All animal-related experiments were approved and organized in accordance with the guidelines of the Experimental Laboratory Animal Committee of Guangdong Pharmaceutical University (permit no: gdpulac2017175), and under the principles of the National Institutes of Health Guide for the Care and Use of Laboratory (12). The animals were housed under controlled temperature (23±2˚C), humidity (55±0%) and lighting conditions (12-h light/dark cycle). Water and food were provided *ad libitum*.

HI brain injury model. Pups were divided into two groups (sham control and HI). A HI brain injury model was established in P7 pups using the Rice-Vannucci method (13), with some modifications. Briefly, P7 pups weighing 5-5.5 g were anesthetized with a 3% isoflurane-oxygen mixture for induction and 2% for maintenance. In pups subjected to the HI model, the left common carotid artery (CCA) was permanently cut off using a bipolar electrocoagulation device (gutta cutter, Jiangsu Kanghua Medical Equipment Co., Ltd., Jiangsu, China). Pups were then transferred to a 37˚C incubator for 10 min until the pups regained consciousness, and were then returned to their dams for 90 min. Subsequently, the pups were placed in a hypoxia chamber containing 8% O_2 in mixture with 92% N_2 for 120 min. Sham controls underwent anesthesia and the left CCA was exposed as in the HI group, but there was no ligation or exposure to hypoxia.

Triphenyl tetrazolium chloride (TTC) staining. At 24 h after completion of occlusion and hypoxic injury, pups were sacrificed, and the whole brains of pups in the HI and sham groups were quickly collected and sectioned coronally into 2-mm slices for TTC staining. Tissue slices were stained with 2% TTC solution (cat. no. 17779; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in a dark incubator at 37˚C for 20 min. The tissue slices were then placed in 4% paraformaldehyde overnight to fix the brains for imaging. Tissue slices were imaged on each side. Each slice of the two hemispheres of infarction area were quantified by ImageJ (version 1.8.0; National Institutes of Health, Bethesda, MD, USA).

Preparation of tissue sections. On the third day after surgery, the pups underwent deep anesthesia by intraperitoneal injection of 10% chloral hydrate (400 mg/kg; cat. no. 302-17-0; Sigma-Aldrich; Merck KGaA) and fixed by transcardiac perfusion of cold PBS followed by ice cold 4% paraformaldehyde in 0.1 M PBS. The brains were removed and further fixed in the same fixative solution at 4˚C overnight, and afterward the brains were dehydrated serially in 10, 20 and 30% sucrose in PBS at 4˚C overnight until sinking occurred. Then, the brains were implanted in Optimal Cutting Temperature compound (cat. no. 4583; Sakura Finetek USA, Inc., Torrance, CA, USA). Serial coronal sections were cut using a freezing microtome at 10-μm intervals and mounted onto poly-L-lysine-coated glass slides.

Immunofluorescence staining. To detect the expression and distribution of Ubqln in the brains of neonatal HI pups, sections were washed with PBS. Following blocking, using blocking buffer (QuickBlock™ Blocking Buffer for Immunol Staining, cat. no. P0260, Beyotime Institute of Biotechnology, Shanghai, China) for 1 h at room temperature to reduce non-specific staining. Then, the sections were incubated with primary antibody against Ubqln (dilution, 1:500; cat. no. 16400-I-AP; Proteintech Group, Inc., Rosemont, USA.) in PBS containing 0.3% Triton X-100 at 4˚C overnight. After sufficient washing with PBS, appropriate secondary antibodies in Alexa Fluor®594 (1:1,000 dilution; cat. no. A-11012; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) were added and incubated for 2 h at 37˚C in the dark, and the sections were washed three times with PBS. Nuclei were stained with the nuclear dye, DAPI (0.1 μg/ml), for 5 min at room temperature and fully rinsed with PBS, then the coverslips were mounted on slides with FluorSave reagent (cat. no. P0126; Beyotime Institute of Biotechnology) and the morphologies observed under a fluorescence microscope (BX51; Olympus Corporation, Tokyo, Japan). Ischemic ipsilateral cortical and hippocampal regions were selected for imaging, and five visual fields were selected for each section (magnification, x100 and x200).

Double immunofluorescent staining. The cellular location of Ubqln was also determined in the brains of HI pups. Frozen sections were heated and washed with PBS then incubated with blocking solution with 10% normal goat serum for 1 h at room temperature. The sections were then incubated with primary antibody against RNA binding protein fox-1 homolog 3 (NeuN) for neurons (dilution, 1:1,000; cat. no. SAB4300883; Sigma-Aldrich; Merck KGaA), glial fibrillary acidic protein (GFAP) for astrocytes (dilution, 1:1,000; cat. no. ab10062, Abcam, Cambridge, USA), allograft inflammatory factor 1 (Iba-1) for microglial cells (dilution, 1:1,000; cat. no. ab15690, Abcam) and Ubqln (dilution, 1:500; cat. no. SAB1305680, Sigma-Aldrich, Merck KGaA) in PBS containing 0.3% Triton X-100 at 4˚C overnight. The sections were then washed with PBS and incubated with the corresponding fluorescence-conjugated secondary antibodies (Alexa Fluor®488 goat anti-mouse IgG (H+L), cat. no. A-11029; Alexa Fluor®594 goat anti-rabbit IgG (H+L), cat. no. A-11012; Invitrogen; Thermo Fisher Scientific, Inc.) for 1 h at room temperature. Nuclei were stained for DAPI (0.1 μg/ml), for 5 min at room temperature. Images were obtained using a fluorescence microscope (BX51; Olympus

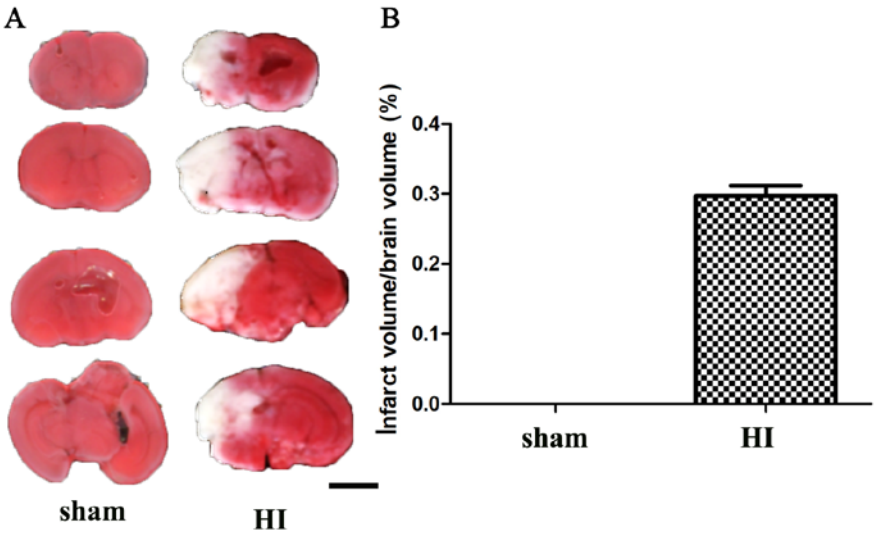

Figure 1. Infarct volumes in neonatal HI brain injury (n=3) and sham (n=3) groups. (A) Representative infarcted areas from HI brain injury following TTC staining (damaged areas in white and undamaged areas in red). Scale bar, 1 cm. (B) Analysis of brain infarction volumes of TTC images. HI, hypoxic-ischemic; TTC, triphenyl tetrazolium chloride.

Corporation). Ischemic ipsilateral cortical and hippocampal regions were selected for imaging, and five visual fields were selected for each section (magnification, x100 and x200).

Western blot analysis. Western blotting was used to determine the level of Ubqln semi-quantitatively following HI treatment. The expression of β-actin was designated as the internal control. For western blot analysis, the total protein of the ipsilateral hemisphere was removed and extracted at 1 and 3 days after HI treatment with a Micro BCA Protein Assay kit according to the manufacturer's instructions (cat. no. P0012S; Beyotime Institute of Biotechnology). The bicinchoninic acid assay (Beyotime Institute of Biotechnology) was used to measure the protein concentration, with BSA (cat. no. P0012S, Beyotime Institute of Biotechnology) as the standard. For each run, 20 mg protein/well lysate was separated by SDS-PAGE on 10% gels and transferred to a polyvinylidene fluoride membrane (EMD Millipore) The membranes were blocked in 5% non-fat milk for 1 h at room temperature and incubated overnight in the presence of the primary antibodies against Ubqln (1:5,000, cat. no. 16400-I-AP; Proteintech Group, Inc) and β-actin (1:10,000; cat. no. T0022; Affinity Biosciences, Cincinnati, OH, USA) at 4˚C. The membrane was fully washed three times with TBS containing 0.05% Tween 20 (TBST) and subsequently reacted with the corresponding secondary antibody (1:10,000, Goat Anti-Rabbit IgG (H+L) HRP; cat. no. S0001; Goat Anti-Mouse IgG (H+L) HRP; cat. no. S0002; both Affinity Biosciences) for 1 h at room temperature. After thorough washing with TBST, the protein bands were developed using enhanced chemiluminescence detection reagents (cat. no. WBKLS0500; Merck KGaA). The optical density of the bands on the films was analyzed using ImageJ version 1.8.0 (National Institutes of Health).

Statistical analysis. Statistical analysis was performed using SPSS 19.0 software (IBM Corp.). The results are expressed as the mean ± standard error from at least three independent experiments. A statistical evaluation was performed with a one-way analysis of variance followed by Duncan's multiple range test, which was used to compare the sham control and HI groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HI brain injury model. To determine the expression of Ubqln in the brains of the HIE mouse model, a mouse pup model was established using the Rice-Vannucci method with some modifications. At 24 h after HI injury, the ischemic infarctions area appeared white and regularly included the neocortex and basal ganglia, as confirmed by TTC staining (Fig. 1). These results suggested that the HIE model had been successfully established.

Expression of Ubqln in the brains of the HIE mouse model. Immunofluorescence staining was performed of the mouse brain tissues to determine the expression of Ubqln in the brains of the HIE model mice. The results demonstrated that Ubqln was expressed predominantly in the cortex and hippocampus (Fig. 2). To analyze the cell type-specific expression of Ubqln in the brains of the HIE mouse model, immunofluorescent double labeling was performed with specific markers for neurons (NeuN), astrocytes (GFAP) and microglia (Iba-1). The results indicated that the mature neuronal marker, astrocyte marker and microglia marker were double-labeled with Ubqln (Fig. 3), which indicated that was predominantly expressed in neurons and microglia during the early stages of mouse brain development.

Western blot analysis of Ubqln in the HIE mouse model. Western blot was then performed to further determine the protein expression of Ubqln in the brains of the two groups. Ubqln expression was significantly decreased in the HI group at 1 and 3 days after HI injury compared with the sham group (Fig. 4). β-actin (43 kDa) was used as an internal control. Therefore, the western blot results indicated that Ubqln expression was markedly decreased following exposure to HI brain injury.

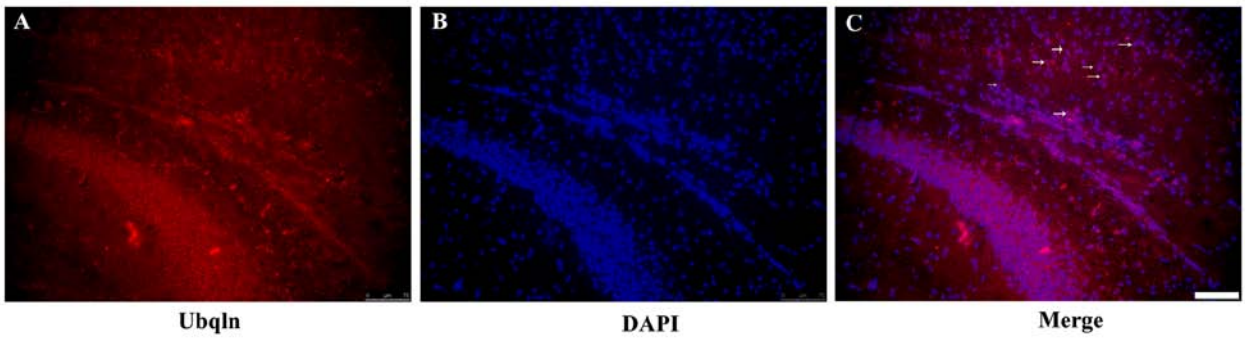

Figure 2. Ubqln expression in the brains of a HIE mouse model. (A) Ubqln expressed predominantly in the cortex and hippocampus. (B) Cell nuclei were stained with DAPI (blue). (C) Merged staining images. Scale bar, 75 μm. Ubqln, ubiquilin-1; HIE, hypoxic-ischemic encephalopathy.

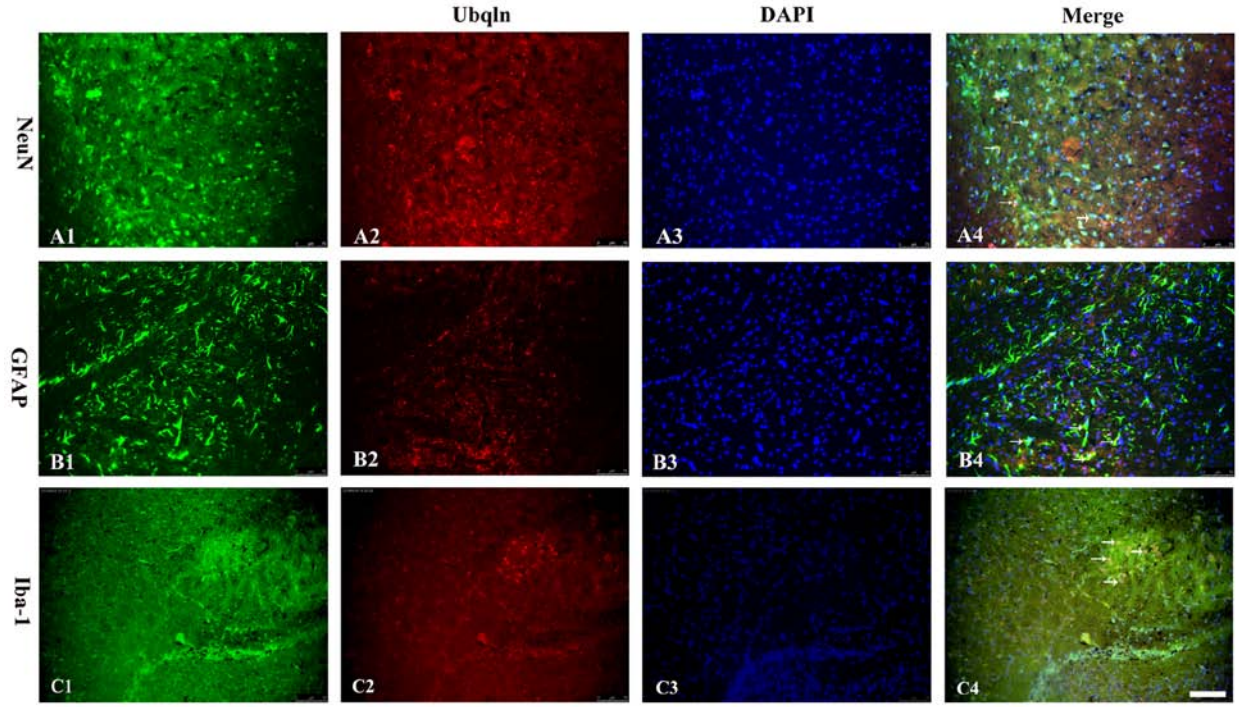

Figure 3. Ubqln is expressed in neurons, astrocytes and microglia in the brains of neonatal HI brain injured mice. Ubqln (red) is expressed in NeuN$^+$ neurons (green; A1-4), GFAP$^+$ astrocytes (green; B1-4) and Iba-1$^+$ microglia (green; C1-4). The cell nuclei were stained with DAPI (blue). Scale bar, 75 μm. Ubqln, ubiquilin-1; NeuN, RNA binding protein fox-1 homolog 3; GFAP, glial fibrillary acidic protein; Iba-1, allograft inflammatory factor 1.

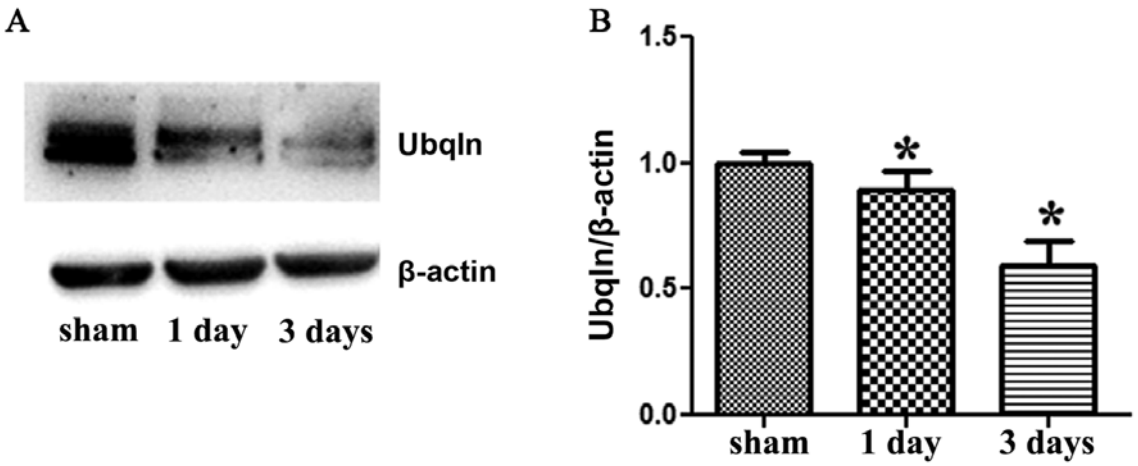

Figure 4. Decreased expression of Ubqln following neonatal HI brain injury in mice. (A) Ubqln expression in the brain following exposure to HI injury at 1 and 3 days following surgery. (B) Densitometry analysis of western blot demonstrating that Ubqln expression was significantly decreased in the HI group compared with the sham group. n=3 for each group. (*P<0.05 vs. sham). HI, hypoxic-ischemic; Ubqln, ubiquilin-1; Con, sham control.

Discussion

This study if the first to report, to the best of our knowledge, Ubqln expression in the brains of a HIE mouse model. The present work demonstrated the following: i) Ubqln was widely expressed in the cortex and hippocampus in brains of the HIE mouse model; ii) Ubqln was expressed in neurons, astrocytes, and microglia in the brains of the HIE mouse model; and iii) Ubqln expression was downregulated after neonatal HI brain injury in mice.

The pathogenesis of HIE is not fully understood, thus studying the underlying molecular mechanism is important for the development of novel prophylactics and therapeutics against neuronal death in neurodegenerative diseases. In particular, the isolation and identification of novel molecules associated neuronal survival/death is important. Currently, increasing evidence links Ubqln to the pathogenic mechanism underlying Alzheimer's disease (AD) and other neurodegenerative diseases (14). Ubqln has been reported to have a critical role in the regulation of the levels, subcellular targeting, aggregation, and degradation of various neurodegenerative disease-associated proteins (15). Despite a number of studies regarding the role of Ubqln in anti-oxidation (16), regulation of autophagy (17), cell protection and involvement in tumorigenesis (18), no precise location and distribution of Ubqln in neonatal HIE has been reported previously. In the present study, Ubqln immunofluorescence staining revealed that Ubqln was expressed abundantly in the brains of neonatal sham and HI pups. The expression of Ubqln and prognosis in the brains of neonatal HI pups was clarified for the first time, to the best of our knowledge, and Ubqln may be a novel molecular marker to predict prognosis in HIE.

Numerous studies have demonstrated that the secreted protein, Ubqln, has an essential role in the regulation of protein degradation, which is involved in the pathophysiology of cancer and neurodegenerative diseases. Ubqln is frequently overexpressed in breast (19), gastric (20) and lung cancers (21). It has been suggested that high Ubqln expression is associated with tumor size, lymph node metastasis, TNM stage and vascular invasion, and is significantly associated with a worse prognosis in patients with gastric and breast cancer (19,20); however, Shah *et al* (21) reported that expression of Ubqln serves as a potential predictive biomarker for therapeutic efficacy in patients of non-small cell lung cancer. In the central nervous system, Ubqln is an AD-associated protein, which is known to modulate amyloid precursor protein processing, amyloid-β secretion, and presenilin-1 accumulation (22). A study by Satoh *et al* (23) showed Ubqln expression in the frontal cortex and hippocampus in brains from patients with AD. Furthermore, Ubqln immunoreactivity is concentrated in Hirano bodies and dystrophic neurites in brains from patients with AD, which suggests that aberrant expression of Ubqln may be a pathologic hallmark of AD. Based on *in vitro* studies, Ubqln expression has been reported in human neuroblastoma cells and rat cortical neurons (24). In the present study, cell localization of Ubqln in the brains of neonatal HI pups was identified. Ubqln was expressed in neurons, astrocytes and microglia *in vivo*. The results of the current study agree with a previous study (24).

Ubqln has an important role in clearing mislocalized mitochondrial proteins upon cell stimulation, and the absence leads to suppression of protein synthesis and cell cycle arrest (25). In the present study, the expression of Ubqln protein was significantly decreased in HI model mice compared with sham controls, as determined by western blot analysis, suggesting that decreased Ubqln may have a role in the development of HIE. Liu *et al* (16,26) demonstrated that Ubqln protects cells from oxidative stress and ischemic stroke causing tissue injury in mice by developing Ubqln transgenic and conditional knockout mouse models to perform gain- and loss-of-function analysis of Ubqln. The yeast two-hybrid system has shown that Ubqln interacts with protein disulfide isomerase (PDI), and observed that Ubqln, together with PDI, is localized in the endoplasmic reticulum (ER) and upregulated in response to hypoxia (27). It has also been demonstrated that Ubqln association with PDI in the ER is involved in tolerance to stress-induced apoptotic cell death (28). In neonatal brains, HI brain injury usually causes cell death via necrosis or apoptosis (29). Previous studies have reported that apoptosis is more frequent in HI brain injury (30,31). Kojima *et al* (32) reported that many genes upregulated following HI injury are associated with cell death signaling, such as the arachidonic acid cascade. By contrast, many downregulated genes affect the expression of target genes, reflecting progressive damage by the HI insult. The lower expression of Ubqln in the brains of neonatal HI pups indicates that Ubqln may contribute to the pathogenesis of HIE by regulating apoptosis.

In summary, immunofluorescence staining and western blot analysis demonstrated the expression and cell location of Ubqln in the brains of neonatal HI pups. Decreased expression of Ubqln was detected following HI brain injury, which suggests that the decreased expression of Ubqln may contribute to the development of HIE. Therefore, further studies should focus on the mechanism underlying the regulation of the changes in Ubqln during HIE.

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Availability of data and materials statements

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

Authors' contributions

LiL contributed to the general administration, statistical analysis, manuscript writing; YL, XT, XR and WZ assisted in the completion of western blotting, behavioral tests and other procedures; JL, LZ and WC assisted in the completion of western blotting, immunofluorescence and other procedures. PZ and WW established animal models and providing technical guidance; LaL and MW provided technical guidance. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The animal-related experiments were approved and organized in accordance with the guidelines of the Experimental Laboratory Animal Committee of Guangdong Pharmaceutical University (permit no: gdpulac2017175).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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