RESEARCH ARTICLES

PINK1 antagonize intracerebral hemorrhage by promoting mitochondrial autophagy

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Abstract

Background: Intracerebral hemorrhage (ICH) causes neurotransmitter release, oligemia, membrane depolarization, mitochondrial dysfunction, and results in the high rate of mortality and functional disability. Here, we focus on PTEN-induced kinase 1 (PINK1), a mitochondrial-targeted protein kinase, and explore its role in ICH progression. **Methods**: The qPCR and Western blot were performed to examine the expression of PINK1 in ICH patients and mouse model. PINK1 gain- and loss-of-function mice were used to evaluate their protective role on brain injury and behavioral disorders. Flow cytometry was carried out, mitochondrial membrane potential and reactive oxygen species production were detected to explore the distribution and neuroprotective function of PINK1. **Results**: PINK1 mRNA was upregulated, however, its protein was downregulated in ICH patients. The reduction of PINK1 was mainly happened in microglial cells in ICH model. Overexpression of PINK1 is able to rescue ICH-induced behavioral disorders. PINK1 protects ICH-induced brain injury by promoting mitochondrial autophagy in microglia. **Conclusion**: PINK1 possesses a neuroprotective role and antagonizes ICH by promoting mitochondrial autophagy, which may be of value as a therapeutic target for ICH treatment.

Introduction

Intracerebral hemorrhage (ICH) is a seriously devastating stroke caused by blood vessel rupture in the brain and the subsequent bleeding into the surrounding tissues.[1,2] ICH accounts for 2,000,000 (about 15%) of all strokes and is associated with a higher mortality rate than either ischemic stroke or subarachnoid hemorrhage.[3,4] Hypertension is the commonest contributing factor to ICH, and other causes including smoking, advanced age, alcohol, amyloid angiopathy, diabetes, coagulopathy, vascular anomalies, tumors, and various drugs.[1,5] ICH imposes a significant economic burden, more than $17 billion in annual direct costs to the U.S. healthcare system, and results in more disability than subarachnoid hemorrhage.[2,4,6]

ICH injuries disrupt neurons and glia, and induce mechanical deformation that causes neurotransmitter release, oligaemia, membrane depolarization, and mitochondrial dysfunction.[7,8] The regions surrounding ICH are characterized by edema, apoptosis, necrosis, and inflammatory cells.[1] The production of coagulation and hemoglobin breakdown are the secondary cascade of ICH, which can activate microglia and induce the breakdown of blood–brain barrier, vasogenic edema, and apoptosis in brain[9–11] Moreover, ICH triggers a series of events leading to secondary insults and severe neurological deficits, which can change motor function, cognition, and emotion-related behavior.[12,13] Given that there are no proven preventative therapies other than control of hypertension and limited modifiable risk factors for ICH, the most promising strategy to therapeutics might be the identification of novel genetic targets with a role in ICH.[14,15]

ICH animal models have significantly advanced our understanding of pathophysiology, thereby identifying numerous therapeutic targets. Rodent models are the most commonly used ICH models, which are created by

intraparenchymal infusion of autologous blood or bacterial collagenase.[16,17] The rapid blood accumulation in the parenchyma of the brain cause increased local pressure and disrupt the normal anatomy. And the following clotting and thrombin production activate proinflammatory response and promote the infiltration of hematogenous inflammatory cells.[18,19]

PTEN-induced kinase 1 (PINK1) is a mitochondrial-targeted serine/threonine-protein kinase, which has a protective role against mitochondrial apoptosis and dysfunction with mitochondrial quality control activating the mitochondrial damage response pathway.[20,21] Therefore, mitochondrial dysfunction is one of the major characters and risk factors of ICH. In this study, we investigated the regulatory role of PINK1 in ICH progression by patient samples and rodent model and explored the underlying anti-ICH mechanism of PINK1 in microglia. To our knowledge, this is the first study exploring that PINK1 protects brain tissue from ICH by promoting mitochondrial autophagy, indicating that PINK1 might work as a therapeutic target for ICH treatment.

Materials and methods

Patient samples

To investigate the pathological role of PINK1 in the brain tissue of ICH patients, brain tissues and clinical records derived from 70 healthy controls and 151 ICH patients were collected and analyzed in this study. All the patients in this study were obtained informed consent following the guidelines approved by the Ethics Committee at the Second Hospital of Hebei Medical University. ICH patients were scanned by computed tomography (CT), to measure the volume of hematoma (see NIHSS and hematoma analyses). Brain tissues were excised and frozen in liquid nitrogen immediately. Brain tissues from all healthy controls and ICH patients were used for mRNA level analysis (qRT-PCR). Brain tissues from healthy controls (#23 and #44) and ICH patients (#19 and #79) were used for protein level analysis (Western blot).

Mouse and ICH model

Animal studies were reviewed and approved by the Second Hospital of Hebei Medical University. Eight-week-old male C57/BL6 wild type (WT) mice and PINK1 knockout (KO) mice were purchased from Shanghai Laboratory Animal Center (Shanghai, China). Mice were kept in a pathogen-free room, fed the normal chow diet, and housed in standard mouse cages on a 12h/12h light–dark cycle. The mouse ICH model was established by intrastriatal injection of autologous blood as described

previously.[22] Mice were narcotized and immobilized on a stereotaxic frame. A 1-mm diameter burr hole was drilled in the skull and blood was infused into the left corpus striatum (15 μl/5 min) using a 26-g stainless steel cannula. Core body temperature was maintained at $37 \pm 0.5°C$ during surgery and for 2 h afterward.

Lentivirus of PINK1

To build the lentiviral vector to express PINK1 in mice, PINK1 was sub-cloned into pLVX (Takara Bio Inc., Kusatsu, Japan). PINK1 shuttle plasmid was co-transfected with pCMV-dR8.2 dvpr (Addgene, #8455) and pCMV-VSV-G (Addgene, #8454) into Lenti-X 293T cells. Viral particles were concentrated by ultracentrifugation and stored in a −80°C freezer.

RNA extraction and qRT-PCR

Total RNA of human brain, mouse brain, and cells was extracted by TRIzol™ Reagent (Invitrogen, Carlsbad, USA). First-strand cDNA was generated by BeyoRT M-MuLV Reverse Transcriptase kit (Beyotime, Shanghai, China). qRT-PCR was conducted using LightCycler® 96 System (Roche, Basel, Switzerland). The relative expression of target genes was normalized by GAPDH and calculated by the $2^{-\Delta\Delta CT}$ method. Primers (5'-3') used in this study: human PINK1, F: GCCTCATCGAGGAAAAA-CAGG, R: GTCTCGTGTCCAACGGGTC; human GAP DH, F: GGAGCGAGATCCCTCCAAAAT; R: GGCTGTT GTCATACTTCTCATGG; mouse PINK1, F: TTCTTCCGC CAGTCGGTAG, R: CTGCTTCTCCTCGATCAGCC; mouse GAPDH, F: AGGTCGGTGTGAACGGATTTG; R: GGGGTCGTTGATGGCAACA.

NIHSS and hematoma analyses

National Institutes of Health Stroke Scale (NIHSS) is a neurological function inspection scale to evaluate the neurologic impairment degree of stroke patients. The 151 ICH patients were divided into six groups based on their NIHSS score (0–4, 5–9, 10–14, 15–19, 20–24, >25), following the evaluation method published recently.[23] Patients with ICH were scanned by CT and linear regression was used to model the mean volume of hematoma (<20, 20–30, >30 cm³) as described previously.[24] The correlation of PINK1 mRNA level with NIHSS and volume of hematoma was analyzed using the GraphPad Prism package.

Behavior analyses

The behaviors of WT, PINK1 gain-of-function, and PINK1 loss-of-function ICH mice were conducted in a quiet and

low-light room by an experimenter masked with respect to the treatment groups. Lentivirus of PINK1 was injected 3 days before the generation of the ICH model. Footfault, circling, postural flexing, and forward placing were performed at 1, 3, 7, and 14 days after ICH induction, according to the previously published protocol.[25] Total neurologic deficit score (NDS, 0 to 16) was calculated by combining the score on the above four tests. All the behavior tests were carried out from 10:00 am to 4:00 pm.

Flow cytometry

The treated mouse was transcranial perfused with 15 ml of $1 \times$ phosphate-buffered saline. Harvested the brain and gently separated it into small pieces. The segments were transferred into a 50 ml tube, 10 ml of trypsin was added, and incubated at 37 °C for 10 min. After incubation, triturated and dissociated the brain clumps, and then added 25 ml RPMI1640 culture medium (Sigma-Aldrich, St. Louis, USA). Filtered the suspension through a 60 μm filter and span at 400 g for 5 min. Resuspend cells with RPMI1640 culture medium and diluted cells to a concentration of 5×10^5 cells/ml. Brain cells were sorted by CytoFLEX S cytometer (Beckmann Coulter, Pasadena, USA) and analyzed by FlowJo software (BD Biosciences). Microglia were identified by CD68 (MCA341, Serotec), oligodendrocytes were identified by OSP (Ab7474, Abcam), astrocytes were identified by GFAP (G3893, Sigma-Aldrich), and the rest of the cells were regarded as neurons. Cellular reactive oxygen species (ROS) of microglia were measured using the dihydroethidium probe as previously described.[26]

Mitochondrial potential measurement

The mitochondrial membrane potential of microglia was measured by MitoProbe™ JC-1 assay Kit (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. The mitochondrial potential was analyzed by ImageJ software as described previously.[27]

Western blot

Brain tissues were lysed by lysis buffer consisting of 150 mM NaCl, 50mM Tris (pH7.4), 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktails. Target protein expression was determined by Western blot as previously described.[28] Primary antibody of PINK1 (ab23707, 1:1000 dilution), PARKIN (ab73016, 1:1500 dilution), and LC3II (ab192890, 1:1000 dilution) were purchased from Abcam (Cambridge, UK); GAPDH (#2118, 1:2000 dilution) primary antibodies were ordered from Cell Signaling Technology, Inc. (Danvers, USA).

Statistical analysis

Prism7 (https://www.graphpad.com/) was used in this study for statistical analysis. Student's t test, one-way, and two-way analysis of variance (ANOVA, post hoc tests with Tukey's method) were used to analyze the differences between treated and control groups. All data were represented as mean ± standard deviation (SD).

Results

Elevation of PINK1 mRNA is positively correlated with NIHSS and volume of hematoma in ICH patients

To evaluate the function of PINK1 in ICH, we detected its transcriptional expression in 151 ICH patients and 70 healthy controls using qRT-PCR. As shown in Figure 1, the PINK1 mRNA level was significantly elevated in ICH patients, about threefold higher than healthy controls ($p < 0.001$). National Institutes of Health Stroke Scale (NIHSS) is a systematic assessment tool to evaluate the neurologic deficit of stroke patients. Based on the NIHSS scale, 151 ICH patients were divided into six groups (0–4, 5–9, 10–14, 15–19, 20–24, >25) and then we analyzed the PINK1 expression among these groups. There was no difference in lower NIHSS scale patients (<15), interestingly, the PINK1 mRNA level was remarkably increased in higher NIHSS scale patients, especially in 15–19 and >25 groups (Fig. 2A). In agreement with inter-group analysis, a significant positive correlation was observed between the NIHSS scale and PINK1 expression in these ICH patients ($R^2 = 0.1884$, $p < 0.001$) (Fig. 2B). Moreover, the PINK1 mRNA level markedly increased following the expanding volume of hematoma (Fig. 2C), which also displayed a significantly positive correlation between PINK1 expression and volume of hematoma (Fig. 2D). These results suggested that the elevation of PINK1 mRNA closely links to the illness severity of ICH, and PINK1 might play as a risk factor in ICH progression.

PINK1 possesses protective activity in the ICH mouse model

PINK1 gain- and loss-of-function mice and ICH model were created to further investigate the regulatory role of PINK1 in ICH progression, and the outcome of mice behavior of different genotype was determined by neurological deficits to score at 1, 3, 7, and 14 days after ICH induction, and footfault, circling, postural flexing, and forward placing deficits of treated mice were worsened over time (Fig. 3A–D). PINK1 KO mice did not exhibited any improvement of behavior deficit, on the contrary,
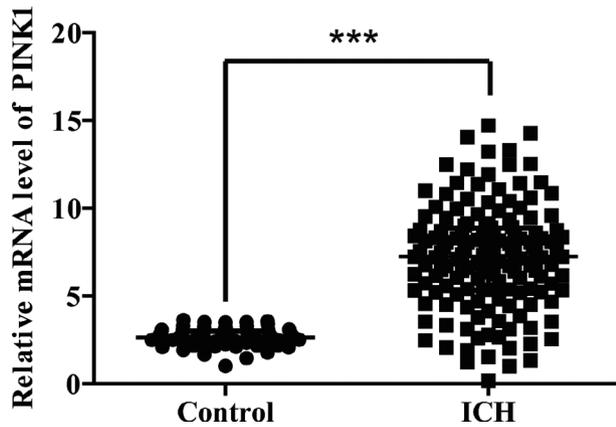
Figure 1. PINK1 is upregulated in ICH patients. PINK1 mRNA level in brain tissue is measured by qPCR in 151 ICH patients and 70 healthy controls. Data are presented as mean $\pm$ SD, *** indicates $p < 0.001$.

their neurological deficits score was poor than WT controls (Fig. 3A–D). Intriguingly, PINK1 overexpression mice exhibited better performance and higher score than those of both WT and KO mice (Fig. 3A-D). The composite neurological deficit score showed the same trend that PINK1 gain-of-function mice had improved behavior deficit, while loss-of-function mice had more serious behavior deficit relative to WT control (Fig. 3E). The above data indicated that PINK1 might be a protective factor instead of a risk factor in the ICH mouse model.

PINK1 protein level is reduced in ICH patients

To resolve this conflict, we returned to human brain tissue samples and detected the PINK1 protein level in ICH patients and healthy controls using Western blot. In contrast to PINK1 mRNA, its protein level was significantly decreased in ICH brain tissues compared to that in healthy control (Fig. 4). This finding demonstrated that PINK1 protein was downregulated in ICH brain tissues and the upregulation of PINK1 mRNA might be due to gene dosage compensation.

PINK1 is mainly downregulated in microglial cells of the ICH mice model

Next, we investigated the expression and distribution of PINK1 in ICH mice brain by FACS and qRT-PCR. After ICH induction, the PINK1 expression level was dramatically decreased in brain tissues compared to that in healthy controls (Fig. 5A). Microglia, oligodendrocytes, and astrocytes were sorted by FACS using CD68, OSP, and GFAP markers, and the rest of the cells were regarded as neurons. We performed qRT-PCR to detect

the expression of PINK1 in different cell types, and found that the comparable level of PINK1 was observed in neurons, oligodendrocytes, and astrocytes; however, PINK1 mRNA was significantly decreased in microglia relative to control (Fig. 5B). The above results suggested that the expression of PINK1 was mainly downregulated in microglial cells of the ICH brain.

PINK1 protects brain tissue from ICH by promoting mitochondrial autophagy

To explore the underlying molecular mechanism of PINK1-induced protective effect in ICH progression, we isolated microglial cells from ICH mouse and performed autophagy analyses by mitochondrial membrane potential assay, flow cytometry, and Western blot. JC-1 staining showed that the ratio of healthy mitochondria in ICH microglial cells was significantly decreased compared to control (28.7% vs. 98.1%), importantly, the ratio of damaged mitochondria in ICH microglial cells was dramatically increased relative to control (71.3% vs. 1.84%) (Fig. 6A). In comparison with the control brain, microglia apoptosis was abundant in the ICH brain, which had an extremely low healthy/damaged mitochondrial ratio (Fig. 6B). Next, we detected ROS production, one of the major damaging factors in ischemic stroke, in control and ICH microglial cells by flow cytometry, and found that the fluorescence intensity of the ICH group was markedly increased compared to that in the control group (Fig. 6C, D). Finally, we measured the protein levels of the PINK1–PARKIN pathway and autophagy markers in control and ICH microglial cells. In line with the mRNA result, PINK1 protein was decreased in ICH microglial cells, intriguingly, comparing with control microglial cells, the protein levels of PARKIN (the E3-ubiquitin ligase of PINK1–PARKIN pathway), LC3II, and mito-LC3II were all significantly reduced in the ICH group (Fig. 6E). All these results indicated that PINK1 protects brain tissue from ICH by promoting mitochondrial autophagy.

Discussion

ICH is a severe public health problem that causes high rates of mortality and morbidity in adults worldwide. Increasing evidence demonstrate that the disruption of membrane potential and mitochondria dysfunction participate in the pathophysiological processes of ICH.[1,2] In the current study, we focused on the PINK1, a serine/threonine-protein kinase located in mitochondrion, demonstrated the expression of PINK1 in ICH patients and mouse model, evaluated the role of PINK1 in ICH progression and behavioral dysfunction by PINK1 gain- and loss-of-function animal model, and explored the
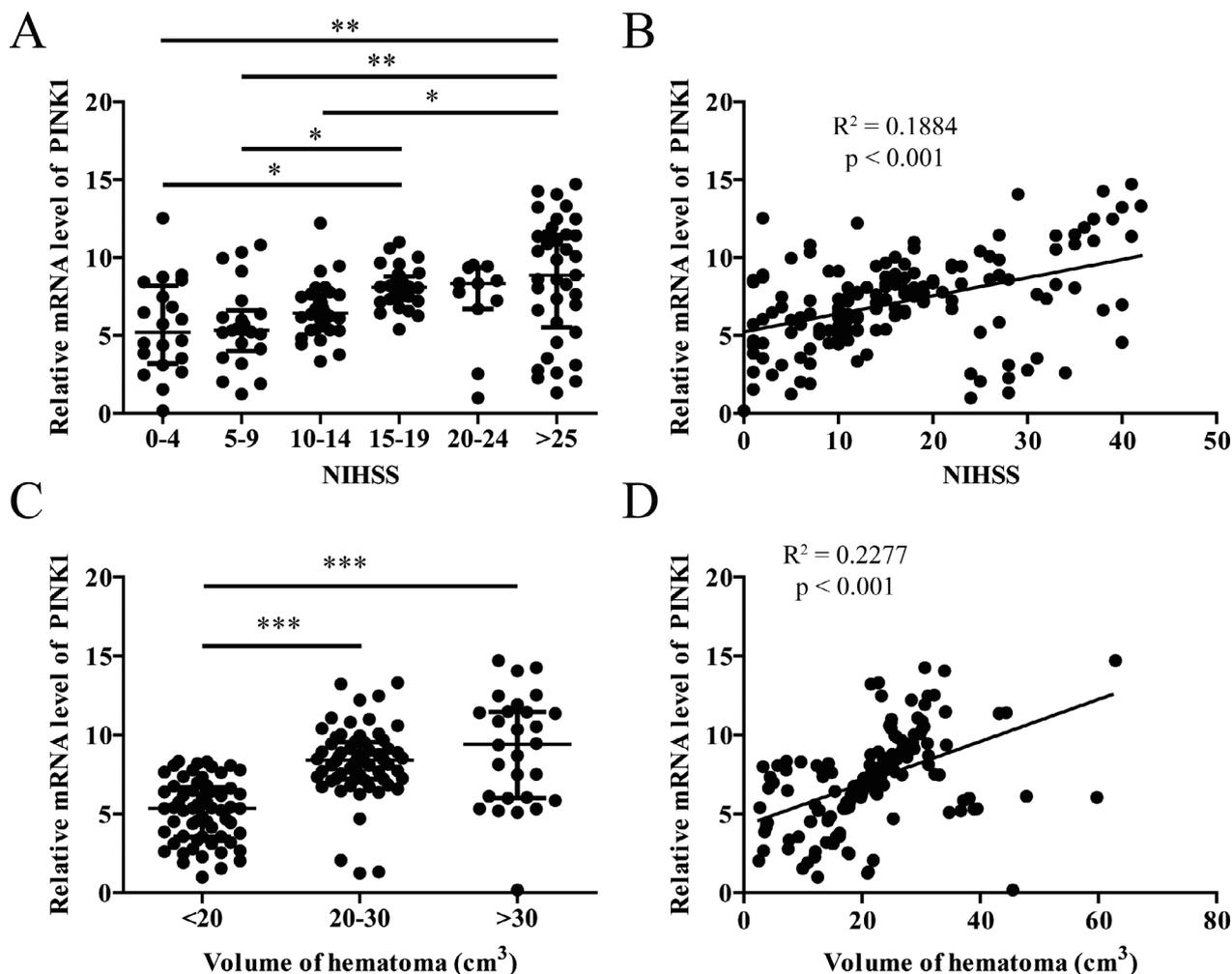
Figure 2. The expression level of PINK1 is positively correlated with NIHSS and volume of hematoma. (A) The expression level of PINK1 in different groups according to NIHSS is evaluated. (B) The correlation between the expression level of PINK1 and NIHSS. (C) The expression level of PINK1 in different groups according to the volume of hematoma is evaluated. (D) The correlation between the expression level of PINK1 and volume of hematoma.

mechanism of PINK1-induced anti-ICH effects. Our findings suggested that PINK1 might work as a therapeutic target for ICH treatment.

PINK1 localizes to mitochondria and maintains a healthy mitochondrial network by stimulating removal of dysfunctional parts through selective autophagy.[29] PINK1 accumulates on dysfunctional mitochondria and its kinase activity is required for PARKIN, a HECT-RING hybrid E3-ligase that can be recruited by PINK1 to decrease mitochondrial membrane potential.[30] PINK1–PARKIN pathway regulates both motility and mitophagy of mitochondria, which are critical for neuronal survival and function.[30] Knockdown PINK1 in *Drosophila* can increase the density and length of mitochondria in motor neurons and neuromuscular junctions.[31] In rat, PINK1 overexpression is able to mitochondrial

retrograde and anterograde transport of hippocampal axons.[32] Hence, PINK1 plays important role in keeping damaged mitochondria away from nerve terminals, thereby facilitating the removal of mitochondria in the soma by mitophagy. Since mitochondrial dysfunction is one of the major features of ICH, we examined the expression of PINK1 in 151 ICH patients and found that the PINK1 mRNA level was significantly increased in them compared to that in healthy people. Moreover, the expression level of PINK1 mRNA was positively correlated with NIHSS and volume of hematoma in ICH patients. Our data indicated that PINK1 might work as a risk factor in ICH, which is in contrast to the previous studies that demonstrated the protective role PINK1 of in stroke and other neuron disease.[21,30,31] Interestingly, we observed dramatically reduction of PINK1 protein in
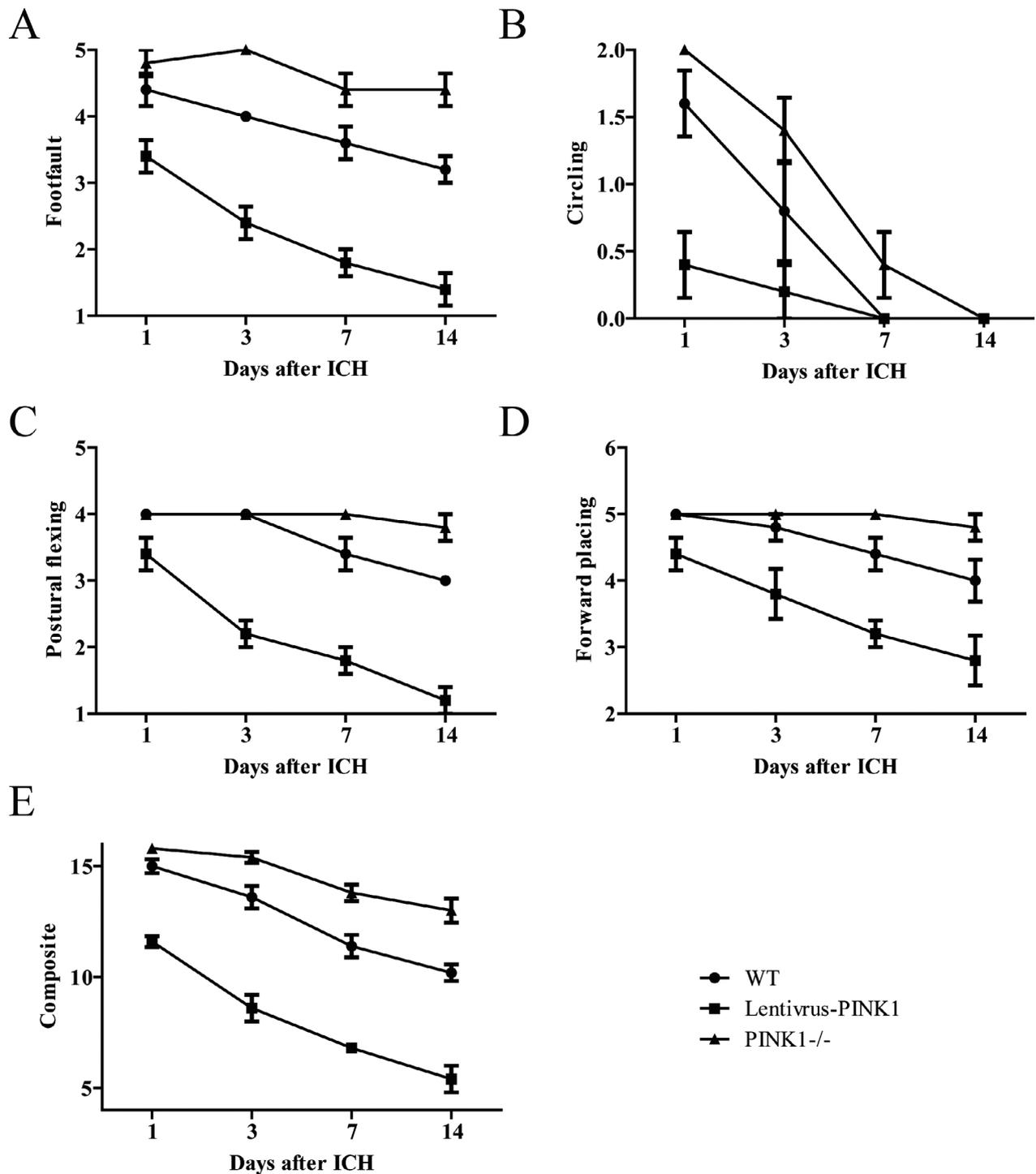
Figure 3. PINK1 shows protective activity in the ICH mouse model. ICH in mice was induced by injecting autologous blood in three different mice as indicated. Lentivirus of PINK1 was injected 3 days before the generation of the ICH model. The outcome of mice behavior was determined by footfault (A), circling (B), postural flexing (C), and forward placing (D) test on days 1, 3, 7, and 14 after ICH (n = 5 for each group). (E) A composite neurological deficits score. The data are presented as mean ± SD.

both ICH patients and mouse ICH model. We speculate that the inverse expression of PINK1's mRNA and protein might due to gene dosage compensation.

A series of behavioral tests have been developed to examine the neurological deficits induced by ICH and assess the outcome of preclinical treatments.[33,34] Hua
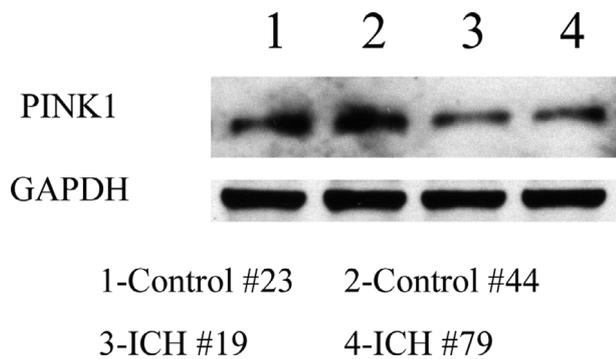
Figure 4. The protein level of PINK1 is decreased in ICH patients. Western blot of the brain tissue samples showed the downregulation of PINK1 in ICH patients.

et al. reported the presence of blood in brain parenchyma with or without direct vascular rupture could induce significantly ICH in the rat model.[13] In this study, we created a mouse ICH model by autologous blood injection into WT, PINK1 gain-, and loss-of-function mice. Three days after ICH induction, mice presented drastic behavioral disorders of footfault, circling, postural flexing, and forward placing. PINK1 knockdown mice had more severe behavioral deficits; however, Lentivirus-mediated PINK1 overexpression could significantly improve the above behavioral disorders. Madeo et al. demonstrated that the repetitive activation of cortical inputs to striatal neurons failed to induce long-term potentiation in PINK1

knockout mice.[35] Furthermore, researchers found that PINK1 deficiency-induced neurogenesis deficits of the hippocampus in the adult mouse, and resulted in more serious stress-induced depression compared to control mice.[36] Our and other groups' findings suggested that PINK1 has the therapeutic potential on brain injury-induced behavioral disorders.

Microglial cells play a critical role in phagocytizing the erythrocytes and debris left in the brain after ICH damage.[37,38] In line with this, after ICH induction, we found the downregulation of PINK1 was mainly happened in microglia instead of other brain cells, such as astrocytes, oligodendrocytes, and neurons. Moreover, we explored that PINK1 protects brain tissue from ICH by promoting mitochondrial autophagy. This study also has several limitations, the increased PINK1 mRNA and decreased PINK1protein the brain of ICH patients might be a compensatory effect of gene regulation, but the regulatory mechanism is still unknown, which might be due to the inhibition of PINK1translation or degradation of existed PINK1 protein; the animal experiments show that the overexpression of PINK1 has a protective effect on ICH; however, how to increase the expression level of PINK1 clinically is still a challenge.

Conclusion

PINK1 protects ICH-induced brain injury and behavioral disorders by promoting mitochondrial autophagy in
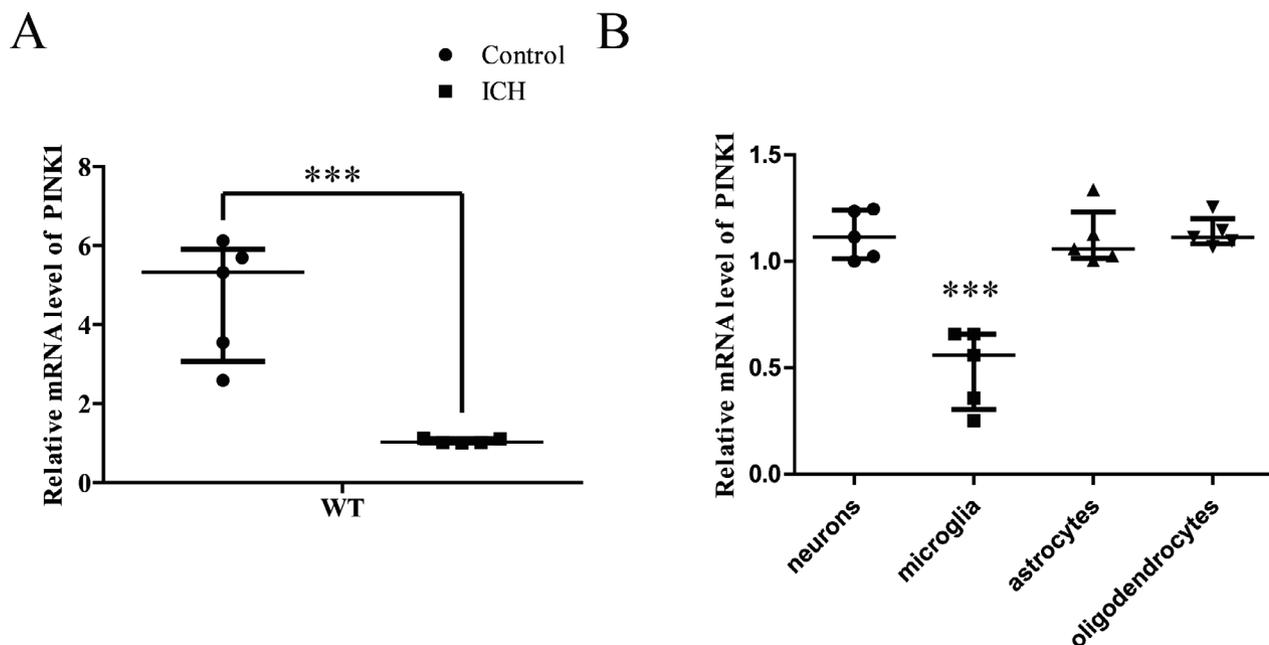
Figure 5. PINK1 is mainly downregulated in microglial cells in ICH mice. (A) PINK1 mRNA level in brain tissue of WT and ICH mice is measured by qPCR. (B) Neurons, microglial cells, astrocytes, and oligodendrocytes are sorted from the brain tissue by FACS, PINK1 mRNA level is determined in different cell types as indicated. Data are presented as mean ± SD, *** indicates $p < 0.001$.

1-microglia in Control
2-microglia in ICH
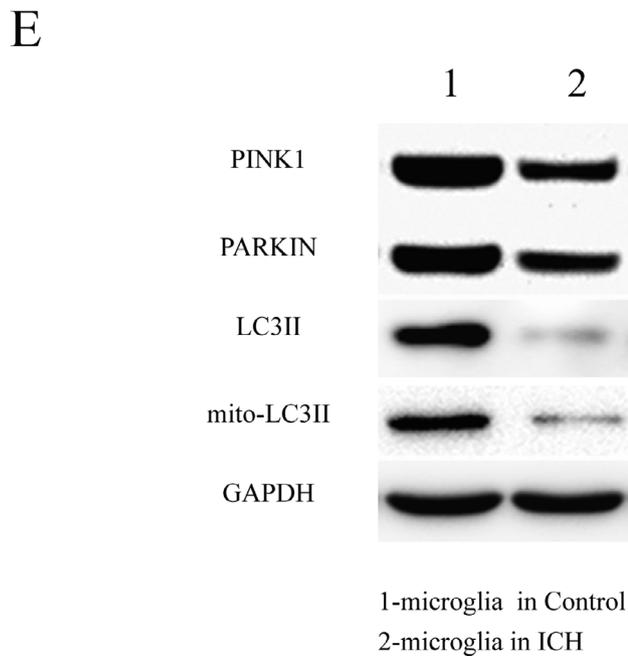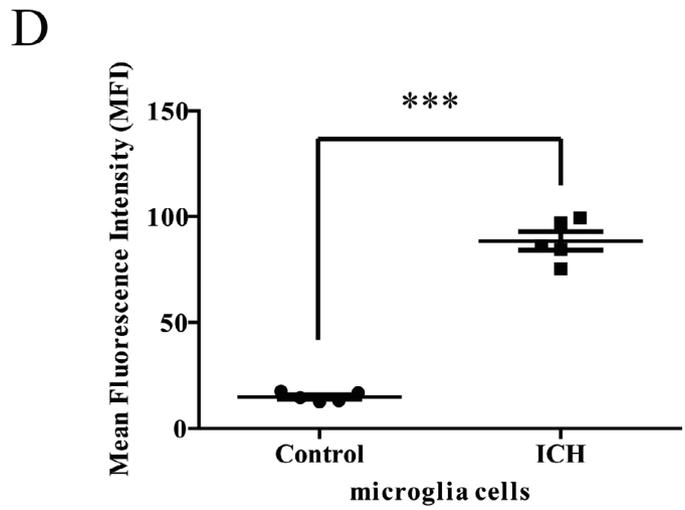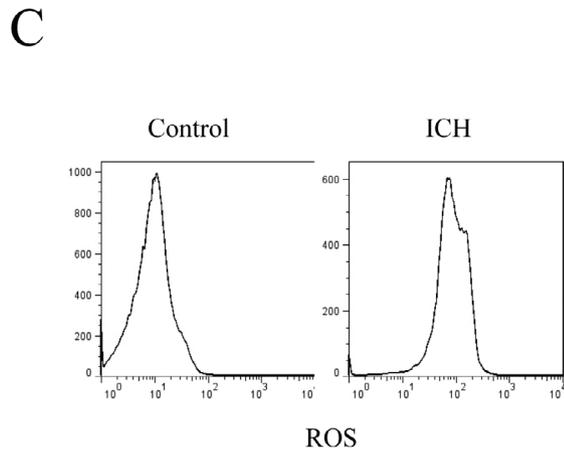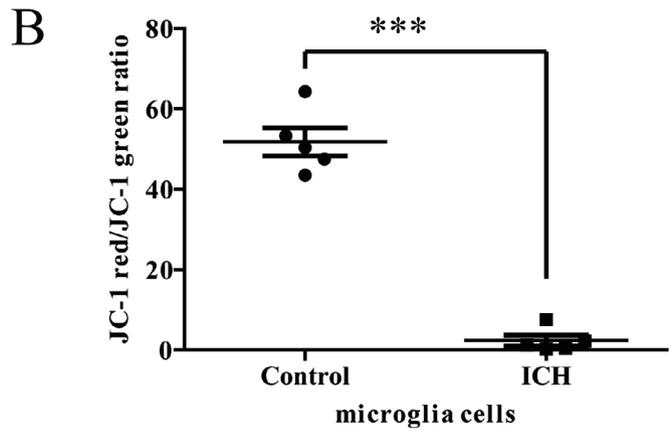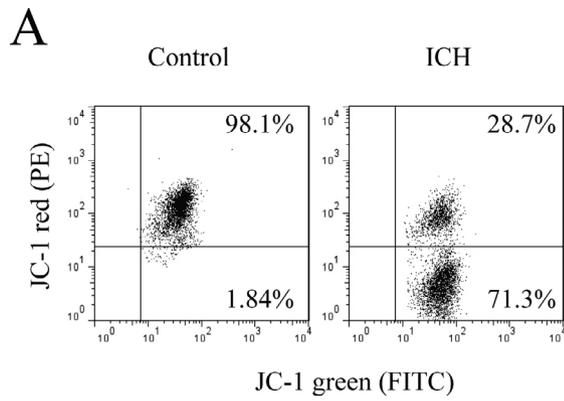
Figure 6. PINK1 protects brain tissue from ICH by promoting mitochondrial autophagy. (A–B) JC-1staining was used to determine the mitochondrial membrane potential. The red fluorescence of the JC-1 probe indicates healthy mitochondria, and the green fluorescence of the JC-1 probe indicates damaged mitochondrial potential. (C–D) ROS production was measured via flow cytometry. (E) Western blot showed the decrease of PINK1, PARKIN, and the autophagy marker LC3II in microglia in ICH mice. Data are presented as mean ± SD, *** indicates $p < 0.001$.

microglial cells. This study shows strong justification for the neuroprotective effects of PINK1 and provides a potential strategy for ICH treatment.

Author Contribution

Data collection and analysis: Jingchen Li, Xiaoyun Wu, Yanbo He, Song Wu, Erkun Guo, Yan Feng, Jipeng Yang, and Jianliang Li; Study design and manuscript writing: Jingchen Li and Jianliang Li, Erkun Guo.

Conflicts of Interest

No conflicts of interest, financial or otherwise, are declared by the authors.

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