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*Original Article*

# Involvement of GMRP1, a novel mediator of Akt pathway, in brain damage after intracerebral hemorrhage

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**Abstract:** GMRP1, also known as BTBD10, has been reported to inhibit apoptosis of neuronal and islet beta cells via Akt pathway. The present study attempted to investigate whether GMRP1 and its mediated Akt pathway were involved in brain injury of rats after intracerebral hemorrhage (ICH). Rat models of ICH had been established successfully. Western blotting was used to investigate the levels of GMRP1 protein in caudate nuclei tissues of hemorrhagic and contralateral sides at 6 h, day 1, day 3, day 5, day 7 after ICH. Phosphorylation of Akt was determined in caudate nuclei mentioned above. TUNEL assay was used to measure the cell apoptosis. GMRP1 protein levels, as well as phosphorylations of Akt, significantly decreased in caudate nuclei of hemorrhagic side, compared with those of contralateral side at day 1, day 3 after ICH. Enhanced cell apoptosis was observed in hemorrhagic side by TUNEL assay. We presented here evidence that decreased GMRP1-mediated Akt pathway contributed to cell apoptosis in hemorrhagic side, suggesting that GMRP1 played an important role in brain damage after ICH.

**Keywords:** GMRP1, intracerebral hemorrhage, apoptosis, Akt

## Introduction

Intracerebral hemorrhage (ICH)-triggered cascade of brain injury can cause tragic outcome. Large number of pathophysiological procedure are involved in the progress. ICH-induced cell death is a preferentially direct damage, of which cell necrosis and cell apoptosis are two different types. Some investigations have shown that apoptosis is an important mechanism of ICH-induced brain injury [1-3].

GMRP1, also known as BTBD10, which is a novel member of BTB/POZ (Broad-complex, Tramtrack, Bric-a-brac/Poxvirus and zinc fingers) domain contained protein family, has been reported to have the ability to inhibit apoptosis of neuronal and islet beta cells via Akt pathway [4-6]. But whether the protein and its mediated Akt pathway could contribute to alleviate brain cell apoptosis is still unknown.

The present study attempted to preliminarily investigate the role of GMRP1 in ICH-induced brain damage and cell apoptosis.

## Materials and methods

### *ICH model*

Animal use protocols were approved by Fudan University. Male Sprague-Dawley rats (300-350g, Experimental Animal Center of Fudan University) were used in the present study. The rats were anesthetized with pentobarbital (45 mg/kg i.p.). The right femoral artery was catheterized for continuous blood pressure monitoring and blood sampling. All rats received an injection of 100 μl autologous whole blood (obtained from femoral artery catheter) into caudate nucleus within 8 minutes, through a 26-gauge needle (coordinates: 0.2 mm anterior, 5.5 mm ventral, and 3.5 mm lateral to bregma) with a microinfusion pump.

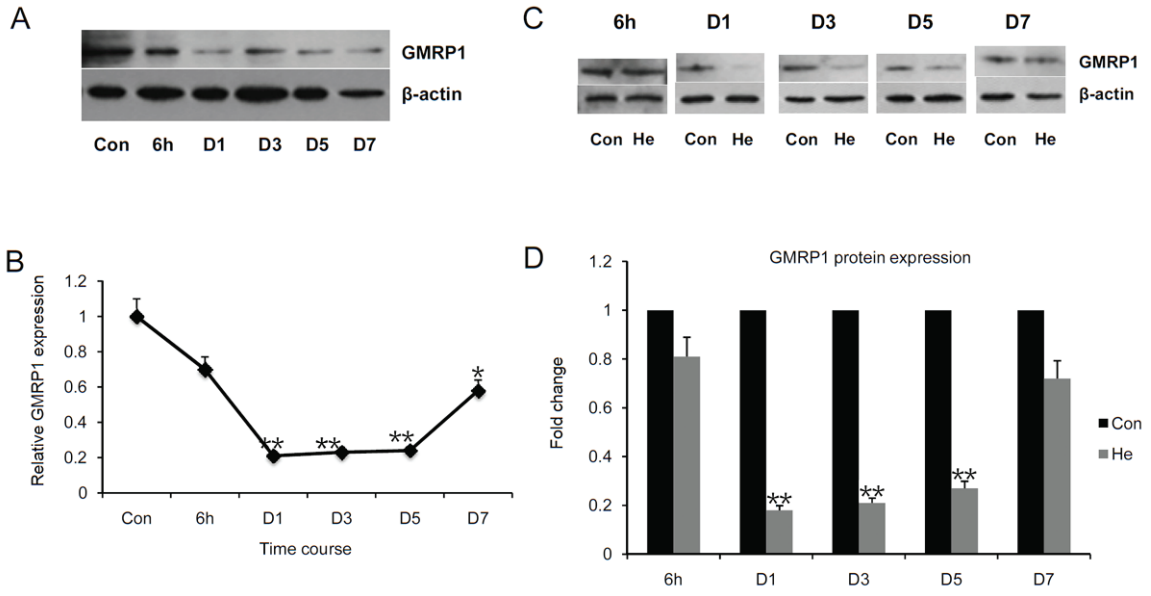


**Figure 1.** GMRP1 protein levels in caudate nucleus tissues of rats after ICH. A: The time course of GMRP1 expression in caudate nuclei tissues of contralateral and hemorrhagic sides by western blotting. B: Relative expression of GMRP1 in caudate nuclei tissues of hemorrhagic sides at 6 h, and day 1, 3, 5, and day 7 after ICH is shown in graph when GMRP1 expression in contralateral sides is set to 1. C: Spatial expression of GMRP1 at different time points after ICH by western blotting. D: Relative expression of GMRP1 in caudate nuclei tissues of hemorrhagic sides is shown in graph when GMRP1 expression in contralateral sides is set to 1. $^{*}p<0.05$, $^{**}p<0.01$. Keys: Con: contralateral sides; He: hemorrhagic sides; 6h: 6 hours after ICH; D1: 1 day after ICH; D3: 3 days after ICH; D5: 5 days after ICH; D7: 7 days after ICH.

*Experimental groups*

These experiments were divided into three parts. In the first part, one control group (needle insertion only) and 5 ICH groups of rats (n=4) were killed at 6 h, day 1, day 3, day 5, day 7, after blood injection for western blotting test of GMRP1 and Akt/p-Akt. In the second part, one control group and 5 ICH groups of rats (n=3) were killed 6 h, day 1, day 3, day 5, day 7, after blood injection for GMRP1 immunohistochemistry analysis. In the third part, TUNEL assay (different section from the same sample of GMRP1 staining) was performed.

*Western blotting*

Western blotting was performed as described previously [7]. Briefly, total protein was extracted from caudate nucleus tissue with Tissue Protein Extraction Reagent (Pierce). Protein concentration was estimated by BCA Protein Assay Kit (Pierce). Samples were run on a polyacrylamide gel and then transferred to pure nitrocellulose membrane. Membranes were probed with 1:1000 dilution of rabbit anti-GMRP1 polyclonal antibody (generated by our lab) and rabbit anti-Akt/p-Akt polyclonal antibody (Santa Cruz), followed by a secondary antibody (peroxidase-conjugated goat anti-rabbit antibody, Santa Cruz). Protein bands were visualized by chemiluminescence with an ECL Luminescence Kit (Pierce) and exposed to X-ray film.

*Immunohistochemistry*

The immunohistochemistry method has been described previously [7]. Briefly, the rats were anesthetized and perfused with 4% paraformaldehyde. Brains were removed and kept in 4% paraformaldehyde for 6 hours, immersed in 25% sucrose for 3 days at 4°C, then dehydrated, embedded in paraffin and sectioned. For GMRP1 immunohistochemistry, rabbit anti-GMRP1 polyclonal antibody (generated by our lab) was used to incubate brain sections, and then the sections were incubated with HRP-conjugated anti-rabbit IgG antibody (Pierce) to stain GMRP1. The sections were then observed and imaged by using a Leica microscope (Leica Microsystems, Watzlar, Germany).

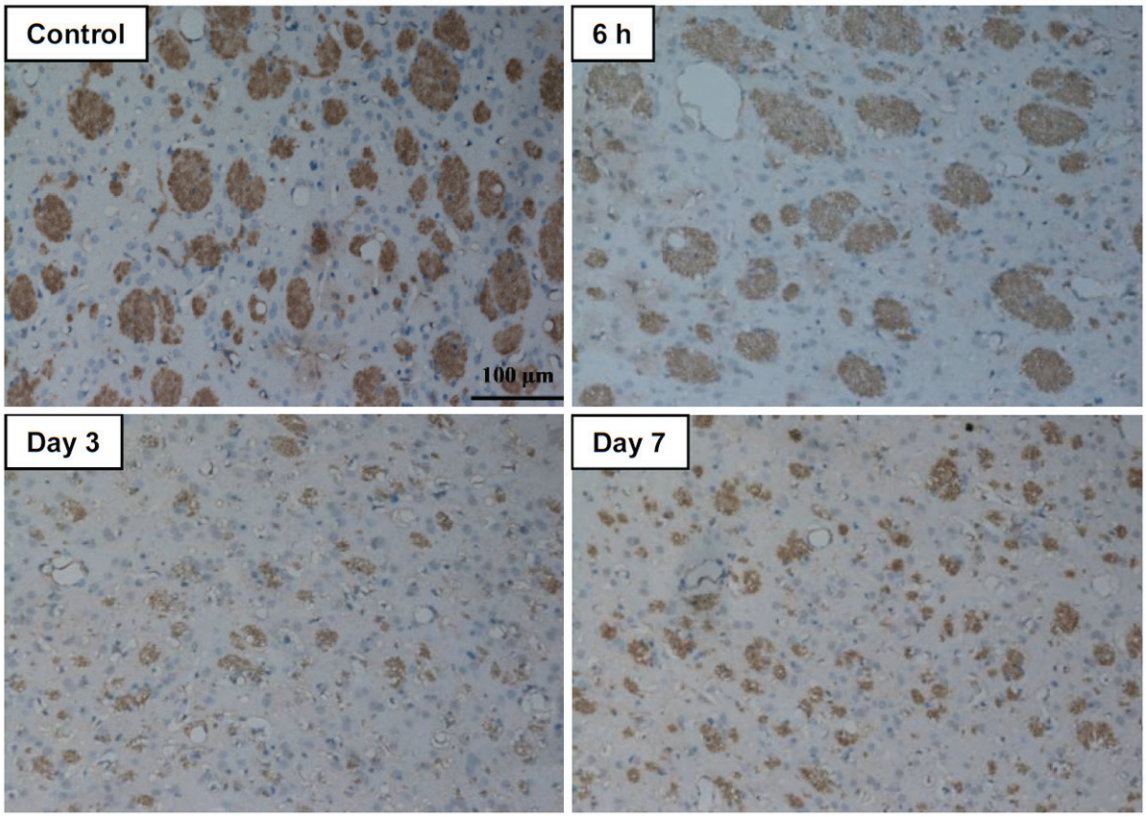


**Figure 2.** Immunohistochemistry analysis of GMRP1 expression in caudate nucleus tissues of ICH and control rats, scale bar: 100 um. Keys: Control: caudate nucleus tissues of control rats; 6h: 6 hours after ICH; Day 3: 3 days after ICH; Day 7: 7 days after ICH.

*TUNEL assay*

TUNEL staining was carried out using a DNA fragmentation detection kit (FragEL; Merck, Darmstadt, Germany) according to the manufacturer's instructions. Cells were counted under 5 high-power field (400×) to gain the average data.

*Statistical analysis*

Values are listed as mean±SD. One-way ANOVA were used with SPSS12.0 software to determine statistical significance, which is set at $P<0.05$.

## Results

*Physiological parameters of ICH and control rats*

Physiological parameters, including mean arterial pressure, blood pH, arterial oxygen and carbon dioxide tensions, hematocrit, and blood glucose, were recorded and controlled within normal ranges. There were no significant differences of these parameters between ICH and control group.

*GMRP1 protein levels in caudate nucleus tissues of rats after ICH*

We first investigate GMRP1 expression in caudate nucleus tissues of hemorrhagic sides at different time after intracerebral hemorrhage. Western blot analysis demonstrated that GMRP1 protein levels declined from 6 h (70% of control, $p>0.05$), tended to the lowest level at day 1(21% of control, $p<0.01$), day 3 (23% of control, $p<0.01$), and day 5 (24% of control, $p<0.01$) , followed by an slight escalation at day 7 (58% of control, $p<0.05$) (**Figure 1A** and **1B**). As for the protein levels of GMRP1 in caudate nuclei tissues at a certain time point after ICH, it was found that GMRP1 expression decreased by 5.6- ($p<0.01$), 4.8- ($p<0.01$),

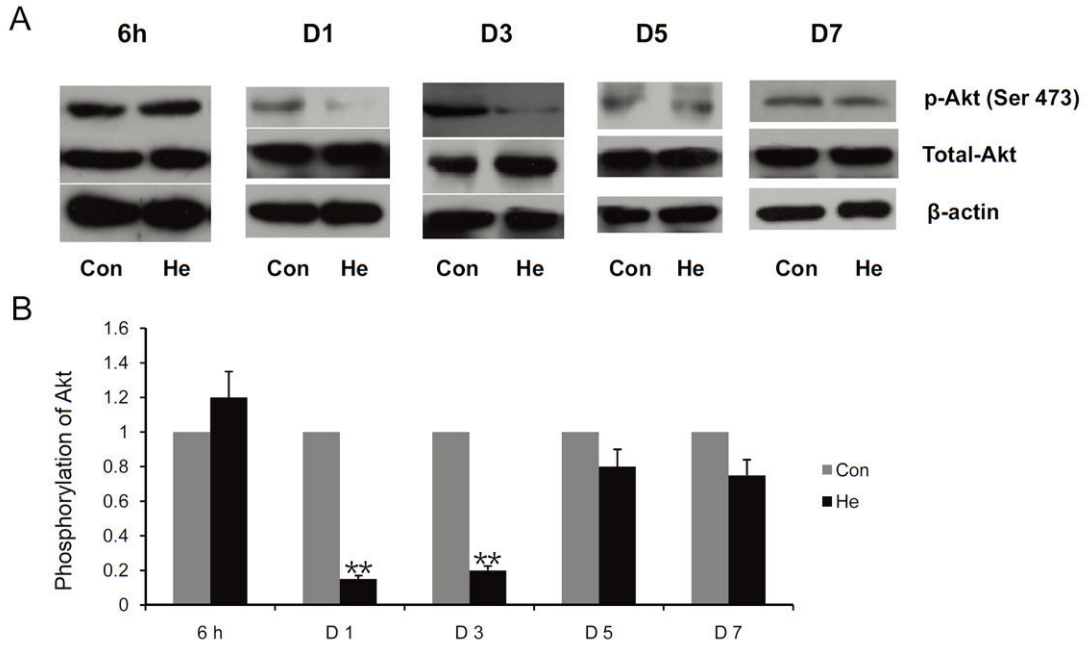


**Figure 3.** Total and phosphorylated Akt (p-Akt) in caudate nucleus tissues of rats after ICH. A: Total and phosphorylated Akt (p-Akt) in caudate nucleus tissues of contralateral and hemorrhagic sides at different time points after ICH by western blotting. B: Phosphorylation of Akt (p-Akt/Total-Akt) in caudate nuclei tissues of hemorrhagic sides is shown in graph when phosphorylation of Akt in contralateral sides is set to 1. $^{**}p<0.01$. Keys: Con: contralateral sides; He: hemorrhagic sides; 6h: 6 hours after ICH; D1: 1 day after ICH; D3: 3 days after ICH; D5: 5 days after ICH; D7: 7 days after ICH.

and 3.7-fold ($p<0.01$) in hemorrhagic sides compared with contralateral ones at day 1, 3, and 5, respectively. However there were no significant differences of GMRP1 protein levels between the two sides at 6 h and day 7 (**Figure 1C** and **1D**).

The results of western blotting were also confirmed by immunohistochemistry analysis. It can be observed that when compared with control sides, the staining density of GMRP1 in caudate nuclei tissues of hemorrhagic sides lowered at 6 h after ICH, reaching at the lowest levels at day3, and then slight increased at day 7 (**Figure 2**). It also can be seen that the GMRP1 protein was mainly located at cytoplasm, not nucleus (**Figure 2**).

*Phosphorylated levels of Akt in caudate nucleus tissues of rats after ICH*

Besides GMRP1 expression, total and phosphorylated Akt were also investigated in caudate nucleus tissues. In agreement with changes of GMRP1, phosphorylated levels of Akt were also found down-regulated in hemorrhagic sides after ICH. In detail, phosphorylated Akt significantly decreased in hemorrhagic sides at day 1 (15% of contralateral, $p<0.01$) and day 3 (20% of contralateral, $p<0.01$) after ICH (**Figure 3**). The levels of phosphorylated Akt also declined at day 5 and 7, but not reaching significances (**Figure 3**).

*Cell apoptosis in caudate nucleus tissues of rats after ICH*

TUNEL positive cells could be hardly seen at contralateral sides of caudate nuclei tissues. However, significant increases of TUNEL positive cells were observed in hemorrhagic sides at 6 h (5.2-fold vs. contralateral side, $p<0.01$), day 1(27.4-fold, $p<0.001$), day 3(23.1-fold, $p<0.001$), day 5, (25.6-fold, $p<0.001$) and day 7 (4.8-fold, $p<0.01$) (**Figure 4**).

## Discussion

The present study demonstrated that down-regulation of GMRP1 and its mediated Akt pathway may be involved in neuronal cell apoptosis in early stage of ICH.

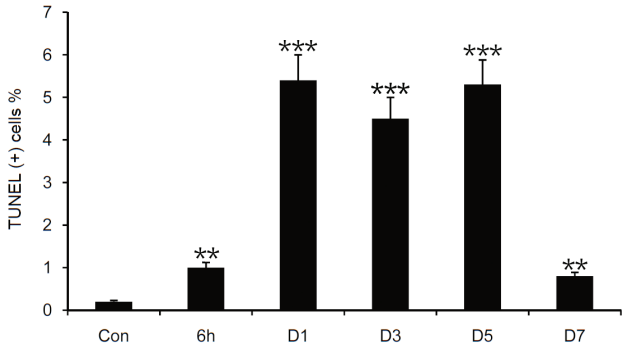


**Figure 4.** TUNEL positive cells in caudate nucleus tissues of contralateral and hemorrhagic sides after ICH. $^{**}p<0.01$, $^{***}p<0.001$. Keys: Con: contralateral sides; 6h: 6 hours after ICH; D1: 1 day after ICH; D3: 3 days after ICH; D5: 5 days after ICH; D7: 7 days after ICH.

BTB/POZ domain-contained proteins have been proved to be able to modulate some important cellular procedure, such as transcription, proliferation, cell morphology maintenance, angiogenesis, and apoptosis [8-11]. They can bind other proteins or self-bind to form homodimer or heterodimer, and then bind to DNA with their zinc finger region, to participate in the function procedure mentioned above. GMRP1, a novel BTB/POZ family member, also known as BTBD10, was first cloned by Chen et al, and proved to be down-regulated in gliomas [4]. In another study of our group, we have presented evidence that knockdown of GMRP1 increased pancreatic beta-cell apoptosis via reducing phosphorylation of Akt, and decreased GMRP1 expression might participate in beta-cell apoptosis of db/db mice [6]. Moreover, experiments on neuronal cells indicated that the reduction of the endogenous BTBD10 level led to a decrease in the phosphorylation levels of Akts, which in turn resulted in neuronal cell apoptosis [5]. In the present study, we observed remarkable down-regulation of GMRP1 protein and Akt phosphorylation after ICH, which decreasing to their extreme level at day 3, suggesting GMRP1-mediated cell apoptosis during early stage after ICH.

In COS7 cells and NSC34 cells, BTBD10 was found to present a unique filamentous cytoplasmic distribution around the nucleus [5], both in overexpressed pattern or in endogenous pattern. We also showed that GMRP1 distributed in the cytoplasm of pancreatic islet cells [6], and in this study, GMRP1 was localized in the same manner in cytoplasm of Sprague Dawley rats' neurons by immunohistochemistry analysis. However, Chen et al. revealed that BTBD10 was located specifically in the nucleus of HEK293 and COS7 cell lines, suggesting that it may function in transcriptional regulation [4]. For NAC-1, another BTB/POZ protein, early research clarified the restricted existence of the protein in nuclei [8]. But later research also detected diffuse distribution both in nuclei and in cytoplasm of NAC-1 in PC12 and Neuro2A cells, implicating complex transcriptional and non-transcriptional role for BTB/POZ proteins [12]. Multiple method of localization study should be further applied to investigate GMRP1 subcellular location.

Apoptosis is progressed cell death. ICH-induced cell apoptosis has been well investigated. Our data showed TUNEL-positive cells could be observed at 6 h after ICH, then increased remarkably, peaked to the top level at day 3, followed with a high level during day 5 to day 7. This time course of apoptosis after ICH is quite in accordance with the results from previous investigations [1, 2]. It is also consistent with changes of GMRP1 and Akt phosphorylation in our study, implying that GMRP1 and its mediated Akt pathway are involved in cell apoptosis in the early stage of ICH.

In summary, we preliminarily presented some evidence that decreased GMRP1-mediated Akt pathway contributed to brain cell apoptosis in the early stage of ICH, suggesting that GMRP1 played an important role in injury after ICH. This finding may explore new sight into the mechanism of brain damage induced by ICH.

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## Conflict of interest statement

No conflicts.

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