TRIM59 induces epithelial-to-mesenchymal transition and promotes migration and invasion by PI3K/AKT signaling pathway in medulloblastoma

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Abstract. Medulloblastoma is the most common malignant brain tumor in children. Despite remarkable advances over previous decades, the long-term survival of patients with medulloblastoma remains poor due to the frequent metastatic nature of this malignancy. The aim of the present study was to examine the role of tripartite motif containing 59 (TRIM59) in cell metastasis in medulloblastoma. It was initially demonstrated that TRIM59 expression was significantly increased in clinical medulloblastoma tissues compared with adjacent non-cancerous tissues and differentially expressed in a series of medulloblastoma cell lines. The knockdown of TRIM59 in D283 cells resulted in epithelial-to-mesenchymal transition (EMT), and decreased cell migratory and invasive capacities. By contrast, the overexpression of TRIM59 in Daoy cells was able to inhibit the EMT process and increase migratory and invasive capacities of the cells. Notably, the knockdown of TRIM59 was able to decrease the protein level of matrix metalloproteinase (MMP)-2 without altering the levels of MMP-9, and conversely the overexpression of TRIM59 was able to increase the protein level of MMP-2. Importantly, the down-regulation of TRIM59 in D283 cells was able to inhibit the levels of phosphorylated (p)-AKT (Ser473), glycogen synthase kinase 3 β (GSK3β; Ser9) and phosphoinositide 3-kinase (PI3K) p85 (Tyr458) without altering the levels of total protein. The data from the present study suggest that TRIM59 induces epithelial-to-mesenchymal transition and promotes migration and invasion by PI3K/AKT signaling pathway in medulloblastoma. This data may provide novel insight into tumor metastasis and pave the way for the development of therapeutic strategies for the treatment of medulloblastoma in the clinic.

Introduction

Medulloblastoma is the most common childhood intracranial malignant tumor. At present, therapies against medulloblastoma comprise surgery, chemotherapy and radiotherapy. Advances in technology have increased the long-term survival of patients with this malignancy by up to ~40-70% (1,2). However, the patients who survive often have long-term, treatment-associated neurological complications (3). Furthermore, the incidence rate of medulloblastoma has also increased gradually over previous decades (3). One particular consensus is that medulloblastoma has a high-metastatic nature (3). Based on a previous clinicopathological study, ~40% of medulloblastoma was disseminated through the cerebrospinal pathways (4). In a previous report assessing metastasis of medulloblastoma, it was revealed that medulloblastoma metastasized to multiple extraneural locations, including bone, bone marrow, lung, liver and lymph nodes (5). Therefore, there is an urgent need to develop novel therapies for medulloblastoma.

The human tripartite motif (TRIM) family has >77 members, of which the majority of proteins are E3 ubiquitin ligases, due to the highly conservative domain, namely, really interesting new gene (RING). Proteins from this superfamily are demonstrated to perform biological activities in a variety of cellular processes, including transcriptional regulation, membrane repair, cytoskeletal remodeling and oncogenesis (6,7). Of note, specific members of this family, including TRIM13, TRIM19 and TRIM25, are involved in human tumorigenesis, including leukemia, prostate and breast cancers via mechanisms of transcriptional regulation (8-11). Thereafter, updated evidence has revealed that multiple TRIM family proteins are critical mediators of human tumorigenesis, including TRIM59, which was initially identified as a protein which interacts with Wnt signaling (12) and was characterized by oncogenic activity in a mouse model of prostate cancer (13).
TRIM59, a surface molecule, was remarkably increased in gastric cancer and prominently associated with poor outcome of patients (13). Ever since the identification of these characteristics in 2011 (13), TRIM59 was identified to be a multiple tumor biomarker in human tumorigenesis (14,15). TRIM59 promotes tumor growth and migration in various cancer types, including non-small cell lung cancer cells (16) and osteosarcoma (17), whereas the knockdown of TRIM59 inhibits cellular proliferation and migration in cervical cancer cells (18). Knockdown of TRIM59 correlates with tumor growth inhibition in prostate cancer (19). However, the exact role of TRIM59 in human medulloblastoma has remained an enigma until now.

The present study aimed to investigate the role of TRIM59 in cell metastasis in medulloblastoma. The expression profile of TRIM59 in clinical medulloblastoma tissues and in a series of medulloblastoma cell lines was initially assessed. Thereafter, the effects of TRIM59 modulation on cancerous cell metastasis were systemically examined in vitro and in vivo. The possible mechanisms that contribute to the biological activity of TRIM59 in medulloblastoma were also investigated and discussed.

Materials and methods

Human tissues and ethics statements. In total, 14 patients with medulloblastoma, who were admitted to Jining No. 1 People's Hospital (Shandong, China) were included in the present study, patient specimens were collected without any prior radiotherapy or chemotherapy between May 2012 and October 2013. The population examined consisted of 14 patients with 9 males and 5 females and the median age at diagnosis was 12 years (range, 8-17 years). For each case, cancerous tissues and the matched adjacent non-cancerous tissues were obtained. All patients gave their full consent to participate in the present study, and a written consent form was obtained from each patient. All of the experiments in the present study were in compliance with the official policies and defined protocols. The research protocol was approved by the Ethical Committee of Jining No. 1 People's Hospital.

Cell lines and reagents. Human medulloblastoma cell lines Daoy, D283, D425, D341 and D458 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The passage number of the cell lines was 10. All cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin/streptomycin. The culture medium was replaced every 2 days. Opti-MEM was purchased from Gibco (Gibco, Thermo Fisher Scientific, Inc.). The primary antibodies were all commercially purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), except for the antibodies used for detection of phosphorylation, which were obtained from Cell Signaling Technology Inc. (Danvers, MA, USA). For the knockdown of TRIM59, two specific short hairpin (sh)RNAs were chemically synthesized by GenePharma (Shanghai, China). The pcDNA3.1-TRIM59 expression plasmid was constructed using a pcDNA3.1 expression vector (Invitrogen; Thermo Fisher Scientific, Inc.). TRIM59 gene amplification was performed from cDNA of the human medulloblastoma cell line D283 using the polymerase chain reaction (PCR). The amplified fragments were digested using HindIII and XhoI (Takara Biotechnology Co., Ltd., Dalian, China).

Cell transfection. A day before transfection, 1x10⁴ cells were plated per well in 2 ml complete growth medium (DMEM with 10% FBS and 100 U/ml penicillin/streptomycin). Cells were 50-60% confluent on the day of transfection. For each well of cells to be transfected, 2 µg DNA expression plasmid or shRNA plasmid was diluted in 250 µl Opti-MEM without serum. For each well of cells, 5 µl Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was added to the media. After gentle mixing the cells were incubated for 5 min at room temperature, prior to the addition 500 µl transfection media and another 5-15 min incubation at room temperature. The cells were transferred to a 37°C, 5% CO₂ incubator for 6 h, before replacing the media with complete media. A total of 48 h post-transfection, subsequent experimentation took place.

Total RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA of each sample was isolated using TRIzol reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The quality and concentration of extracted RNAs were identified by measuring the absorbance at 260 nm. First-strand cDNAs were generated with the PrimeScript RT Master Mix Perfect Real Time kit (Takara Biotechnology Co., Ltd.) by mixing the components and incubating at 42°C for 1 h. All qPCRs were performed with SYBR Premix Ex Taq kit (Takara Biotechnology Co., Ltd.) on an ABI PRISM 7500 Real-Time system (Thermo Fisher Scientific, Inc.). Initial denaturation took place at 95°C for 5 min, followed by annealing at 60°C for 30 sec, and a final extension at 72°C for 5 min. These conditions were cycled 40 times. The primers used are listed below, and GAPDH was included as the internal control. Each experiment was performed in triplicate at least three times. The primer sequences used are as follows: TRIM59 forward, 5'-TACGAGAGCAGCAGCTTGAA-3'; and reverse, 5'-ACGGGTGTGAACCTCAGGAG-3'; GAPDH forward, 5'-GGTGGCACATCGGCAAACAGC-3'; and reverse, 5'-AAGGGTGTAACGCACTA-3'. The sequence of the short hairpin RNAs against Trim59 were as follows: shTrim59#1, 5'-CAATTACAGGCAACATTTAAA-3'; shTrim59#2, 5'-TCCTCTTGTTGATCTGCATCTCATC-3'; sh (normal control) NC: 5'-GGGGTAAACTCAAGTCAGGAG-3'.

Western blot analysis. Total protein was extracted using a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40 and 1 mM EDTA, pH 7.5) containing a Complete protein inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) to generate the whole protein lysate following centrifugation at 14,000 x g at 4°C for 15 min. An equal amount of 50 µg protein/lane was loaded into each lane in a 12% SDS-PAGE gel. The proteins were then transferred onto a polyvinylidene fluoride (PVDF) membrane by electrophoresis. Following blocking using 5% milk in Tris-buffered saline with 0.1% Tween for 1 h at 25°C, the membranes were incubated with primary antibodies against Trim59 (1:1,000; catalog no. ab166793; Abcam, Cambridge,
UK) overnight at 4°C. A secondary antibody conjugated with horseradish peroxidase (goat-anti rabbit; 1:5,000; catalog no. SC-2030; Santa Cruz Biotechnology Inc.) that recognizes the primary antibody was then added at room temperature for 1 h, and the immunoreactivity was determined with enhanced chemiluminescent autoradiography (Thermo Fisher Scientific, Inc.). β-actin was used as a loading control. Each experiment was repeated at least three times.

**Immunofluorescent assay.** Briefly, 1x10⁵ D283 cells treated with 2 µg Trim59 shRNA or shNC for 48 h were seeded on the sterile coverslips in a 24-well plate in DMEM with 10% FBS. After 24 h, the cells were rinsed with PBS and fixed for 20 min in 4% paraformaldehyde at 25°C. Following washing with PBS, the cells were blocked in 5% FBS for 1 h and then incubated with a primary antibody against F-actin (1:500; Santa Cruz Biotechnology Inc.) at 4°C overnight. Following washing with PBS, the cells were incubated with the corresponding secondary antibody conjugated with rhodamine phalloidin (goat-anti rabbit; 1:5,000; Santa Cruz Biotechnology Inc.) in the dark for 1 h and counterstained at 25°C for 30 min with DAPI (1:1,000). Finally, the samples were mounted and observed, and images were captured using an inverted microscope (IX71; Olympus Corporation, Tokyo, Japan).

**Wound-healing and Borden chamber assays.** The wound-healing assay and Boyden chamber assay were performed according to previous literature (20). Briefly, 5x10⁵ D283 cells were plated on 6-well plates to form a confluent monolayer. Wounds made with sterile pipette tips were observed after 6 h. The rate of wound recovery was then calculated at each time point (0 and 24 h). A migration assay was carried out using Boyden chambers (tissue culture-treated; diameter, 6.5 mm; pore size, 8 µm; Transwell, Corning Incorporated, Corning, NY, USA) containing polycarbonate membrane. For the invasion assay, the upper surface of the insert was pre-coated with Matrigel (BD Biosciences, San Jose, CA, USA) to mimic basement membrane. Thereafter, 100 µl 1x10⁶ cells in serum-free DMEM was added to the upper chamber, and 600 µl DMEM with 10% FBS was added to the lower chamber. The cells were incubated for 12 h at 37°C. The migrated cells on the under-surface of the membrane were fixed and stained with crystal violet for 10 min at room temperature. Images of five random regions were captured, and the number of cells was counted to calculate the mean number of migrated cells per plate.

**Statistical analysis.** The data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS software package (version 16.0; SPSS, Inc., Chicago, IL, USA). Trim59 mRNA statistical comparisons were made using unpaired Student's t-test. Comparisons between two groups were analyzed using Mann-Whitney U test. Multiple group comparisons were analyzed using one-way analysis of variance test with post hoc contrasts by Student-Newman-Keuls test. All the experiments were repeated at least three times. *P*<0.05 was considered to indicate a statistically significant difference.

**Results**

**TRIM59 is upregulated in medulloblastoma.** The expression profile of TRIM59 was initially determined. In the 15 cases of clinical medulloblastoma tissues, it was detected that the relative mRNA of TRIM59 was ~2-fold in cancerous tissues compared with adjacent non-cancerous tissues (Fig. 1A). qPCR also validated the high expression of TRIM59 in medulloblastoma tissues compared with adjacent noncancerous tissues (Fig. 1B). Western blot analysis further indicated that TRIM59 protein levels were consistently upregulated in cancerous tissues compared with adjacent non-cancerous tissues (Fig. 1C). In a series of medulloblastoma cell lines, it was notable that TRIM59 was differentially expressed in multiple cell lines, with the highest expression observed in D283 cells and the lowest level in Daoy cells (Fig. 1D). These data suggested that TRIM59 was upregulated in medulloblastoma.

**Knockdown of TRIM59 in D283 cells inhibits epithelial-to-mesenchymal transition (EMT).** Two specific shTrim59s were synthesized and used to infect D283 cells. As compared with the negative control shRNA (shNC), the two different specific shTrim59s were effective in depleting TRIM59 gene expression, with the second shTrim59 exhibiting higher efficiency (Fig. 2A). Western blot analysis also validated that shTrim59#2 was more effective in depleting TRIM59 expression (Fig. 2B). Therefore, the second shTrim59 was used for subsequent analyses. Notably, it was observed that knockdown of TRIM59 inhibited D283 cell pseudopodia formation and led to oval morphological shapes (Fig. 2C), therefore indicating epithelial characteristics following the depletion of TRIM59. Furthermore, it was demonstrated that the expression of the epithelial marker E-cadherin was increased, while mesenchymal markers vimentin, SMA and N-cadherin were decreased following the knockdown of TRIM59 in D283 cells. Epithelial-to-mesenchymal transition (EMT) signaling molecules ZEB1, Slug and Twist were consistently depleted by shTrim59 (Fig. 2D). Immunofluorescent assay also revealed that knockdown of TRIM59 inhibited pseudopodia formation of D283 cells, and the expression of cytoskeleton protein F-actin was markedly decreased, compared with shNC (Fig. 2E). These data strongly indicate that TRIM59 is a mediator of EMT in D283 cells.

**Knockdown of TRIM59 inhibits motility of D283 cells.** In the wound-healing assay, it was observed that after 24 h, the wound was barely observable in the shNC-treated D283 cells, whereas a significant wound remained in shTrim59-infected D283 cells (Fig. 3A). Quantification of wound-recovery area further elucidated that only half of the wound was recovered in shTrim59 group, whereas the wound was almost fully recovered area in the control group. Knockdown of TRIM59 markedly suppressed the cell mobility ability by 50% in D283 cells (Fig. 3B). Similarly, cell migration was significantly inhibited by shTrim59 as indicated by the Transwell migration assay, compared with shNC (Fig. 3C). An average of ~40 cells in the TRIM59-knocked down group migrated to the lower surface, whereas ~100 control D283 cells transmigrated. Knockdown of TRIM59 inhibited the cell migratory ability by...
60% in D283 cells (Fig. 3D). Furthermore, as indicated by the Transwell invasion assay, there was a significant decrease in the number of cells that invaded following the knockdown of TRIM59 (Fig. 3E and F). Therefore, the depletion of TRIM59 is attributable for the inhibition of migration and invasion of D283 cells.

Upregulation of TRIM59 induces epithelial-to-mesenchymal transition in Daoy cells. The pcDNA3.1-TRIM59 expression plasmid was utilized to upregulate the protein level of TRIM59 in Daoy cells (Fig. 4A). Overexpression of TRIM59 led to EMT-mediated morphological changes, for example TRIM59-overexpressing Daoy cells exhibited a shuttle shape or multiple angle shape (Fig. 4B). This suggests that EMT formation was mediated by the overexpression of TRIM59. Western blot analysis also revealed that following the overexpression of TRIM59, the expression of epithelial marker E-cadherin was decreased, whereas the expression of...
mesenchymal markers, vimentin, SMA and N-cadherin was increased, compared with the expression in the cells that were transfected with the control vector (Fig. 4C). The transcription factors ZEB1, Slug and Twist, which suppress epithelial genes and activate mesenchymal genes, were consistently upregulated by TRIM59 (Fig. 4D). Together with the data acquired from TRIM59 knockdown, it may be concluded that TRIM59 may be a mediator of EMT in medulloblastoma cells.

Upregulation of TRIM59 promotes motility in Daoy cells. Furthermore, in TRIM59-overexpressing Daoy cells, the wound recovery process was accelerated, as indicated by the closure of the wound after 18 h (Fig. 5A and B). In the Transwell migration assay, the cells that had migrated to the lower surface were increased following overexpression of TRIM59 (Fig. 5C). The analysis of the migrated cells further demonstrated that >150 TRIM59-overexpressing Daoy cells migrated, which was by contrast with only 100 control cells that exhibited migratory abilities. Overexpression of TRIM59 promoted the migratory ability by 45% in Daoy cells (Fig. 5D). Similarly, invasion was significantly increased in TRIM59-overexpressing Daoy cells compared with control Daoy cells (Fig. 5E). A total of ~100 TRIM59-overexpressing Daoy cells were stained, whereas only 40 control cells were observed under the lower surface of the membrane. Overexpression of TRIM59 promoted the invasive ability by 70% in Daoy cells (Fig. 5F). These data strongly suggest that the overexpression of TRIM59 significantly promotes cell motility in Daoy cells.

TRIM59 positively regulates MMP-2 and phosphoinositide 3-kinase (PI3K)/AKT signaling. MMPs are upregulated in the process of tumor invasion-metastasis cascade (21). Western blot analysis revealed that the major MMP, MMP-2 but not MMP-9, was downregulated following...
the knockdown of TRIM59 in D283 cells, and upregulated by the overexpression of TRIM59 in Daoy cells compared with shNC cells (Fig. 6A), which supported the findings of the aforementioned invasion assays. Notably, it was observed that following the knockdown of TRIM59, the levels of p-AKT (Ser473), p-GSK3β (Ser9) and p-PI3K p85 (Tyr458) were consistently decreased, while the total protein levels remained largely unaltered. The phosphorylation level of AKT at the site Thr308 also remained unchanged (Fig. 6B). These data suggest that the PI3K/AKT signaling cascade is activated by TRIM59 in medulloblastoma.

Discussion

Medulloblastoma is the most common malignant pediatric tumor in the brain, and it is often resistant to traditional therapy. Medulloblastoma is also characterized by frequent extraneural metastasis (5). Therefore, novel therapeutic strategies for intercepting critical regulatory pathways in cancer development and progression are warranted. Here, we present in vitro and in vivo data that TRIM59 displays potent metastatic activity via the PI3K/AKT signaling pathway in medulloblastoma and may be a potential therapeutic target for the treatment of medulloblastoma.

In the present study, TRIM59 was initially observed to be upregulated in medulloblastoma tissues and differentially expressed in a series of medulloblastoma cell lines. Notably, it was observed that the knockdown of TRIM59 in D283 cells resulted in prominent epithelial features, which was accompanied with decreased cell migratory and invasive capacity. By contrast, the overexpression of TRIM59 in Daoy cells induced mesenchymal features, which was accompanied with increased cell migratory and invasive abilities. The corresponding regulation of MMP-2 by TRIM59 further supported the metastatic potency of TRIM59 in medulloblastoma cells. The present data suggested that TRIM59 was able to induce the EMT process in medulloblastoma.

EMT is a critical manifestation of epithelial cell plasticity, where multiple regulatory molecules are involved, including the Zeb family and the Snail family (22). The EMT process is induced by various cellular procedures, including increased expression of mesenchymal markers (N-cadherin and vimentin), decreased protein levels of epithelial markers (E-cadherin) and the overexpression of ECM molecules (fibronectin) (23). EMT formation is also associated with changes in cell morphology (the cells exhibited a shuttle shape or multiple angle shape) (24). The EMT process has been associated with human tumorigenesis in a wide range of literature. For instance, it was shown that in ovarian cancer, the activation of EMT is associated with chemoresistance, the latter of which can cause cancer recurrence and metastasis following conventional treatment against ovarian cancer (25,26). Additionally, benzophenone-3 has been demonstrated to increase metastasis potential in lung cancer via the induction of EMT (27). Following full review of the EMT data in the present study, together with the cell migration and invasion assays, it is concluded that TRIM59 may promote metastasis in medulloblastoma.

The PI3K/AKT signaling pathway has been identified as a key driver of proliferation, migration and angiogenesis in human tumorigenesis, including medulloblastoma, where the activation of PI3K/AKT signaling has been associated with enhanced tumor growth, metastasis and chemoresistance (28-30). In addition, the present study also identified that PI3K/AKT signaling was involved in TRIM59-mediated cell metastasis. Following the knockdown of TRIM59, the levels of p-AKT, p-GSK3β and p-PI3K p85 were decreased, suggesting the positive regulation of PI3K/AKT signaling by TRIM59. A previous study indicated that the PI3K inhibitor GDC-0941 displayed promising in vitro and in vivo efficacy for targeted medulloblastoma therapy (31). In addition, PI3K/AKT signaling serves as an integration node in a network of tumor-promoting signaling pathways (32). It is therefore likely that any compound or reagent targeting TRIM59 and consequently inhibiting the PI3K/AKT signaling pathway may serve as a promising therapeutic strategy for the treatment of medulloblastoma.

In conclusion, the present study identified TRIM59 as a critical mediator of cell metastasis in medulloblastoma. The loss-of-function and gain-of-function assays highlighted the strong metastatic potential of TRIM59 in medulloblastoma. TRIM59 may be able to induce the EMT process and promote cell migration and invasion via the PI3K/AKT signaling pathway. The present study provided data to indicate that TRIM59 may serve as a molecular target that is useful for targeted therapeutic strategies.
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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
RG and LJC conceived the study; RG, GQL, XW and CZ collected the cancer tissues; RG and GQL performed the experiment; RG and LC calculated the data and wrote the paper.

Ethics approval and consent to participate
All patients gave their full consent to participate in the present study, and a written consent form was obtained from each patient. All of the experiments in the present study were in compliance with the official policies and defined protocols. The research protocol was approved by the Ethical Committee of Jining No. 1 People’s Hospital.

Consent for publication
A written consent form was obtained from each patient.

Competing interests
The authors declare that they have no competing interests.

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