

Matrine Inhibits Neuroblastoma Cell Proliferation and Migration by Enhancing Tribbles 3 Expression

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Neuroblastoma is a major contributor of cancer-specific mortality. Although remarkable enhancement has been achieved in the treatment of neuroblastoma in patients with early stage disease, limited progress has been made in the treatment of patients with high-risk neuroblastoma. Thus, innovative approaches are required to achieve further improvements in neuroblastoma patient survival outcomes. The major alkaloid obtained from *Sophora flavescens* Ait, matrine, has been shown to counteract malignancy in various kinds of cancers. In the current study, we evaluated the effects of matrine on the migration and proliferation of neuroblastoma cells. Cell cycle analysis coupled with Transwell and wound healing experiments showed that matrine triggers G₂/M cell cycle arrest and suppresses neuroblastoma migration. This effect of matrine is due to upregulation of TRB3 expression followed by inhibition of the PI3K/AKT activation. Consistent with the in vitro data, growth of xenograft cancer was also suppressed by matrine. Our results indicate that matrine inhibits neuroblastoma cell proliferation and migration by enhancing TRB3 expression, suggesting that matrine may serve as a promising agent for the treatment of neuroblastoma.

Key words: Neuroblastoma; Matrine; Tribbles 3 (TRB3); Proliferation; Migration

INTRODUCTION

Originating from paravertebral sympathetic ganglia or the adrenal medulla, neuroblastoma is the most prevalent solid tumor of sympathetic nerves in the initial stages of childhood and has noticeably different clinical outcomes^{1,2}. Furthermore, the median diagnosis age for neuroblastoma is 18 months, with 40% of patients being diagnosed prior to the age of 1 year³. Nearly half of neuroblastoma patients are identified as highly vulnerable, with a limited overall survival (OS) of less than 40%⁴. Although many improvements have been made in the diagnosis and treatment of neuroblastoma, a substantial number of children with incurable neuroblastoma continue to experience many long-term side effects. Hence, continued efforts are required to characterize the deregulated genes and pathways to facilitate development of novel therapeutic strategies for the treatment of neuroblastoma.

Matrine, a major alkaloid obtained from *Sophora flavescens* Ait, counteracts malignancy in various types of cancers^{5,6}. Acting as a suppressor of proliferation and stimulator of cell death, matrine has been successfully

utilized for the treatment of retinoblastoma, osteosarcoma, colorectal cancer, cholangiocarcinoma, nasopharyngeal carcinoma, gastric cancer, liver cancer, prostate cancer, neuroblastoma, breast cancer, and pancreatic cancer⁷⁻¹². However, there are few reports that address modulation of migration and proliferation of neuroblastoma cells by matrine.

Tribbles 3 (TRB3) is a pseudokinase that binds to and inhibits the activation of the serine–threonine kinase AKT¹³. Gain-of-function mutations of *TRB3* result in enhanced resistance to insulin and diabetes-related complications¹⁴. These findings indicate that TRB3 upregulation results in resistance to insulin¹⁴. After binding to and modulating the activity of mitogen-activated protein kinase (MAPK) kinase, TRB3 blocks MAPK stimulation via input signals¹⁵. It has been demonstrated that TRB3 disrupts insulin signaling by interacting with AKT¹⁶. Furthermore, treatment of various malignancies by counteracting drugs enhances tumor apoptosis through upregulation of TRB3 followed by repression of the AKT pathway.

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In this study, the effect of matrine on migration as well as proliferation of neuroblastoma was investigated. Our results reveal an innovative mechanism by which matrine suppresses migration and proliferation of neuroblastoma via inactivation of the phosphoinositide 3-kinase (PI3K)/AKT pathway modulated by TRB3, suggesting that matrine can function as a potential drug to treat neuroblastoma.

MATERIALS AND METHODS

Cell Culture, Proliferation, and Viability Assays

Neuroblastoma cell lines (SKNLP, LAN1, and SKNJD) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were grown in Roswell Park Memorial Institute (RPMI)1640 medium supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 µg/ml) at 37°C and 5% CO₂. Mycoplasma polymerase chain reaction (PCR) tests were performed routinely. The cells (5,000 cells per well) were plated in flat-bottomed 96-well microplates. Sixteen hours after seeding, fresh medium containing either different concentrations of matrine (Sigma-Aldrich, St. Louis, MO, USA) or solvent control (DMSO; Sigma-Aldrich) was added. Cells were further incubated for the indicated times, followed by MTS treatment for 1 h. The plates were then assayed by measuring the absorbance at 490 nm.

Clonogenic Experiment, Analysis of Cell Cycle, and Cell Death

Clonogenic experiments were performed by resuspending cells in RPMI-1640 (1 ml) supplemented with 10% FBS and 0.3% low-melting point agarose. Subsequently, plates with matrine (1 g/L) and agarose (0.6%) were utilized to plate cells at a density of 1,000 cells per plate. Colonies of surviving cells were counted after 2 weeks by staining with Giemsa stain, and clones including 50 cells or more were counted. For evaluation of the cell cycle, cells were exposed to the indicated concentrations of matrine for 24 h. Propidium iodide (PI) staining and flow cytometry (FCM) was performed to analyze the DNA. For cell death analysis, cells were incubated with 1 g/L matrine for 24 h. Externalization of phosphatidylserine was tested using an Annexin-V/PI Apoptosis Detection Kit (Invitrogen) according to the manufacturer's instruction.

Wound Healing Assay and In Vitro Migration Assay

To perform wound healing experiments, cells were plated in six-well plates (5 × 10⁵/well). Cells were allowed to attach for 24 h, and monolayers were scratched manually with micropipette tips. The cell debris was removed by replacing the media with fresh media containing matrine

(1 g/L), and healing was assessed after 48 h. Transwell experiments were performed by plating 2 × 10⁴ cells on Transwell inserts that were rehydrated with media without serum for no less than 1 h. Complete media supplemented with serum served as a chemoattractant in the chamber below. Matrine (1 g/L) treatment was performed to suppress migration. After 24 h of incubation, cells in the upper surface of the insert membrane were removed by wiping with a cotton swab, and cells in the lower surface were fixed with methanol, stained with crystal violet, and counted by microscopy.

Western Blotting and Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

With regard to Western blotting, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors¹⁷. Proteins (20 µg) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% nonfat milk in Tris-buffered saline, the membrane was washed and incubated with the indicated primary [epithelial (E)-cadherin, cyclin-dependent kinase inhibitor 1B (p27), TRB3, phosphorylated (p)-AKT, AKT, and β-actin] and secondary antibodies and detected using the Luminescent Image Analyser LSA 4000. For mRNA quantitative analysis, qRT-PCR was performed with SYBRTM Green Real Time PCR Master Mix¹⁸. Primers of TRB3 and β-actin are as follows: TRB3, 5'-TGCCCTACAGGCACTGAGTA-3' (forward) and 5'-GTCCGAGTGAAAAAGGCGTA-3' (reverse); β-actin, 5'-GGACTTCGAGCAAGAGATGG-3' (forward) and 5'-AGCACTGTGTTGGCGTACAG-3' (reverse).

Transfection and Small Interfering RNA (siRNA)

Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions^{19,20}. Knockdown experiments were performed 24 h prior to matrine treatment using 200 pmol of siRNA. The control scrambled siRNA and siRNA for human *TRB3* were obtained from Thermo Fisher Scientific (Beijing, P.R. China).

Immunohistochemistry (IHC) Analysis

IHC assay was performed using anti-TRB3 antibodies as previously described²¹. Briefly, formalin-fixed, paraffin-embedded mouse neuroblastoma tissue specimens (5 mm) were deparaffinized in xylene and graded alcohol and subjected to a heat-induced epitope retrieval step in citrate buffer solution. The sections were then blocked with 5% bovine serum albumin (BSA) for 30 min and incubated with indicated antibodies at 4°C overnight, followed by incubation with secondary antibodies for 90 min at 37°C. Detection was achieved with 3,3'-diaminobenzidine (DAB;

Sigma-Aldrich) and counterstained with hematoxylin, dehydrated, cleared, and mounted as in routine processing.

Mouse Models

Animal experimental procedures were conducted in accordance with the internationally accepted principles for laboratory animal use and care. All animal studies were conducted according to protocols approved by the Animal Ethics Committee of the Shanghai University of Traditional Chinese Medicine. Severe combined immunodeficient (SCID)/beige mice aged 4–5 weeks were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, P.R. China) and housed in the Animal Resource Facility. The mice were injected with SK-N-LP cells (1×10^6) via the tail vein, and 4 days later were randomized into four groups to receive treatment with intraperitoneal injection of vehicle or 40 mg/kg matrine ($n=6$ for each group; once every 2 days for 21 days).

Statistical Analysis

Data are presented as the mean \pm standard deviation (SD). Statistical comparisons were made by Student's *t*-test. A value of $p < 0.05$ was considered to indicate statistically significant data.

RESULTS

Matrine Suppressed Proliferation and Colony Generation and Caused G₂/M Cell Cycle Arrest of Neuroblastoma Cell Lines

To examine whether matrine (Fig. 1A) could suppress neuroblastoma proliferation, MTS assay was conducted in SK-N-LP, LA-N-1, and SK-N-JD cells exposed to different concentrations of matrine for 72 h. The findings revealed that matrine suppressed proliferation of neuroblastoma cells (Fig. 1B). Consequently, 1 g/L of matrine was used for the subsequent experiments. Matrine remarkably suppressed colony generation of neuroblastoma (Fig. 1C). To further confirm suppression of neuroblastoma proliferation by matrine treatment, cell cycle as well as cell death were assessed in SK-N-LP, LA-N-1, and SK-N-JD cells. Cells were treated with matrine for 24 h followed by fluorescence-activated cell sorting (FACS) assay. Cell cycle analysis revealed that matrine contributed to the G₂/M cell cycle arrest in SK-N-LP, LA-N-1, and SK-N-JD cell lines (Fig. 1D), indicating that proliferation was suppressed by matrine via cell cycle arrest.

Matrine Suppressed Neuroblastoma Migration

To determine whether matrine also suppresses neuroblastoma migration, wound healing and Transwell assays were carried out. Matrine noticeably suppressed migration in SK-N-LP and LA-N-1 cells (Fig. 2A). In concordance, matrine treatment reduced neuroblastoma cell penetration in Transwell experiments (Fig. 2B).

Matrine Upregulated TRB3 Expression in Neuroblastoma Cells

To discern the molecular mechanisms underlying matrine-mediated G₂/M cell cycle arrest as well as migration suppression, Western blotting analysis was conducted to identify modulators related to G₂/M progression as well as cell migration. Our findings indicated that E-cadherin and p27 expression was noticeably enhanced upon matrine exposure in a time-dependent manner (Fig. 3A). Moreover, based on Western blotting and qRT-PCR assays, we found that matrine upregulated TRB3 at both the protein and mRNA levels in a dose- and time-dependent manner in SK-N-LP, LA-N-1, and SK-N-JD cells (Fig. 3B and C). In general, the findings above indicate that matrine promoted expression of TRB3.

Matrine Suppressed Cell Migration and Proliferation via TRB3 Upregulation

To understand how matrine counteracted malignancy via TRB3 upregulation in neuroblastoma, TRB3 was knocked down in SK-N-LP cells with or without matrine treatment. Western blot analysis was performed to confirm knockdown of TRB3 (Fig. 4A), followed by MTS, wound healing, cell cycle, and Transwell assay. TRB3 knockdown attenuated the suppression of proliferation by matrine treatment (Fig. 4B). Additionally, TRB3 knockdown reversed the G₂/M cell cycle arrest triggered by matrine (Fig. 4C). Last, wound healing and Transwell assay demonstrated that TRB3 knockdown promoted migration in the matrine-treated group as well as the control group and counteracted migration suppression by matrine treatment (Fig. 4D and E). Together, these findings indicate that migration as well as proliferation of neuroblastoma were suppressed by matrine via TRB3 upregulation.

TRB3 Modulated the PI3K/AKT Pathway Inactivation Triggered by Matrine

It has been previously reported that AKT stimulation enhances proliferation and promotes progression of cell cycle via activating cyclin-dependent kinase (CDK) as well as cyclins. Moreover, AKT activation also plays a role in cell migration. Therefore, Western blotting analysis was performed to evaluate AKT expression upon matrine treatment. As expected, matrine treatment resulted in a dramatic decrease in AKT phosphorylation at Ser473 (Fig. 5A). Since TRB3 could suppress AKT stimulation, we tested whether TRB3 participated in PI3K/AKT inactivation via matrine. AKT phosphorylation was analyzed in SK-N-LP cells transfected with either TRB3 siRNA or control siRNA in the presence or absence of matrine. As shown in Figure 5B, TRB3 knockdown blocked matrine-induced AKT dephosphorylation. The above findings suggest that TRB3-mediated AKT

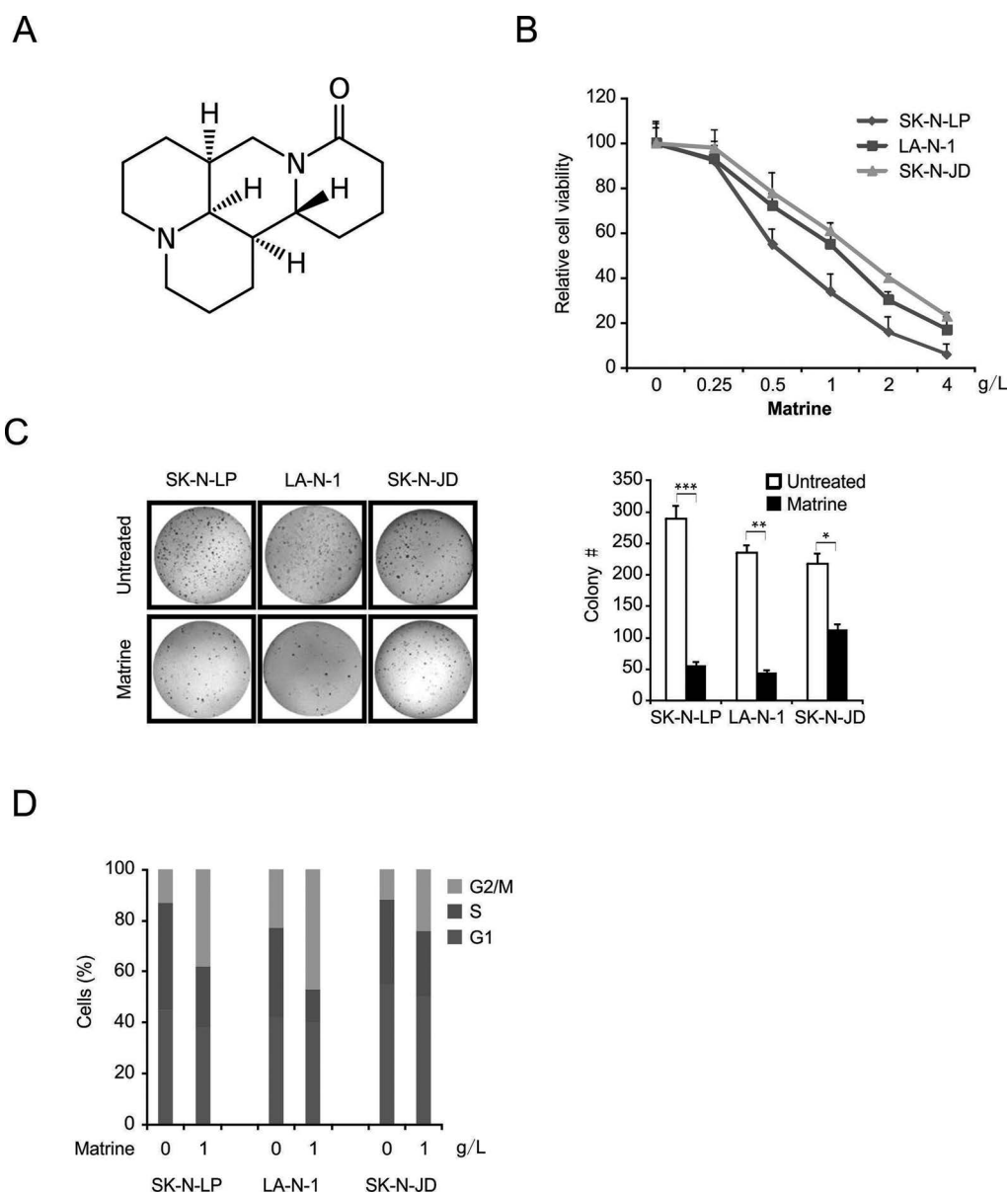


Figure 1. Matrine inhibits neuroblastoma cell proliferation and colony formation, and arrests the cell cycle at the G₂/M phase. (A) Chemical structure of matrine. (B) SK-N-LP, LA-N-1, and SK-N-JD cells were treated with different concentrations of matrine for 72 h and assessed by MTS. (C) Soft agar colony formation assays for SK-N-LP, LA-N-1, and SK-N-JD cells treated with or without matrine. (D) Matrine induced G₂/M accumulation in SK-N-LP, LA-N-1, and SK-N-JD cells. (C) Results are represented as mean \pm standard deviation (SD). *** p < 0.01; ** p < 0.01; * p < 0.05 compared to untreated.

stimulation could account for suppression of migration and proliferation by matrine in neuroblastoma.

Antineuroblastoma Activity of Matrine In Vivo

To assess neuroblastoma counteraction by matrine in vivo, SCID/beige mice ($n=6$ in each group) were injected intravenously with SK-N-LP cells (1×10^6). Vehicle and matrine (40 mg/kg) were intraperitoneally administered every other day for 3 weeks. Our findings indicate that

matrine treatment remarkably inhibited cancer growth (Fig. 6A). No decrease in body weight was caused by matrine injection in both experimental and control groups (Fig. 6B). IHC assays indicated that matrine triggered TRB3 upregulation in cancer specimens (Fig. 6C). Western blot analysis confirmed that matrine exposure resulted in TRB3 upregulation and AKT dephosphorylation in vivo (Fig. 6D). These data indicate that matrine displayed antitumor effects in vivo with no obvious side effects.

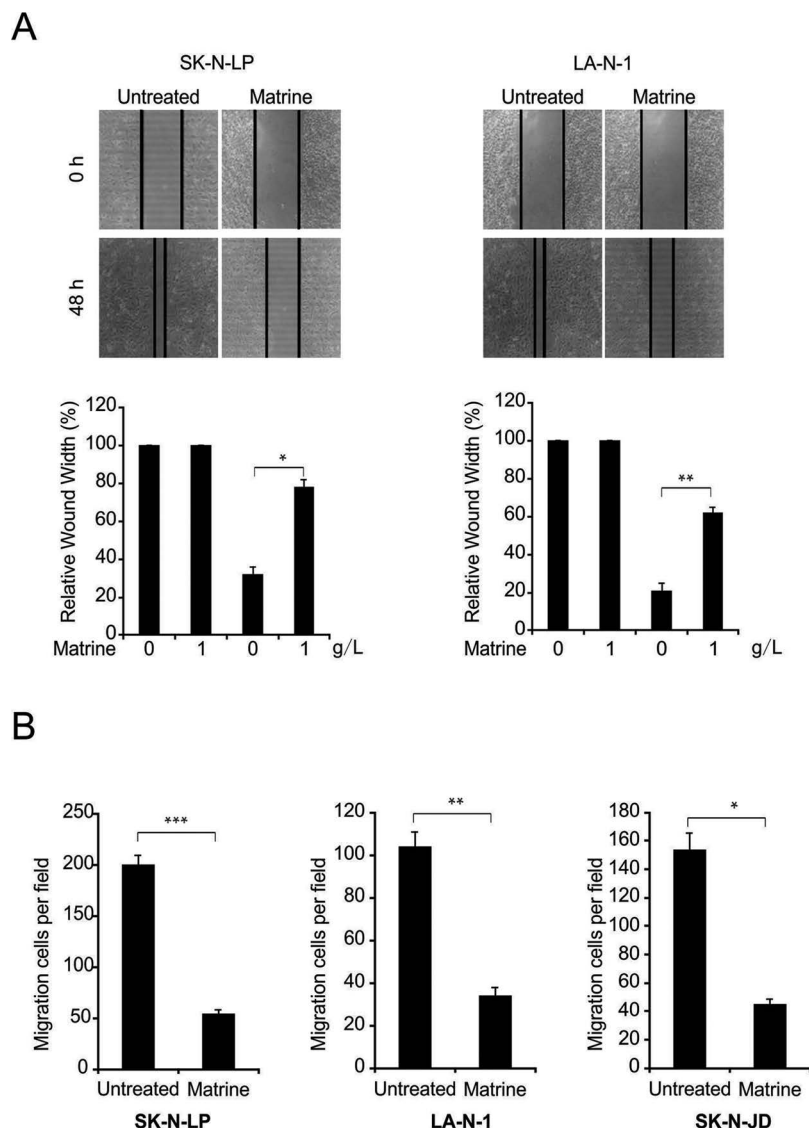


Figure 2. Matrine suppresses cell migration in neuroblastoma cells. (A) Inhibition of cell migration by matrine was confirmed by the wound healing assay. (B) Treatment with matrine reduced the number of migrated cells in the Transwell assay. Results are represented as mean ± SD. *** $p < 0.01$; ** $p < 0.01$; * $p < 0.05$ compared to untreated.

DISCUSSION

Neuroblastic cancer is a highly heterogeneous family of developmental neoplasms of the sympathetic nerves, such as neuroblastomas^{4,22}. Diffuse neuroblastomas of babies and local, well-differentiated neoplasms tend to be benign²³. Nevertheless, high-risk cancers such as metastatic neuroblastomas of patients more than 18 months old and several local neuroblastomas are highly vulnerable to recurrence²⁴. Unfortunately, there are no available therapies to cure such refractory neuroblastomas. In this study, we showed that matrine suppresses migration as well as proliferation of neuroblastoma via inactivation of

the PI3K/AKT pathway modulated by TRB3, suggesting that matrine is a potential drug for the treatment of neuroblastoma.

Matrine is currently approved to serve as an ancillary drug to avoid cachexia in China²⁵. It has been clinically proven that the quality of life as well as the immune system of cancer patients are remarkably improved by a combination of standard treatments and matrine^{26,27}. Moreover, it has been previously reported that matrine is able to suppress proliferation of diverse kinds of tumor cells^{28,29}. In the current study, we found that in addition to causing G₂/M cell cycle arrest, matrine suppresses proliferation, migration, and colony generation by neuroblastoma cells.

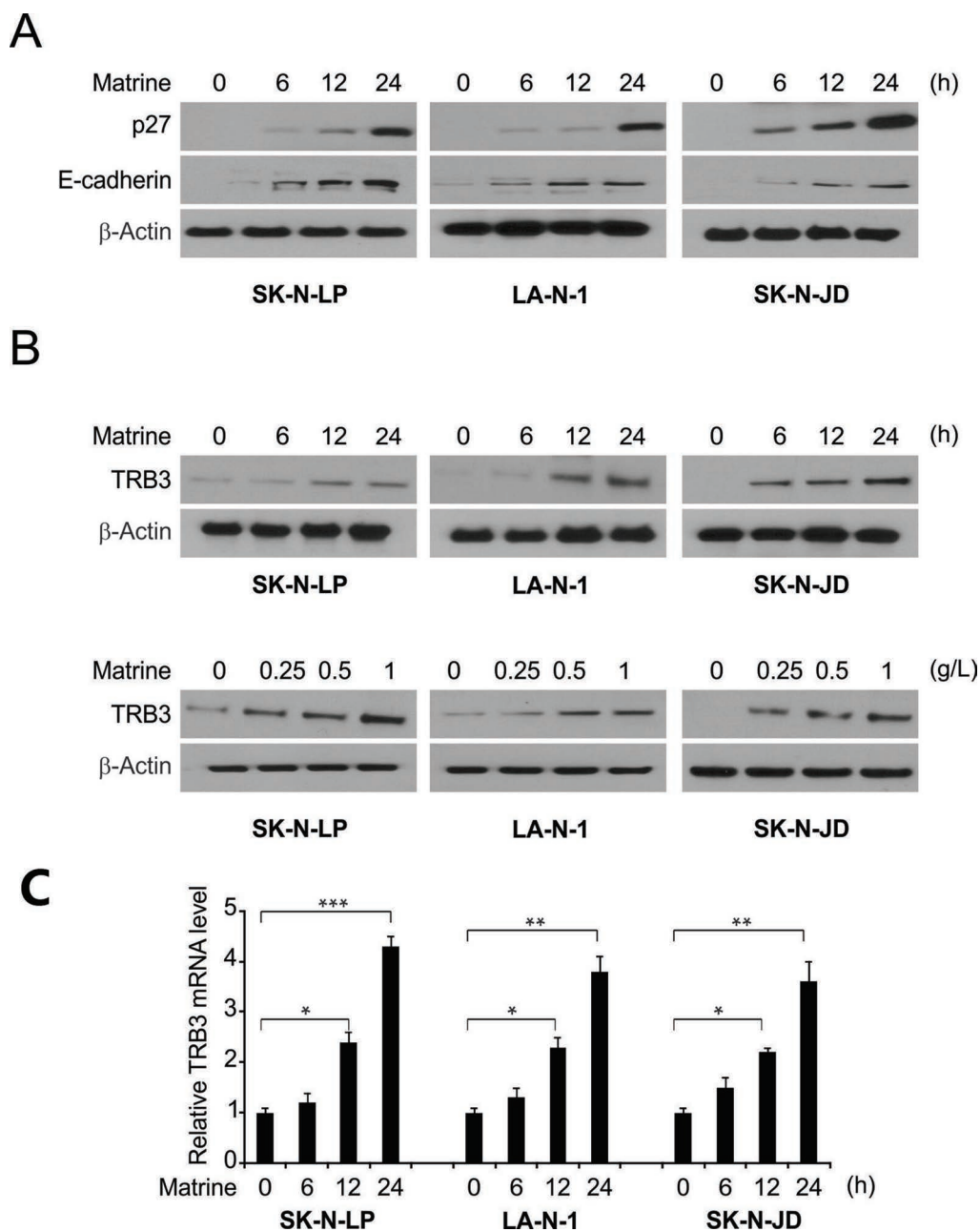


Figure 3. Matrine treatment leads to tribbles 3 (TRB3) upregulation. (A) Cyclin-dependent kinase inhibitor 1B (p27) and epithelial (E)-cadherin proteins were upregulated upon matrine treatment. β -Actin was used as a loading control. (B) Matrine induced TRB3 expression in a time- and dose-dependent manner (top and bottom, respectively). (C) Treatment with matrine led to transcription induction of TRB3. Results are represented as mean \pm SD. *** p < 0.01; ** p < 0.01; * p < 0.05 compared to 0 h.

Our results are consistent with those of previous studies that have suggested that matrine could function as a promising ingredient for counteracting malignancy.

TRB3 is a mammalian homolog of *Drosophila tribbles* and functions as an inhibitor of AKT activation¹⁴. It has been previously demonstrated that TRB3 participates in the suppressive activity of fenofibrate on proliferation of mesangial cells triggered by elevated concentrations

of glucose³⁰. TRB3 is also essential for cell cycle arrest modulated by homocysteine in the endothelium³¹. Additionally, it reversibly regulates the AKT pathway in vitro, thus modulating differentiation of muscle cells in skeletal muscle cell lines³². Although the molecular mechanisms underlying these observations are still unclear, it is possibly due to an enhancement of cell death that occurs in macrophages that arise from monocytes or kidney

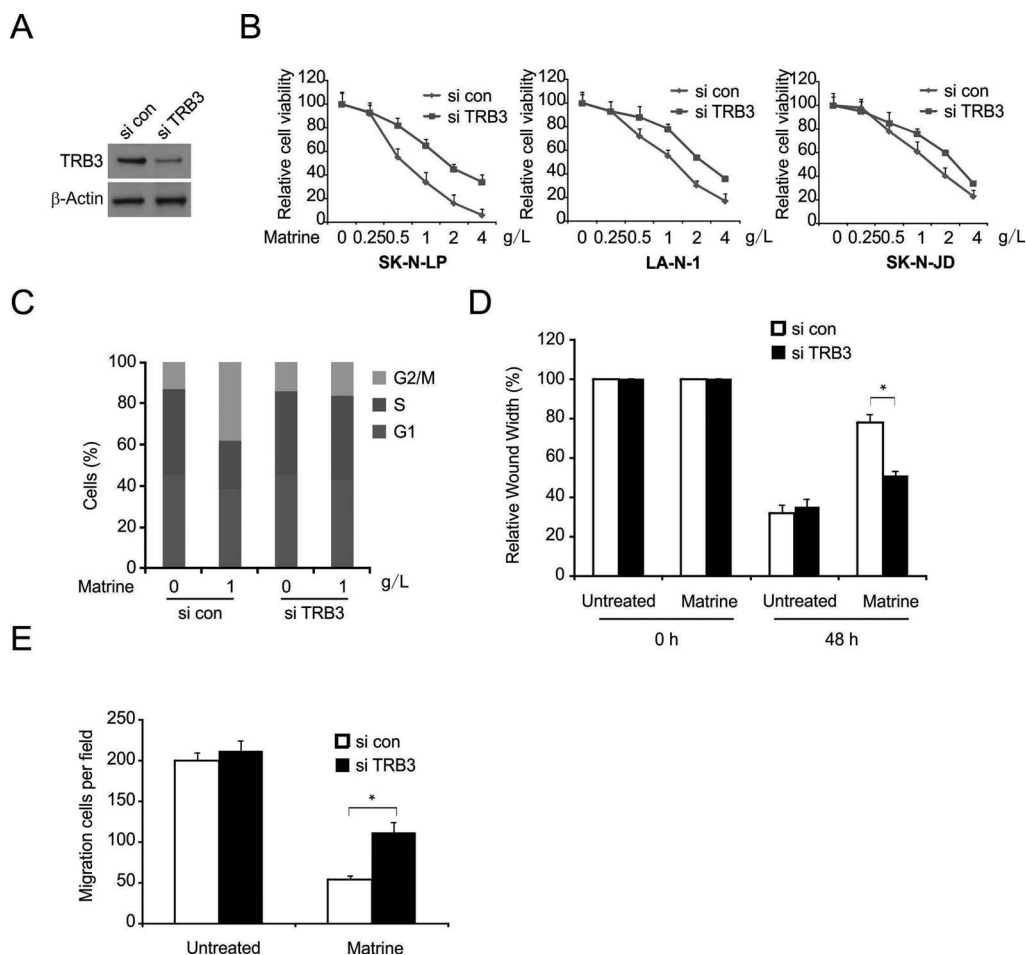


Figure 4. Knockdown of TRB3 attenuates matrine-induced proliferation inhibition, G₂/M phase accumulation, and migration suppression. (A) SK-N-LP cells were transfected with TRB3 small interfering RNA (siRNA) or control siRNA for 24 h. Western blotting assays were performed to detect the expression of TRB3 and β -actin. (B) Indicated cells were transfected with TRB3 siRNA or control siRNA and treated with matrine. Viable cells were detected by MTS assay. (C) SK-N-LP cells were transfected with TRB3 siRNA or control siRNA and treated with matrine for 24 h. The cells were analyzed by flow cytometry to evaluate the cell cycle distribution. SK-N-LP cells were transfected with si TRB3 or control siRNA and then treated with matrine. Wound healing (D) and Transwell (E) assays were conducted to evaluate cell migration. (D, E) Results are represented as mean \pm SD. * $p < 0.05$ compared to si CON.

tubular cells³³. In this study, we have shown that matrine upregulates TRB3 expression in neuroblastoma cells. TRB3 plays an indispensable role in the maintenance of glucose homeostasis as well as proliferation of cells. Previous studies have demonstrated that upregulation of TRB3 enhanced glucose intolerance and inhibited insulin signaling through the insulin receptor substrate 1 (IRS-1)/PI3K/AKT pathway. In addition, it has been demonstrated that TRB3 can interact with mothers against decapentaplegic 3 (Smad3) in order to positively modulate biological reactions mediated by transforming growth factor (TGF)- β -SMAD, suggesting that TRB3 can interact with diverse kinds of signals³⁴. Other studies have also shown that TRB3 silencing counteracts cell death triggered by albumin in tubular cells and attenuates cardiomyopathy related to type 2 diabetes in rats, via recovery

of AKT phosphorylation³⁴. Further, TRB3 knockdown protects photoreceptors from endoplasmic reticulum (ER) stress-induced apoptosis in rats with retinal detachment. In the present study, we found that migration as well as proliferation of neuroblastoma cells were suppressed by matrine via TRB3 upregulation. It is widely accepted that AKT sustains cell survival through suppression of cell death^{35,36}. It has been demonstrated that TRB3 binds to and inhibits activity of AKT. Consequently, we hypothesized that TRB3 causes cell death triggered by matrine through modulation of the AKT pathway. Our findings demonstrate that dephosphorylation of AKT at S473 is triggered by matrine, thus indicating that TRB3 suppressed AKT stimulation by matrine treatment. Further, we observed that matrine displayed favorable antitumor effects in vivo with no obvious side effects.

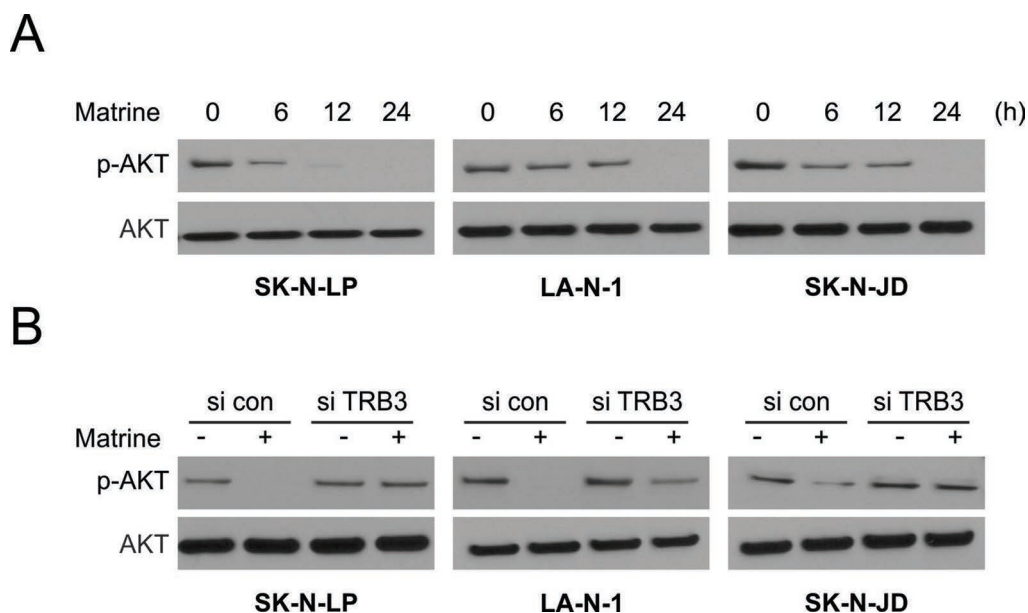


Figure 5. Knockdown of TRB3 attenuates matrine-induced serine–threonine kinase AKT inhibition. (A) The neuroblastoma cell lines were treated with matrine at indicated time points, and phosphorylated (p)-AKT and AKT protein levels were analyzed by Western blotting. (B) The neuroblastoma cell lines were transfected with TRB3 siRNA and treated with matrine for 24 h. Western blotting assays were used to detect the expression of p-AKT and AKT.

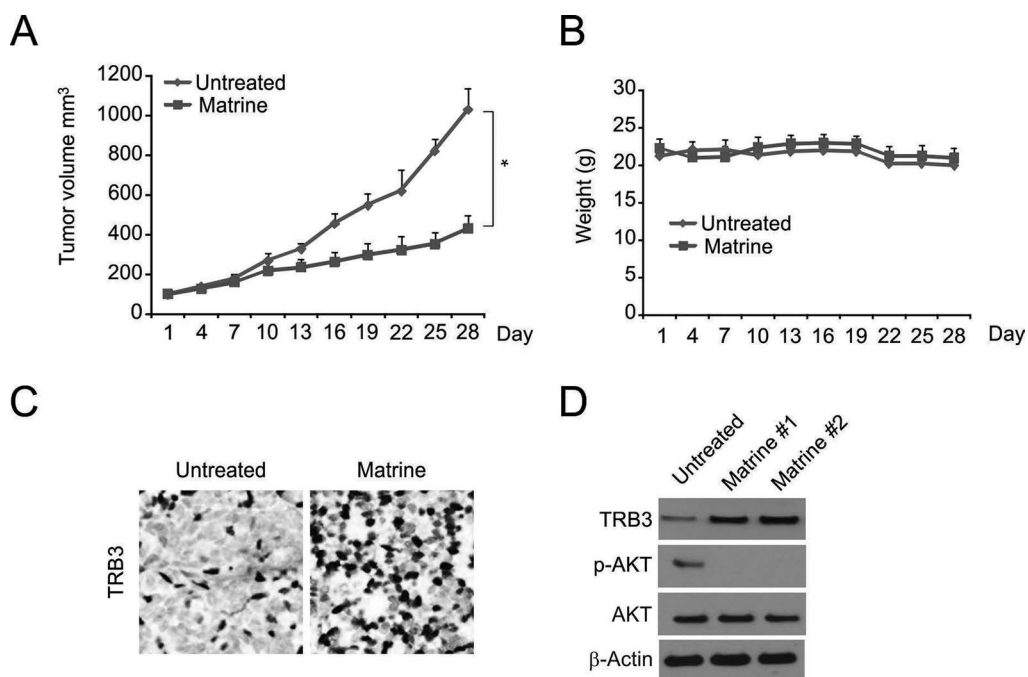


Figure 6. In vivo antineuroblastoma efficacy of matrine. (A) SK-N-LP cells were intravenously injected into severe combined immunodeficient (SCID)/beige mice, and 4 days later the mice were randomized to receive vehicle or matrine treatment ($n=6$ for each group). The tumor volume was measured over 28 days. (B) The body weight of mice was monitored every 2 days for 28 days. (C) Immunohistochemistry (IHC) of the tumor using anti-TRB3 antibody following vehicle or matrine treatment. (D) Western blotting assays using lysates of isolated tumors and TRB3 and β -actin antibodies. (A) Result is represented as mean \pm SD. $*p < 0.05$ compared to untreated.

In summary, our work demonstrates that matrine suppresses proliferation, triggers G₂/M cell cycle arrest, and inhibits cell migration through TRB3 upregulation and a consequential inactivation of the PI3K/AKT pathway in neuroblastoma. Thus, induction of TRB3 by matrine provides an innovative therapeutic strategy that can be utilized in the treatment of neuroblastoma.

ACKNOWLEDGMENTS: *The authors declare no conflicts of interest.*

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