

# Myristoylated alanine rich protein kinase C substrate is a potential cancer prognostic factor that regulates cell migration and invasion in glioblastoma

WEI XIANG\*, TAO PENG\*, YANG MING, SHENJIE LI, KE WANG,  
HAORUN WANG, LIGANG CHEN and JIE ZHOU

Department of Neurosurgery, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan 646000, P.R. China

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**Abstract.** Myristoylated alanine-rich C-kinase substrate (MARCKS) serves an important role in various pathological processes in several malignancies. However, little is known about the specific role and molecular mechanism of MARCKS in glioblastoma (GBM). In the present study, it was found that the expression of MARCKS was significantly upregulated in GBM, and was associated with a poor clinical outcome in patients with GBM. Knockdown of MARCKS suppressed the migration and invasion of GBM cells *in vitro*. Western blotting showed that the knockdown of MARCKS reduced the expression of phosphorylated phosphoinositide 3-kinase and protein kinase B, as well as zinc finger protein SNAIL expression, thereby modulating the expression of its downstream epithelial-mesenchymal transition (EMT)-associated factors, including E-cadherin, vimentin, N-cadherin and  $\beta$ -catenin in GBM cells. These results indicate that MARCKS functioned in the migration and invasion of GBM, and therefore may provide a potential therapeutic target in GBM therapy.

## Introduction

Glioblastoma (GBM; also known as grade IV astrocytoma) is the most aggressive and lethal type of brain tumor, according

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*Correspondence to:* Dr Ligang Chen or Dr Jie Zhou, Department of Neurosurgery, The Affiliated Hospital of Southwest Medical University, 25 Taiping Street, Luzhou, Sichuan 646000, P.R. China  
E-mail: chengligang.cool@163.com  
E-mail: zj000718@yeah.net

\*Contributed equally

*Abbreviations:* EMT, epithelial-mesenchymal transition; MARCKS, myristoylated alanine-rich C kinase substrate; PI3K, phosphoinositide 3-kinase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; siRNA, small interfering RNA

*Key words:* epithelial-mesenchymal transition, glioblastoma, MARCKS, phosphoinositide 3-kinase/protein kinase B

to the World Health Organization (WHO) criteria (1). Due to the high invasive potential of GBM cells, they easily infiltrate into the healthy brain tissues and ultimately result in tumor recurrence and patient mortality (2). Despite continuous developments in GBM treatment, the median survival time of GBM patients is still only around 14 months (3). Therefore, assessment and identification of the molecular events underlying the biological behavior of invasive tumor cells may provide novel markers for GBM treatment and improve patient prognosis.

Myristoylated alanine rich protein kinase C substrate (MARCKS) was first identified over 20 years ago in brain synaptosomes (4). It is involved in cellular processes, such as motility through control of the actin cytoskeleton, motility and membrane trafficking (5,6). MARCKS is a well conserved protein that is ubiquitously expressed in various tissues. Several studies have demonstrated that MARCKS is involved in the pathological processes of various malignancies, including tumor invasion, apoptosis and therapeutic resistance (7-11). However, the role of MARCKS in glioma tumorigenesis remains poorly understood. It has been reported that MARCKS expression is inversely correlated with GBM cell proliferation, suggesting that MARCKS may be regarded as a tumor suppressor (12). However, another study on MARCKS in glioma observed that higher MARCKS expression leads to increased tumor invasion (13). These data suggest that the function of MARCKS in glioma is multifaceted and complex.

In the present study, MARCKS expression in GBM specimens was determined and its biological roles in glioma tumorigenesis were characterized. It was shown that MARCKS expression was upregulated in GBMs, and patients with high MARCKS protein expression had shorter survival times. In addition, it was demonstrated that inhibition of MARCKS *in vitro* suppressed cell migration and invasion, resulting in the decreased expression of its downstream epithelial-mesenchymal transition (EMT)-associated genes. These data indicate that MARCKS may be a prognostic biomarker and potential therapeutic target for GBM.

## Materials and methods

*Tissue samples.* A total of 62 tumor samples and 30 normal brain tissues from patients with GBM (38 males and 24 females;

age range, 17-67; median, 46.3 years), confirmed by a pathologist according to the WHO criteria (1), were collected from the Neurosurgery Department of the Affiliated Hospital of Southwest Medical University (Sichuan, China) between January 2012 and January 2015. Normal brain tissue from the peritumoral area was obtained during the tumor resection procedures. These tissues were examined by a pathologist and confirmed to be free of tumor cells. None of the included 62 patients with GBM had undergone preoperative chemotherapy or radiotherapy, and complete follow-up data were collected. All patients with GBM were followed up from 4 to 30 months. The overall survival (OS) was defined as from the date of histological diagnosis of GBM to the date of death or last known alive. The present study was approved by the Ethics Committees of the Affiliated Hospital of Southwest Medical University and informed consent was obtained from all the patients whose clinical tissue samples were used for research purposes.

**Survival analysis.** Survival data were collected for all 62 patients with GBM, who were grouped into low or high expression groups, according to the mean mRNA expression level of MARCKS (mean value, 1.148). The Kaplan-Meier method was used to evaluate patient survival.

**RNA extraction and reverse transcription, quantitative polymerase chain reaction (RT-qPCR).** Total RNA was isolated from GBM and normal brain tissues using an RNA extraction kit (Tiangen Biotech Co., Ltd., Beijing, China), according to the manufacturer's instructions. The concentration and purity of total RNA was measured on a Nanodrop ND-100 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Total RNA was converted into cDNA using ReverTra Ace<sup>®</sup> qPCR RT Master mix (Toyobo Life Science, Osaka, Japan), according to the manufacturer's protocol. Subsequently, qPCR was conducted using a SYBR-Green Realtime PCR Master mix (Toyobo Life Science), according to the manufacturer's protocol. The PCR primers for GAPDH and MARCKS were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and the sequences were as follows: MARCKS sense primer, 5'-AGCCCGGTAGAGAAGGAGG-3', and antisense primer, 5'-TTGGGCGAAGAAGTCGAGGAG-3'; GAPDH sense primer, 5'-ATCATCAGCAATGCCTCTG-3' and antisense, 5'-ATGGACTGTGGTCATGAGTC-3'. GAPDH was used as an internal control. The relative gene expression data was analyzed by the 2<sup>-ΔΔC<sub>q</sub></sup> method (14).

**Cell culture and treatment.** Human GBM U87 MG and LN-229 cell lines (American Type Culture Collection, Manassas, VA, USA) were provided by Dr Y.W. Liu of the Nanfang Hospital of Southern Medical University (Guangzhou, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) with 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences) at 37°C with 5% CO<sub>2</sub>. For drug treatment, U87 and LN229 glioma cells were treated with 50 μM phosphoinositide 3-kinase (PI3K) inhibitor LY294002 (Tocris Bioscience, Bristol, UK) for 24 h at 37°C.

**Cell transient transfection with small interfering (si)RNAs.** MARCKS siRNA (si-MARCKS) was designed and chemically

synthesized by Sangon Biotech Co., Ltd. The sequence of si-MARCKS was as follows: Sense, 5'-GCCAGTTCTCC AAGACCGTT-3' and antisense, 5'-CGGUCUUGGAGAACU GGGCTT-3'. The sequence of the si-negative control (si-NC) was also designed by Sangon Biotech Co., Ltd. (sense, 5'-UUC UCCGAACGUGUCACGUTT-3' and antisense, 5'-ACGUGA CACGUUCGGAGAATT-3'). U87 and LN229 cells were seeded onto a 6-well plate (Corning Incorporated, Corning, NY, USA) and grown to 60-70% confluence 24 h before transfection. si-MARCKS and si-NC (50 nmol/l) were then transfected into cells using Lipofectamine<sup>®</sup> 2000 siRNA transfection reagent (Fermentas; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Lipofectamine<sup>®</sup> 2000 siRNA transfection reagent alone was added to the culture medium of the U87 and LN229 cells to serve as the control group (Blank). Cells were collected after 24 h for further experiments.

**Matrigel invasion assays.** Cell invasion was assessed using a Transwell assay with 24-well Transwell plates (8 μm pore size; BD Biosciences, Franklin Lakes, NJ, USA); chamber membranes were precoated with 45 μg Matrigel (BD Biosciences) to form a matrix barrier. For the invasion assay, 5x10<sup>4</sup> transfected cells were suspended in 200 μl serum-free DMEM and added to the upper chambers. DMEM (600 μl) with 10% FBS was placed in each of the lower chambers. Following incubation for 8 h at 37°C in a 5% CO<sub>2</sub> atmosphere, cells remaining on the upper membrane were removed carefully with cotton wool. Cells that had invaded through the membrane were fixed in pure methanol and stained with 0.5% crystal violet (Beyotime Institute of Biotechnology, Haimen, China) for 8 min at room temperature, rinsed in PBS and subsequently counted in 10 microscopic fields (magnification, x100) and photographed using an inverted phase contrast microscope (Leica Microsystems GmbH, Wetzlar, Germany). Experiments were independently repeated three times.

**Scratch migration assay.** Transfected U87 and LN229 cells were cultured in DMEM with 1% FBS in two 6-well plates until fully confluent. A straight scratch was carefully made through the central axis of the plate using a 20 μl micropipette tip. Images were acquired every 8 h of the same scratched region until the scratch closed completely. Images were captured using an inverted phase contrast microscope (magnification, x50).

**Western blot analysis.** Western blot analysis was performed as previously described (15,16) with primary rabbit polyclonal antibodies including those against: MARCKS (cat. no. ab52616; 1:5,000; Abcam, Cambridge, UK), PI3K, phosphorylated (p)PI3K (Tyr458; cat. no. 9655; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), Akt (cat. no. 4691; 1:1,000, Cell Signaling Technology, Inc.), pAkt (Ser473; cat. no. 4060; 1:2,000; Cell Signaling Technology, Inc.), β-catenin (1:1,000; cat. no. 8480; Cell Signaling Technology, Inc.), N-cadherin (1:1,000; cat. no. 13116; Cell Signaling Technology, Inc.), vimentin (1:1,000; cat. no. 5741; Cell Signaling Technology, Inc.), E-cadherin (1:1,000; cat. no. 3195; Cell Signaling Technology, Inc.), Zinc finger protein SNAI1 (Snail; 1:1,000; cat. no. 3879; Cell Signaling Technology, Inc.), Zinc finger protein SNAI2 (Slug; 1:1,000;

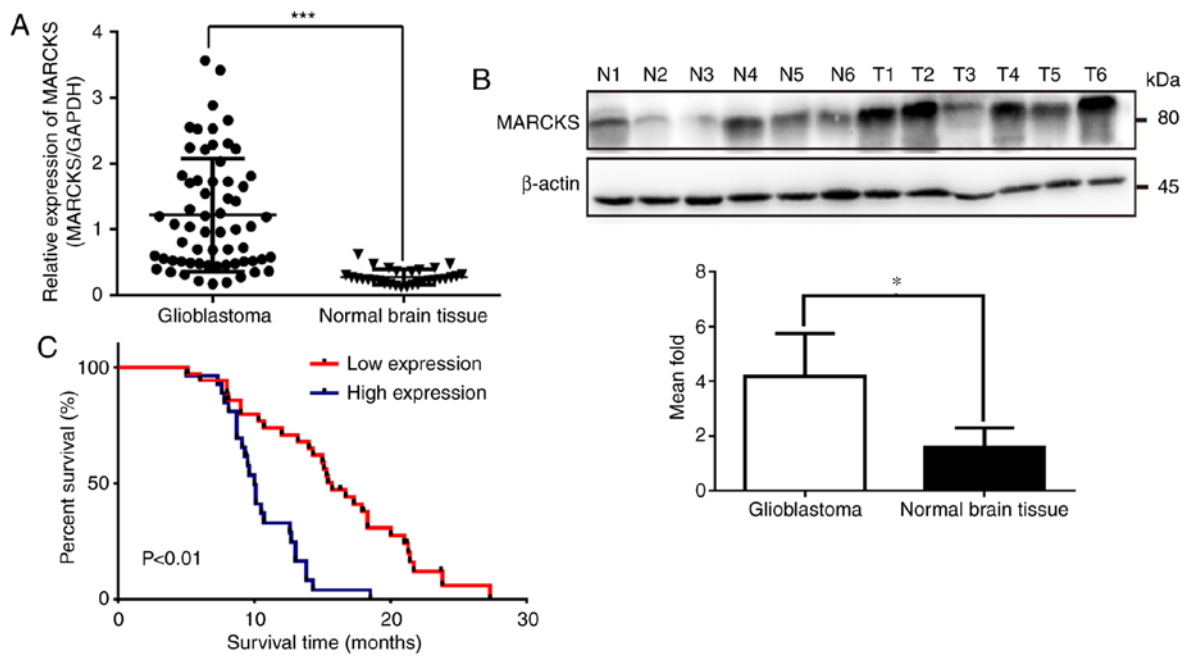


Figure 1. MARCKS expression in GBM and its association with patient survival. (A) The expression of MARCKS mRNA in 62 GBM specimens and 30 normal brain tissue specimens was analyzed by quantitative polymerase chain reaction. Data are presented as the mean  $\pm$  standard deviation (n=3). GAPDH was used as an internal control. \*\*\*P<0.001. (B) The expression of MARCKS protein was measured in six GBM samples and six normal brain samples by western blotting. An unpaired t test was used for this assay. \*P<0.05. (C) The cut-off point was the mean mRNA expression level of MARCKS in all 62 GBM tissue samples, and the Kaplan-Meier method was used to analyze the overall survival of patients in the high and low expression groups. There was a significant difference in the probability of patient survival (P<0.01). MARCKS, myristoylated alanine-rich C kinase substrate; GBM, glioblastoma; N, normal; T, tumor.

cat. no. 9585; Cell Signaling Technology, Inc.) and  $\beta$ -actin (1:1,000; cat. no. 4970; Cell Signaling Technology, Inc.). The horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:1,000; cat. no. 7074; Cell Signaling Technology, Inc.) was used. Protein signals were detected using an chemiluminescent detection system (Pierce; Thermo Fisher Scientific, Inc.). The gray-scale value was quantified by ImageJ software (version 1.8.0; National Institutes of Health, Bethesda, MD, USA) to calculate relative protein expression.

**Statistical analysis.** All experiments were repeated at least three times and data were expressed as the mean  $\pm$  standard deviation. Differences in MARCKS expression between two groups were compared using the Mann-Whitney U test. One-way analysis of variance or Student's t-test was used for comparisons between groups, followed by the Least Significant Difference test. P<0.05 was considered to indicate a statistically significant difference. Statistical analyses were performed using SPSS 20.0 (IBM Corp., Armonk, NY, USA). The Kaplan-Meier estimate was used to evaluate and compare the prognosis of patients with GBM using GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA).

**Results**

**MARCKS is overexpressed in GBM tissues and is associated with patient survival.** To assess the role of MARCKS in GBM tumorigenesis, RT-qPCR was performed to examine the expression of MARCKS mRNA in 62 GBM tissues and 30 normal brain tissues. The results showed that MARCKS mRNA expression markedly increased in GBM tissues compared with normal brain tissues (\*\*\*P<0.001; Fig. 1A).

Table I. The level of expression of MARCKS between glioblastoma and normal brain tissues.

Group	Cases	mRNA expression		P-value
		High	Low	
Glioblastoma	62	40	22	<0.001
Normal brain tissue	30	9	21	

Additionally, MARCKS protein expression was upregulated in six GBM samples, when compared with six normal brain tissues, as determined by western blot analysis (\*P<0.05; Fig. 1B). Furthermore, to investigate the relationship between the expression level of MARCKS and the outcome of patients with GBM, MARCKS expression in all 62 GBM tissues was categorized as high or low expression according to the mean value (Table I). Kaplan-Meier analysis revealed that the patients with GBM and high MARCKS expression had considerably worse outcomes than those who had low MARCKS expression (P<0.01; Fig. 1C).

**Downregulation of MARCKS suppresses GBM cell migration and invasion in vitro.** To determine the function of MARCKS in GBM, the expression of MARCKS was knocked down in U87 and LN229 cell lines, which were established from GBM via the transfection of siRNA. The interference efficiency of siRNA was detected by qPCR and western blot analysis. The data showed that the expression of MARCKS in the

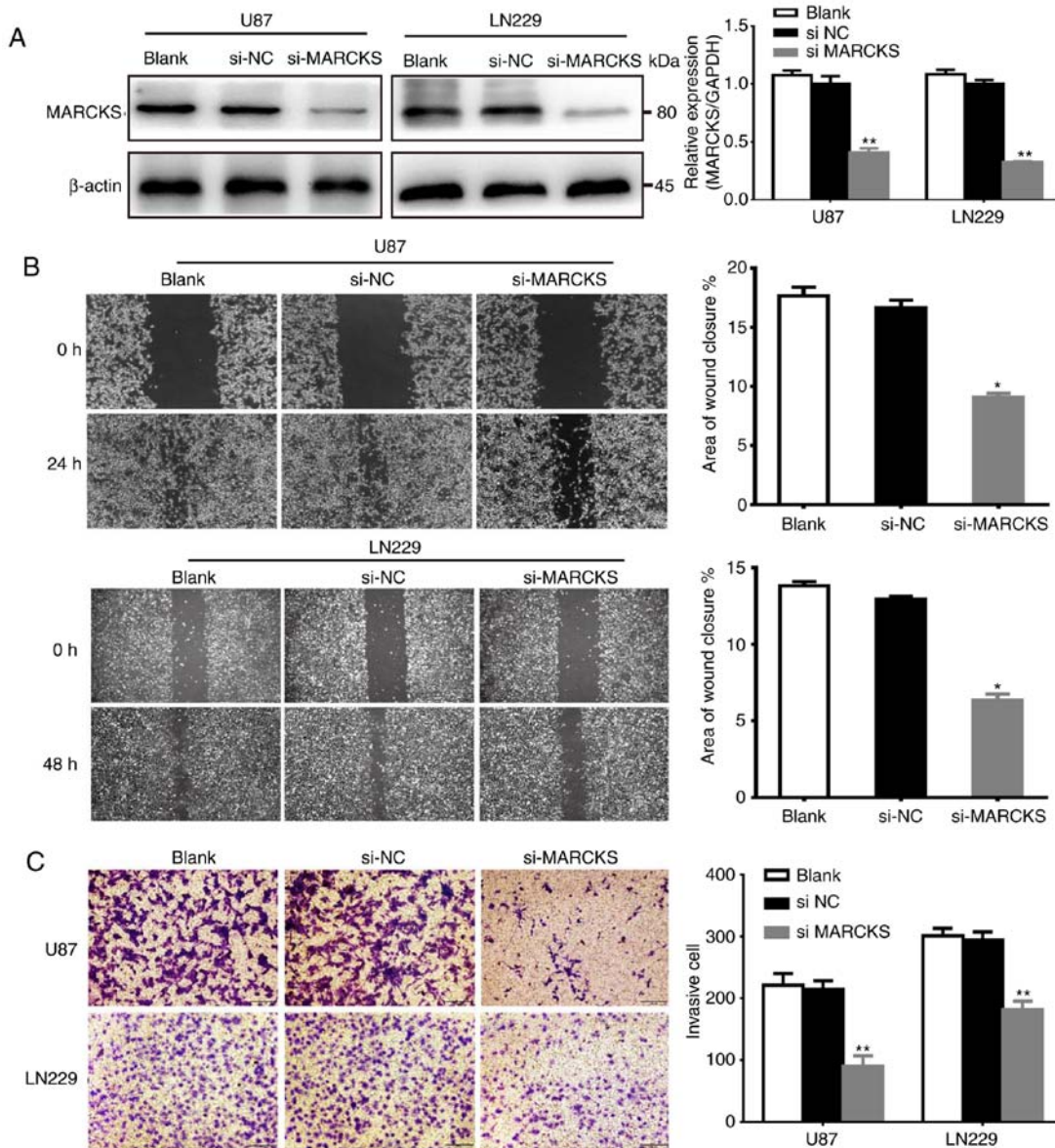


Figure 2. Downregulation of MARCKS reduces cell migration and invasion ability *in vitro*. (A) MARCKS mRNA (right) and protein (left) expression in U87 and LN229 cells was significantly reduced by si-MARCKS transfection. The data of the bar graph are presented as the mean  $\pm$  standard deviation (n=3). \*\*P<0.01 vs. si-NC. (B) Scratch assays indicated that U87 and LN229 cells transfected with si-MARCKS had significantly reduced cell migration abilities. The bar graph shows the difference in the wound healing capacity of the U87 and LN229 cells at 24 and 48 h, respectively. \*P<0.05. Magnification, x50. (C) Transwell Matrigel invasion assays showed that transiently silencing MARCKS by si-MARCKS remarkably decreased the invasion ability of U87 and LN229 cells *in vitro*, compared with the si-NC and Blank groups. The bar graph shows differences in the number of invasive cells. Magnification, x100. \*\*P<0.01 vs. si-NC. MARCKS, myristoylated alanine-rich C kinase substrate; si, small interfering RNA; NC, negative control.

si-MARCKS group was significantly downregulated in the U87 and LN229 cell lines, compared with the si-NC and the untransfected group (\*\*P<0.01; Fig. 2A).

Subsequently, the effects of MARCKS downregulation on the invasion and migration of both U87 and LN229 cells was investigated *in vitro* with Matrigel invasion and scratch assays. The data showed that downregulation of MARCKS markedly decreased the invasive ability of GBM cells, compared with the si-NC group. Furthermore, si-MARCKS also significantly inhibited the migratory capacity of cells, compared with the si-NC-transfected and Blank cells (\*P<0.05, \*\*P<0.01; Fig. 2B and C).

*MARCKS regulates the expression of EMT-associated genes in GBM.* To further investigate the mechanism of MARCKS

in GBM cell invasion and migration, the protein expression of EMT-associated genes in U87 and LN229 cells with down-regulated MARCKS expression was examined by western blot analysis. Knocking down MARCKS decreased the expression of  $\beta$ -catenin, vimentin and N-cadherin, while increasing that of E-cadherin (\*P<0.05, \*\*P<0.01; Fig. 3A).

*MARCKS inhibits the expression of E-cadherin by upregulating Snail expression in GBM cells.* It has been reported that the expression of E-cadherin may be inhibited by EMT-associated transcription factors, such as Snail and Slug in cancer cells (17-20). To study whether MARCKS modulates E-cadherin expression by affecting the expression of these EMT-associated transcription factors, the expression

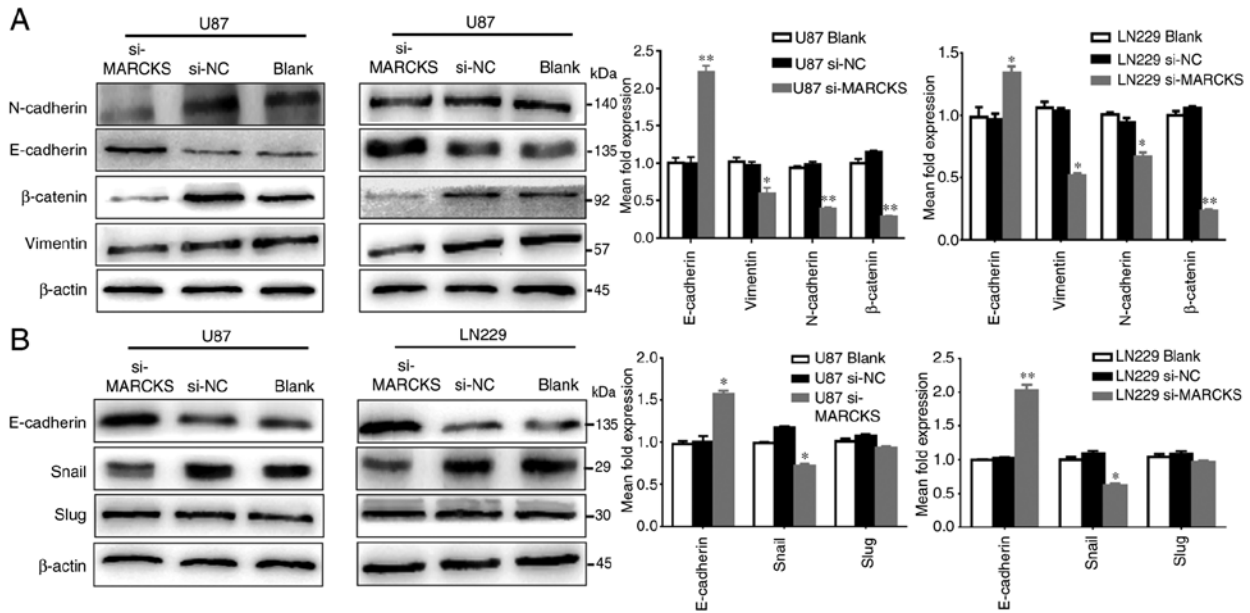


Figure 3. MARCKS regulates the expression of EMT-associated genes in GBM cells. (A) Knockdown of the expression of MARCKS by si-MARCKS in U87 and LN229 cells enhanced E-cadherin expression, but decreased the expression of EMT-marker genes including  $\beta$ -catenin, N-cadherin and vimentin. \* $P < 0.05$ , \*\* $P < 0.01$  vs. si-NC. (B) In si-MARCKS-transfected U87 and LN229 cells, the protein expression of Snail was downregulated and E-cadherin was upregulated. Slug expression was unchanged. \* $P < 0.05$ , \*\* $P < 0.01$  vs. si-NC. MARCKS, myristoylated alanine-rich C kinase substrate; si, small interfering RNA; NC, negative control; Snail, zinc finger protein SNAI1; Slug, zinc finger protein SNAI2.

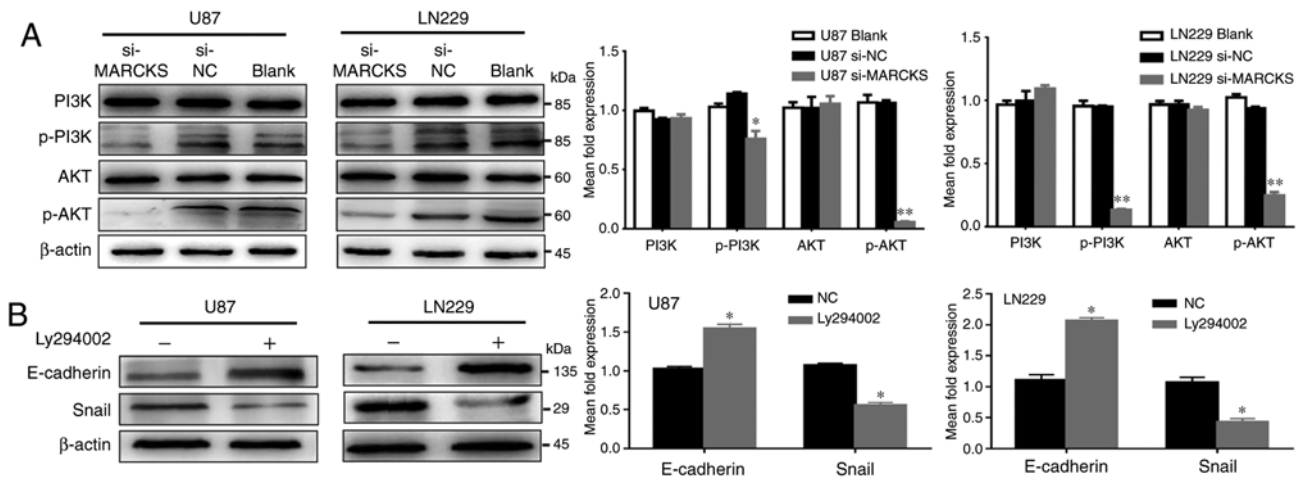


Figure 4. MARCKS regulates the migration and invasion of GBM cells through the PI3K/Akt/Snail/E-cadherin pathways. (A) MARCKS expression downregulation significantly decreased the expression of phosphorylated PI3K and Akt, whereas total expression remained unchanged.  $\beta$ -actin was used as a loading control. \* $P < 0.05$ , \*\* $P < 0.01$  vs. si-NC. (B) U87 and LN229 cells with MARCKS knockdown were treated with the PI3K inhibitor LY294002 for 24 h. Expression of Snail and E-cadherin was detected using immunoblotting.  $\beta$ -actin was used as a loading control. \* $P < 0.05$  vs. si-NC. MARCKS, myristoylated alanine-rich C kinase substrate; si, small interfering RNA; NC, negative control; Snail, zinc finger protein SNAI1; p-, phosphorylated; Akt, protein kinase B; PI3K, phosphoinositide 3-kinase.

of MARCKS in U87 and LN229 cells was knocked down and western blotting was used to examine the alterations in E-cadherin, Snail and Slug protein expression. It was found that the downregulation of MARCKS in U87 and LN229 cells significantly inhibited Snail expression and increased E-cadherin expression, whereas the expression of Slug remained unchanged (\* $P < 0.05$ ; Fig. 3B).

*MARCKS modulates GBM cell invasion and migration through the PI3K/Akt/Snail/E-cadherin pathways.* A previous study reported that inhibition of the PI3K/Akt pathway, which

is known to be an upstream signaling pathway involved regulating EMT signals, could downregulate the expression of Snail (21,22). The present study investigated the role of MARCKS in the PI3K/Akt pathway and found that phosphorylated PI3K and Akt expression was significantly downregulated in U87 and LN229 cells following the knockdown of MARCKS, but total PI3K and Akt protein expression (\* $P < 0.05$ , \*\* $P < 0.01$ ; Fig. 4A). In addition, the suppression of PI3K in U87 and LN229 GBM cells using LY294002, also decreased Snail and increased E-cadherin expression, similar to the effects of MARCKS downregulation (\* $P < 0.05$ ; Fig. 4B).

## Discussion

Although the expression of MARCKS in glioma has been reported, the biological effects, functions and underlying molecular mechanisms of MARCKS in the invasion and migration of GBM cells have not yet been well-characterized (12,13). In the present study, using RT-qPCR and western blotting, it was found that MARCKS was significantly upregulated in GBM specimens compared with normal brain tissues. Furthermore, higher levels of MARCKS were associated with worse outcomes in patients with GBM, suggesting that MARCKS may be a prognostic factor. These results were consistent with those of previous studies, which demonstrated that MARCKS expression is upregulated in breast cancer, osteosarcoma and hepatocellular carcinoma (23-25). These data imply that MARCKS may play an oncogenic role in GBM tumorigenesis.

Several studies have reported that MARCKS is associated with the proliferation, invasion and migration of several tumors, including lung cancer, prostate cancer and hepatocellular carcinoma (6,25,26). Furthermore, Micallef *et al* (13) showed that MARCKS serves as a mediator of attachment and invasion in epidermal growth factor receptor variant III-expressing GBM cells (13). However, the molecular mechanisms underlying its effects on tumor cell invasion and migration remain elusive. Therefore, in the current study, the role of MARCKS in GBM cell invasion and migration was first determined. It was shown that downregulated MARCKS expression inhibited GBM cell invasion and migration *in vitro*.

Furthermore, EMT, which may be associated with alterations in: Epithelial marker expression, such as E-cadherin; mesenchymal marker expression, such as  $\beta$ -catenin, vimentin, and N-cadherin; and the expression of several key transcription repression factors, such as Snail and Slug. These proteins serve an important role in cancer cell invasion and migration (27-31). Thus, the underlying mechanisms involved in MARCKS-induced cell invasion and migration were determined by examining the effects of MARCKS on these EMT-associated proteins. Decreased Snail protein expression and a concomitant increase in E-cadherin expression was observed following the downregulation of MARCKS in GBM cell lines. These data indicated that MARCKS may have modulated the expression of E-cadherin via Snail, therefore resulting in EMT regulation.

Many studies have demonstrated that the PI3K/Akt pathway is widely involved in human cancer migration, proliferation and survival. Activation of the PI3K/Akt pathway increases Snail and suppresses E-cadherin expression, thereby inducing EMT and promoting invasion and migration (32-35). The results of the present study found that the expression of p-PI3K and p-Akt was downregulated following MARCKS knockdown, and the treatment of U87 and LN229 cells with LY294002 had a similar effect on E-cadherin and Snail expression, indicating that MARCKS may be an upstream effector regulating the PI3K/Akt pathway in GBM. Inactivation of the PI3K/Akt pathway was perhaps responsible for the si-MARCKS-mediated inhibition of GBM cell invasion and migration. Therefore, these results indicated that MARCKS may conduce to GBM EMT via the PI3K/Akt pathway.

In summary, and to the best of our knowledge, the present study is the first to demonstrate the function of MARCKS in

GBM, and to demonstrate that MARCKS promoted GBM cell invasion and migration through activation of the PI3K/Akt pathway, which may have inhibited EMT by increasing the expression of Snail and reducing E-cadherin expression. Furthermore, it was shown that MARCKS may be a poor prognostic marker in GBM. Therefore, it was concluded that MARCKS may serve an important role in GBM tumorigenesis and represents a potential therapeutic target for the treatment of GBM.

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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

JZ, WX, YM and LC conceived and designed the experiments. JZ, WX, TP, LGC, YM, SL, KW, HW performed the experiments. JZ, WX, TP, LC and YM analyzed the data. JZ, WX, TP, LC provided materials and collected the clinical data. JZ, WX, TP, YM and LC wrote the paper. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

## Ethics approval and consent to participate

All procedures performed in studies involving human participants were in accordance with the ethical standards of the Ethics Committees of the Affiliated Hospital of Southwest Medical University and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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