Original Article

MAGI3 Suppresses Glioma Cell Proliferation via Upregulation of PTEN Expression*

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Abstract

Objective To investigate the role and molecular mechanism of membrane-associated guanylate kinase inverted 3 (MAGI3) in glioma cell proliferation.

Methods The expression levels of MAGI3 and PTEN were assessed in glioma samples by Western blotting. MAGI3 was stably transfected into C6 glioma cells to obtain C6-MAGI3 cells. Then, the proliferation, the expression levels of MAGI3 and PTEN, and Akt phosphorylation were evaluated in C6 and C6-MAGI3 cells. Xenograft tumor models were established by subcutaneous injection of C6 and C6-MAGI3 cells into nude mice, and the growth rates of xenografts in the mice were compared. The potential role of MAGI3 expression in PI3K/Akt signaling activation was further investigated by examining the correlation between MAGI3 expression and the expression of PI3K/Akt signaling downstream target genes in a glioma dataset using gene set enrichment analysis (GSEA).

Results Expression levels of MAGI3 and PTEN were significantly downregulated in gliomas. Overexpression of MAGI3 in the glioma C6 cell line upregulated PTEN protein expression, inhibited the phosphorylation of Akt, and suppressed cell proliferation. MAGI3 overexpression also inhibited the growth of C6 glioma tumor xenografts in nude mice. Analysis based on the GEO database confirmed the negative correlation between activation of PI3K/Akt pathway and MAGI3 mRNA levels in human glioma samples.

Conclusion The loss of MAGI3 expression in glioma may enhance the proliferation of glioma cells via downregulation of PTEN expression, leading to the activation of the PI3K/Akt pathway. MAGI3 is a potential glioma suppressor.

Key words: Glioma; PTEN; MAGI3; PDZ; Protein-protein interaction; PI3K/Akt

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INTRODUCTION

Glioma, the most common type of brain tumor, is a highly invasive malignancy associated with a poor prognosis. The median survival time for patients with high-grade glioma is about 14-15 months[1-2]. Among the dysregulated signaling pathways in gliomas, such as the EGFR, STAT3, JNK, and PI3K/Akt signaling pathways[3-6], the aberrant activation of PI3K/Akt-PTEN signaling is crucial for the malignant features of gliomas, such as rapid tumor growth, invasiveness, resistance to cytotoxic treatments, and massive neovascularization[7-8].

PTEN is a major negative regulator of the PI3K/Akt signaling pathway, and malfunctioning of PTEN (loss of PTEN expression, gene mutation, or gene deficiency) plays a causal role in the aberrant activation of the PI3K/Akt-PTEN pathway[9]. In patients with glioma, a reduced expression of PTEN and an increased Akt activity have been correlated with the invasive behavior of the tumor and the reduced survival time[10]. Re-expression of wild-type PTEN in originally PTEN-deficient glioma cells suppresses cell growth *in vitro* as well as tumorigenicity in nude mice[11-13]. Most human gliomas show high levels of activated Akt[14]. Interestingly, although less than half of these cases carry *pten* mutations or homozygous deletions[15-16], approximately two thirds of gliomas lack detectable PTEN protein expression[15]. Thus, it is possible that other unknown mechanisms may result in the decreased expression of the PTEN protein. Hence, elucidation of the underlying mechanism may improve our understanding of glioma carcinogenesis.

PTEN has been reported to be a fast turnover protein that is easily degraded[17]. The carboxyl-terminus of PTEN, containing PEST sequences and PDZ-binding motifs, plays an important role in the regulation of protein stability and degradation[18]. The PDZ-binding motif mediates the interaction with several PDZ domain-containing proteins, such as members of the membrane-associated guanylate kinase inverted (MAGI) protein family (MAGI1, MAGI2, and MAGI3). The expression of PTEN protein was reported to be regulated by binding with MAGI proteins, which modulated the kinase activity of Akt[18-21]. MAGI3 is extensively expressed in glial cells[22], and the binding to MAGI3 proteins enhances the ability of PTEN to suppress Akt activation[20]. However, the impact of MAGI3 on gliomagenesis has not been reported to date. Thus, we propose the hypothesis that MAGI3 might play a role in gliomagenesis.

In this study, the role of MAGI3 in the proliferation of glioma cells and in the regulation of PI3K/Akt-PTEN activation was investigated. MAGI3 was overexpressed in C6 glioma cells and its potential role in the regulation of PTEN expression and glioma cell proliferation was examined *in vitro*, as well as *in vivo* in a xenograft mouse model. The results were also validated by examining the correlation between MAGI3 expression levels and PI3K/Akt signaling activation in human glioma specimens from GEO databases.

MATERIALS AND METHODS

Tissue Samples

Glioma samples were collected from Tiantan Hospital, Beijing during 2013. Patients were included after they provided written informed consent. The study was approved by the Ethics Committee of Capital Medical University. After surgical resection, the tissues were immediately snap-frozen in liquid nitrogen and stored at -80 °C. Diagnosis and classification of glioma samples were based on the WHO guidelines. None of the patients included in the study had a family history of glioma or secondary malignancies, and none had received radiotherapy or chemotherapy before surgery. Twelve glioma cases were analyzed: three with histopathologically confirmed grade II, five with grade III, and four with grade IV.

Cell Culture and Transfection

The rat C6 glioma cell line was obtained from the European Collection for Animal Cell Culture (ECACC; Porton Down, Salisbury, UK). The cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in a 37 °C/5% CO_2 incubator. All cell culture media and related reagents were purchased from Hyclone (Logan, UT).

For the establishment of stable MAGI3-expressing cells, C6 cells were seeded into a six-well plate and then transfected with GFP control or GFP-MAGI3 vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. At 24 h after transfection, the cells were selected with 1 mg/mL G418 (Calbiochem, San Diego, CA) in culture medium for an additional

2 weeks. Expression of the GFP-MAGI3 protein was confirmed by Western blotting. The C6 cells stably transfected with GFP control or GFP-MAGI3 vector were named as C6-ctrl or C6-MAGI3, respectively.

Cell Proliferation Assay

Cells were cultured in 96-well microplates at a density of 3000 cells per well. At 24, 48, 72, and 96 h after plating, CCK-8 (Dojindo, Kumamoto, Japan) was added to each well according to the manufacturer's instructions and the cells were cultured for another 1 h. Optical density (OD) values were quantified by measuring at 450 nm with an EnSpire label microplate reader (PerkinElmer, Waltham, MA).

Xenograft Tumor Mouse Models

C6-ctrl or C6-MAGI3 cells (5×10^5) suspended in 0.1 mL PBS were injected subcutaneously into the flanks of Balb/c nude mice (aged 4-5 weeks), with 12 mice per group. Xenografts were measured daily and tumor volume was estimated as volume = (length× $width^2$)/2. The mice were sacrificed on day 15 post-injection. The tumors were harvested and individually weighed. All animal experiments were performed after obtaining approval from the Administrative Panel on Laboratory Animal Care of Capital Medical University.

Western Blotting

Cell lysis and Western blotting were performed as described previously[23]. The anti-PTEN, anti-GFP, anti-p-Akt^{S473}, and anti-Akt antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-MAGI3 antibody was purchased from BD Biosciences (San Jose, CA). Anti-β-actin, anti-GAPDH, and HRP-conjugated secondary antibodies were obtained from ZSGB-BIO (Beijing, China).

Gene Set Enrichment Analysis

The association between the expression level of MAGI3 and biological processes was analyzed using Gene Set Enrichment Analysis (GSEA v2.0, http://www.broad.mit.edu/gsea/) as previously reported[24]. Gene set permutations were performed 1000 times for each analysis. The gene sets related to the PI3K/Akt signaling pathway are exemplified by the KEGG PI3K/Akt signaling pathway (http://www.genome.jp/kegg/pathway/hsa/hsa04151.html). False discovery rate (FDR) <0.05 was considered to be statistically significant.

Statistical Analysis

Data are presented as mean±standard error. Repeated measures analysis of variance was used to compare the proliferation of C6 cells and xenograft tumor. Student's *t*-test was used to analyze the statistical results. Statistical analysis was performed using SPSS 19.0 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism 5 (GraphPad Software, Inc, San Diego, CA). $P<0.05$ was considered to be statistically significant.

RESULTS

MAGI3 Expression is Downregulated in Human Glioma

To explore the function of MAGI3 in glioma, MAGI3 expression was assessed in human normal and glioma tissues at ONCOMINE (https://www.oncomine.org). The results showed that MAGI3 transcript levels are significantly downregulated in glioma based on microarray gene expression data of human gliomas and normal brain samples (Figure 1A) (ONCOMINE, Murat Brain). To confirm this finding, we examined the protein expression of MAGI3 in human glioma specimens by Western blotting. As shown in Figure 1B, MAGI3 expression was downregulated at the protein level in gliomas compared to that in adjacent tissues.

Overexpression of MAGI3 Inhibits Proliferation and Reduces Tumorigenicity of C6 Glioma Cells

MAGI3 was stably transfected into C6 glioma cells, and cell proliferation was assessed for determining whether glioma carcinogenesis can be driven by MAGI3. The results showed that C6-MAGI3 cells grew at a significantly slower rate than C6-ctrl cells, indicating that overexpression of MAGI3 inhibited glioma cell proliferation (Figure 2A). To further verify the effect of MAGI3 expression *in vivo*, a xenograft tumor model was established by subcutaneous injection of C6-ctrl or C6-MAGI3 cells into nude mice. Consistently, MAGI3 overexpression inhibited the growth of C6 xenografts in nude mice (Figure 2B). Accordingly, the weight of tumors was significantly lower in C6-MAGI3 xenografts than those in the controls (Figure 2C). Taken together, these results demonstrate that MAGI3 inhibits the growth of glioma cells both in vitro and *in vivo.*

MAGI3 Upregulates PTEN Protein Expression in Glioma Cells

MAGI3 was shown to promote PTEN protein stability and inhibit the activation of PI3K/Akt signaling pathway in HEK 293 cells[20]. Therefore, we explored whether MAGI3 had a similar effect in glioma cells. As shown in Figure 3A, Cell lysates of C6-ctrl or C6-MAGI3 cells were analyzed by Western blotting. MAGI3 overexpression significantly upregulated PTEN expression at the protein level in C6 cells.

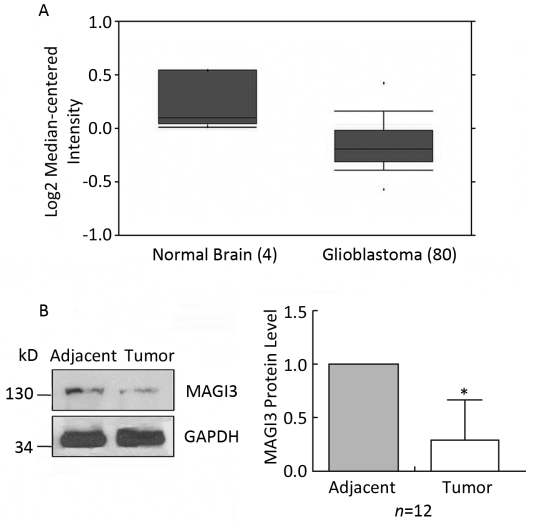


Figure 1. Downregulation of MAGI3 expression in glioma. (A) Comparative analysis of MAGI3 mRNA expression in gliomas and non-tumor tissues. The mRNA level of MAGI3 in glioma (Oncomine). Oncomine microarray data analysis of MAGI3 expression in glioma (n=80) *vs.* normal brain tissue (n=4) is shown. Student's t-test was performed using the oncomine software; $^{*}P$<0.05. The horizontal lines and the points represent the mediansand the end of the ranges, respectively. (B) MAGI3 protein expression in glioma samples. Representative Western blots of MAGI3 expression in 12 independent glioma samples (left panel). The signal intensity was quantified by densitometry and normalized to GAPDH. Data are expressed as fold change compared to adjacent normal tissues. Results represent the mean±SD of 12 samples. $^{*}P$<0.05 with respect to adjacent tissues (right panel).

Then, we subsequently measured the turnover rate of PTEN protein upon binding to MAGI3. HEK 293 cells transfected with PTEN constructs in the absence

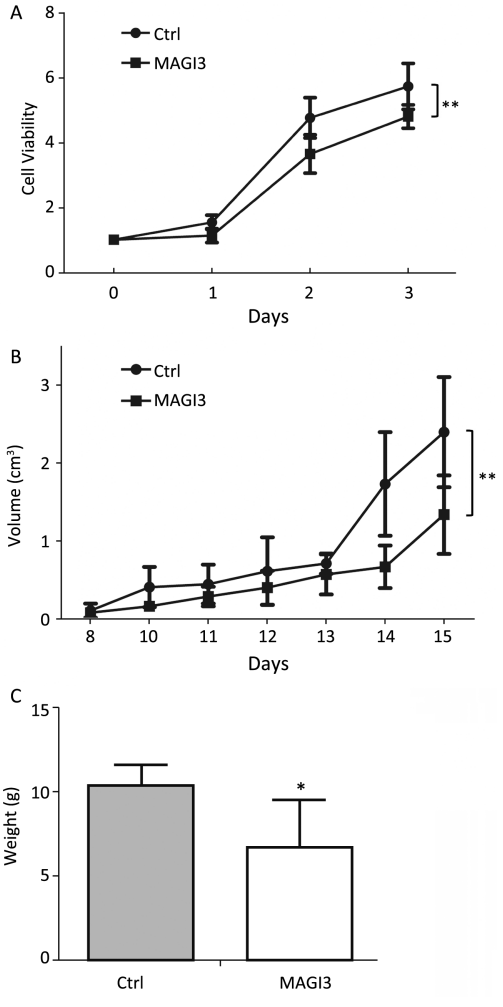


Figure 2. MAGI3 inhibits C6 glioma cell growth *in vitro* and in xenograft tumors. (A) Effect of MAGI3 expression on the proliferation of glioma cells. Viability of C6 cells was assessed by measuring the absorbance at 450 nm. Values are presented relative to the absorbance of cells seeded after 12 h. The repeated measures analysis of variance was used to determine significance, $^{**}P$<0.01. (B) Growth rate of C6 xenograft tumors. Tumor volume was expressed as the mean±SD of 12 tumors in each group. The repeated measures analysis of variance determined statistical significance ($^{**}P$<0.01). (C) Weight of the tumors produced by C6 glioma cells. Student's paired t-test was used to determine significance (*), set at P<0.05.

or presence of MAGI3 constructs were treated with the protein synthesis inhibitor CHX (cycloheximide 100 ng/mL). The cells were harvested at different time points as indicated in Figure 3B, and the protein levels of PTEN and MAGI3 were assessed by Western blotting. The protein expression levels of PTEN significantly decreased in a time-dependent manner when PTEN was expressed alone. When MAGI3 was overexpressed, however, the PTEN protein turnover rate was significantly reduced. These data indicated that PTEN protein stabilization was enhanced by interaction with MAGI3. Furthermore, results of Western blotting of human glioma specimens showed that PTEN expression was decreased in gliomas in which MAGI3 was downregulated (Figure 3C).

MAGI3 Negatively Regulates PI3K/Akt-PTEN Signaling Pathway in Gliomas

To examine the effect of MAGI3 on the activation of PI3K/Akt signaling in C6 cells, Akt phosphorylation induced by serum was examined by Western blotting. C6-ctrl or C6-MAGI3 cells were serum starved overnight, and then stimulated with 10% serum for 15 min before harvesting. Cell lysates were analyzed by Western blotting with anti-MAGI3, anti-Akt, and anti-p-Akt antibodies. Quantitative measurement of p-Akt was normalized to total Akt (Figure 4A). The levels of phosphorylated Akt in C6-MAGI3 cells were significantly lower than those in C6-ctrl cells, indicating that MAGI3 expression abolished the activation of PI3K/Akt pathway.

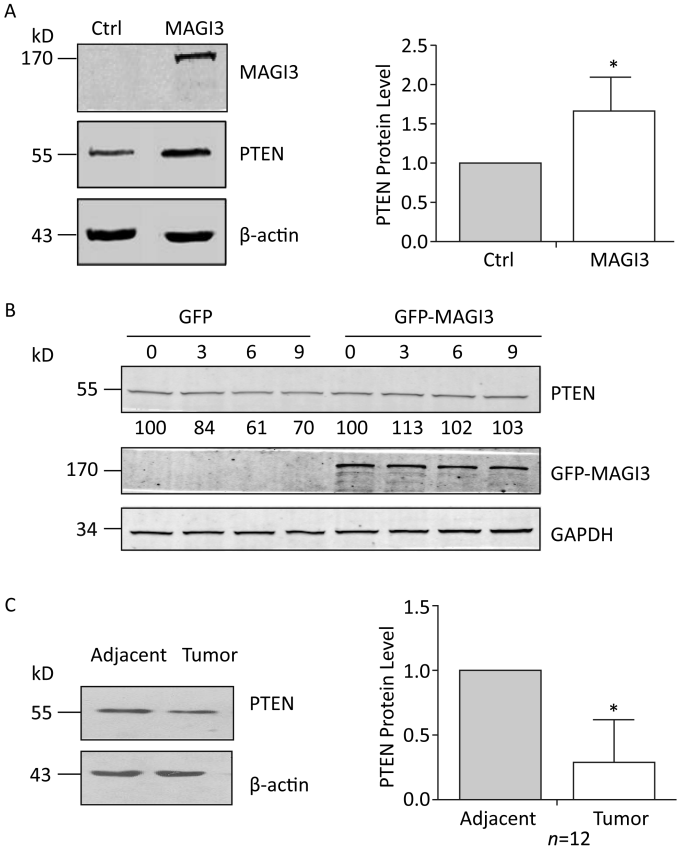
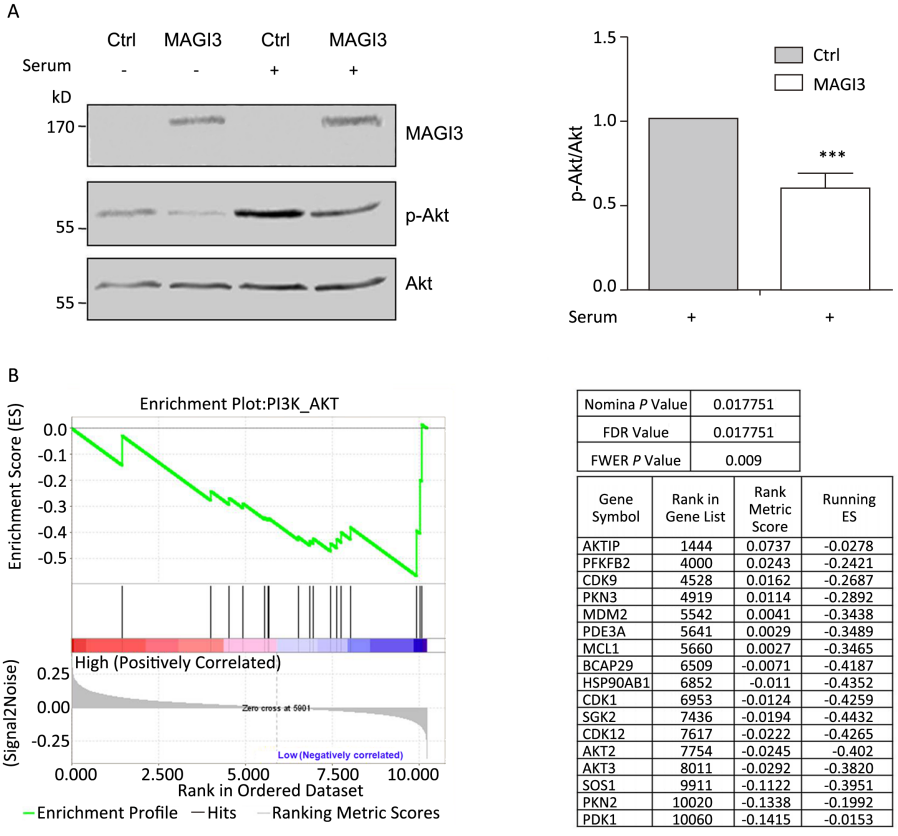


Figure 3. MAGI3 enhances PTEN expression at the protein level. (A) MAGI3 overexpression upregulates PTEN protein expression. Data are presented as means±SD of three separate experiments. (B) MAGI3 overexpression prolongs PTEN half-life time. The data shown in the figure were quantified by densitometry and normalized to GAPDH. (C) PTEN protein expression is downregulated in human glioma samples. Representative Western blotting of PTEN expression in 12 independent glioma samples (left panel). Results represent the mean±SD of 12 samples. $^{*}P<0.05$ with respect to adjacent tissues (right panel).

To verify these results, we examined the correlation between MAGI3 expression and PI3K/Akt signaling activation in human glioma specimens from the GEO database (GSE4412) using GSEA(http://www.broad.mit.edu/gsea). The expression level of a priori defined positive PI3K/Akt pathway gene set was used as an indicator of activation of the PI3K/Akt pathway. The results of GSEA revealed a negative correlation between MAGI3 expression and activation of PI3K/Akt pathway in gliomas (Figure 4B), indicating that MAGI3 expression could retard PI3K/Akt signaling activation. To further validate these results, we downloaded the glioma expression profiles of mRNA from the GEO database (GSE4290) and selected samples with PTEN expression level similar to that in normal tissues. We then examined the correlation between MAGI3 expression and the downstream genes of the PI3K/Akt pathway, such as MDM2 and MCL1. As shown in Table 1, MAGI3 expression was negatively correlated with the expressions of MDM2 and MCL1 in gliomas. Taken together, these results indicated that MAGI3 might play a role in the regulation of activation of PI3K/Akt pathway in glioma cells.



Nomina *P* Value	0.017751
FDR Value	0.017751
FWER *P* Value	0.009

Gene Symbol	Rank in Gene List	Rank Metric Score	Running ES
AKTIP	1444	0.0737	-0.0278
PFKFB2	4000	0.0243	-0.2421
CDK9	4528	0.0162	-0.2687
PKN3	4919	0.0114	-0.2892
MDM2	5542	0.0041	-0.3438
PDE3A	5641	0.0029	-0.3489
MCL1	5660	0.0027	-0.3465
BCAP29	6509	-0.0071	-0.4187
HSP90AB1	6852	-0.011	-0.4352
CDK1	6953	-0.0124	-0.4259
SGK2	7436	-0.0194	-0.4432
CDK12	7617	-0.0222	-0.4265
AKT2	7754	-0.0245	-0.402
AKT3	8011	-0.0292	-0.3820
SOS1	9911	-0.1122	-0.3951
PKN2	10020	-0.1338	-0.1992
PDK1	10060	-0.1415	-0.0153

Figure 4. MAGI3 suppresses PI3K/Akt signaling in glioma cells. (A) MAGI3 overexpression retards activation of PI3K/Akt signaling in glioma cells. Data are expressed as fold change compared to control. *** *P*<0.001 with respect to control (right panel). The results shown are representative data from at least three independent experiments. (B) GSEA enrichment plot of KEGG cancer pathway genes associated with PI3k/Akt pathway in human gliomas from the GEO profile (GSE4412). Genes in the KEGG cancer signaling pathway showed significant enrichment in gliomas with low MAGI3 expression (FDR=0.0117). The top portion of the figure plots the enrichment scores (ES) for each gene. The bottom portion of the plot shows the value of the ranking metric moving down the list of ranked genes. The table enumerates the genes in the pathway for which a majority of probe sets were significantly enriched in gliomas with low MAGI3 expression compared with those in gliomas with high MAGI3 expression. FDR (false discovery rate); FWER *P* value (FWER, family wise-error rate). FDR<0.05 was considered to be statistically significant.

Table 1. Correlation Analysis in the mRNA Levels of MAGI3 and Related Genes of PI3K/Akt Pathway

Group	PTEN	MAGI3	MDM2	MCL1
Non-tumor (*n*=23)	528.2±98.4	802±226.5	676.8±172.8	165.8±95.3
Tumor (*n*=81)	569.4±187.2	517.8±181.5	1930±2997.9	542.8±311.5
	P=0.16	*P*<0.001	*P*<0.001	*P*<0.001

DISCUSSION

Loss of PTEN function, including the downregulation of PTEN protein expression, is one of the major factors leading to glioma formation[25]. MAGI3 was shown to promote the stability and upregulate the expression of PTEN protein in HEK 293 cells via binding of the PDZ4 domain of MAGI3 to the carboxyl-terminus of PTEN[20]. In the present study, we identified a positive correlation between the expression levels of MAGI3 and PTEN in clinical glioma samples. Overexpression of MAGI3 in glioma cells resulted in the upregulation of PTEN expression at the protein level, suggesting that MAGI3 regulated the stability and thus the expression of PTEN protein in glial cells.

We also showed that MAGI3 could regulate Akt activation and inhibit the proliferation of glioma cells *in vitro* and *in vivo*. Our results suggest that MAGI3 might regulate PI3K/Akt signaling by modulating the expression of PTEN, thereby resulting in the inhibition of glioma cell proliferation. Besides proliferation, PI3K/AKT signaling pathway regulates diverse cellular processes, including migration and invasion of glial cells[26]. MAGI3 is downregulated in gliomas and modulates PI3K/AKT-PTEN signaling, which suggests that MAGI3 might also play a role in the migration and invasiveness of glioma cells, However, this hypothesis needs to be explored further in a future study. Our results were further validated in clinical glioma specimens from patients, which indicated that MAGI3 expression is an important factor for glioma growth, and its low expression could be used as an important indicator for progression of glioma.

MAGI3 is widely expressed in many tissues including the brain[27]. Analysis of tumor datasets with oncomine also showed low expression levels of MAGI3 in many other tumors, such as colorectal cancer (GSE9348), esophageal squamous cell carcinoma (GSE23400), renal clear cell carcinoma (GSE781), and lung cancer (GSE3398). The expression of MAGI3 is significantly downregulated in colon cancer cells[28], and consequently, the downregulation of PTEN expression in colon cancer was shown to lead to the aberrant activation of the PI3K/Akt pathway, resulting in colon tumorigenesis[29]. In other words, MAGI3 expression in colon cancer might also affect the growth of colon tumors by inhibiting the expression of PTEN and promoting the activation of PI3K/Akt signaling pathway. These findings suggest that MAGI3 might have similar tumor suppressive functions in colon cancer to those in glioma cells. Therefore, results of the present study provide new clues not only for gliomas but also for other malignancies such as colon cancer.

In conclusion, the present study identified MAGI3 as a potential glioma suppressor that exerts an inhibitory effect on glioma cell proliferation and PI3K/Akt signaling via regulation of PTEN expression, which may help improve our understanding of the pathogenesis of glioma.

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