Methylation-induced silencing of maspin contributes to the proliferation of human glioma cells

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Abstract. Maspin, a member of the serpin superfamily of serine protease inhibitors, has been reported to be involved in cancer initiation and progression. However, the expression of maspin and its expression regulation in glioma remain unknown. In the present study, we aimed to investigate the function of maspin in glioma cells and its regulatory mechanism. We found that the expression of maspin was silenced in glioma cells and tissues. Although maspin had no effect on the migration and invasion of human glioma cells *in vitro*, overexpression of maspin inhibited cell growth in U87 cells. We showed that the methylase inhibitor 5-Aza-2'-deoxycytidine induced the expression of maspin in glioma cell lines. Furthermore, both U87 and U251 cells showed hypermethylation in the *maspin* promoter. In addition, bisulphite sequencing analysis indicated that 16 CpG sites in the promoter were completely methylated in glioma cells and cancerous tissues, while CpG dinucleotides in the maspin promoter were unmethylated in normal brain tissues. Our data suggest that methylation-induced silencing of maspin contributes to the proliferation of human glioma cells, and maspin may be a potential therapeutic target in glioma.

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Introduction

Gliomas are the most common primary central nervous system tumors and account for more than 46% of all brain tumors (1). Despite comprehensive treatment, the prognosis of patients with glioma remains poor (2). The prognosis of glioma patients is associated with various clinical and biological factors, such as disease stage, patient age and genetic and epigenetic molecular features of the tumor (3). In general, changes in the expression of oncogenes and tumor-suppressor genes are associated with glioma initiation and progression. However, the involved genes are ambiguous.

Epigenetic regulation plays a key role in the dysregulation of gene expression in tumors. Epigenetic regulation is defined as a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence (4), including DNA methylation and histone modification. Aberrant promoter methylation of CpG island-associated genes is the most common epigenetic alteration associated with the inactivation of tumor suppressor and other genes in human cancers (5-7).

Maspin, identified in 1994, is a member of the serpin superfamily of serine protease inhibitors (8). Multiple studies have shown that maspin plays a tumor-suppressor role in various cancers, including breast cancer (9), prostate cancer (10) and other cancers (11). Although the *maspin* gene is frequently silenced in breast cancer cells (9), maspin deletions and mutations have not been reported (8,12). Maspin can be silenced by an epigenetic mechanism that involves aberrant methylation in breast cancer cells (13). Findings have suggested that DNA methylation is probably involved in regulating the expression of maspin in cancers (14,15). Although maspin is expressed in normal brain tissue (16), the expression level of maspin in glioma has been rarely reported, and the role of the maspin gene in glioma is unknown.

In the present study, the expression level and promoter methylation status of maspin in glioma were investigated. We also explored the effect of maspin on the proliferation and migration of glioma cells.

Materials and methods

Human tissue samples. All human normal brain and glioma tissues were collected from patients treated at the Department of Neurosurgery, The Second Affiliated Hospital of Soochow University. Normal brain tissues were obtained from patients with cerebral trauma. Glioma tissues were obtained and verified following diagnosis of the clinical and pathological grade. Prior consent was obtained from all patients, and the study was approved by the institutional research boards of the affiliated institutions.

Cell culture. The human glioma cell lines U87, U251, and U343, and the human prostate cancer cell line PC3 were purchased from the Cell Bank of the Chinese Academy of Science. PC3 cells were used as the opposite control for maspin expression and methylation status studies. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/ml penicillin and 100 U/ml streptomycin; Gibco, Grand Island, NY, USA). The cells were grown in a 37˚C incubator with 5% CO_2.

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR). Total RNA from tissues and cell lines was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the isolated RNA was reverse transcribed into complementary DNA using the RevertAid™ First Strand cDNA Synthesis kit (Fermentas, Vilnius, Lithuania) following the manufacturer's instructions. The primers for the PCR were as follows: *maspin* forward, 5'-GCTTTTGCC GTTGATCTGTTC-3' and reverse, 5'-GATCTGACCTTT CGTTTTCTTC-3'; and *GAPDH* forward, 5'-GGAAGGTGA AGGTCGGAGTC-3' and reverse, 5'-GAGGCATTGCTG ATGATCTTGA-3'. The PCR conditions were as follows: an initial denaturation at 94˚C for 5 min followed by 33 cycles of 94˚C for 30 sec, 52˚C for 30 sec, and 72˚C for 30 sec, and a final extension at 72˚C for 7 min. The RT-PCR products were analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA), and the images were collected and saved. The housekeeping gene *GAPDH* was used as the internal control.

Western blot analysis. Tissues and cells were lysed in lysis buffer, and whole proteins were extracted by incubation with the western blot assay buffer (Beyotime, Nantong, China). Protein concentration was measured using a BCA protein assay kit (Beyotime, Nantong, China). A total of 50 μg of each protein sample was subjected to 10% SDS-PAGE, and the proteins were transferred to nitrocellulose membranes. The membranes were blocked with 5% skim milk in TBST for 1 h at room temperature, and then incubated with a 1:1,200 dilution of anti-maspin monoclonal antibody (BD Pharmingen, San Diego, CA, USA) overnight at 4˚C. The HRP-conjugated secondary antibody was incubated for 1 h at 37˚C. Specific bands were visualized using the enhanced chemiluminescence detection kit for HRP (Biological Industries, Kibbutz Beit Haemek, Israel) and Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY, USA). Quantitative data were obtained using Quantity One software.

Lentivirus infection and confirmation of maspin expression. Lentiviral vectors expressing maspin (LV-maspin) and a non-silenced control lentivirus (LV-NC) were designed and constructed by GenePharma (Shanghai, China). Cell infection was conducted according to the recommendations of GenePharma. U87-LV-maspin (U87-maspin) cells represent U87 cells infected with LV-maspin, and the negative control U87-LV-NC (U87-NC) cells represent U87 cells infected with LV-NC. GFP expression was observed under a fluorescence microscope after infection. For further validation, the expression of maspin was confirmed by RT-PCR and western blotting.

Migration and invasion assay. For the migration assay, U87, U87-NC and U87-maspin cells were suspended in 120 μl of serum-free medium. A total of $2x10^4$ cells/well were seeded into the upper chambers of the 24-well Transwell inserts with 8-μm pores (Corning, Corning, NY, USA). Next, 600 μl of DMEM containing 10% FBS was added to the lower chambers. After 48 h, the cells on the upper surface were removed, and the cells on the lower surface were fixed using a crystal violet cell colony staining kit (Genemed, Shanghai, China). The stained cells were counted in five randomly selected fields per filter under a microscope (x400 magnification). For the invasion assay, the Transwell inserts were coated with Matrigel (40 μl/well; BD Biosciences, San Jose, CA, USA). The following procedures were the same as those for the migration assay.

Proliferation assay. The number of viable cells was assayed using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) as per the manufacturer's protocol. U87, U87-NC and U87-maspin cells were seeded onto the 96-well plates at a density of $2x10^3$ cells/well. After 24 h, 10 μl of CCK-8 solution was added to each well. After 2 h, the optical density (OD) at 450 nm was measured using a microplate reader (Bio-Rad).

BrdU incorporation assay. Cell proliferation was also determined using 5-ethynyl-2-deoxyuridine (EdU) using the Cell-Light™ EdU Apollo® 567 In Vitro Imaging kit (RiboBio, Guangzhou, China) according to the manufacturer's protocol. Briefly, cells seeded in 96-well culture plates were incubated with 50 μM EdU for 3 h before fixation, permeabilization, and Apollo staining. Cell nuclei were stained with Hoechst 33342 at a concentration of 5 μg/ml for 30 min. The cells were observed using a fluorescence microscope (Olympus, Tokyo, Japan).

5-Aza-2'-deoxycytidine treatment. U87 and U251 cells were treated with various concentrations of 5-Aza-2'-deoxycytidine (5-Aza-dC; Sigma-Aldrich, St. Louis, MO, USA). U87 cells were treated with 12 μM 5-Aza-dC for 96 h. U251 cells were treated with 15 μM 5-Aza-dC for 96 h. After treatment with 5-Aza-dC, the maspin mRNA expression level was investigated in the U87 and U251 cell lines.

DNA extraction and bisulphite modification. Genomic DNA from cells and tissues was isolated using the Genomic DNA purification kit (Thermo, Waltham, MA, USA).

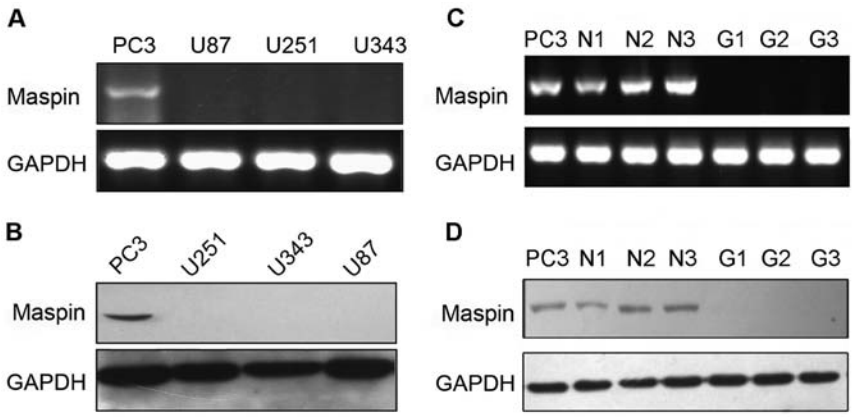

Figure 1. Differential maspin expression in glioma cell lines and tissues. (A) Maspin expression in PC3 (control), U87, U251 and U343 cells as assessed by RT-PCR. (B) Maspin expression in the PC3, U251, U343 and U87 cell lines as determined by western blot analysis. (C) Maspin expression in PC3 cells (control), 3 normal brain tissues (N1, N2 and N3) and 3 glioma tissues (G1, G2 and G3) as assessed by RT-PCR. (D) Maspin expression in PC3 cells, 3 normal brain tissues (N1, N2 and N3) and 3 glioma tissues (G1, G2 and G3) as determined by western blot analysis.

Bisulphite modification was performed using the EZ DNA Methylation-Gold™ kit (D5005; Zymo Research, Orange, CA, USA) following the manufacturer's instructions.

Methylation-specific PCR (MSP). The primers for maspin promoter methylation analysis by PCR were designed by MethPrimer (http://www.urogene.org/methprimer/). The primers were as follows: methylated maspin (Mmaspin) forward, 5'-TTTTATCGAATATTTTATTTTTCGG-3' and reverse, 5'-GATAACTCACCTAAACAACACCG-3'; and unmethylated Maspin (Umaspin) forward, 5'-TTTTATTTTAT TGAATATTTTATTTTTTGG-3' and reverse, 5'-CAATAACT CACCTAAACAACACCAC-3'. The DNA for this PCR was treated as previously described. The PCR reaction conditions were as follows: 94˚C for 5 min; 40 cycles of 94˚C for 30 sec, 57˚C for 30 sec, 72˚C for 30 sec; and a 72˚C extension for 7 min. MSP products were analyzed by Quantity One software.

Bisulphite genomic sequencing. DNA CpG islands of the maspin promoter were analyzed by EMBOSS Cpgplot (http://www.ebi.ac.uk/). The parameters for CpG island searching were set at Mayor >100 bp, CpG/expected CpG >0.6, GC% >50% for the EMBOSS Cpgplot. The primers for this PCR were designed from the predicted sequence of the maspin promoter CpG island using MethPrimer as previously described. The primers were 5'-GAGAAATTTGTAGTGTTATTATTATTATAT-3' (forward) and 5'-ATAACTCACCTAAACAACACC-3' (reverse), amplifying a 321-bp product including 16 expected CpG sites. The PCR conditions were as follows: 94˚C for 5 min; 40 cycles of 94˚C for 30 sec, 57˚C for 30 sec, 72˚C for 30 sec; and a 72˚C extension for 7 min. Bisulphite-sequencing PCR products were analyzed using Quantity One software. The PCR products were purified directly using the TIANgel Midi Purification kit (Tiangen Biotech, Beijing, China). The purified products were sequenced by Suzhou Genewiz, Inc. (Suzhou, China).

Statistical analysis. Data are presented as the mean ± standard error of the mean (mean ± SEM). Statistical analyses were conducted using Statistical Package for the Social Sciences (SPSS) software version 19.0 (Chicago, IL, USA). The differences between groups were analyzed using the Student's t-test or one-way analysis of variance (ANOVA). Differences were considered statistically significant at a P-value <0.05.

Results

Expression of maspin is silenced in glioma. We first investigated the expression of maspin in glioma cells and tissues. The mRNA and protein levels of maspin in the glioma cell lines and tissues were explored by RT-PCR and western blotting, respectively. PC3 cells were used as the positive control for maspin expression. As shown in Fig. 1A and C, maspin transcripts were present in the PC3 cells and normal brain tissues, but not in the glioma cell lines and tissues. Consistent with this result, maspin protein was not detected in the above glioma cell lines and tissues (Fig. 1B and D). These results indicated that the expression of maspin was silenced in glioma.

Establishment of stable maspin-overexpressing cells. To explore the function of maspin, the coding region of maspin was cloned and then subcloned into an overexpression vector (with GFP tag). To perform the cell function experiments, the stable glioma cell lines with overexpression of maspin and the control cells were screened (Fig. 2A). Expression of maspin in the parental U87, U87-NC and U87-maspin cells was verified by RT-PCR and Western blotting. As shown in Fig. 2B-E, the mRNA and protein levels of maspin were both significantly upregulated in the U87-maspin cells compared with the control cells (P<0.01). These results indicated that the stable cell line with overexpression of maspin and the control cell line were successfully established.

Overexpression of maspin does not affect the migration and invasion of the U87 cells. We next investigated the effect of maspin overexpression on cell migration and invasion. Parental U87 cells, U87 cells infected with the control lentivirus (U87-NC) and U87 cells with maspin overexpression (U87-maspin) were used for the Transwell migration and invasion assays. The numbers of invasive U87, U87-NC and U87-maspin cells were 45.0±4.0, 46.0±4.6 and 44.3±2.5, respectively. As shown

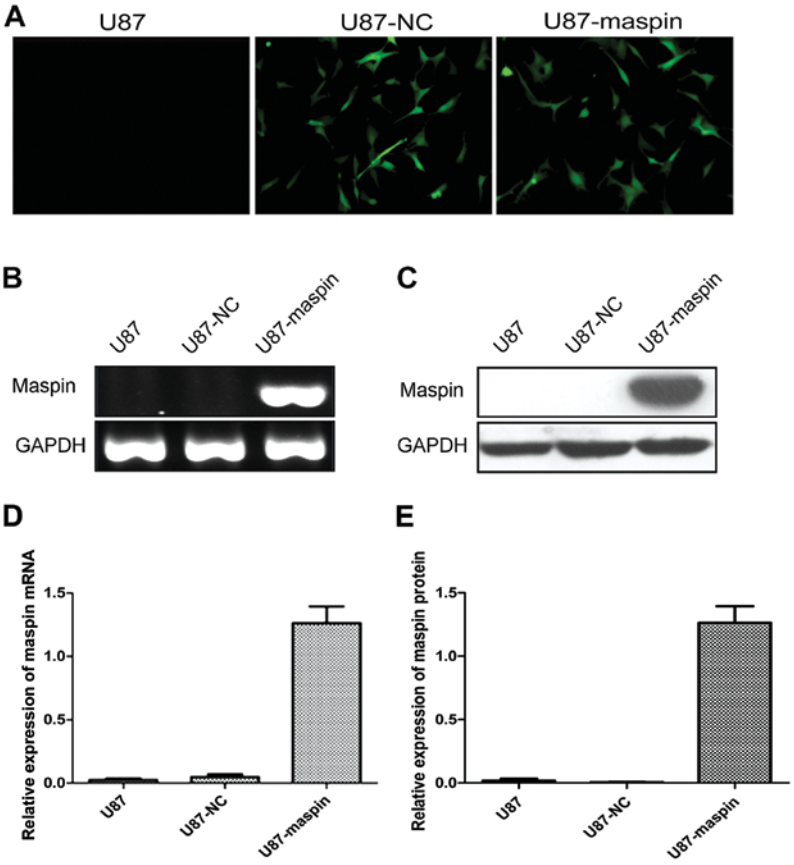

Figure 2. Establishment of maspin-overexpressing glioma cell lines. (A) GFP expression was observed under a fluorescence microscope after infection. U87 cells as the blank control (left), U87-NC cells infected with lentiviral vectors expressing LV5-NC as the negative control (middle), and U87-maspin cells infected with lentiviral vectors expressing maspin (right). Maspin expression was analyzed by RT-PCR and western blotting after infection in the U87 cells. (B) The maspin mRNA expression level in cell lines was measured by RT-PCR. (C) The Maspin protein level was detected by western blotting. (D) The relative expression level of maspin mRNA in the U87, U87-NC and U87-maspin cells. (E) The relative expression level of maspin protein in the U87, U87-NC and U87-maspin cells. A statistically significant difference in the mRNA and protein expression levels of Maspin was observed between the U87-maspin and the other control cells ($P<0.01$).

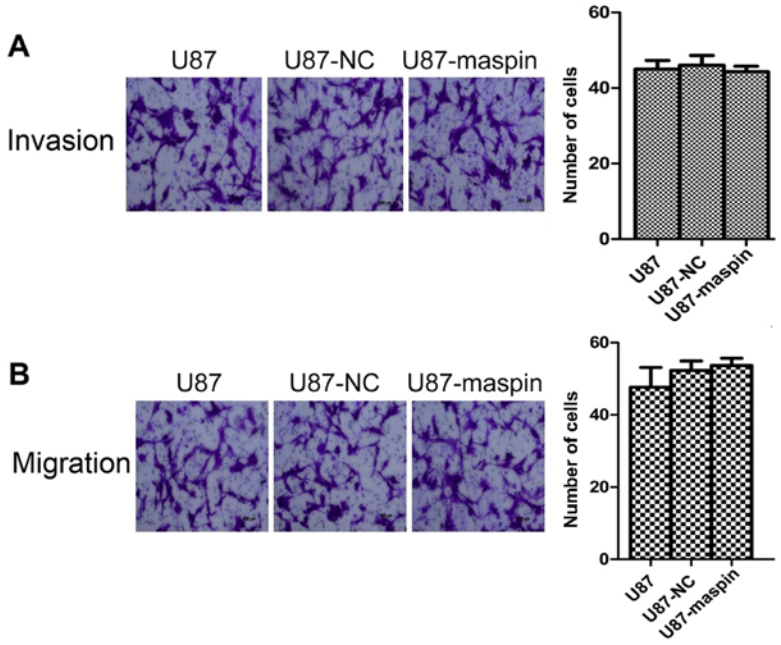

Figure 3. Effect of maspin on the migration and invasion of glioma cells. (A) Matrigel invasion and (B) migration assays were performed using the U87, U87-NC and U87-maspin cells. Cells that migrated to the bottom surface of the membrane were stained with Giemsa. Representative images subjected to the migration and invasion assays of the U87, U87-NC and U87-maspin cells. There was no significantly differences between the U87-maspin cells and control cells regarding the migration and invasion capabilities ($P>0.05$).

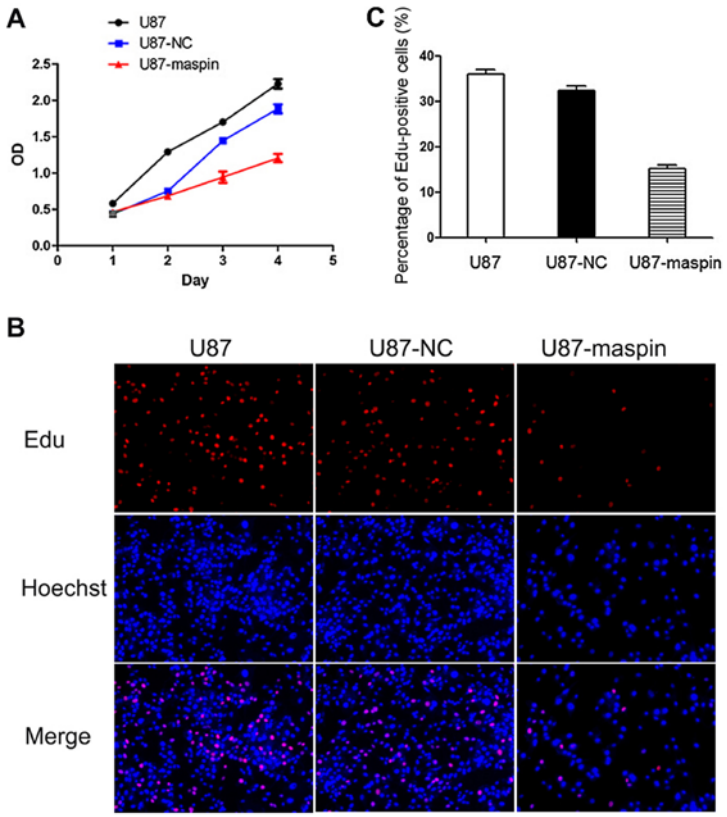

Figure 4. Effect of maspin on the proliferation of glioma cells. (A) Overexpression of maspin inhibited cellular proliferation in the U87 cells using CCK-8 tests (P<0.01). Expression of maspin inhibited U87 cellular DNA replication (magnification, x200). (B) Proliferating cells were labelled with EdU (red). Cell nuclei were stained with Hoechst 33342 (blue). The images are representative of the results obtained. (C) The percentage of EdU-positive cells was quantified. The percentage of EdU-positive cells was significantly downregulated in the U87-maspin cells compared to the other control cell lines (P<0.01).

in Fig. 3A, no significant difference was noted between the U87-maspin cells and control cells regarding the invasion capability (P>0.05). The numbers of migrated U87, U87-NC and U87-maspin cells were 47.7±9.5, 52.3±4.5 and 53.7±3.5, respectively. The number of U87-maspin cells that transferred through the chambers was almost the same as that of the control group (P>0.05; Fig. 3B). These data indicate that maspin had no effect on the invasion and migration of the U87 cells.

Effects of maspin overexpression on the cell proliferation of U87 cells. We next investigated the effect of maspin on the proliferation of glioma cells. To this end, the CCK-8-based assay was used. As shown in Fig. 4A, U87-maspin cells grew markedly slower than the control group starting from the second day. Thus, maspin inhibited cell proliferation in the U87 cells *in vitro*. To further characterize the effect of maspin on the proliferation of glioma cells, the EdU proliferation assay was also performed (Fig. 4B). The percentage of EdU-positive cells was quantified. The percentage of EdU-positive cells was significantly downregulated in the U87-maspin cells when compared with the control cell lines (P<0.01; Fig. 4C). These results indicated that maspin contributed to the reduced proliferation of the U87 cells.

5-Aza-dC induces the expression of maspin in glioma cell lines. Gene sequences of maspin were analyzed using EMBOSS Cpgplot (http://www.ebi.ac.uk/). After bioinformatic analysis, a CpG island in the 5' promoter region and 5 CpG islands in the gene body were predicted (Fig. 5A). The presence of a CpG-rich island in the 5' promoter region suggested the possibility that the maspin gene might be regulated through DNA methylation. To demonstrate a functional association between maspin promoter methylation and its gene inactivation, a DNA-demethylating agent, 5-Aza-dC, was used to treat the U87 and U251 cells. After treatment with 5-Aza-dC, a significant increase in maspin mRNA expression was noted in both the U87 and U251 cells (Fig. 5B).

DNA hypermethylation in the maspin promoter. Since maspin could be induced by 5-Aza-dC in the U87 and U251 cells, it could be suggested that the aberrant promoter methylation might contribute to maspin inactivation in glioma. To test this hypothesis, MSP was used to examine the methylation status of the maspin promoter. The results from MSP showed that the maspin promoter was methylated in the U87 and U251 cells but not in the PC3 cells (Fig. 5C). Furthermore, to determine a more detailed map of the methylation in the maspin promoter, we performed bisulphite sequencing around the promoter region of the maspin gene in the glioma cell lines and tissues. The maspin gene was amplified with bisulphite-treated DNA dinucleotides in the promoter, including 16 putative CpG sites. In the U87 and U251 cells as well as three glioma tissues, 16 CpG dinucleotides of the promoter were found to be

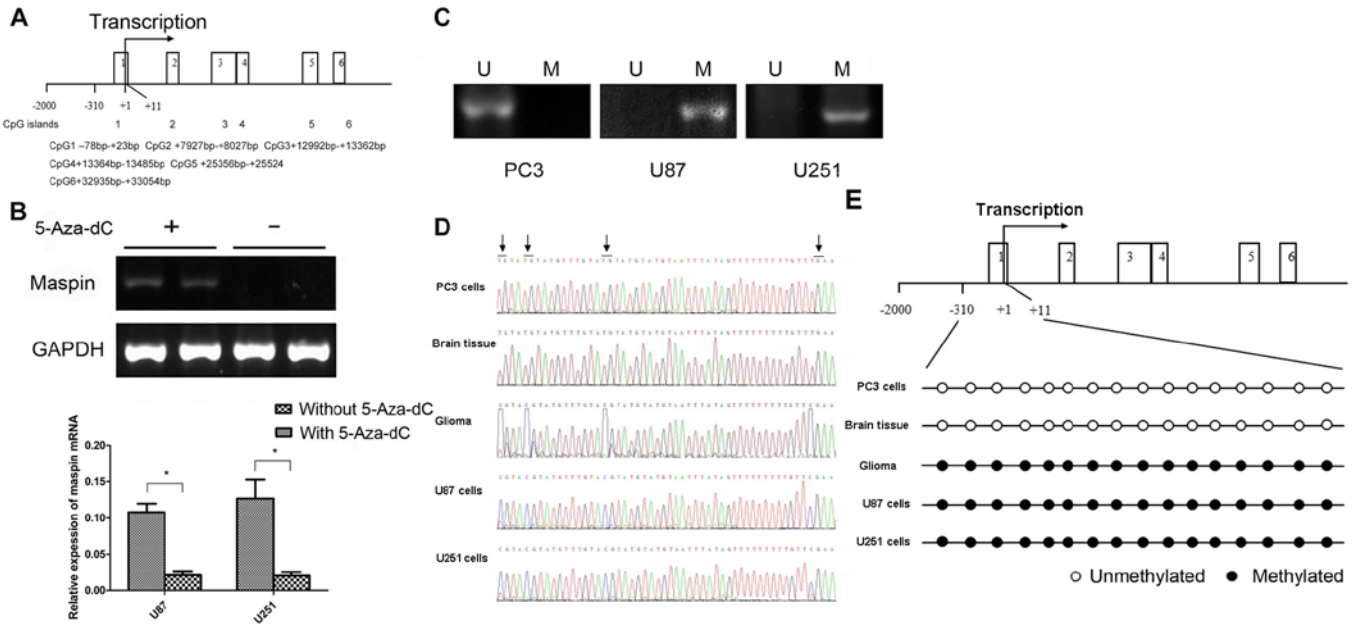

Figure 5. Regulation of maspin expression by DNA methylation. (A) The gene sequences of maspin were analyzed using EMBOSS Cpgplot; 6 CpG islands were found, and one of them was in the 5' promoter region. (B) The effect of 5-Aza-dC on maspin expression. Restoration of maspin expression in U87 and U251 cells after treatment with 5-Aza-dC. (+) represents cells treated with 5-Aza-dC. (-) represents cells untreated with 5-Aza-dC. Maspin mRNA expression was analyzed by RT-PCR, and a statistically significant difference was observed ($P<0.01$). (C) MSP was used to determine the methylation status of maspin. U, unmethylated; M, methylated. The glioma cell lines U87 and U251 were methylated in the DNA promoter region of maspin, and PC3 was shown to be unmethylated. (D) Representative bisulphite sequencing results of maspin promoter methylation in the PC3 cells, normal brain tissues, glioma tissues, U87 and U251 cells. (E) Bisulphite sequencing detected methylation in the PC3 cells, normal brain tissues, glioma tissues, U87 and U251 cells, showing a partially methylated sequence (arrows indicate CpG sites).

methylated, whereas in the PC3 cells and three normal brain tissues, the CpG dinucleotides in the maspin promoter were unmethylated. Representative sequencing results are shown in Fig. 5D and E. Thus, DNA methylation is involved in the suppression of maspin promoter activity in glioma.

Discussion

Exploration of the molecular mechanisms involved in glioma initiation and development affords the opportunity to identify molecules that may provide new targets for the therapy of malignant tumors. Many known oncogenes and tumor-suppressor genes, including c-Myc, p53 and RB, have been identified and characterized in glioma and contribute to our understanding of this malignancy (17-19). In the present study, we investigated, for the first time, the role of maspin in glioma.

Maspin was first discovered as a tumor suppressor in breast cancer (8). Studies have further revealed that treatment with exogenous recombinant maspin in breast and prostate cancer cells leads to less invasive and aggressive phenotypes (20,21). Loss of maspin expression in these cancers is correlated with tumor invasiveness and poor prognoses (22,23). By contrast, maspin expression correlates with better prognosis and better overall patient survival (23,24). Consistent with these clinical data, functional studies have demonstrated the tumor-suppressive functions of maspin in biological processes in cancer cells, including cell differentiation, cell migration, invasion, apoptosis and angiogenesis (25-27). Several candidate maspin targets have been identified under various experimental conditions, including histone deacetylase 1 (HDAC1) (28) and pro-urokinase type plasminogen activator (pro-uPA) (29). In addition, interferon regulatory factor 6 (IRF6) (30), β1-integrin (31), collagen I (32) and glutathione S-transferase (GST) (33) have been identified as maspin-associated proteins. For example, studies have suggested that, in prostate carcinoma cells, the loss of endogenous inhibition of HDAC1 by maspin may lead to the silencing of other tumor suppressors such as GSTp, thus representing a significant gain of function in tumor progression (33).

Although ample studies have reported that maspin may play important roles in the formation and progression of human tumors, the role of maspin in glioma remains unknown. Our results, for the first time, showed that maspin was silenced in glioma-derived U87, U251 and U343 cells and glioma tissues. To explore the functions of maspin in glioma cell lines, we infected lentiviral vectors overexpressing maspin into U87 cells. The results revealed that maspin had no effect on the migration and invasion *in vitro* of U87 human glioblastoma cells. Nevertheless, overexpression of maspin significantly inhibited tumor cell proliferation. In breast cancer, maspin is the only pro-apoptotic serpin implicated in apoptosis regulation. Intracellular maspin can translocate to the mitochondria to induce cytochrome *c* release and caspase activation or modulate the expression of Bcl-2 family members (34,35). In human prostate cancer, the progressive decrease in invasive cancer is associated with the capability of maspin to reduce tumor growth, osteolysis and angiogenesis. Furthermore, there is evidence that maspin inhibits prostate cancer-induced bone matrix remodelling and induces prostate cancer glandular redifferentiation (25,26). Although our result was reconcilable

with those research data, the different roles of maspin in different tumors suggest that maspin may demonstrate different activities in different cell types.

We next explored the mechanisms underlying maspin silencing in glioma. Alterations in DNA methylation, usually in the CpG island region of the gene promoter, is one of the common reasons that leads to gene silencing, and could contribute to oncogenesis (36,37). A putative CpG island in the 5' promoter and 5 CpG islands in the maspin gene body were found by EMBOSS Cpgplot. In this study, U87 and U251 cells were treated with 5-Aza-2-dC, and the mRNA expression of maspin was restored in both cell lines. Based on these findings, it could be reasonably speculated that DNA methylation in the CpG island region of the maspin promoter is involved in the regulation of the expression of maspin, influencing the proliferative abilities of the glioma cells. The molecular mechanism by which DNA methylation silences gene expression is not fully understood. DNA methylation may directly interfere with the binding of transcription factors, resulting in the transcriptional repression of the associated gene (37). In addition, methyl-binding domain-containing proteins (MBDs) may bind to areas of dense DNA methylation and recruit histone deacetylases and transcriptional repressor complexes that are refractory to transcription (38).

To clarify the mechanism of maspin inactivation in these two cell lines, MSP was used to examine the methylation status of the maspin promoter. Both U87 and U251 cell lines showed methylation of the maspin promoter. To confirm the results of MSP, we performed bisulphite sequencing analysis of a 321-bp fragment, including 16 expected CpG sites. The 16 CpG sites in the promoter were completely methylated in the U87 and U251 cells and glioma tissues, while in normal brain tissues, the CpG dinucleotides were unmethylated. Taken together, these findings suggested that promoter methylation is an important mechanism involved in the inactivation of maspin in glioma. Combined with the effect of CpG island methylation on maspin silencing in breast cancer cells (13), our research result appears to be convincing. However, other epigenetic processes, such as histone deacetylation, also played a role in the gene silencing in the breast cancer cells (39), and maspin expression may be regulated by hormone receptors in prostate cells (40). Moreover, some researchers have reported that the differential expression of maspin in tumor progression may be mediated by factors such as p53 and IKK-α (41-43). It is possible that other mechanisms, such as histone deacetylation, may also modulate maspin expression in glioma cell lines. Clearly, more studies are needed to clarify the issue.

In conclusion, we found that methylation-induced silencing of maspin contributes to the proliferation of human glioma cells, and maspin may be exploited as a potential therapeutic target for glioma.

Acknowledgements

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