Stathmin expression in glioma-derived microvascular endothelial cells: A novel therapeutic target

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Abstract. The purpose of this study was to investigate stathmin expression and its mechanisms of action in GDMEC. Microvascular endothelial cells were isolated from human gliomas (n=68) and normal brain specimans (n=20), and purified by magnetic beads coated with anti-CD105 antibody. The expression of stathmin mRNA and protein were detected by RT-PCR and western blotting, respectively. Stathmin expression was silenced by application of specific siRNA in high grade GDMEC. The proliferation, apoptosis and invasion behavior of GDMEC were investigated. The stathmin positive rate of endothelial cells in normal brain, grade I-II glioma and grade III-IV glioma was 20, 66 and 95.5%, respectively (P<0.05). When cells were treated with siRNA to silence stathmin, cell viability was reduced, the apoptosis rate increased and the migration of vascular endothelial cells was suppressed significantly (P<0.05). Down-regulation of stathmin suppressed neoangiogenesis of glioma and provides a potential target for glioma treatment.

Introduction

Glioma is the most common and aggressive form of primary brain tumors. High grade gliomas (anaplastic glioma/AA, WHO grade III, and glioblastoma multiforme/GBM, WHO grade IV) displays unique biological features especially network of neoplastic blood vessels, invasion and metastasis. The current standard treatment schemes for high grade glioma include gross total resection, radiotherapy, and concomitant and/or sequential oral chemotherapy with temozolomide. Although these treatments may significantly prolong survival, they are not curative.

With the development of molecular biology and RNAi techniques, the molecular genetic markers and chemotherapy resistance mechanisms of glioma have been extensively investigated. In 1971, Folkman (1) proposed that tumor growth and metastasis depend on angiogenesis, and that inhibition of angiogenesis may be an effective strategy for cancer treatment. Since microvasular endothelial cell is the key player in the process of neoangiogenesis, molecular genetic investigations of microvasular endothelial cells have been extensively carried out. Most of the researches have been concentrated on VEGF and its analogs within the VEGF receptor-mediated signaling cascades (2,3).

Stathmin (synonymous with oncoprotein 18/OP18), also known as metablastin, is a member of the microtubule destabilizing protein family. It regulates microtubule dynamics during cell cycle progress (4,5). The activity of stathmin is regulated by phosphorylation during the cellular transition from interphase to metaphase. The non-phosphorylated stathmin promotes the depolymerization of microtubules by sequestering tubulin (6) while the phosphorylated stathmin leads to increasing microtubule stabilization which promotes formation of a mitotic spindle (7).

Stathmin is overexpressed in various human malignancies, such as leukemia, neuroblastoma and mesothelioma (8). Most studies have demonstrated that expression of stathmin is associated with tumor progression and unfavorable long-term prognosis. However, the expression of stathmin in human GDMEC (glioma-derived microvascular endothelial cells) and NDMEC (normal brain tissue-derived microvascular endothelial cells) has not been previously investigated.

Recent studies have revealed significant differences in stathmin between VEC (vascular endothelial cells) lines and newly isolated tissue-specific microvascular endothelial cell (9-12). However, there were several studies of NDMEC and GDMEC, which showed that GDMEC have stronger ability of angiogenesis and invasion (13,14). We have developed a reliable and reproducible method for microvascular endothelial cell isolation from human glioma of different histological grade and have examined the expression of stathmin in human

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GDMEC and NDMEC. In addition, we evaluated the effect of siRNA-mediated silencing of the stathmin gene.

Materials and methods

Tissue collection. Glioma samples were obtained from 68 patients (30 male, 38 female; age range: 13-74 years) who had surgical resection of glioma between 2008 and 2010. Normal brain tissue were obtained from 20 patients (12 male, 8 female; age range: 20-45 years) who underwent decompressive surgical procedures for severe head injury within the same time period. The protocol for this study was approved by the local Ethics Committee of our Hospital. Gliomas were of different histological grades: 10 pilocytic astrocytomas (WHO I), 14 low-grade astrocytomas (WHO II), 24 anaplastic astrocytomas (WHO III), and 20 glioblastomas (WHO IV).

Cell isolation and culture. Fresh tumor tissue was washed with PBS containing penicillin-streptomycin and then minced into pieces ~1 mm^3. Following digestion with 0.25% trypsin (Sigma-Aldrich) at 37˚C for 30 min, further digested by 1 mg/ml collagenase (Invitrogen) at 37˚C for 1 h, and the mixture was filtered with sterile steel mesh. After centrifugation at 1000 rpm for 5 min, the cells were suspended with complete DMEM medium, then placed in 2% gelatin-coated tissue culture flasks at 37˚C for 7 days to yield an enriched cell population. Cells were purified by incubating with magnetic MACS (Miltenyi Biotec, Germany) coated with a monoclonal anti-CD105 antibody according to the manufacturer's instructions (15). GDMEC and NDMEC of second passage were used for study. Characterization of microvasular endothelial cells was performed by FVIII-Rag stain with cellular immunity (Fig. 1A).

Immunohistochemistry. One part of each tissue was fixed with formalin and then embedded with paraffin, and sliced into 4-μm sections. Following deparaffinization and hydration, the slides were treated with 0.3% peroxide for 15 min, after which the sections were blocked with 1.5% blocking serum (Invitrogen) for 2 h at room temperature. A rabbit anti-human stathmin polyclonal primary antibody (CST, USA) was applied in overnight incubation. After washing with PBS thrice and incubating with goat anti-rabbit secondary antibody at 37˚C for 30 min, the slides were treated with DAB (ZhongShan, Co. Ltd., Beijing, China). Finally, the slides were lightly counterstained with hematoxylin, dehydrated and mounted. The mean percentage of stathmin-positive cells of each sample was determined by counting at least 5 random visual fields with microscope at x400 magnification.

RNA interference. High grade GDMEC at 10^5 cells/ml were transfected in duplicate with 80 nM siRNA pools (2 siRNA duplexes) targeting the stathmin. Lipofectamine 2000 and siRNA were diluted in 50 μl Opti-MEM I at a ratio of 1:25 and incubated at room temperature for 5 min, respectively. Equal volume of the siRNA and Lipofectamine 2000 solutions were mixed and incubated at room temperature for another 15 min with gentle shaking, then the mixture was added into the cell culture. After overnight incubation, the cells were cultured in complete medium for 2 days.

Cell proliferation assays. High grade GDMEC were plated in 96-well plates (6,000/well) and transfected as mentioned above. Transfected and non-transfected high grade GDMEC were incubated for 24, 48, or 72 h, respectively. Then cell proliferation was analyzed by MTT colorimetric assay. The cells were incubated with MTT solution (1 mg/ml in RPMI-1640) for 4 h at 37˚C, then medium was decanted, and 150 μl DMSO was added into each well. Absorbance was measured at 570 nm using an ELISA Reader (RT-2100C, Rayto, USA). Experiments were performed in triplicate.

Cell apoptosis assay. After designated treatment, cells were washed, harvested, and counted, then cells ($1x10^5$) were re-suspended in 100 μl binding buffer, before 10 μl of Annexin V and 5 μl of PI were added, and incubated in the dark for 15 min at room temperature, according to the manufacturer's instructions (Biosea, China). The apoptosis rate (%) was determined with a cytometer (Epics Altra II, Beckman Coulter, USA). Cells were also viewed under an inverse fluorescent microscope. The experiments were repeated thrice.

Western blotting. Total protein was extracted and then electrophoresis was performed by SDS-PAGE and transferred onto PVDF membrane (Millipore, Bedford, MA, USA), followed by blocking with 5% skimmed milk dissolved in TBS contained 1% Tween-20 for 1 h at room temperature. The membrane was incubated with primary antibody at 4˚C overnight, and incubated in alkaline phosphatase-conjugated secondary antibody for 1 h at room temperature after washing with TBST three times. After washing, the bands of protein were detected with NBT/BCIP substrates (ZhongShan, Co. Ltd). The specific antibodies were anti-stathmin rabbit polyclonal antibody (Cell Signal Tech), rabbit polyclonal Bcl-X antibody, rabbit polyclonal Bcl-2 antibody, rabbit polyclonal Bax antibody, and rabbit polyclonal pro-Caspase-3 antibody (Santa-Cruz Biotech).

Reverse transcription PCR. Total RNA was extracted with the TRIzol reagent (Invitrogen) following the manufacturer's protocol. Total RNA was retro-transcribed using MMLV reverse transcriptase. Oligo(d)T (Promega, USA) including 2 μg RNA of the obtained cDNA was then amplified using primers (SanGon, Shanghai, China) for the human stathmin and GAPDH sequences. The sequences and the reaction conditions are described in Table I. PCR products were separated on 2% agarose gel and quantified by densitometry.

Invasion assay. Appropriate Matrigel (BD Biosciences, Bedford, MA, USA) was added to the upper chamber of the transwell apparatus with 8-μm pore size membrane (Costar, Cambridge, MA, USA). After the Matrigel solidified at 37˚C, serum-free DMEM containing $1x10^5$ siRNA-transfected and control high grade GDMEC in 100 μl were added into the upper chamber. The lower chamber was loaded with 500 μl medium with 10% FBS. After incubation at 37˚C for 24 h, membranes coated with matrigel were wiped with a cotton swab and fixed with 100% methanol for 10 min. The membranes with cells were soaked in 0.1% crystal violet for 10 min and then washed with distilled water. The number of cells attached to the lower surface of the polycarbonate filter was counted at x400 magnification

Table I. The sequences of primer for RT-PCR.[a]

Gene	Sequences	Procedure
Stathmin	F: 5'-ACTGCCTGTCGCTTGTCT-3' R: 5'-GTCTCGTCAGCAGGGTCT-3'	95°C for 3 min 95°C for 30 sec, 57°C for 30 sec, 72°C for 40 sec,
GAPDH	F: 5'-GGTGAAGGTCGGAGTCAACGG-3' R: 5'-CCTGGAAGATGGTGATGGGATT-3'	30 cycles; Final elongation step at 72°C, 10 min.

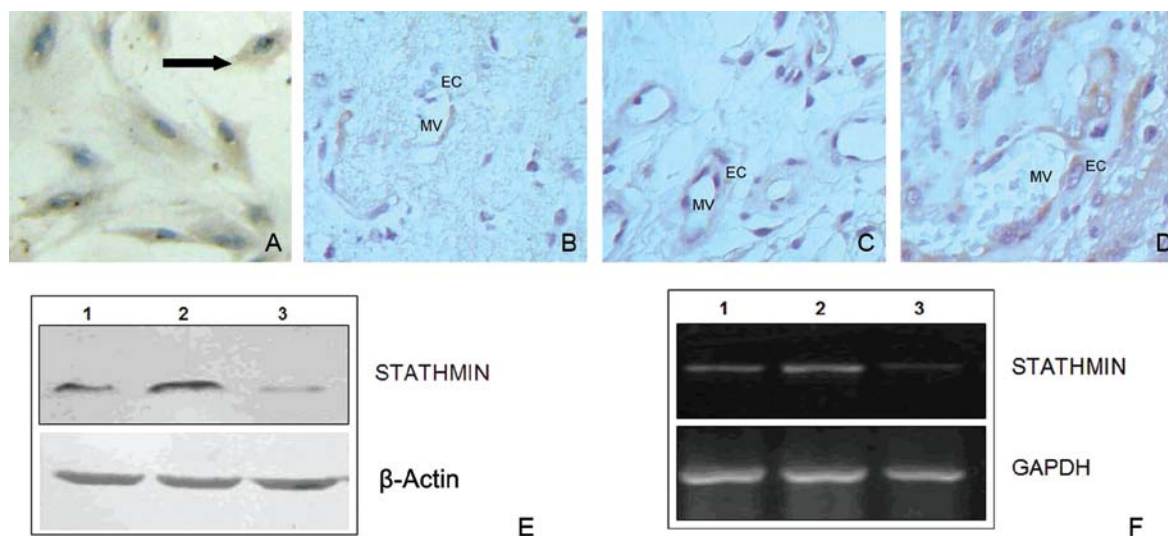[a]All sequences were designed with PrimerPremier 5.0.

Figure 1. Immunohistochemical staining for FVIII-Rag in cultured GDMEC (DAB staining appears brown-colored; original magnification, x400). (B-D) Analysis of stathmin expression in human glioma by immunohistochemistry. Stathmin was detected mainly in the cytoplasm of VEC from microvessels and in glial cells (original magnification, x400; counterstaining with H&E). MV, microvessel; EC, endothelial cell. (B) Expression of stathmin in normal brain. (C) Expression of stathmin in low-grade glioma (WHO grade I-II). (D) Expression of stathmin in high grade glioma (WHO grade III-IV). (E) Western blot analysis of stathmin protein expression in GDMEC and NDMEC, lanes: 1, GDMEC from WHO I-II tumors; 2, GDMEC from grade III-IV tumors; 3, NDMEC. (F) RT-PCR analysis of stathmin mRNA in GDMEC and NDMEC, lanes: 1, GDMEC from WHO I-II tumors; 2, GDMEC from WHO III-IV tumors; 3, NDMEC.

Table II. The statistical results of immunohistochemistry.[a]

Group	Stathmin (-)	Stathmin (+)	P-value
Normal Brain	16	4	<0.001
Glioma	10	58	
Glioma			
Grade I-II	8	16	<0.05
Grade III-IV	2	42	

[a]The tissue was regarded as negative when the positive cell rate was <10%.

under a light microscope. Results were expressed as the mean of triplicate experiments.

Statistical analysis. Statistical analysis was performed using SPSS 16.0 statistical software (SPSS, Chicago, IL), differences between groups were analyzed by χ^2 test or t-test and P<0.05 was considered as statistically significant.

Results

Stathmin expression in VEC is associated with histological grade of glioma. Immunohistochemical analysis demonstrated that the percentages of stathmin-positive VEC in normal human brain tissue, WHO grade I-II glioma, and WHO grade III-IV glioma were 20, 66 and 95.5%, respectively. The numbers of stathmin-positive VEC increased with increasing histological grade of tumor (Fig. 1B-D, Table II). The expression of stathmin protein and transcription of stathmin mRNA were significantly increased in GDMEC from high-grade glioma compared with GDMEC from low grade glioma and NDMEC (Fig. 1E and F).

Stathmin silencing may suppress proliferation and induce apoptosis of GDMEC. Application of siRNA to silence stathmin in GDMEC resulted in reduced expression of stathmin protein as well as inhibited mRNA transcription (Fig. 2A and B). Cell proliferation was significantly suppressed
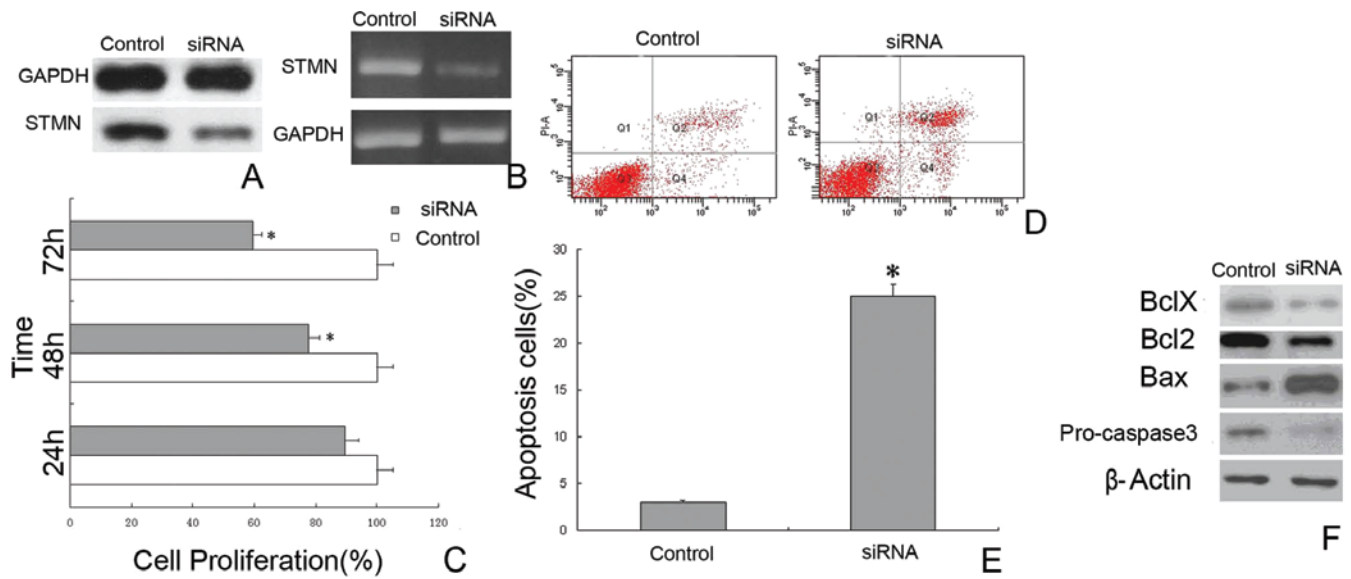
Figure 2. (A) Western blot analysis on stathmin expression of siRNA transfection. (B) RT-PCR analysis on stathmin transcription of siRNA transfection. (C) Proliferation of GDMEC in the siRNA group is suppressed, the difference between the siRNA and control groups is statistically significant (*P<0.05). (D and E) The apoptosis rate of siRNA-transfected GDMEC is significantly higher than the control group (*P<0.05). (F) Western blot demonstrates that expression of apoptosis associated proteins. Bcl-X, Bcl-2 and pro-caspase-3 were down-regulated while Bax was up-regulated in the siRNA-transfected group.
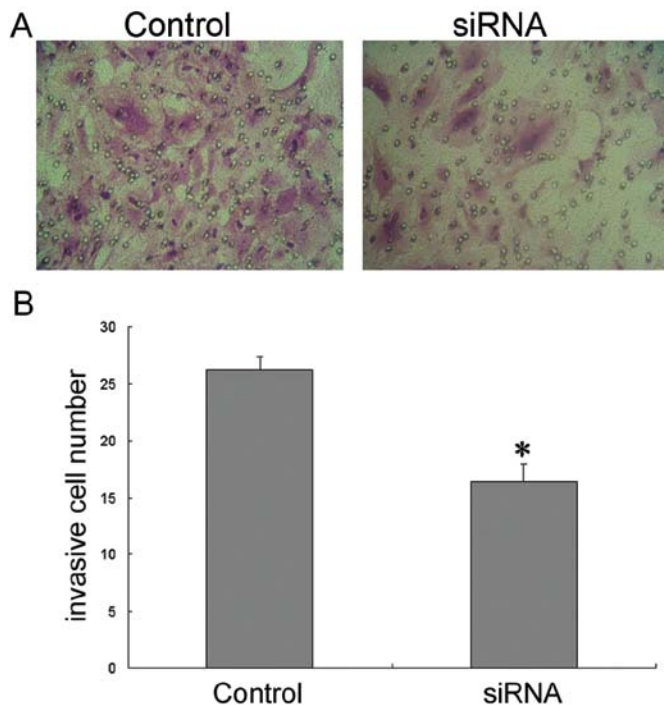

Figure 3. (A) Matrigel transwell assays for analysis of GDMEC invasion (original magnification, x400). (B) Graph shows invading cell numbers in the siRNA and the control groups. The difference between groups is statistically significant (*P<0.05).

(Fig. 2C) and the rate of apoptosis was increased (Fig. 2D and E). Proteins inducing apoptosis were up-regulated while anti-apoptosis proteins were down-regulated (Fig. 2F). These results demonstrated that siRNA-mediated silencing of stathmin could suppress proliferation and induce apoptosis in GDMEC.

siRNA mediated silence of stathmin may suppress the migratory ability of GDMEC. The results of Matrigel Transwell analysis showed that the invasive capacity of GDMEC derived from high grade glioma was significantly reduced by siRNA transfection and stathmin silencing (Fig. 3). Cell number of siRNA group passing through the matrigel was significantly lower than the control group. The results demonstrated that siRNA-mediated stathmin silence suppressed metastasis of GDMEC and inhibited invasion of gliomas.

Discussion

Angiogenesis is a crucial step in solid tumor growth and metastasis (16). Currently it is agreed that the growth of tumors can be divided into two stages (17). Stage I indicates the slow non-vascular growth of small cell clusters without neovascularisation, and stage II indicates the angiogenesis phase when tumor blood vessels could provide nutrition for a larger cell cluster. In stage II, the tumor grows rapidly and scattered cells may infiltrate surrounding tissue and metastasize along neocapillaries. Angiogenesis provides not only the prerequisite for the survival but also a route for tumor cell invasion and metastasis. Microvascular endothelial cell is an indispensable component of the vascular system and plays a pivotal role in the above described processes.

The cytoskeleton experiences dramatic structural rearrangements during the various stages of angiogenesis, including proliferation, migration, and differentiation of microvascular endothelial cells into vascular structures (18,19). Stathmin is an important protein that regulates cellular microtubule dynamics, and promotes mitotic spindle formation during mitosis (20-23). A potential role for stathmin in angiogenesis is that stathmin expression may be regulated by vascular endothelial zinc finger 1 (Vezf1). When antisense oligonucleotides targeted

Vezf1 were applied, the inhibition of stathmin expression was observed (24).

A recent study by Mistry *et al* shows that application of anti-stathmin ribozyme to human umbilical vascular micro-vasular endothelial cells results in inhibition of angiogenesis (25). In this study, we confirmed that the level of stathmin expression in GDMEC was significantly higher than NDMEC. Immunohistochemical analysis showed that glioma-derived microvasular endothelial cells exhibited moderate to strong stathmin staining (mainly in the cytoplasm), whereas stathmin staining was weak in microvasular endothelial cells from normal brain. Further, we found that the stathmin expression was significantly higher in cultured GDMEC compared with NDMEC at both transcriptional and translational level. The expression of stathmin in VEC was intimately associated with the WHO grade.

As WHO grade III-IV gliomas have a locally aggressive behavior and the VEC from this grade strongly express stathmin, we selected high grade GDMEC for the next investigation. The transcription and expression of stathmin was suppressed by transfection of cells with specific siRNA, which was also able to reduce the proliferation rate and the invasive potential of GDMEC. All these findings indicate that stathmin might play an important role in glioma neoangiogenesis and may exert its action by different mechanisms. Also, stathmin is a potential target of neoangiogenesis suppression in glioma treatment.

Further elucidation of the mechanisms of tumor angiogenesis may provide more precise and effective anticancer therapies. Comparative studies of tumor-derived microvasular endothelial cell and microvascular endothelial cells from non-neoplastic tissues seem to be best suited for such differential expression studies. Our findings suggested that stathmin plays an important role in glioma progression by supporting neoangiogenesis. Stathmin is a promising therapeutic target molecule in human glioma.

Acknowledgements

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