Mechanism of SEMA3B gene silencing and clinical significance in glioma

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ABSTRACT. The aim of the current study was to explore mechanisms of SEMA3B gene expression and its clinical significance in glioma, and provide a theoretical foundation for investigating individualized treatment in glioma. Paraffin-embedded tissues from 43 patients with a confirmed clinical diagnosis of glioma following neurosurgery at the First Affiliated Hospital of Zhengzhou University from December 2013 to April 2014 were selected randomly. An additional three normal brain tissues were obtained following encephalic decompression excision due to acute craniocerebral injury in the same period, which were used as the control group. Immunohistochemical staining for vascular endothelial growth factor was performed on the glioma tissues from the 43 patients. Genomic DNA was extracted for bisulfate conversion and sequencing. SEMA3B was fully expressed in the three normal brain tissues, and incompletely expressed in the 43 glioma tissues, with a lack of expression in 48.8% (21/43) of samples. Moreover, 58% of high-grade gliomas (grade III and IV) lacked SEMA3B expression, which was significantly more than those that lacked expression (20%) in low-grade gliomas (grade I and II), indicating that, as the clinical pathological grade increased, SEMA3B expression decreased. The occurrence and development of malignant tumors is a product of multiple genes and other factors. Here, we provide theoretical basis for

glioma development and prognosis involving DNA-methylation driven silencing of SEMA3B, and thus, SEMA3B is a potential target for directed treatments against glioma.

Key words: SEMA3B; Glioma; Immunohistochemistry; DNA methylation

INTRODUCTION

Tumor occurrence and development is a process involving multiple genes and other factors, and has multiple stages. Malignant cancer can be considered a genomic disease, mainly characterized by the activation of proto-oncogenes (growth factors and their receptors, signal transduction factors, and gene transcriptional regulatory factors, etc.) and the inactivation of tumor suppressor genes. DNA methylation is an important molecular mechanism in the inactivation of tumor suppressor genes, and occurs in the promoter region of target genes (Karayan-Tapon et al., 2008). The overproliferation of glioma cells accelerates the progression and metastasis of the cancer, thereby causing further gene mutations, inducing excessive or abnormal gene expression, and inducing the inactivation of apoptosis signals within the tumor cells. In addition to these aberrant changes, the apoptosis of excessively proliferating cells, cell repair mechanisms, and the inhibition of tumor growth are induced.

The SEMA3B gene is now considered the 19th candidate tumor suppressor gene (TSG) (Nair et al., 2007). SEMA3B is involved in signal transduction pathways and its biological functions are associated with the combination of some receptors, which plays an important role in the formation of new blood vessels, in tumorigenesis, and in immune responses. In the process of its tumor suppression function, SEMA3B interacts with associated proteins to ultimately form a signal transduction pathway, which helps to reduce the occurrence of glioma, and thus this pathway may provide new targets for the treatment of glioma.

Glioblastoma multiforme (GBM) has been found to be the most common and most aggressive primary malignant brain tumor, and affects 25,000 patients yearly (Deorah et al., 2006). Currently, treatments for this disease include surgical resection and radiation therapy (RT) in combination with postoperative medication. However, the survival rate of GBM patients is still low. It is reported that the overall survival of GBM is 14.6 months and that the two-year postoperative survival rate is 26.5%. The proliferation and diffuse infiltration of microvessels are two biological characteristics of glioma cells, which are also related to difficulties in curing this disease. Additionally, the tumor cells infiltrate normal brain tissue; therefore, it is challenging to remove all tumor cells during surgery. Presently, there are many specific treatments for these fatal tumor cells (Cohen et al., 1993). Furthermore, patients who cannot undergo surgery or bear postoperative recurrence and/or metastasis may consider drug therapies that can control tumor growth to a certain extent. Therefore, it is necessary to develop new drugs that can specifically target tumor cells, have low toxicity towards healthy cells, are safe, and exhibit strong antitumor effects for the treatment and prevention of recurrence of GBM.

MATERIAL AND METHODS

Patients and tissue samples

Paraffin-embedded blocks of glioma tissues clinically diagnosed after surgical removal were randomly selected from the Neurosurgery Department at the First Affiliated Hospital of

Zhengzhou University from December 2013 to April 2014. Selected tissues were from patients that had no preoperative tumor treatment, and the postoperative pathological diagnosis for each tissue was glioma, of which there were 4, 25, 20, and 20 cases of grade-I, -II, -III, and -IV gliomas, respectively. Thirty-six cases were male, 33 cases were female. The ratio of males to females was 1.07:1, and the patient ages ranged from 17 to 70 years (mean 48.37 ± 12.06 years). All tissue blocks were cut into 5-µm sections by professional personnel. Additionally, three normal brain tissues removed by intracranial pressure due to acute traumatic brain injury were selected as the control group. Tissues from the control group were cryopreserved using liquid nitrogen and stored until subsequent use. Written informed consent was obtained for all study participants.

Reagents and instruments

The following reagents and instruments were used in the study: Ultra Sensitive TMS-Hypersensitive Kit (Maixin Biotechnology Company, Fuzhou, China); DAB Coloration Kit (Maixin Biotechnology Development Company, Fuzhou, China); Plasmid Extraction Kit (Sangon Biological Engineering Co., Ltd., Shanghai, China); SK8161; sappanwood blank cell-staining fluid (Maixin Biotechnology Development Company, Fuzhou, China); CpG Assay (200) (Qiagen, Germany); DNA Extraction Kit (Qiagen, Germany); $NaHSO_3$ (analytically pure, Sangon); Taq DNA polymerase SC0010 and dNTPs (Sangon); hydroquinone (Sangon); 3730 Sequencing Analysis Meter (Applied Biosystems, Carlsbad, CA, USA); SW-CJ-1D Clean Bench (Sue Clean Equipment Factory, Jiangsu); DK-8D Electric Heating Thermostatic Water Bath (Senxin Experimental Instrument Co., Ltd., Shanghai, China); YXJ-2 Centrifuge (Xiangyi Centrifugal Machine Instrument Co., Ltd.); Liquid-Moving Machine (scope 100-1000, 20-200, 0.5-10 mL) (BBI Life Sciences, Shanghai, China).

Immunohistochemical staining of vascular endothelial growth factor (VEGF) in glioma tissue

The Ultra Sensitive TMS-Hypersensitive Kit was used to stain sliders as per the instructions of the manufacturer. The DAB reagent was prepared simultaneously with the last of these three PBS washes; the PBS was then removed, and 100 µL fresh DAB liquid was added to each section. Next, the presence of yellow staining was observed after 3-10 min under microscopy.

Genomic DNA extraction from the paraffin embedded samples

Genomic DNA was extracted from the paraffin-embedded sample blocks following manufacture instructions (note: Buffer AL and methyl alcohol were mixed in advanced).

DNA bisulfate conversion

The DNA was first melted for use in the bisulfate reaction. Next, 800 µL enzyme-free water was added to dissolve each uniform mixture of hydrogen sulfite. The solutions were mixed for 5 min until the full dissolution of sodium hydrogen sulfite, and then the bisulfate reaction mixtures were prepared in 200-µL PCR tubes according to the volumes shown in Table 1.

Table 1. Bisulfate reaction mixtures.

Component	Volume per reaction (µL)
DNA solution (1-2 µg)	10
Rnase-free water	10
Bisulfite mix (dissolved)	85
DNA protect buffer	35
Total volume	140

The PCR tubes were covered; the bisulfate reaction mixtures were mixed vigorously; the reaction mixtures were incubated at room temperature; and then the DNA bisulfate conversions were carried out in a thermal cycler with conditions shown in Table 2.

Table 2. Thermal cycle conditions for bisulfate conversion.

Step	Time (min)	Temperature (°C)
Denaturation	5	95
Incubation	25	60
Denaturation	5	95
Incubation	85	60
Denaturation	5	95
Incubation	175	60
Store	Overnight	20

After thermal cycling and completion of the bisulfate conversions, the reaction mixtures were moved into 1.5-mL centrifuge tubes; 310 µL fresh Buffer BL that included 10 µg/mL carrier RNA was added into each sample. The tubes were mixed and centrifuged briefly, 250 µL 96-100% ethyl alcohol was added to each sample and mixed for 15 s before centrifuging briefly to remove droplets in the tubes. EpiTect spin columns were placed into collection tubes; the complete bisulfite reaction mixtures were transferred to the EpiTect spin columns; the spin column/collection tubes were centrifuged at the maximum speed; the flow through was removed; and then the spin columns were put back into the collection tubes. Next, 500 µL Buffer BW was added to each spin column; centrifuged again at maximum speed for 1 min; the flow through was removed; and the columns were put back into the collection tubes. Then, 500 µL Buffer BD was added to each spin column; the columns were incubated for 15 min at room temperature; centrifuged at maximum speed for 1 min; the flow through was removed; and the columns were put back into the collection tubes. Next, 500 µL Buffer BW was added to each spin column; the columns were centrifuged at the maximum speed for 1 min; this last step was repeated; the spin columns were placed into 2-mL collection tubes; centrifuged at the maximum speed for 1 min; and any residual liquid was removed. The spin columns (cap opened) were put into new 1.5-mL clear centrifuge tubes; heated at 56°C for 5 min; put into clear centrifuge tubes; 20 µL Buffer EB was added to the center of each membrane; and then finally the columns were centrifuged at 12,000 rpm (15,000 *g*) for 1 min to elute the purified DNA.

Gene sequencing

Positive bands were cut off for recycling DNA as per the instructions of the manufacturer, and then gene was sequenced under the following conditions (Table 3).

Table 3. Reaction system.

Degeneration	25-time cycles	Constant temperature
96°C for 1 min	96°C for 10 s: 50°C for 5 s: 60°C for 4 min	4°C

Finally, the products were purified and detected.

Statistical method

The GraphPad Prism 5 Software (San Diego, CA, USA) was used for one-way analysis of variance (ANOVA) and correlation analyses, and data are reported as means ± SD. Results were considered to be statistically significant at $P < 0.05$.

RESULTS

Methylation of the SEMA3B gene promoter in glioma

Studies have shown that methylation in the promoter regions of TSGs is the most common cause of their inactivation, which indicates that the most direct way to analyze mechanisms of TSG silencing in glioma is to analyze genetics and epigenetics. Thus, this research may allow us to better understand and explain glioma development. As mentioned, the most direct epigenetic mechanism for TSG silencing is DNA methylation, and one such TSG that can be affected by this is SEMA3B. Therefore, we analyzed the CpG methylation status of the SEMA3B gene in glioma tissues using the CpG methylation pyrophosphate test of the 3p21.3 area (Figure 1), and conducted a quantitative analysis for the degree of methylation at the target locus through detecting the rate of C/T at the CpGs at that locus. Our results revealed that the SEMA3B gene may be a potential methylation locus in glioma.
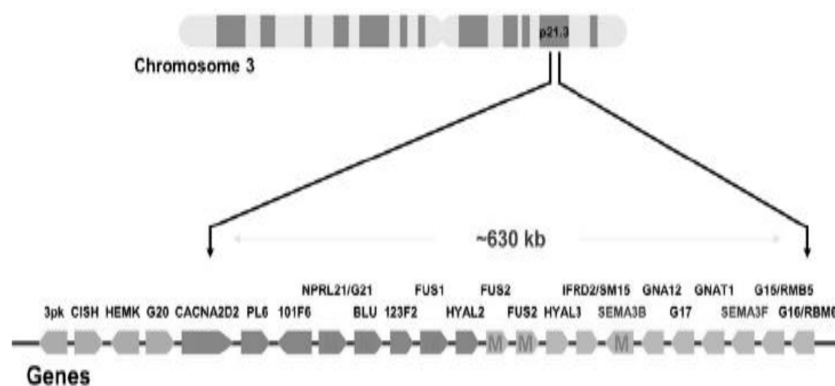

Figure 1. Chromosomal location of the SEMA3B gene.

Through the detection of methylation in the SEMA3B promoter region in tissues from 43 glioma cases and from 3 normal brain tissues (Table 4), we found that the three normal brain tissues were unmethylated and that 21 glioma cases were methylated with a methylation rate of 48.8% (21/43). Moreover, we analyzed the specific cites of methylation using PCR and pyrosequencing methods, and found five methylated loci (Figure 2).

Table 4. Clinical information of glioma patients from whom tissue samples were used for the methylation analysis.

ID	Gender	Age	PD	Grade	Methylated	Unmethylated
1	F	28	NB	-	-	-
2	M	30	NB	-	-	-
3	M	45	NB	-	-	-
4	F	30	PA	I	-	-
5	F	17	PA	I	-	-
6	M	48	MOA	II	+	-
7	M	69	ODG	II	-	-
8	M	65	LGA	II	-	-
9	F	53	MOA	II	-	-
10	F	53	LGA	II	-	-
11	F	43	MOA	III	+	-
12	F	55	AA	III	+	-
13	F	65	AMOA	III	+	-
14	M	50	AO	III	+	-
15	F	54	LGA	III	-	-
16	M	61	GBM	IV	+	-

NB = normal brain tissue; PD = pathological diagnosis; PA = hair-cell type astrocytoma; LGA = dispersivity astrocytoma; ODG = less glioma; MOA = oligodendroglioma; AMOA = variant and hybrid oligodendroglioma; AA = variant astrocytoma; AO = variant oligodendroglioma; GBM = colloid mother tumor; (+) positive; (-) negative.
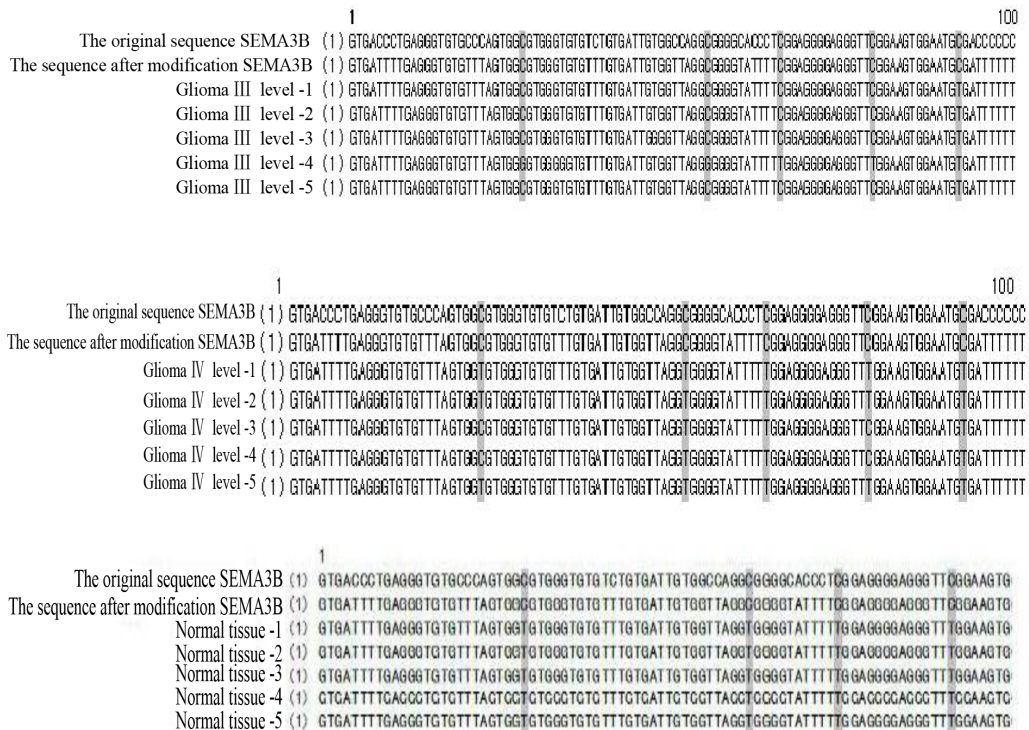

Figure 2. SEMA3B sequence alignment following bisulfite conversion of representative grade-III and -IV glioma tissues and normal brain tissues.

Methylation status of SEMA3B and the clinical pathological characteristics of glioma patients

We found that methylation in the promoter region was not detected in grade-I glioma tissues, while the methylation rates of grade-II, -III, and -IV gliomas were 25% (2/8), 67% (12/18), 47% (7/15), respectively, and the data are shown in Table 4. Through the comparison of methylation rates in gliomas of high and low grades, we discovered that 58% (19/33) of SEMA3B promoters in the high-grade glioma group were methylated, whereas only 20% (2/10) were methylated in the low-grade glioma group. A general increasing trend in the methylation of the SEMA3B gene was observed with increasing pathological grade of the samples, which also indirectly suggests that SEMA3B gene silencing and methylation in the promoter region are correlated. Statistical analysis revealed that the degree of methylation in normal brain tissue and low-grade glioma tissues was significantly different from that in high-grade glioma tissues (Table 5). Therefore, we can consider methylation in the promoter region of the SEMA3B gene as one of the mechanisms of its downregulation in glioma.

Table 5. Clinical pathological characteristics of 3 normal brain tissues and tissues from 43 cases of glioma and SEMA3B methylation status from these tissues.

Characteristic	Types	Percentage (%)	Unmethylated (%)	Methylated (%)	Methylation rate (%)
Gender	Male	26 (56.5)	9 (19.6)	17 (36.9)	65.4
	Female	20 (43.5)	6 (13.0)	14 (30.5)	70.0
Age	≤60	35 (76.1)	15 (32.6)	20 (43.5)	57.1
	>60	11 (23.9)	2 (4.3)	9 (19.6)	81.8
Tissues	Normal brain tissues	3 (6.5)	0 (0)	3 (100.0)	100.0
	I/II	10 (21.7)	8 (17.4)	2 (4.3)	20.0
	III/IV	33 (71.7)	14 (30.4)	19 (41.3)	57.6
Total	Glioma	43 (93.5)	17 (37.0)	26 (56.5)	60.5

DISCUSSION

The SEMA3B gene belongs to the semaphorin/collapsin family, and mainly exists in the form of a secreted protein or membrane protein. Alterations in the human 3p21.3 chromosome area are often implicated in high incidences of cancer. For example, loss of heterozygosity of this area is more frequent in cancers such as in lung cancer, breast cancer, ovarian cancer, and cancers of other organs. The SEMA3B gene usually shows higher expression levels in normal tissues than those found in tumors. However, SEMA3B expression has been shown to be higher in highly metastatic tumors such as in lung cancer, but lower in melanoma cells, bladder cancer cells, and prostate cancer cells. Moreover, the genome in glioblastoma cells has shown a wide range of low methylation in specific areas (Kozlowski et al., 2010). This methylation pattern increases genetic instability, silences TP53 and PTEN TSGs, and activates oncogenes. Methylation usually occurs in the promoter CpG island of a gene, and methylation in these regions is related to expression of TSGs (McDonnell et al., 2001), such as those involved in DNA repair (Quiros et al., 2011), cell cycle (Rickles et al., 2001) and cell apoptosis (Saitoh et al., 1995; Liang et al., 2013), invasion (Suchorska et al., 2011), and migration.

Research has shown that SEMA3B gene expression is absent or reduced by more than 80% in lung cancer cell lines, and has homozygous deficiency in tissues from lung cancer. It was previously found that methylation in the promoter region of SEMA3B can reach 92% in gallbladder carcinoma, which indicated that high methylation of the SEMA3B gene participates in gallbladder

carcinoma development (Jarjour et al., 2011). Expression of SEMA3B has been shown to increase cell apoptosis and inhibit the growth of human lung cancer cells, breast cancer and ovarian cancer cells, and of the A9 sarcoma cell line in mice. These findings strongly implicate SEMA3B in the inhibition of tumor cell growth. Additionally, SEMA3B plays a role in the process of mediated neural axon growth. Recent research on a variety of tumor cells has shown that the 5'-CpG island in the promoter of SEMA3B is highly methylated, which leads to low or absent expression of the gene in tumor cell lines. Notably, in small-cell lung cancer and non-small cell carcinoma, the deficient expression of the SEMA3B gene can predict the degree of malignancy and aggressiveness of tumors. *In vitro* transfection of the SEMA3B gene can reduce the proliferation and invasion ability of tumor cells, and can ablate the tumorigenicity of cancer cells in nude mice. Moreover, SEMA3B expression can inhibit the growth of malignant cells, which may explain the close relationship between SEMA3B gene expression and the occurrence of cancer.

In the current study, we detected the expression of SEMA3B by adopting RT-PCR methods. We found that in the three normal brain tissues, SEMA3B was fully expressed, whereas in the 43 glioma tissue samples, the SEMA3B expression rate was 48.8% (21/43). Interestingly, with increases in clinical pathologic grade, we observed decreases in SEMA3B expression. Specifically, we found that the deficiency rate of SEMA3B expression in high-grade glioma (grades III and IV) was 58%, which was significantly higher than the deficiency rate (20%) in low-grade glioma (grades I and II), indicating that higher clinical pathological grades were associated with more significant decreases in SEMA3B gene expression. These differences in SEMA3B expression are likely to affect glioma prognosis, and may also participate in the development of these tumors. The physiological deficiency of the SEMA3B gene may have resulted in the loss of its expression, and this may directly cause the disease or aggravate the development of the illness, which further illustrates that SEMA3B plays an important role as a TSG in glioma. The normal physiological function of SEMA3B is reduced in gliomas, which enhances their degree of malignancy. We also demonstrated that the deficiency rate in low-grade gliomas was significantly lower than that in high-grade gliomas, which may indicate that the high-grade cases will progress with faster growth patterns, and may be more easily transformed in the direction of malignancy. We can thus predict that high expression of SEMA3B is associated with a positive prognosis in glioma patients.

In conclusion, DNA methylation continues to draw more and more attention in early diagnosis, prognostic evaluation, and targeted clinical treatments of different human cancers, and plays an important role in tumorigenesis and development. DNA methylation, the most common epigenetic event, does not change primary gene sequences, but rather can lead to changes in gene expression and to chromatin structure. Taken together, the results of the present study suggest that DNA methylation may be a potential target in gene-targeted therapies, and may be valuable in early diagnosis, prognostic evaluation, disease progress, and treatment of glioma.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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