Changes in Transcriptional Factor Binding Capacity Resulting from Promoter Region Methylation Induce Aberrantly High GDNF Expression in Human Glioma

Zheng-Quan Yu · Bao-Le Zhang · Qing-Xian Ren · Jian-Cun Wang · Ru-Tong Yu · De-Wei Qu · Ze-Hao Liu · Ye Xiong · Dian-Shuai Gao

Abstract Glial cell line-derived neurotrophic factor (GDNF), which belongs to transforming growth factor β superfamily, plays important roles in glioma pathogenesis. *Gdnf* mRNA is aberrantly increased in glioma cells, but the underlying transcription mechanism is unclear. Here, we found that although the base sequence in the promoter region of the *gdnf* gene was unchanged in glioma cells, there were significant changes in the methylation level of promoter region I ($P<0.05$) in both high- and low-grade glioma tissues. However, the methylation degree in promoter region II was notably decreased in low-grade glioma tissue compared to normal brain tissue ($P<0.05$), and the demethylation sites were mainly located in the enhancer region. Conversely, methylation was markedly increased in high-grade glioma tissue ($P<0.05$), and the sites with decreased methylation level were mainly located in the silencer region. The binding capacities of several transcriptional factors, such as activating protein 2, specificity protein 1, ETS-related gene 2, and cAMP response element binding protein, which specifically bind to regions with altered methylation status decreased along with the pathological grade of glioma, and the differences between high-grade glioma and normal brain tissue were significant ($P<0.05$). The results suggest that changes in transcriptional factor binding capacity are due to changes in promoter region methylation and might be the underlying mechanism for aberrantly high *gdnf* expression in glioma.

Keywords GDNF · Promoter · Glioma · DNA methylation · Transcription factor

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Introduction

Glial cell line-derived neurotrophic factor (GDNF) was originally isolated, purified, and cloned from rat B49 glial cell line by Lin et al. [1] in 1993. It belongs to the transforming growth factor family β (TGF-β) and exerts protective effects in many cell types as an important bioactive nutritional factor [2]. In humans, the *gdnf* gene is mapped to chromosome 5p12-p13.1 and is a single-copy gene comprised of two promoters and five exons. Early studies suggested that GDNF was merely a type of growth-promoting factor that influenced the survival and differentiation of dopaminergic neurons in the embryonic mesocerebrum [3, 4]. Later, it was shown that GDNF showed nutritional and protective effects in the periphery including sympathetic, parasympathetic, sensory, and motor neurons.

Recent studies have demonstrated that GDNF acts as a potent proliferation-promoting factor in glioma cells [5]. *Gdnf* levels are significantly higher in human glioma cells compared to normal glial cells, and GDNF has been shown to participate in tumor cell metastasis [6].

In recent years, the mechanisms underlying the proliferation- and metastasis-promoting effects of GDNF have been studied thoroughly, but few groups have investigated the mechanism underlying the abnormally high expression of the *gdnf* gene in glioma cells. Markedly increased expression of any gene is usually the result of mutation or epigenetic modification [7]. Methylation in DNA promoter region is one of the most important epigenetic modifications, which regulates gene expression through the changes in the methylation level of DNA promoter region [8, 9]. We previously found that the DNA copy number and coding sequence of *gdnf* were not altered in glioma cells; therefore, we hypothesized that the abnormally high expression might be due to the change of methylation modification in its promoter region. Specifically, it was reported that promoter region methylation of the gene closely related with glioma can affect the ability of transcription factors to bind to its promoter region.

To test the above hypothesis and clarify the methylation status of the *gdnf* promoter region and transcription factor binding in glioma, we compared the methylation status of promoter regions I and II of *gdnf* in glioma and normal brain tissues using bisulfite sequencing PCR (BSP). We also employed chromatin immunoprecipitation (ChIP) coupled with PCR to detect the binding of four transcription factors [activating protein 2 (AP-2), specificity protein 1 (SP1), gene 2 (ERG-2), and cAMP response element binding protein (CREB)], to the *gdnf* promoter region in glioma and normal brain tissues. Our results provide evidence for altered methylation and transcription factor binding in glioma and shed light on the mechanism of aberrantly high *gdnf* expression in glioma.

Materials and Methods

Tissue Source

Six human brain tissue samples (collected from patients with acute brain trauma given with intracranial decompression) and 12 glioma tissue samples (World Health Organization grades 1–4) acquired from the Affiliated Hospital of Suzhou University were used for BSP, real-time PCR, enzyme-linked immunosorbent assay (ELISA), and single-nucleotide polymorphism (SNP) analysis. Biopsied glioma tissues from patients who had not undergone cancer therapy were randomly sampled. Samples in pathological grades 1–2 and 3–4 were classified as low- and high-grade glioma groups, respectively.

Cell Culture

Human U251 glioma cell line were cultured at 37 °C in a humidified 5 % CO_2 atmosphere in Dulbecco's modified Eagle's medium (Gibco, Shanghai Invitrogen Biotechnology Co., Ltd., China) supplemented with 10 % (*v/v*) fetal bovine serum (Gibco). Exponentially growing cells were used for the following experiments. Then, the cells were incubated in 0, 1, 5, 10, and 20 μmol/l 5-aza-CR (Sigma-Aldrich, St. Louis, MO) for 48 h (37 °C, 5 % CO_2) to detect its demethylation effect.

RNA Extraction and Reverse Transcription

Total RNA was extracted from tissue samples, and its concentration and purification were measured using ultraviolet spectrophotometry. Reverse transcription was performed according to the M-MLV Operating Instructions from Fermentas (Burlington, ON, Canada). Briefly, the procedures are as follows: the reaction system consisted of 20 μl in an RNase-free PCR tube that included 1 μg RNA, 1 μl Oligo (dT) primer (0.5 μg/μl), 1 μl random primer (0.2 μg/μl), and 10 μl diethylpyrocarbonate water. The components were mixed and heated to 65 °C for 10 min then rapidly transferred to an ice bath to cool before adding 4 μl 5× reaction buffer, 0. 5 μl RiboLock™ Ribonuclease inhibitor (20 U/μl), 2 μl dNTP mix (10 mM), and 1 μl reverse Tra Ace. The mixture was heated to 42 °C for 60 min, then 70 °C for 10 min. The acquired cDNAs were either stored at −20 °C or immediately used for PCR after determination for the reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Real-Time PCR

Real-time PCR primers were designed for *gdnf* and *GAPDH* (Table 1). Serial dilutions of plasmids (pMD19T, Takara,

Table 1 PCR primer sequences for real-time PCR

Primer name	Primer sequence	Product (bp)	Annealing temperature (°C)	Efficiency (%)	R^2
hGAPDH-F hGAPDH-R	GAAGGTGAAGGTCGGAGTC GAAGATGGTGATGGGATTTC	226	59	98.5	0.990
hGDNF-F hGDNF-R	TGACAAAGTAGGGCAGGCATGT ATCCACACCTTTTAGCGGAATGC	116	59	96.9	0.996

Table 2 PCR primer sequences for SNP detection

Primer name	Primer sequence	Product (bp)	Annealing temperature (°C)
1-F	5′CAGAGAATCTCAAAGGTGCA3′	960	60
1-R	5′ACGACATCCCATAACTTC3′		60
2-1-F	5′CTCCAGAGAGCATCCTAATT3′	900	58
2-1-R	5′TCTGCTGGCTGAAGTCAGA3′		58
2-2-F	5′TCATTGCCTGCCATGTAATAC3′	1,200	60
2-2-R	5′CTAGGTTGGACATTAACTCCA3′		60

Shanghai, China) containing segments of interest were used to generate the standard curve, and the linear correlation for each dilution was confirmed. The copy number of the plasmids containing segments of interest was calculated according to the following formula: the copy number of the plasmids in per microliter = plasmid concentration (in nanogram per microliter) $\times\ 10^{-9}\times 6.02\times 10^{23}$/molecular weight of the target fragment (in gram per mole). The PCR mixture was prepared using 2× SYBR Green PCR Master Mix (Shanghai R&S Biotechnology Co., Ltd., Shanghai, Jiangsu, China) according to sample number and parallel number. The reaction system was as follows: 10 μl 2× SYBR Green PCR Master Mix, 1 μl primer mix, 1 μl template, and 8 μl RNase-free water. Then, each aliquot was transferred to PCR tubes and briefly centrifuged before undergoing quantitative real-time PCR on an Eppendorf Realplex PCR instrument (Hamburg, Germany) according to the following protocol: 95.0 °C, 2 min; 95.0 °C, 15 s, 59.0 °C, 20 s; 72.0 °C, 20 s; 40 cycles. After PCR, product specificity was determined with melting curve analysis. Threshold cycle (Ct) values were calculated according to PCR reaction curves, and relative quantitative analysis was performed according to *gdnf* (copy/microliter)/*hGDNF* (copy/microliter) method.

Enzyme-Linked Immunosorbent Assay

GDNF protein level was measured by biotin double-antibody sandwich ELISA (Shanghai Yifeng Biotechnology Co., Ltd., Shanghai, Jiangsu, China) using a GDNF antibody that did not cross-react with TGFs, BDGF, or NGF. Standards (0, 0.75, 1.5, 3, 6, and 12 ng/ml) mixed with biotin-labeled antibody were added into wells of 96-well plate in triplicate. In standard wells, 50 μl standard and 50 μl streptomycin–horseradish peroxidase (HRP) were added; in sample wells, 40 μl sample, 10 μl anti-GDNF antibody, and 50 μl streptomycin–HRP were sequentially added into each well. Next, the plate was sealed with a membrane, gently agitated to mix, and then incubated at 37 °C for 60 min. After the membrane was removed, the solution was removed, and the plate was dried by patting. The plate was washed with 1× washing buffer five times, and the plate was dried by patting. Next, 50 μl each of chromogenic agents A and B was added into each well, and the plate was gently agitated to mix evenly before 10-min incubation in the dark at 37 °C to allow color development. Finally, 50 μl stop buffer was added to each well to quench the reaction. The absorbance value [optical density (OD)] of each well was measured at 450 nm, and the blank control well was set as zero. The linear regression equation

Table 3 BSP primer sequences

Primer name	Primer sequence	Product (bp)	Annealing temperature (°C)
250-1-1-F	5′GAAGGGATTAGGGTTAGAATTTTT3′	469	60
250-1-1-R	5′CCCAAACAAAAACRATATTTCT3′		60
250-1-2-F	5′ATGYGTTTGATTTTATTTTTAAAGA3′	507	60
250-1-2-R	5′CTACRCRAACAAACRAAA3′		60
250-2-1-F	5′AGAAGGTTTAGGTAGTTTTTGG3′	484	57
250-2-1-R	5′CCCTCCAATAACTTTTCTAATT3′		57
250-2-2-F	5′ GAATTAGAAAAGTTATTGGAGGG 3′	516	58
250-2-2-R	5′ AAACCCTAAAATTCCCRA 3′		58
250-2-3-F	5′ GTTTYGGGAATTTTAGGG 3′	517	58
250-2-3-R	5′ AAACRACRACCAAAACTC 3′		57

Table 4 Primer sequence for ChIP-PCR

Gene name	Primer sequence	Annealing temperature (°C)	Primer length (bp)
gdnf p2.1	F 5′GCGCCCTCATGTCTTCACG3′ R 5′GGGAGCACGAGACTGGTTTG3′	60	115
gdnf p2.2	F 5′GGCAGCTCCTTTTCTCGC3′ R 5′CTTCCCGCTCGGGTGTCT3′	60	71
gdnf p1.1	F 5′AATGCAGTCTTTGCCTAACAG3′ R 5′CATGACAAGCTCTAGCGGAAC3′	60	211
gdnf p1.2	F 5′CTTAACGTGCATTCTGCGGT3′ R 5′GACAGCCACGACATCCCATA3′	60	104

of the standard curve was calculated using the concentrations of standards and corresponding OD values, and the sample concentrations were calculated using the regression equation.
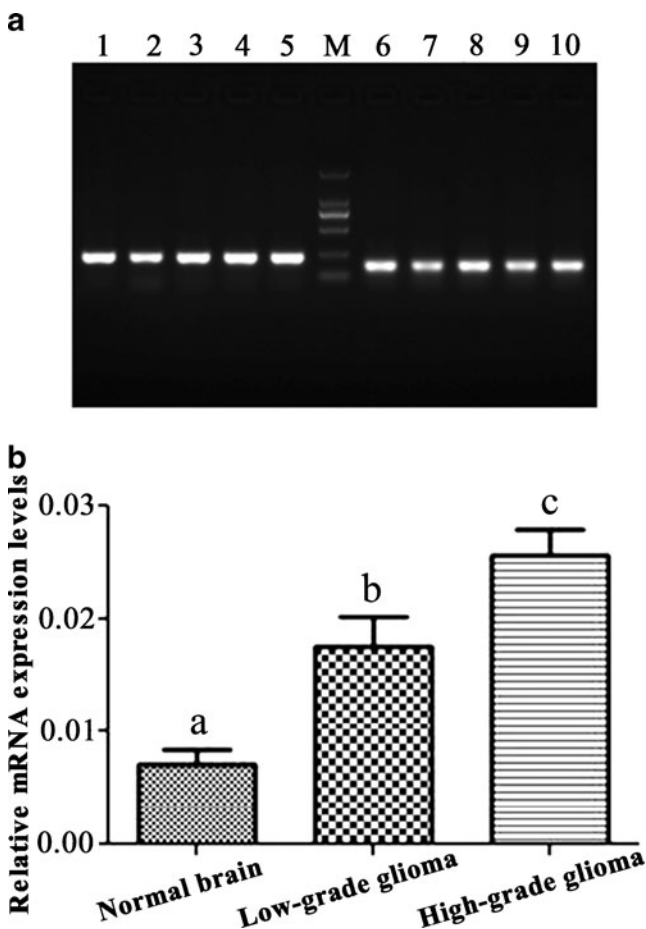

Fig. 1 *Gdnf* mRNA levels in glioma tissue and normal brain tissue. a *hGAPDH* and *gdnf* PCR results showing band lengths similar to the predicted values; *M* DL2000 marker; lanes *1–5* hGAPDH amplification using sample cDNA as a template, *6–10 gdnf* using sample cDNA as a template. **b** *Gdnf* mRNA levels. One-way ANOVA showed that *gdnf* mRNA expression was significantly different among normal brain tissue and low- and high-grade glioma tissues ($P<0.05$)

Single-Nucleotide Polymorphism Detection

DNA samples were extracted using QIAamp DNA Mini and QIAmp Blood Mini kits (Qiagen, Shanghai, China). Three pairs of primers (Table 2) were designed to amplify DNA segments in *gdnf* promoter regions I and II, and the products were purified and sent to Sangon Biotech (Shanghai, China) for sequencing. Through alignment analysis of the acquired sequences, we could determine whether there were abnormal SNP sites in *gdnf* promoter regions I and II of GDNF in glioma tissues (−1991 to +151 and +3952 to +4904).

BSP

DNA was extracted as described in "Enzyme-Linked Immunosorbent Assay" and treated with bisulfite in strict accordance with the EZ DNA Methylation-Gold™ kit instructions (Zymo, Irvine, CA, USA). Five pairs of BSP primers (Table 3) covering promoter regions I and II were designed to amplify the treated DNA templates, and the PCR reaction protocol was as follows: denaturation at 98 °C for 4 min; 98 °C for 15 s, 60 °C for 15 s, 72 °C for 30 s, repeat 30 cycles; 72 °C for 10 min.

PCR products were separated by agarose gel electrophoresis, and the target bands were retrieved using a gel extraction kit (Axygen, Union City, CA, USA) and ligated with pMD19-T (Takara Bio, Otsu, Japan), and then, they were transformed into DH5α competent cells. The positive clones determined by bacterium medium PCR were sent to Sangon Biotech for sequencing. The methylation status of each sample was analyzed based on the sequencing result using the QUMA web server at http://quma.cdb.riken.jp/.

ChIP-PCR

ChIP was performed according to the manufacturer's instructions of the EZ-ChIP chromatin immunoprecipitation kit (Millipore, Billerica, MA, USA). After the ChIP experiment, DNA binding to the target antibody was detected by real-time PCR. The PCR reaction system
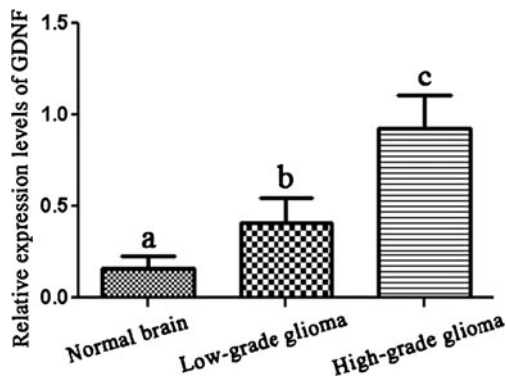
Fig. 2 GDNF protein expression in glioma and normal brain tissue. One-way ANOVA showed that GDNF protein expression was significantly different among normal brain tissue and low- and high-grade glioma tissue ($P<0.05$)

contained 12.5 µl SYBR Premix Ex Taq™ II (Takara Bio, Otsu, Japan), 1 µl each of upstream and downstream primers (10 µmol/l), and 3 µl DNA template. The total volume of 25 µl was reached by adding sterilized double-distilled water. Amplification was performed following a two-step PCR reaction protocol (predenaturation, 95 °C, 2 min; PCR reaction, 95 °C,

5 s; 60 °C, 4 s, 40 cycles), and relative quantitative analysis was carried out using the $2^{-\Delta\Delta Ct}$ method [4]. The primers (Table 4) were synthesized by Kangcheng Biotech Co., Ltd. (Shanghai, China).

Statistical Analysis

Data were analyzed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA) and expressed as mean ± standard deviation. The means of two samples were compared using independent samples t tests, and the means of multiple samples were compared using single factor analysis of variance (ANOVA). A value of $P<0.05$ was considered as significantly different.

Results

Gdnf Transcription is Significantly Increased in Glioma Tissue

The relative expression level of *gdnf* mRNA in normal brain and low- and high-grade glioma tissues was determined with real-time PCR. *Gdnf* mRNA was detectable in all three groups (Fig.
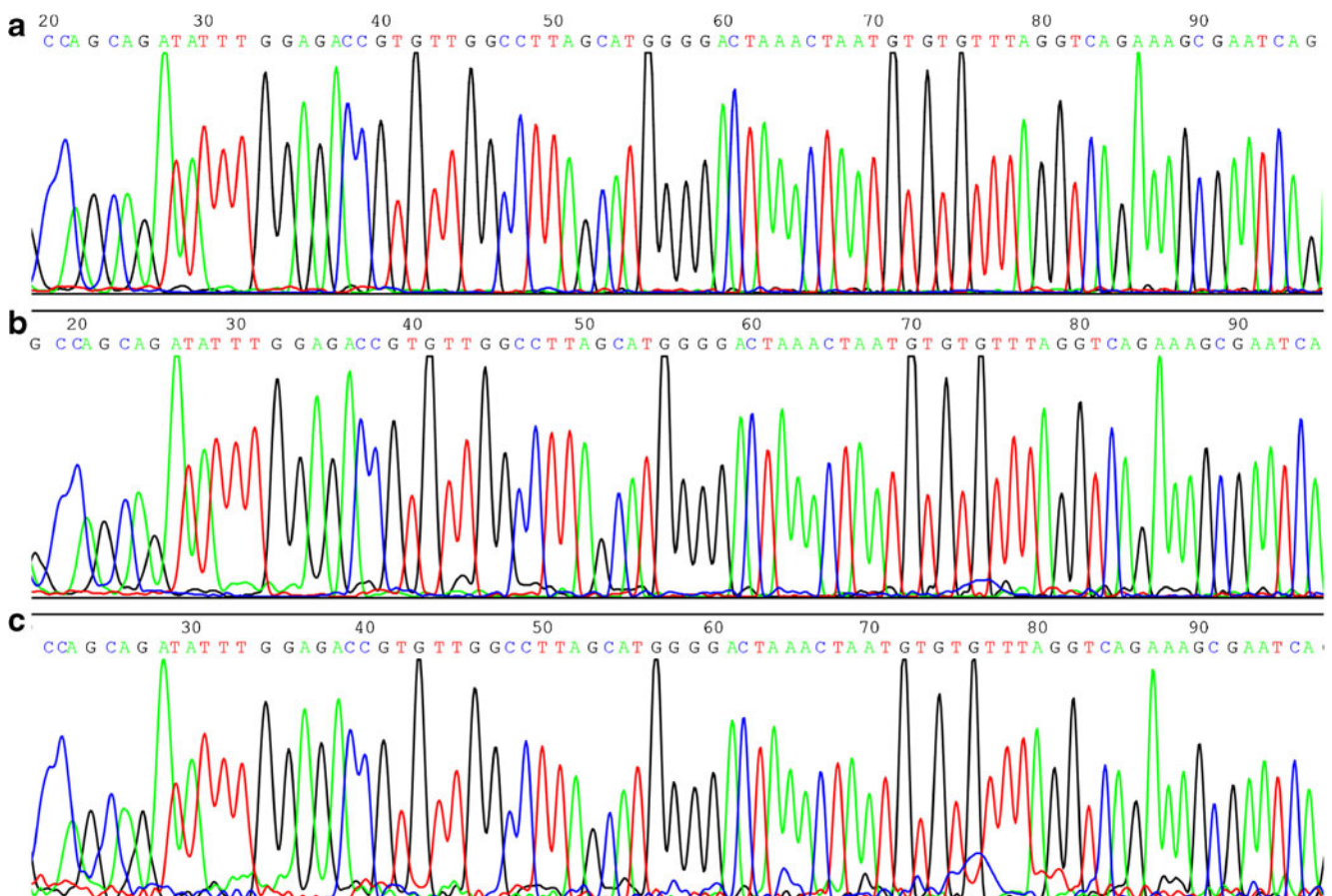

Fig. 3 The partial sequencing results of *gdnf*. a Normal brain tissue, b low-grade glioma tissue, c high-grade glioma tissue. The base sequence in the promoter was unchanged in low- and high-grade gliomas versus in normal brain tissue
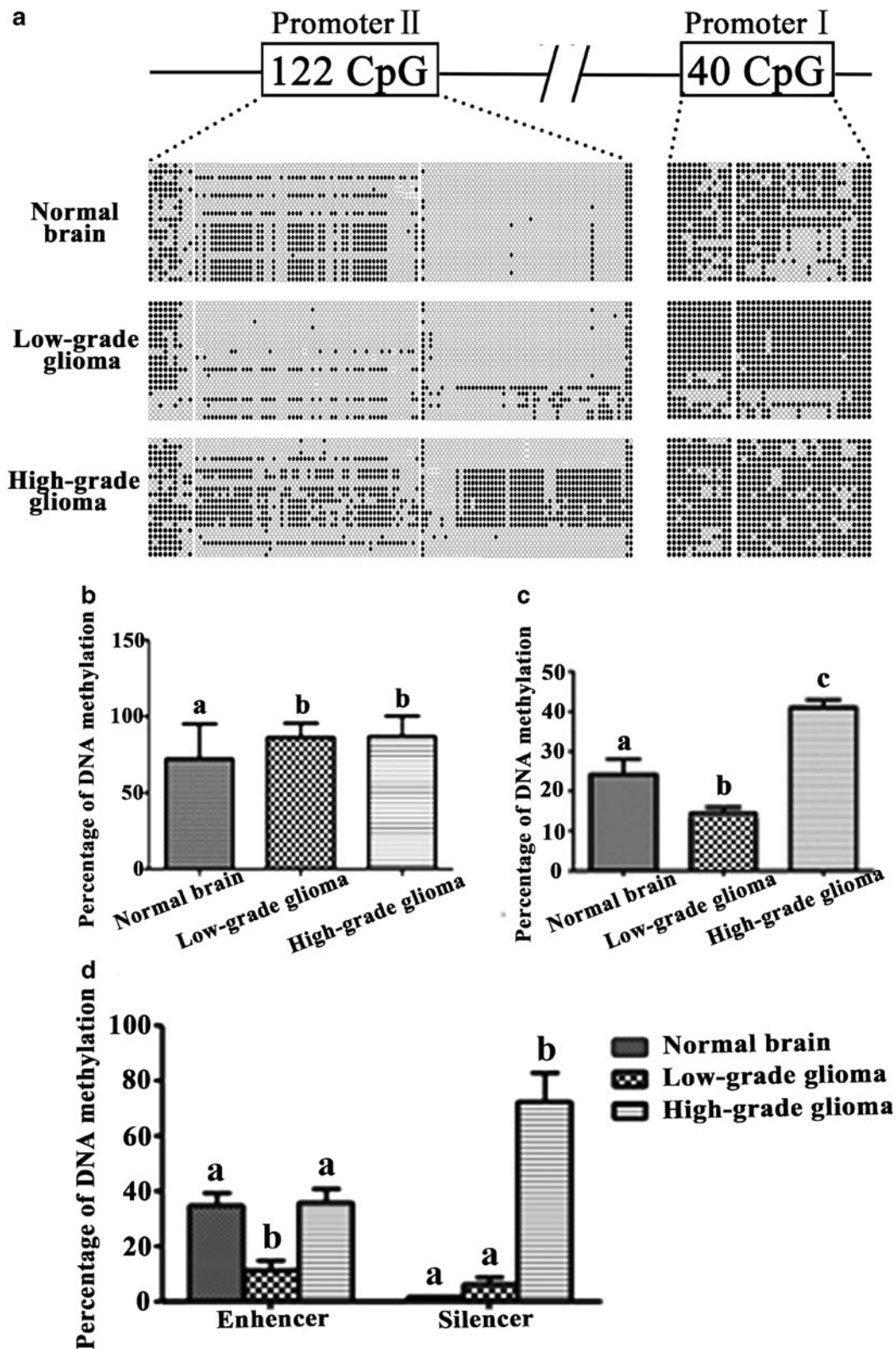
Fig. 4 Promoter region methylation in the *gdnf* gene. **a** Methylation status of each site in the two promoter regions detected by BSP; *black circles* indicate a methylated site, and *white circles* indicate a nonmethylated site. **b** Methylation status of promoter region I in normal brain tissue and low- and high-grade glioma tissues. **c** Methylation status of promoter region II in normal brain tissue and low- and high-grade glioma tissue. **d** Methylation status of *cis*-acting elements in promoter region II in normal brain tissue and low- and high-grade glioma tissues. One-way ANOVA showed that *gdnf* mRNA expression was significantly different among normal brain tissue and low- and high-grade glioma tissues ($P<0.05$)

1a), and the difference between glioma and normal brain tissues was statistically significant ($P<0.05$). *Gdnf* mRNA levels also increased with pathological grade of glioma tissue ($P<0.05$) (Fig. 1b).

GDNF Protein is Significantly Increased in Glioma Tissue

The relative protein level of GDNF was assessed with ELISA. Consistent with the mRNA results, the difference among the three types of tissue was statistically significant ($P<0.05$). GDNF protein was notably higher in glioma tissue compared to normal brain tissue and increased along with higher pathological grade ($P<0.05$) (Fig. 2).

The *gdnf* Promoter Region Base Sequence is Unchanged in Glioma

To clarify the underlying mechanism of *gdnf* overexpression in glioma tissue, we cloned, amplified, and sequenced the promoter region of the *gdnf* gene in normal brain and low- and high-grade gliomas. Contrastive analysis revealed that the base sequence in low- and high-grade glioma tissues was unchanged versus normal brain tissue (Fig. 3).

Methylation is Altered in Promoter Regions I and II of the *gdnf* Gene in Glioma Tissue

Next, we investigated the methylation status of promoter regions I and II of the *gdnf* gene using BSP (Fig. 4a). We found that methylation was significantly enhanced in promoter region I of the *gdnf* gene in glioma tissue compared to normal brain tissue ($P<0.05$), but there was no significant difference between low- and high-grade glioma tissues (Fig. 4b). Conversely, methylation of promoter region II was significantly decreased in low-grade glioma ($P<0.05$) and notably increased in high-grade glioma ($P<0.05$) (Fig. 4c). Through further analysis of the CpG islands in promoter region II, we found that sites with significantly decreased methylation in low-grade glioma were mainly located in the enhancer region ($P<0.05$), whereas those sites with significantly increased methylation in high-grade glioma were primarily localized in the silencer region ($P<0.05$) (Fig. 4d).

Changes of Methylation Level in Promoter Region I of the *gdnf* Gene Influenced its Expression in U251 Glioma Cell Line

In order to investigate the effect on methylation level of *gdnf* gene in promoter region I and its mRNA expression, the cell model was established by using 5-aza-CR to alter DNA methylation in U251 human glioma cell line. The results showed that the methylation level of *gdnf* gene in promoter region I began to decline after application with 5-aza-CR, the methylation level dropped significantly when adding 5 µmol/l 5-aza-CR ($P<0.05$) (Fig. 5a). In contrary, the expression of *gdnf* mRNA researched the highest (Fig. 5b). The methylation level of *gdnf* gene in promoter region I was no longer changed when higher concentrations of 5-aza-CR (10 or 20 µmol/l) were added, but the expression of *gdnf* mRNA began to decline ($P<0.05$) (Fig. 5b).

Transcription Factor Binding to the *gdnf* Gene

We predicted transcription factors that might bind to *gdnf*'s promoter region using web-based tools (http://www.gene-regulation.com; Table 1 in Supplementary Material). We found that binding sites for AP-2, CREB, EGR-2, and SP1 were located in the region of the promoter where we had observed abnormal methylation. ChIP-PCR was employed to detect the binding of these four transcription factors to promoter regions I and II of the *gdnf* gene. The results showed that the binding capacity of the GDNF for these four transcription factors was altered to different degrees in glioma tissue (Tables 2 and 3 in Supplementary Material).
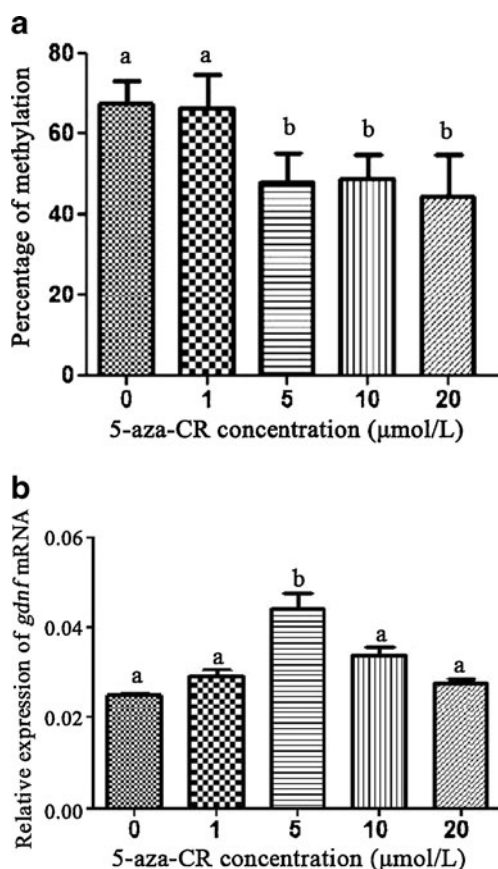

Fig. 5 Effect of methylation state in *gdnf*'s promoter region I on its mRNA expression after treating with different doses of 5-aza-CR in U251 glioma cells. **a** Effect of different doses of 5-aza-CR on methylation state in *gdnf*'s promoter region I. **b** Effect of different doses of 5-aza-CR on the mRNA expression level of *gdnf*' gene

The statistical results of these experiments are summarized in Fig. 6.

Discussion

GDNF is an important neurotrophic factor that has been intensely studied since its discovery in the late twentieth century. Due to its effect on dopaminergic neurons, GDNF has attracted considerable attention as a potential therapy for Parkinson's disease [10]. Recent studies have demonstrated a relationship between GDNF and glioma; *gdnf* expression level is significantly higher in glioma compared to normal glial cells [11]. To clarify the underlying mechanism of aberrant *gdnf* expression in glioma tissue, we investigated

the methylation status of the promoter region of the *gdnf* gene and assessed the binding of specific transcription factors to the promoter region.

The *gdnf* gene has two promoter regions I which is upstream of exon 4 and II which is found to be an upstream region of exon 1. Baecker et al. [12] found that the transcriptional activity of promoter region II was stronger and thus concluded that region II played an important role in transcription initiation. To determine if mutations in the promoter region were involved, we assessed *gdnf* promoter region sequences in different grade gliomas; however, we did not identify any SNP sites that could discriminate glioma tissue from normal brain tissue. Considering other work in our lab that revealed that there was no mutation in the *gdnf* coding region sequence in glioma (data not shown), we
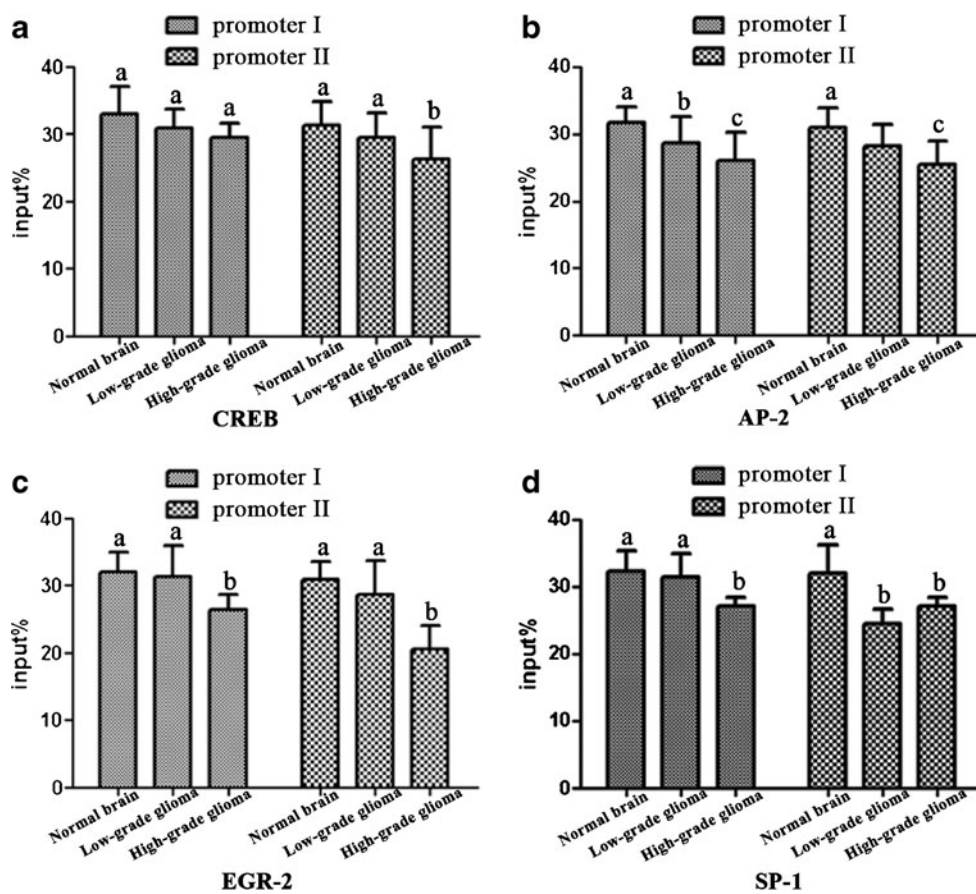

Fig. 6 Transcription factors binding capacity on promoter regions I and II of the *gdnf* gene. **a** AP-2 binding to promoter region I decreased along with greater pathological degree of glioma; the differences among the three groups were statistically significant (*P*<0.05). AP-2 binding to promoter region II also decreased along with higher pathological degree of glioma, and the differences among the three groups were statistically significant (*P*<0.05). **b** CREB binding to promoter region I varied among the three groups (*P*>0.05). A significant difference in CREB binding capacity was only observed in high-grade glioma tissue, which was decreased versus normal brain tissue (*P*<0.05). **c** ERG-2 binding to promoter region I was not significantly different between normal brain tissue and low-grade glioma tissue, but

it significantly decreased in high-grade glioma tissue versus normal brain tissue and low-grade glioma tissue (*P*<0.05). There was no significant difference in ERG-2 binding capacity on promoter region II between normal brain tissue and low-grade glioma tissue, but it was significantly decreased in high-grade glioma tissue (*P*<0.05). **d** SP1 binding to promoter region I showed no significant difference between normal brain tissue and low-grade glioma tissue, whereas it significantly decreased in high-grade glioma tissue versus normal brain tissue (*P*<0.05); the binding capacity of SP1 on promoter region II significantly decreased in glioma tissue and was the lowest in low-grade glioma tissue; the differences among them were statistically significant (*P*<0.05)

surmised that the observed abnormal *gdnf* expression might be caused by epigenetic modifications.

A 2010 study showed that methylation on the 5′-side region of the *gdnf* gene promoted tissue development, neurogenesis, and neural proliferation; furthermore, this modification was closely related to upregulated transcription [13]. This led us to hypothesize that the increase in *gdnf* expression in glioma cells might be due to changes in the methylation of the promoter region of the target gene. We assessed the methylation status of both *gdnf* promoter regions in glioma tissue with BSP and found that promoter region I methylation was increased in glioma tissue. After the promoter of cancer suppressor gene Rb was found to have altered CpG island methylation, several genes were subsequently found, including p16, DAPK, and EBER1. Due to high methylation in the promoter region of these genes, they were silenced in specific tumors [14, 15].

Hypermethylation of *gdnf* promoter region I does not result in GDNF gene silencing; rather, *gdnf* transcription is highly active in glioma cells. Thus, we speculated that the "deactivation" of promoter region I was compensated by activation in promoter region II. In assessing the methylation status of promoter region II, we found that it was significantly decreased in low-grade glioma tissue and significantly elevated in high-grade glioma. Further sequence analysis revealed that the hypermethylated sites were located in the enhancer element of promoter region II in low-grade glioma tissue and in the silencer element of promoter region II in high-grade glioma tissue. Based on available methylation information, we speculated that promoter region II hypomethylation resulted in enhancer activation, whereas promoter region II hypermethylation caused silencer inhibition. Both of the modifications would ultimately induce *gdnf* gene transcription; however, how these modifications could do so was still unclear.

In addition, we investigated the effect on methylation level of *gdnf* gene in promoter region and its mRNA expression in the cell model mentioned in the "Materials and Methods." The results showed that 5-aza-CR can change the methylation level of *gdnf* gene in promoter region. The change of methylation level of *gdnf* gene in promoter region I influenced the expression of *gdnf* mRNA in glioma cell. Interestingly, we found the methylation level of *gdnf* gene in promoter region I was no longer changed when higher concentrations of 5-aza-CR (10 or 20 μmol/l) were added, but the expression of *gdnf* mRNA began to decline. It was suggested that the demethylation of the silencers in promoter region II may be the reason for the downregulation of the *gdnf* mRNA after treating with the high concentrations of 5-aza-CR.

Several studies have shown that DNA methylation is related to the binding affinity of transcription factors, thus providing new insights for exploring the abnormal gene expression in tumor cells [16]. Through bioinformatic analysis, we identified four transcription factors AP-2, CREB, EGR-2, and SP1 that could bind to the methylated promoter region and detected alterations in their binding using ChIP-PCR. The binding capacities of AP-2, CREB, EGR-2, and SP1 declined with increasing pathological degree of glioma, and the difference between high-grade glioma and normal brain tissue was significant ($P<0.05$). These results indicate that transcription factor binding to the *gdnf* promoter is inhibited by methylation to some extent. AP-2, a DNA-binding protein that binds to a special sequence, regulates the transcription of many genes and participates in cell proliferation, differentiation, apoptosis, and carcinogenesis [17]. Here, we found that the binding capacity of AP-2 on promoter regions I and II of the *gdnf* gene decreased along with the increase in the pathological degree of glioma. However, it is difficult to identify the critical site involved, as there are many AP-2 binding sites in the promoter region. CREB is a nuclear transcription that binds to target DNA via its C-terminal basic amino acids [18]. Studies have shown that overexpression of CREB can induce cell apoptosis, and it is known to participate in tumor pathogenesis through its regulation of cAMP response elements on the promoters of many tumor-related genes [19]. There are two specific CREB binding sites on the enhancer and silencer elements of *gdnf*'s promoter region, respectively, making it an ideal candidate for studying transcription factor binding and *cis* element methylation. We found that CREB binding to the promoter region II of *gdnf* gene was decreased in high-grade but not low-grade glioma. Considering the methylation alterations in the *cis* elements in the promoter region, we hypothesized that abundant CREB binding in low-grade glioma was due to enhancer hypomethylation, while the decrease in high-grade glioma was due to silencer hypermethylation. Biological analysis of the promoter *gdnf* gene showed that EGR-2 merely bound to the enhancers in the core promoter region of *gdnf* gene, which provided us an ideal opportunity to investigate enhancer methylation and transcription factor binding activity. Analysis of enhancer element methylation in the promoter region of the *gdnf* gene showed that the promoter was hypo- and hypermethylated in low- and high-grade glioma tissues, respectively. EGR-2 binding to the promoter was only significantly decreased in high-grade glioma tissue, suggesting that the decrease was due to methylation in the enhancer region. SP1 plays important roles in various tumor cells during growth and metastasis through modulation of oncogenes and tumor suppressor genes [20, 21]. Our findings show that the binding capacity of SP1 on the promoter region of *gdnf* gene is decreased in glioma. Interestingly, the binding capacity of SP1 on the promoter region of *gdnf* gene increased with glioma progression; the specific mechanism underlying this phenomenon remains to be clarified.

Collectively, we investigated the methylation status of the promoter region of *gdnf* gene in glioma tissue and assessed transcription factor binding to the promoter region. We found that the methylation status of the promoter region of *gdnf* gene varied in different grades of glioma, and transcription factor binding to the methylated promoter region also changed with disease stage. We posit that transcription factor binding to the promoter region of *gdnf* gene, especially the *cis* elements, was affected by altered methylation of the promoter region of the *gdnf* gene, which resulted in aberrant regulation of *gdnf* gene transcription and overexpression. Our results provide a novel avenue to explore the molecular mechanism governing *gdnf* overexpression in glioma.

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