

Original Article

Methylation status of promoter 1 region of GDNF gene in human glioma cells

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Abstract: Objective: This study aimed to investigate the methylation status of promoter 1 region of glial cell line-derived neurotrophic factor (GDNF) in human glioma cells and to explore the effect of GDNF methylation on the expression of GDNF in glioma. Methods: GDNF gene mutation was detected by sequencing in 10 patients with glioma and 5 healthy controls. Bisulfite modification for analysis of DNA methylation was done to detect the methylation status of promoter 1 region of GDNF in 20 patients with glioma (10 with poorly differentiated and 10 with well differentiated) and 5 healthy controls. Results: There was no mutation at the promoter 1 region of GDNF gene in glioma. The incidence of methylation of GDNF gene at the promoter 1 region in healthy control, patients with poorly differentiated glioma and those with well differentiated glioma was 72.25%, 86.25% and 86.75%. The incidence of GDNF methylation in glioma was significantly higher than that in the normal brain ($P < 0.05$); while there was no significant difference between well differentiated glioma and poorly differentiated glioma. Conclusions: Hypermethylation occurs in the promoter 1 region of GDNF and may influence the expression of GDNF in glioma.

Keywords: Glioma cell, glial cell line-derived neurotrophic factor, promoter, methylation

Introduction

Glioma is the most common primary tumor in the brain [1, 2], and its occurrence and development are complex. Abnormal or silence expression of genes secondary to gene mutation and epigenetic modifications play important roles in the occurrence and development of tumors [3]. Of these modifications, methylation of the promoter of genes related tumors is often found to affect the expression of these genes. In normal cells of humans, the CpG island of the promoter is usually in a non-methylated status, which assures the normal expression of a specific gene [4]. However, sporadic CpG dinucleotides in the genome are often methylated, which is better to maintain the stability of chromosome structure. The methylation status is relatively stable in the genome, but it changes significantly in cancer cells [5]. To elucidate the methylation status of promoter 1 of glial cell line-derived neurotrophic factor (GDNF), the initial sequence of promoter 1 of

GDNF and that after bisulfite modification were compared between glioma and normal brain tissues, and the change in the methylation status of promoter 1 of GDNF was further analyzed. Our findings may provide evidence for further elucidation of change in the epigenetic modification of GDNF in the glioma.

Materials and methods

Sample collection and reagents

The normal brain tissues were collected from 5 patients with acute brain trauma who received intracranial decompression and glioma tissues was collected from 20 patients with glioma (WHO grades 1-4) in the Central Hoospital of Wenzhou City. Primary sequencing for SNPs and for BSP methylation was done in samples randomly collected from patients who did not receive pharmacotherapy. Glioma of pathologic grade 1-2 was classified as low grade one and that of pathologic grade 3-4 as high grade one.

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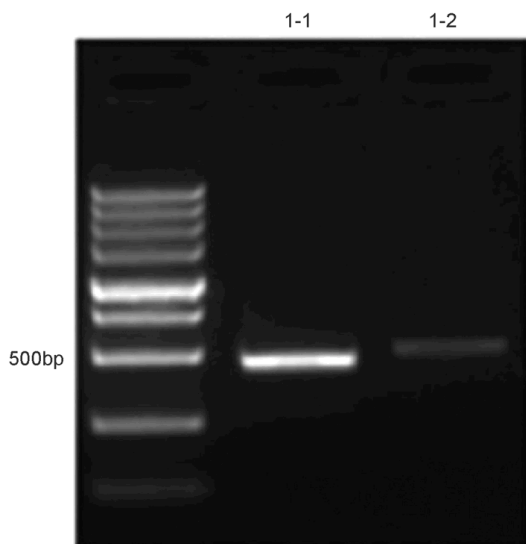


Figure 1. Products from PCR after bisulfite treatment.

Following reagents were used in the present study: QIAamp DNA Mini and Blood Mini Handbook (QIAGEN; Germany), DNA Gel Extraction Kit (Shanghai Genebase Gene-Tech Co., Ltd), EZ DNA Methylation-Gold™ Kit (Zymo Research Biotech, USA).

Sequencing for SNP analysis

QIAamp DNA Mini and Blood Mini Handbook (QIAGEN) was used for DNA extraction. In brief, tissues were cut into blocks (about 25 mg) and then placed in 1.5 ml-tubes, followed by addition of Buffer ATL (180 μ L) and protein K (20 μ L). Then, this mixture was incubated at 56°C until the blocks were absent. Centrifugation was done, and the supernatant was removed, followed by addition of Buffer AL (200 μ L). After incubation at 70°C for 10 min, centrifugation was done, and the supernatant was removed. Following addition of absolute ethanol (200 μ L), centrifugation was performed, and the mixed solution was transferred into 2-ml QM5C, followed by centrifugation at 8000 rpm for 1 min. Then, QM5C was placed into a 2-ml collector, followed by addition of Buffer AW1 (500 μ L) and subsequent centrifugation at 8000 rpm for 1 min. QM5C was placed into a 2-ml collector, and centrifugation was done at 14000 rpm for 3 min following addition of Buffer AW2 (500 μ L). QM5C was placed into a 1.5 ml-EP tube and allowed to stay at room temperature for 1 min following addition of Buffer AE (200 μ L). Centrifugation was done for 1 min at 8000

rpm, and solution was harvested. Primers targeting the promoter I region of DNA was designed as follows: 5'CAGAGAATCTCAAAGGT-GCA3' (forward), 5'ACGACATCCCATAACTTC3' (reverse). Pre-denaturation was done at 98°C for 4 min, 30 cycles of denaturation at 98°C for 15 s, annealing at 60°C for 15 s and extension at 72°C for 30 s, and a final extension at 72°C for 10 min. The products were subjected to gel electrophoresis, and the gel was retrieved for detection. Sequencing was done to detect the abnormal SNPs of promoter I region of GDNF gene.

Sequencing for BSP methylation

QIAamp DNA Mini and Blood Mini Handbook (QIAGEN) was used for DNA extraction, and procedures were abovementioned above. Bisulfite treatment was done with EZDNA Methylation-Gold™ Kit. In brief, 1 μ g of DNA (20 μ L) was collected from each sample, and then mixed with 130 μ L of CT Conversion Reagent, followed by centrifugation. The mixture was incubated at 98°C for 10 min, 64°C for 2.5 h and 4°C for 16 h. Then, 600 μ L of M-Binding Buffer was added to Zymo-Spin IC Column and then placed in a collection tube. Products collected after centrifugation (150 μ L) were added to 600 μ L of above Binding Buffer, followed by vortexing. Centrifugation was done at >10000 rpm for 30 s. The supernatant was removed. Then, 200 μ L of wash buffer was added, followed by centrifugation for 30 s at >10000 rpm. Following addition of desulphonation buffer (200 μ L) into the column, incubation was done at room temperature (20°C-30°C) for 15-20 min, followed by centrifugation at >10000 rpm for 30 s. After addition of 200 μ L of wash buffer, centrifugation was done for 30 s at >10000 rpm. The column was transferred into a new 1.5-ml tube, followed by addition of 20 μ L of Elution Buffer into the column. The column was allowed to stay for 3-5 min, followed by centrifugation at >10000 rpm for DNA elution. Then, DNA was immediately used for expansion by PCR or stored at -20°C. Two pairs of primers were designed targeting the promoter I region of BSP as follows: Primer 1: 5'GAAGGGATTAGGGTTAG-AATTTTT3' (forward), 5'CCCAAACAAAACRATA-TTTCT3' (reverse); Primer 2: 5'ATGYGTTTGATTT-TATTTTTAAAGA3' (forward), CTACRCRAACAAAC-RAAA3' (reverse). Reaction mixture was composed of 10 \times buffer (5 μ L), 50 mM MgCl₂ (2 μ L),

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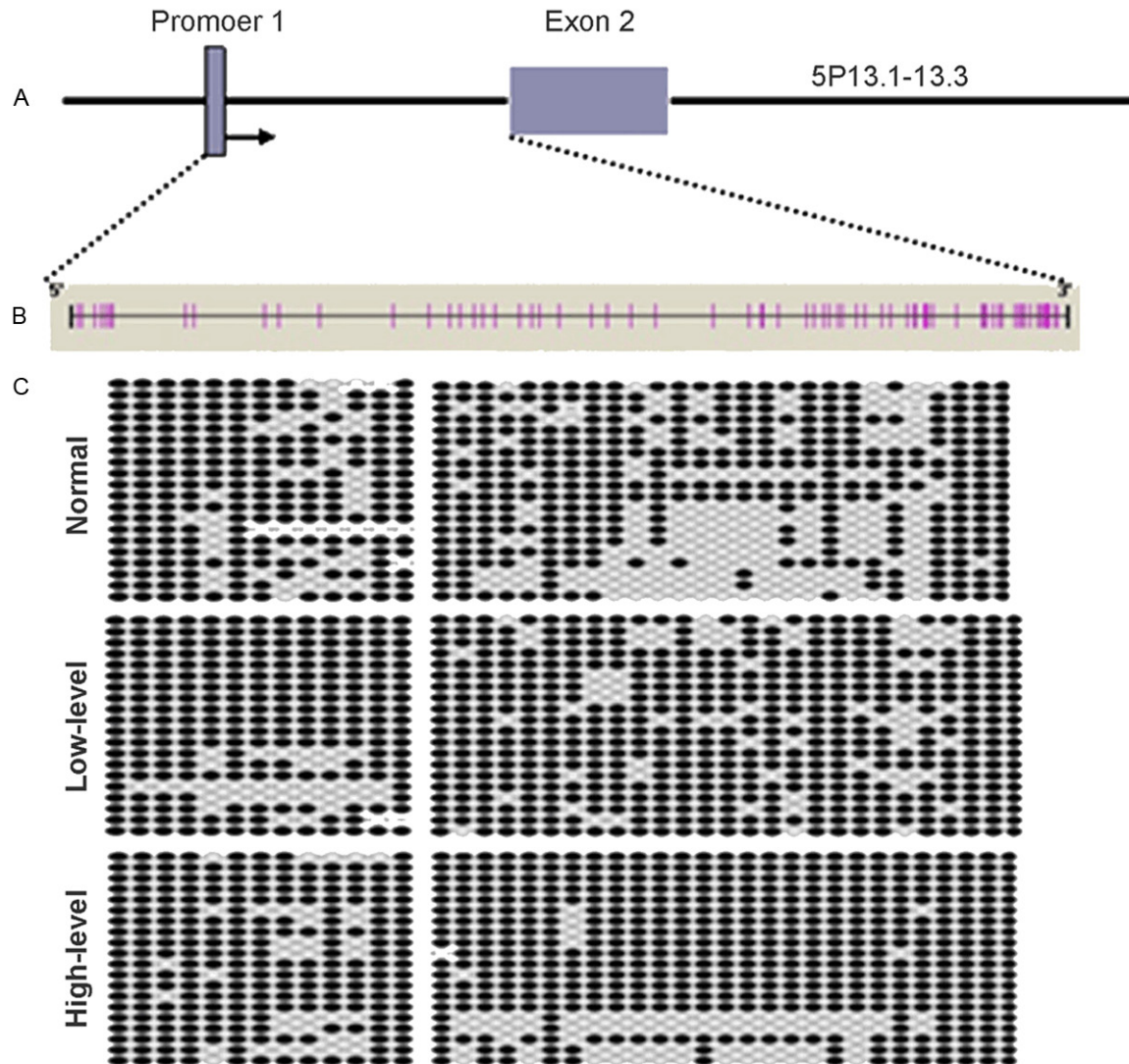


Figure 2. Sequencing of promoter 1 region of GDNF gene after bisulfite treatment. The promoter I region locates at about 1000 bp preceding the exon 2 of GDNF gene (A). There were 40 CpG sites in the promoter I region (B). After bisulfite treatment, sequencing was done (black: methylation; white: non-methylation) (C).

10 mM dNTP (1 μ L), forward primer (2 μ L), reverse primer (2 μ L), DNA (5 μ L), Platinum Taq (Invitrogen; 0.3 μ L) and ddH₂O (32.7 μ L). PCR was done at 95°C for 5 min, 42 cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 40 s, and a final extension at 72°C for 10 min. Products after PCR were connected to pMD19-T (Takara) at room temperature for 2 h. Then, 10 μ L of products after connection was used to infect competent DH5a cells which was then smeared on the Lysogeny Broth (LB) containing the antibiotic ampicillin (Amp), followed by incubation at 37°C over night. Eight colonies were selected from each plate for identification by PCR. Five positive colonies were seeded into 3 ml of

liquid LB, followed by incubation at 37°C over night. Plasmid Miniprep Kit (Axygen) was used for plasmid extraction. Then, plasmids were subjected to sequencing. The extent of methylation was analyzed on the database (<http://quma.cdb.riken.jp/>).

Statistical analysis

Statistical analysis was done with SPSS version 16.0. Data were expressed as mean \pm standard deviation (SD). Comparisons of data among groups were done with one way analysis of variance. A value of $P < 0.05$ was considered statistically significant.

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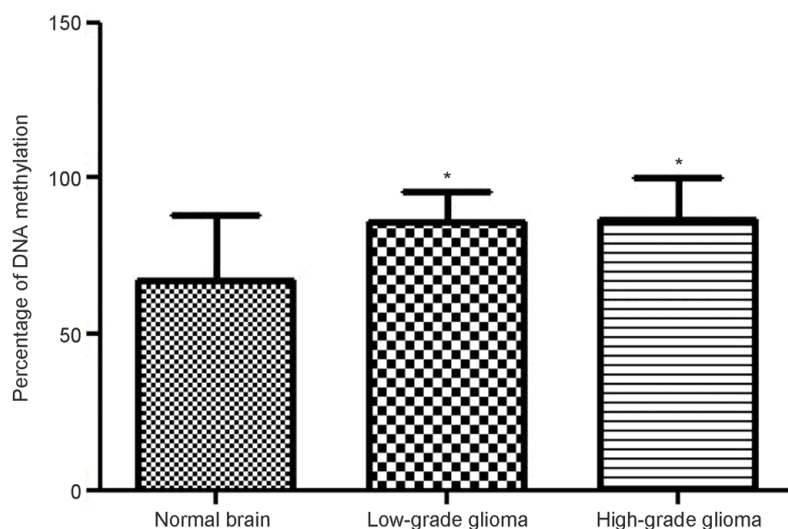


Figure 3. Methylation status of promoter 1 region of GDNF gene.

Results

Sequencing

There was no GDNF gene mutation in glioma and normal brain tissues.

PCR after bisulfite treatment

After expansion with primer 1 and primer 2, the promoter 1 region was identified at 469 bp and 507 bp, respectively (**Figure 1**).

Methylation status of promoter 1

The promoter 1 region locates at about 1000 bp preceding the exon 2 of GDNF gene (**Figure 2A**). It was predicted that there were 40 CpG sites in the promoter 1 region (**Figure 2B**). After bisulfite treatment, sequencing was done (**Figure 2C**) (black: methylation; white: non-methylation). Statistical analysis showed the proportion of tissues with methylation of promoter 1 of GDNF gene was 72.25%, 86.25% and 86.75% in normal brain tissues, low grade glioma and high grade glioma. Significant difference was noted between normal brain tissues and low or high grade glioma, but there was no marked difference between low grade glioma and high grade glioma (**Figure 3**).

Discussion

GDNF belongs to the TGF- β superfamily and is a new neurotrophic factor first identified, puri-

fied and cloned from B49 glioma cell line in 1993 by Lin et al [6]. GDNF gene is mapped to 5P13.1-P13.3. It has neurotrophic effect on multiple types of cells and has been regarded an important bioactive neurotrophic factor [7, 8]. In our study, results showed the primary GDNF sequence was identical between glioma and normal brain tissues, and the methylation of promoter 1 region of GDNF region increased markedly. This indicates that GDNF gene is not mutated and the highly methylated promoter 1 region of GDNF gene is likely an important factor causing silencing of promoter 1 region in the glioma.

Epigenetic modifications include DNA methylation, histone modification, nucleosome positioning and non-coding RNA. Of these modifications, DNA methylation has been deeply studied in mammals [9, 10]. DNA methylation induced gene silencing, together with histone modification and modifications of nuclear chromatin-associated proteins, is able to regulate the expression of genes. In cancer cells, the epigenetic modifications are characterized by whole genomic methylation and high CpG methylation of the promoter [11]. In cancer cells, high DNA methylation may cause gene silencing, which has been confirmed [12, 13]. Since the CpG methylation was first identified at the promoter of Rb tumor suppressor gene, methylation is identified in a lot of genes. Studies have shown that DNA methylation of p16, MLH1 and BRCA1 may cause these gene silencing [14, 15]. In our study, high methylation was found at the promoter 1 region of GDNF gene, which might cause promoter 1 region silencing in the glioma. However, a lot of findings suggest that GDNF expression is higher than that in normal brain tissues [16], which seem to be inconsistent with the fact of silencing of promoter 1 region due to its methylation.

GDNF gene is one with two promoters including promoter 1 and promoter 2. Suter-Crazzolaro et al. [17] found that promoter 1 region locating at

the up-stream of exon 2 had sites with high transcriptional activities. However, Baecker et al. [18] proposed promoter 2 played a major role in the transcription initiation. In different tissues and at different stages of development and diseases, the role of each promoter is distinct. In our study, results showed the promoter 1 region was highly methylated in GDNF. Some studies also reveal that the GDNF expression increases markedly in glioma [16]. Thus, we speculate that there might be low methylation of promoter 2 region of GDNF gene. In glioma, the methylation of promoter 1 region of GDNF causes the silencing of promoter 1 region, but that of promoter 2 region leads to the activation of promoter 2 region. In several studies on transcriptional factors of GDNF promoters, findings showed there were inhibitors targeting the promoters, which may cause low activity of these promoters, and the activity of these transcriptional factors of promoters plays important roles in the transcriptional expression of GDNF gene.

In the present study, GDNF gene was sequenced, and results showed there were no mutations on GDNF gene. After bisulfite treatment, the promoter 1 region of GDNF gene was highly methylated, as shown by sequencing. Our findings provide evidence for further investigations on the epigenetic modifications in glioma.

Acknowledgements

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Disclosure of conflict of interest

None.

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