Tumor suppressor candidate gene, *NDRG2* is frequently inactivated in human glioblastoma multiforme

BIN ZHOU[1,2*], ZHUO TANG[1,3*], YANCHUN DENG[1*], SHUANGXING HOU[1], NAN LIU[1,4], WEI LIN[5], XINPING LIU[6] and LIBO YAO[6]

[1]Department of Neurology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710032; [2]Department of Traditional Chinese Medicine, 451 Hospital of PLA, Xi'an, Shaanxi 710054; [3]Department of Internal Medicine, The 518th Hospital of PLA, Xi'an, Shaanxi 710043; [4]Department of Neurology, 451 Hospital of PLA, Xi'an, Shaanxi 710054; [5]Department of Neurosurgery, Xijing Hospital Fourth Military Medical University; [6]Department of Biochemistry and Molecular Biology, Fourth Military Medical University, Xi'an, Shaanxi 710032, P.R. China

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Abstract. N-myc downstream regulated gene 2 (*NDRG2*) is highly expressed in numerous normal tissues, while it is marginally expressed or undetectable in various tumors, including lung and colon cancer. In order to investigate the expression of NDRG2 in human glioma and its downstream regulatory mechanisms, quantitative polymerase chain reaction (qPCR), immunohistochemistry and western blot analyses were used to assess NDRG2 mRNA and protein expression in different grades of human glioma and adjacent normal tissues. The methylation status of the NDRG2 promoter region was also determined using bisulfite sequencing. NDRG2 mRNA expression was observed to be significantly lower in glioma tissues than in adjacent normal tissues ($P<0.05$). Furthermore, a significant negative correlation was found between the glioma tumor grade and NDRG2 expression ($P<0.05$), at the mRNA and protein levels. Moreover, the methylation rate of the NDRG2 promoter region was 46.3% in the glioma tissues compared with 18.2% in the adjacent normal tissues ($P<0.05$). These findings show that NDRG2 expression is downregulated in human glioma and that the level of NDRG2 expression negatively correlates with the glioma grade. Furthermore, these findings indicate that NDRG2 downregulation may be due to aberrant methylation of the NDRG2 promoter region and subsequent transcriptional inactivation.

Correspondence to: Dr Yanchun Deng, Department of Neurology, Xijing Hospital, Fourth Military Medical University, Changlexi Road, Xi'an, Shaanxi 710032, P.R. China
E-mail: ychun_deng@163.com

*Contributed equally

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Introduction

Glioma is the most common human brain tumor and is associated with high mortality and disability. Traditional therapies, including surgery, radiotherapy and chemotherapy have not achieved satisfactory outcomes. As with other types of tumor, genetic mutation is likely to be the cause of glioma. It is possible to treat glioma through regulating the expression of certain genes. The human N-myc downstream regulated gene (*hNDRG*) family is a family of differentiation-associated genes, which consists of four members, *NDRG1*, *-2*, *-3* and *-4* (1). *NDRG2* was originally cloned from a normal human whole brain cDNA library through subtractive hybridization in our laboratory (Experimental Teaching Center of Basic Medicine, The Fourth Military Medical University, Xi'an, China) in 1999 when analyzing different genes in gliomas and their associated normal tissue. The *NDRG2* gene is located on chromosome 14q11.2 (2) and it has been reported that *NDRG2* may function as a tumor suppressor gene (3). *NDRG2* is highly expressed in numerous normal tissues, whereas its expression is low or undetectable in various tumors, including lung and colon cancer. Furthermore, transfection of *NDRG2* into human cancer cell lines results in growth inhibition (4-6). Therefore, in the present study, it was hypothesized that *NDRG2* is a tumor suppressor gene, which may be involved in tumorigenesis and tumor progression. *NDRG2* expression was assessed in human gliomas, and the methylation status of the CpG islands within the *NDRG2* promoter region was analyzed in the glioma samples and their adjacent tissues. Furthermore, the correlation between *NDRG2* expression and the methylation status of the *NDRG2* promoter was investigated. The findings of the present study support a role for *NDRG2* as a tumor suppressor gene and provide a basis for further investigation into the mechanism underlying *NDRG2*-induced tumor growth inhibition.

Materials and methods

Tissue samples. The present study was approved by the Ethics Committee of the Fourth Military Medical University (Xi'an, China). Fifty-three glioma tissue samples and 26 adjacent normal tissue samples were collected from patients who underwent surgery at the Xijing and Tangdu Hospital of the Fourth Military Medical University (Xi'an, China) between November 2006 and September 2007. The patients included 31 males and 22 females. The median patient age was 42 years (range, 13-70 years). None of the patients had received radiotherapy or chemotherapy prior to surgery. Among the patients included in the present study, there were 24 cases of astrocytoma, 19 cases of anaplastic astrocytoma and 10 cases of glioblastoma multiform astrocytoma. Tumors were graded according to the pathological classification criteria of glioma established by the World Health Organization (7). There were 24, 19 and 10 cases of grade II, III and IV tumors, respectively. All patients provided informed consent according to institutional guidelines and remained under continuous medical supervision and assistance in accordance with the Declaration of Helsinki. All samples were snap-frozen in liquid nitrogen and stored at -70˚C until analysis, or fixed in 10% formaldehyde and embedded in paraffin for subsequent analysis.

Cell culture. U251 human glioblastoma cells were obtained from American Type Culture Collection (Rockville, MD, USA) and were cultured in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA, USA) with 10% fetal bovine serum in a humidified 5% CO_2 atmosphere at 37˚C. U251 cells served as a typical glioblastoma sample for the subsequent methylation analysis.

Quantitative polymerase chain reaction (qPCR). Total RNA was extracted from each tissue sample (50-100 μg) using TRIzol® reagent (Invitrogen Life Technologies) according to the manufacturer's instructions. Total RNA was reverse-transcribed using a ThemoScript™ RT-PCR system (Invitrogen Life Technologies). Primers were designed from the conserved region of their respective cDNA sequences and were synthesized by Beijing Aoke Biotechnology Co., Ltd. (Beijing, China). The primer sequences were as follows: Forward, 5'-GAG ATA TGC TCT TAA CCA CCC G-3' and reverse, 5'-GCT GCC CAA TCC ATC CAA-3' for *NDRG2* (GenBank accession no. AF159092; amplicon size, 90 bp); and forward, 5'-ATC ATG TTT GAG ACC TTC AAC A-3' and reverse, 5'-CAT CTC TTG CTC GAA GTC CA-3' for *β-actin* (GenBank accession no. NM001101; amplicon size, 318 bp). The ΔΔCT method (8) was used to calculate the fold gene expression relative to the lowest tumor grade in the specific analysis. Human β-actin mRNA served as the reference transcript.

Western blot analysis. Total protein was extracted from the 100-μg tissue samples from each of the eight cases of grade II, III and IV tumors using 1 ml lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.25% sodium taurodeoxycholate, 50 mM NaF, 5 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 mM Na_3NO_4, 20 μg/ml aprotinin, 1 μg/ml leupeptin and 1 μg/ml pepstatin) as described previously (9). Equal quantities of protein were resolved using discontinuous SDS-PAGE (6% lamination gel and 12% separation gel). Proteins were transferred from the gel onto a pyroxylin membrane (Takara, Dalian, China) for 2.5 h at a constant voltage of 100 V. The membranes were blocked with Tris-buffered saline (TBS; pH 7.0) containing 5% non-fat dried milk for 1 h at room temperature. The membrane was subsequently washed in TBS containing Tween-20 (TBST) and incubated overnight at 4˚C with anti-NDRG2 (Abnova Co., Ltd., Taipei, Taiwan) and -tubulin antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Following agitation for 1 h using a Vortex Genie 2 (Scientific Industries, Inc., Bohemia, NY, USA) the membranes underwent four 5 min washes with TBST. The membranes were incubated with two horseradish peroxidase-conjugated secondary antibodies (Beijing Zhongshan Jinqiao Biotechnology Co. Ltd., Beijing, China) at room temperature and were agitated for 1 h in a shaker and washed with TBST four times. Immunoreactive bands were visualized using an enhanced chemiluminescence (ECL) method and ECL reagents (Pierce Biotechnology, Rockford, IL, USA).

Immunohistochemistry. In order to detect the expression of NDRG2, the tissue samples were subjected to immunohistochemistry as previously described (10). In brief, 5-μm sections of paraffin-embedded, formalin-fixed tissue samples were treated with 3% H_2O_2 at room temperature for 10 min to inhibit any endogenous peroxidase activity. Sections were washed three times with phosphate-buffered saline (PBS) and pretreated for antigen retrieval by microwaving in 10 mM citrate buffer (pH 6.0) for 15 min at high power. Sections were subsequently incubated with primary antibodies against NDRG or Tubulin (Santa Cruz Biotechnology) overnight at 4˚C and underwent three 5 min washes with PBS containing 0.1% Tween-20. Following washing, slides were incubated with biotin-labeled secondary antibodies (Santa Cruz Biotechnology, Inc.) for 10 min at room temperature and washed again. The staining was visualized using an avidin-biotin complex and counterstaining was performed using hematoxylin (11).

Methylation detection using bisulfite sequencing PCR. DNA was extracted from the tumor tissues and U251 cells using a DNA extraction kit (Tiangen Biotechnology Co., Ltd., Beijing, China) according to the manufacturer's instructions and was stored at -20˚C. Normal lymphocyte DNA was modified using a SssI methyltransferase kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instructions. Genomic DNA was modified using bisulfite (Sigma-Aldrich, St. Louis, MO, USA) according to the method described by Herman *et al* (12). Non-methylated genomic DNA from normal human peripheral blood lymphocytes served as negative control. Genomic DNA was obtained from human lymphocytes, whose CpG sites had been completely methylated, using the SssI methyltransferase and served as positive control. Deionized water was the blank control. Two overlapping fragments from the *NDRG2* promoter region, spanning 32 CpG sites between nucleotides 20,564,110 and 20,562,476 (Genbank accession no. NC_000014) were PCR-amplified from sodium bisulfite-modified DNA using the following primers: Forward, 5'-AAG GAG AGT TTA TTT TAG GGT GTG-3' and reverse 5'-TAC CCA AAA TCC TAAT ACC

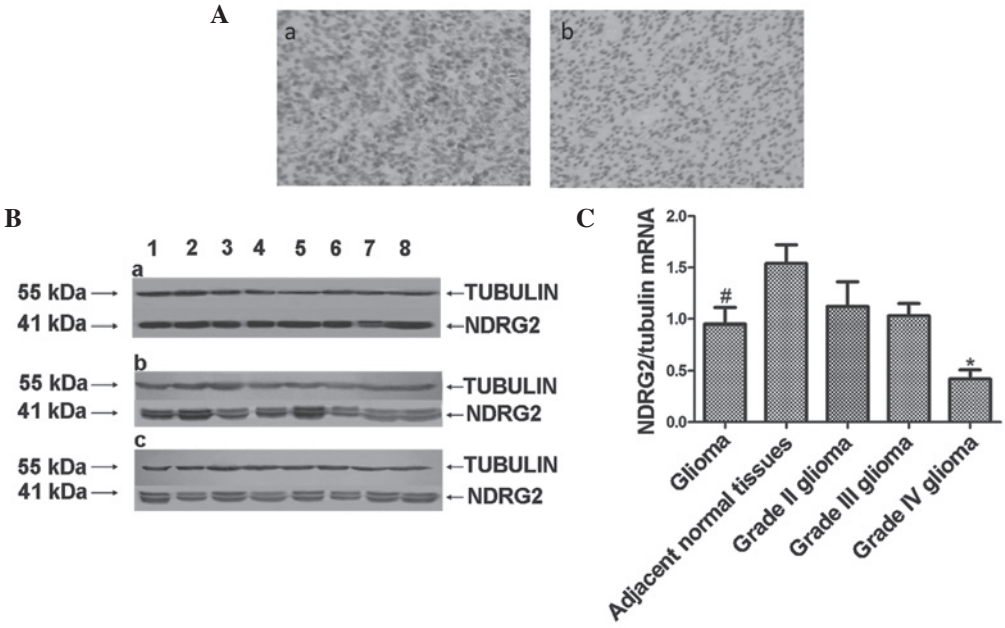

Figure 1. NDRG2 mRNA expression in glioma samples. NDRG2 protein and mRNA expression was determined using immunohistochemistry, western blot analysis and qPCR. (A) Immunohistochemistry was performed in order to determine the level of NDRG2 protein expression in (a) adjacent normal tissues and (b) glioma tissues. NDRG positive staining: brown (magnification, x40) (B) NDRG2 protein expression in grade (a) II, (b) III and (c) IV glioma tissues assessed using western blot analysis. (C) NDRG2 mRNA expression in grade II, III and IV glioma tissues assessed using qPCR. $^{\#}$P<0.05 vs. adjacent normal tissues. *P<0.05 vs. adjacent normal tissues and grade II glioma. *NDRG2*, N-myc downstream regulated gene 2; qPCR, quantitative polymerase chain reaction.

TCT C-3' (9). The amplified sequence was 530 bp and included 32 CpG sites. The recovered PCR products were ligated into the pMD19-T Vector (Takara Bio Inc., Shiga, Japan) and transformed into *E. coli* competent cells (GM109). Extracted plasmids were digested using the *Pst*I and *Eco*RI restriction endonucleases (Takara) according to the manufacturer's instructions. Restriction digests were ethanol precipitated prior to gel analysis.

Statistical analysis. Data are presented as the mean ± standard deviation. Paired t-tests were performed to assess the differences in NDRG2 mRNA expression between tumor and adjacent normal tissue samples. Mean mRNA levels were compared between the three tumor grades using variance analysis and P<0.05 was considered to indicate a statistically significant difference. Mean protein levels were compared between the three tumor grades using variance analysis. The proportion of promoter methylation in normal and tumor tissues among the three grades was analyzed using Pearson's χ^2 test. The number of methylation sites in each sample was compared between grades using the Kruskal-Wallis H test.

Results

NDRG2 expression is reduced in glioma. Immunohistochemistry was used to analyze NDRG2 expression in 26 human glioma samples (grade II, n=9; grade III, n=9; and grade IV, n=8). As shown in Fig. 1A, the expression of NDRG2 was observed to be significantly higher in adjacent normal tissues (Fig. 1A-a) compared with glioma tissues (Fig. 1A-b) and NDRG2 was observed to be primarily expressed in the cytoplasm. NDRG2 staining was also found to decrease with increasing glioma malignancy (data not shown). Western blot analysis was performed to further assess the protein expression of NDRG2 in glioma tissues. The protein expression of NDRG2 in the grade II and III glioma samples was found to be 1.75- and 1.7-fold of that in the grade IV glioma samples, respectively. This difference in NDRG2 protein expression among the glioma grades was considered to be statistically significant (P<0.05; Fig. 1B).

Furthermore, NDRG2 mRNA expression was assessed and the findings were in accordance with those that were identified via immunohistochemistry and western blot analysis. In 26 human brain normal tissue samples, the expression of NDRG2 mRNA was found to be 1.7-fold that in the glioma samples (P<0.05). Furthermore, NDRG2 mRNA expression in the grade II and III glioma samples was observed to be 2.7- and 2.5-fold of that in grade IV glioma samples. Moreover, a significant negative correlation was identified between NDRG2 mRNA expression and the glioma grade (P<0.05; Fig. 1C).

NDRG2 promoter methylation detection in glioma. To further investigate the mechanisms underlying the decrease in NDRG2 expression observed in the glioma tissue samples, NDRG2 promoter methylation was detected in glioma tissue samples using bisulfite sequencing PCR. In bisulfite sequencing PCR, cytosine (C) residues in methylated CpG sites of the NDRG2 promoter are not deaminated into uracil (U); therefore, remain as a C or guanine in the PCR product. C residues of non-methylated CpG sites are deaminated into U residues and are transformed into thymine or adenine in the PCR product. Sequencing revealed that none of the CpG sites in the NDRG2 promoter region in the genomic DNA from normal human lymphocytes were methylated; however, following treatment with the SssI methyltransferase, all of the CpG sites were methylated, thus, validating the experimental procedures. Methylated CpG sites were detected in 19 out of 41 of the

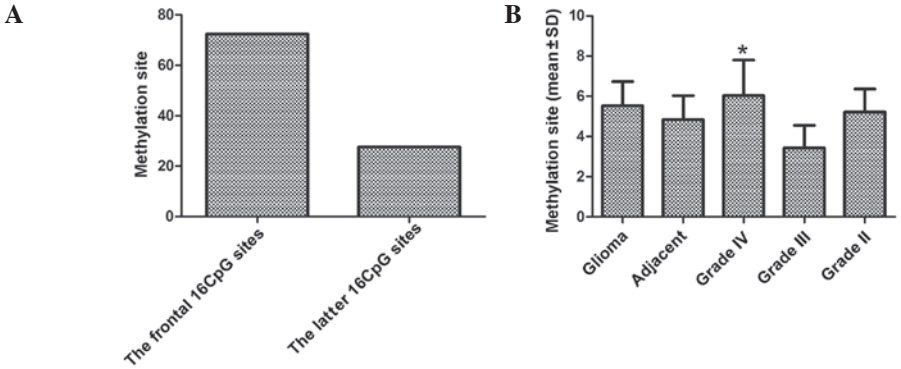

Figure 2. (A) Distribution and (B) number of N-myc downstream regulated gene 2 methylation sites in glioma and adjacent normal tissues. *P<0.05 vs. Grade III and II. SD, standard deviation.

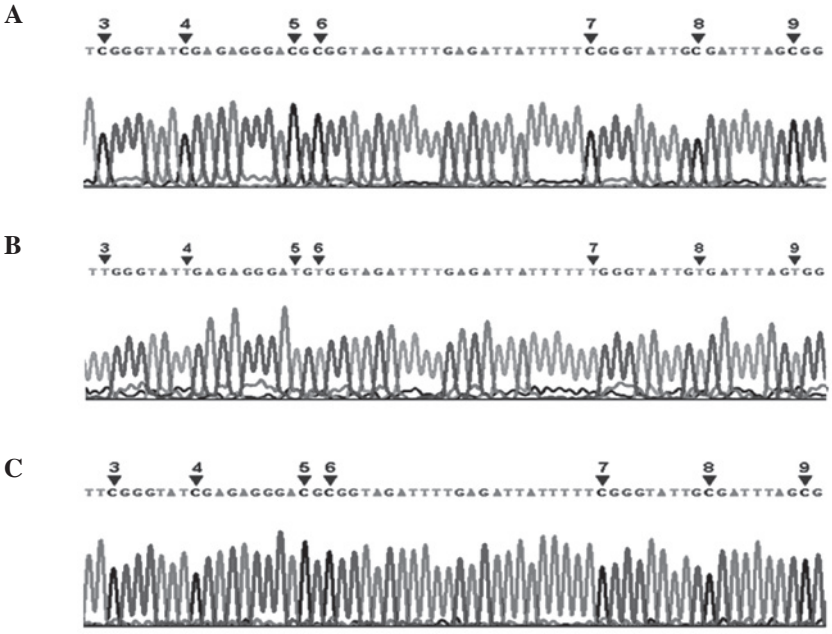

Figure 3. N-myc downstream regulated gene 2 promoter methylation in different tissues and cells detected using DNA sequencing. (A) Glioma tissues, (B) adjacent normal tissues and (C) U251 human glioblastoma cells.

Table I. N-myc downstream regulated gene 2 methylation in glioma and adjacent normal tissue samples.

Group	Samples (n)	Methylated (n)	Methylation rate (%)
Glioma tissue	41	19	46.3[a]
Adjacent normal tissue	22	4	18.2
Grade IV	15	10	66.7
Grade III	12	4	33.3
Grade II	14	5	35.7

Data were analyzed using Pearson's χ^2 test. [a]P<0.05 vs. adjacent normal tissue.

glioma tissue samples (46.3%), compared with four out of the 22 adjacent normal tissues (18.2%), which was considered to be a significant difference (P<0.05; Table I). In addition, 66.7% of the 15 grade IV glioma samples, 33.3% of the 12 grade III glioma samples and 35.7% of the 14 grade II glioma samples were observed to be methylated.

Distribution of methylation sites. Further assays were performed in order to analyze the distribution of methylated CpG sites in the NDRG2 promoter region. As shown in Fig. 2A, methylation was predominantly observed in the frontal 16 CpG sites, which were 63 of 87 (72.4%) as compared with 24 of 87 (27.6%) in the latter 16 CpG sites (P<0.05). The average number of methylation sites in 19 methylation-positive glioma tissues was 5.53±1.21, compared with 4.85±1.18 in the four adjacent normal tissue samples. No significant difference was observed in the average number of methylation sites in glioma tissue compared with that in the normal tissue (P>0.05). The average number of methylation sites in the 10 methylated grade IV, four methylated grade III and five methylated grade II samples was 6.04±1.76, 3.44±1.12 and 5.22±1.14, respectively (Fig. 2B). The average number of methylation sites in the

grade IV samples compared with that in the grade III and II samples was significantly different (P<0.05).

DNA sequencing. To further investigate the methylation of the NDRG2 promoter region, DNA sequencing of the NDRG2 promoter was performed in different tissues and cells. Sequencing revealed that the CpG sites in the NDRG2 promoter region were methylated in the U251 cells and glioma tissues (Fig. 3).

Discussion

Immunohistochemistry and qPCR analysis revealed that NDRG2 expression was significantly decreased in glioma tissues compared with adjacent normal tissues and that the decrease in NDRG2 expression was associated with increased glioma malignancy. These findings indicate that decreased NDRG2 expression may have a role in the occurrence and development of glioma. Furthermore, bisulfite sequencing PCR revealed that NDRG2 promoter methylation was responsible for the decrease in NDRG2 expression in glioma. These findings may have important implications for the use of NDRG2 as a potential target for gene therapy in patients with glioma.

NDRG2 is a novel tumor suppressor candidate gene. Previous studies have shown that NDRG2 expression is low in numerous types of tumor tissue and cancer cell lines (11,13-15). Moreover, induced expression of NDRG2 in cultured cancer cells has been reported to inhibit the cell cycle at G_1-phase, indicating that NDRG2 expression inhibits cancer cell proliferation (16). However, the mechanisms underlying the downregulation of NDRG2 in glioma and NDRG2-induced inhibition of cell proliferation are yet to be elucidated. Based on a novel epigenetic theory, in the present study, it was hypothesized that the downregulated NDRG2 expression observed in tumors, including glioma, may be due to hypermethylation of the NDRG2 promoter region, as no *NDRG2* coding sequence mutant has been detected in tumors. The majority of previous investigations of NDRG2 expression have been performed in tumors and their adjacent normal tissues. In the present study, NDRG2 expression and methylation were anlalyzed among three glioma grades (II, III, IV), as well as in adjacent normal tissues.

Research has shown that NDRG2 expression in certain types of tumor, including glioma, is significantly lower compared with that in corresponding normal tissue. In the present study, a negative correlation was observed between NDRG2 mRNA expression and the tumor grade. Furthermore, a negative correlation was identified between NDRG2 protein expression and tumor grade (P<0.05; Fig. 1). In addition, in the glioblastoma group (grade IV), NDRG2 expression was significantly decreased, although still detectable, demonstrating that NDRG2 may have potential as a biomarker to determine prognosis. These data indicate that *NDRG2* is a candidate glioma suppressor gene, and NDRG2 may be important in the development and progression of gliomas.

Previous studies have shown a correlation between CpG island methylation and tumorigenesis. CpG island methylation results in tumorigenesis via transcriptional silencing and loss of function through inducing changes in chromatin structure (17-19). In the present study the methylation rate in the NDRG2 promoter region was observed to be higher in glioma tissues compared with that in adjacent normal tissues. Furthermore, the methylation rate of grade IV glioma was markedly higher when compared with grade III and II glioma (Table I). One grade II sample exhibited a high number of methylation sites, however, the reason for this was unknown. No significant difference was observed between the number of methylation sites in gliomas compared with those in adjacent normal tissues. This may be due to the small sample size; however, a greater percentage of methylation was observed in the grade IV samples than in the grade III samples. These findings indicate that methylation of the NDRG2 promoter region is important in the development and progression of glioma.

In the present study, methylation was detected at a higher frequency in the frontal 16 CpG sites compared with the latter 16 CpG sites. This indicates that the sequence including the frontal 16 CpG sites of the NDRG2 promoter may have a significant effect in the inhibition of gene transcription. Methylation sites in the NDRG2 promoter region were also sequenced in a glioblastoma sample, adjacent normal tissue sample and U251 human glioblastoma cells. It was hypothesized that hypermethylation of the NDRG2 promoter may lead to repressed NDRG2 protein expression and aberrant cell proliferation, which may contribute to the pathogenesis of malignant glioma. Although it is statistically higher than that of normal tissue, the methylation rate of glioma samples is <50%, indicating that other mechanisms, for example the regulation of the histone structure, may contribute to NDRG2 downregulation in glioma.

In conclusion, the present study demonstrated that *NDRG2* may be a tumor suppressor gene and methylation of the NDRG2 promoter region may be one of the mechanisms by which NDRG2 expression is downregulated in gliomas. This mechanism of glioma tumorigenesis may provide an important insight for glioma treatment. Furthermore, it has been reported that demethylation using 5-Aza-2'-deoxycytidine or S-adenosyl-methionine is capable of restoring the expression of certain genes (13). Therefore, demethylating agents may have potential for use in glioma treatment.

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