

Identification of *MGMT* promoter methylation sites correlating with gene expression and *IDH1* mutation in gliomas

Jie Zhang¹ · Jian-hui Yang¹ · Jia Quan¹ · Xing Kang¹ · Hui-juan Wang¹ · Peng-gao Dai¹

Received: 27 January 2016 / Accepted: 12 July 2016 / Published online: 28 July 2016
© International Society of Oncology and BioMarkers (ISOBM) 2016

Abstract O⁶-methylguanine-DNA methyltransferase (*MGMT*) gene promoter methylation was reported to be an independent prognostic and predictive factor in glioma patients who received temozolomide treatment. However, the predictive value of *MGMT* methylation was recently questioned by several large clinical studies. The purpose of this study is to identify *MGMT* gene promoter CpG sites or region whose methylation were closely correlated with its gene expression to elucidate this contradictory clinical observations. The methylation status for all CpG dinucleotides in *MGMT* promoter and first exon region were determined in 42 Chinese glioma patients, which were then correlated with *MGMT* gene expression, *IDH1* mutation, and tumor grade. In whole 87 CpG dinucleotides analyzed, three distinct CpG regions covering 28 CpG dinucleotides were significantly correlated with *MGMT* gene expression; 10 CpG dinucleotides were significantly correlated with glioma classification ($p < 0.05$). Isocitrate dehydrogenase 1 (*IDH1*) mutation and *MGMT* gene hypermethylation significantly co-existed, but not for *MGMT* gene expression. The validation cohort of gliomas treated with standard of care and comparison of the CpGs we identified with the current CpGs used in clinical

setting will be very important for gliomas individual medicine in the future.

Keywords Glioma · *MGMT* · Methylation · *IDH1* · Pyrosequencing · Correlation analysis

Introduction

Gliomas are the most common primary brain tumors that are classified into four different malignancy grades according to the World Health Organization (WHO) classification [1]. Glioblastoma multiforme (GBM) belongs to WHO grade IV and is the most common and malignant human brain tumor. The median survival for GBM is only 12 to 18 months [2]. Anaplastic astrocytoma (WHO grade III) and diffuse astrocytoma (WHO grade II) have relatively low degree of malignancy and an inherent tendency for recurrence and malignant progression. WHO grade I tumors are rare. Currently, the most effective treatment method for glioma is surgery combined with radiation and/or chemotherapy [3]. Temozolomide (TMZ), an alkylating anticancer drug, is currently the most effective chemotherapy for treatment of glioma. However, it is known that not all patients benefit to the same extent for TMZ treatment.

MGMT is a highly efficient DNA methyltransferase that repair DNA alkylation damage caused by a variety of alkylating agents, especially TMZ [4]. Loss of *MGMT* gene expression frequently occurs in various human malignancies [5]. Studies have shown that transcriptional silencing caused by *MGMT* gene promoter methylation plays a crucial role for gliomas response to alkylating agents [6, 7]. For *MGMT* promoter methylation, most trials revealed a positive relationship between promoter methylation and therapeutic response of glioblastoma patients treated with alkylating agents like

Jie Zhang and Jian-hui Yang contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s13277-016-5153-4) contains supplementary material, which is available to authorized users.

✉ Peng-gao Dai
daipg@nuw.edu.cn

¹ National Engineering Research Center for Miniaturized Detection Systems, School of Life Sciences, Northwest University, Xi'an, People's Republic of China

TMZ [6, 8–11]. While some other studies failed to show this correlation [12], this might be due to the current CpG islands detected are of different importance for the silencing of the *MGMT* gene in some certain populations, which is supported by the finding that the position of the methylated CpG islands is highly variable in *MGMT* deficient tumor cell lines [13]. The question, which CpG island in the *MGMT* promoter is most reliable and efficient in silencing needs to be addressed.

In this study, we screened the whole CpG dinucleotides methylation located in *MGMT* promoter and first exon region using pyrosequencing to obtain quantitative results for all CpG sites in 42 Chinese glioma patients. Then, we statistically analyzed the relationship between location-specific CpG methylation and *MGMT* gene expression levels. Besides, correlation between *IDH1* mutation and *MGMT* promoter methylation was also analyzed.

Materials and methods

Samples

Tissue samples were collected from 42 patients with astrocytoma, oligodendroglioma, and GBM (WHO grades I, II, III, and IV) treated at the department of Neurosurgery, Tangdu Hospital, affiliated with The Fourth Military Medical University in Xi'an city, China. None of the patients had any other forms of cancer. Histological typing of the tissues was performed according to the WHO grading system of brain tumors using standard histological and immunohistological methods [1]. The study was approved by the Tangdu Hospital, and informed consent was obtained from all patients.

Bisulfite treatment

DNA from frozen tumors was extracted using E.Z.N.A tissue DNA kit (OMEGA Inc.). Two micrograms of DNA was converted using the EpiTect bisulfite kit (QIAGEN Inc.), according to the manufacturer's instructions. Cytosine and its counterpart 5-methylcytosine behave differently when single-stranded DNA is treated with sodium bisulfite. While cytosine residues react with this reagent and are converted to uracil, 5-methylcytosine remains inert under the same conditions. In a subsequent polymerase chain reaction (PCR), the uracil residues are transcribed to thymine and 5-methylcytosine to cytosine [14].

PCR

Genomic DNA sequences of *MGMT* were downloaded from the UCSC Genome Browser database. PCR primers were designed using Methprimer. PCR was performed using a

Mastercycler Gradient (Eppendorf Inc.). We used 25 μ l volume reactions based on the number of amplicons required for analysis in the PyroMark Q24 Instrument (QIAGEN Inc.). Each reaction contained 400 nM of each primer, 30 ng bisulfite-treated human genomic DNA, 10 \times HS reaction buffer containing 1.5 mM $MgCl_2$, 1 U Hotstart enzyme (Genscript), and 250 μ M dNTP mixture. The PCR program consisted of an initial polymerase activation step at 95 $^{\circ}C$ for 15 min followed by 50 cycles of denaturation at 95 $^{\circ}C$ for 30 s, primer annealing at 58–60 $^{\circ}C$ for 30 s, extension at 72 $^{\circ}C$ for 40 s, and a final extension step at 72 $^{\circ}C$ for 5 min. The PCR primers and annealing temperatures are shown in Table S1.

Pyrosequencing

PCR products from bisulfite-treated genomic DNA samples were analyzed by pyrosequencing to quantify site-specific methylation. The sequence primers are summarized in Table S1. Ten microliters of the amplification product was incubated with 1 μ l streptavidin sepharose high performance beads (GE Healthcare Inc.) in 40 μ l binding buffer. The template strands were purified and rendered singlestranded using a PyroMark Q24 vacuum workstation (QIAGEN Inc.). Beads were released into 20 μ l annealing buffer containing 12.5 pmol of the respective sequencing primer, and sequencing primers were annealed to the target by incubation at 80 $^{\circ}C$ for 2 min. Quantitative DNA methylation analysis was performed on a PyroMark Q24 system with the Qiagen kit, and the results were analyzed using the PyroMark Q24 software (version 2.0.6, Qiagen Inc.). We are presenting a pyrosequencing diagram in Fig. 1a.

MGMT mRNA expression analysis

Total RNA was isolated using the RNeasy mini kit (Qiagen Inc.), according to the manufacturer's instructions. RNA quality was assessed using the NanoDrop 2000 (Thermo Fisher Scientific Inc.). mRNA expression levels of *MGMT* were determined with the one-step PrimeScript RT-PCR kit (TaKaRa Inc.) by qRT-PCR using LightCycler 480 (Roche Inc.). The qRT-PCR program consisted of a reverse transcription step at 42 $^{\circ}C$ for 5 min, RT enzyme deactivation at 95 $^{\circ}C$ for 10 s followed by 50 cycles of denaturation at 95 $^{\circ}C$ for 5 s, and primer annealing at 57 $^{\circ}C$ for 20 s. The expression level of β -actin was used for normalization. All assays were run in triplicate. Relative quantification analyses were performed using the formula: $2^{-(Ct \text{ value of } MGMT - Ct \text{ value of } \beta\text{-actin})}$ [15]. The primers and probes are as follows: *MGMT* forward primer, 5'-CCTGGCTAA-TGCCTATTTC-3'; *MGMT* reverse primer, 5'-TGAACGACTCTTGC TGGAAAAC-3'; *MGMT* probe, 5'-FAM-ACCAGCCC

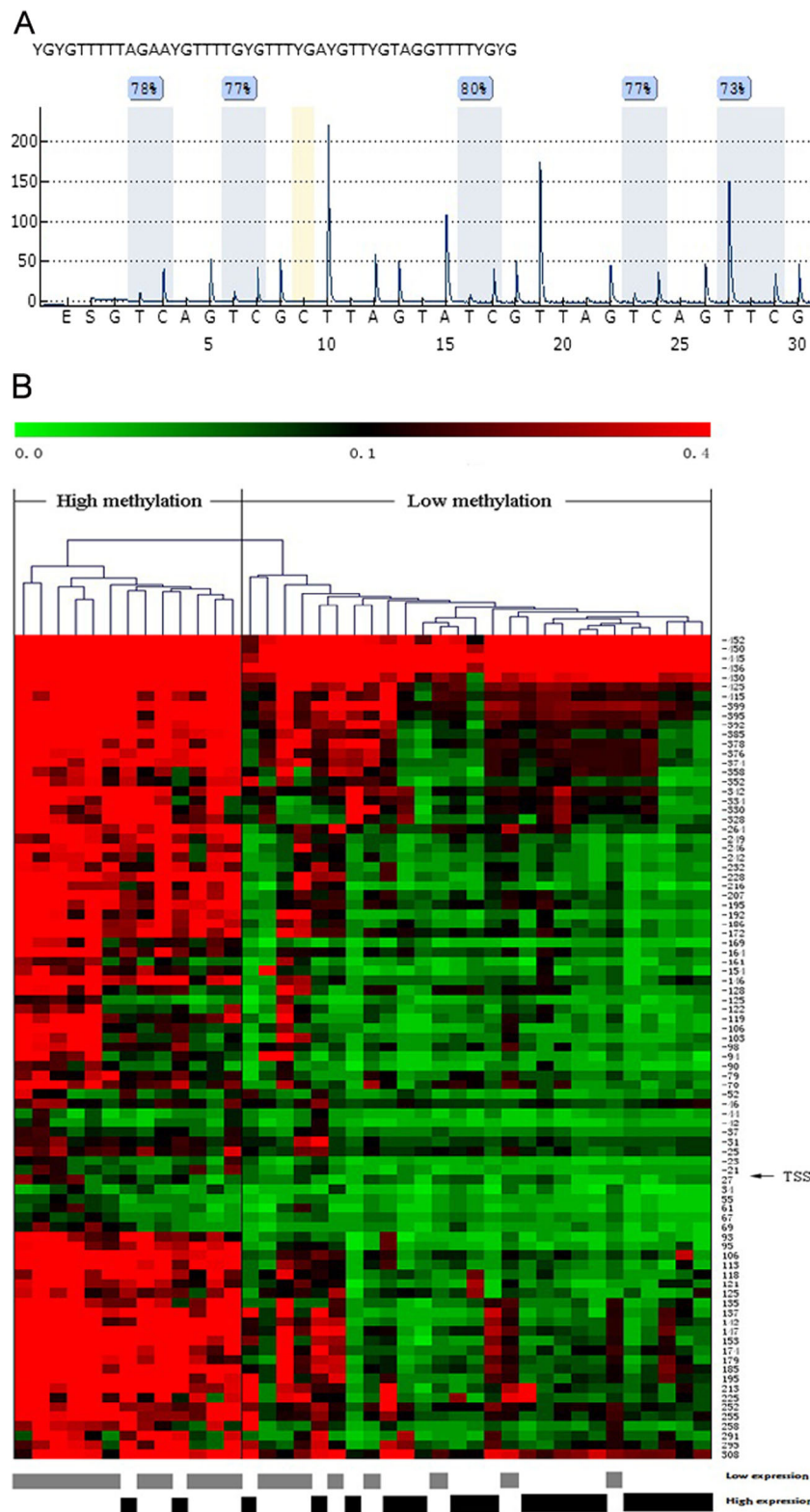


Fig. 1 a The pyrosequencing diagrams present quality control for completion of bisulfate treatment. **b** Hierarchical clustering of 40 glioma patients with 87 CpG sites were analyzed by pyrosequencing. Green, black, and red represent methylation levels of CpG sites. By hierarchical clustering, the samples are classified into two distinct patient groups: high methylation and low methylation. The expression

level of *MGMT* gene for each patient is shown at the bottom. **b** Box plot of *MGMT* gene methylation levels and expression. The box plot represents quantiles, medians, and extreme points. The squares and dots represent the average methylation levels of 87 CpG sites for each patient. The *MGMT* gene expression and methylation levels had significant differences, $p < 0.001$

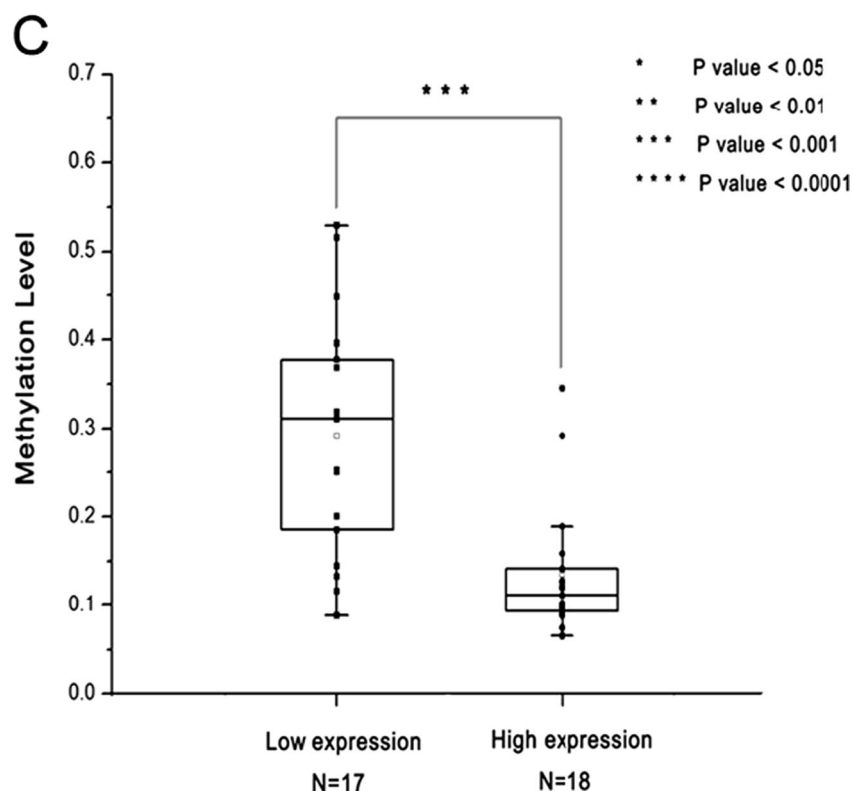


Fig. 1 (continued)

GAGGCTATCGAAGAGTTC-BHQ1-3'; β -actin forward primer, 5'-CAGAGCCTCGCCTTTGCC-3'; β -actin reverse primer, 5'-CACATGCCGGAGCCGTT-3'; β -actin probe, 5'-FAM-ACCAGCCCGAGGCTATCGAA GAGTTC-BHQ1-3'.

Detection of *IDH1* mutations

Pyrosequencing was used to determine *IDH1* mutation status. Sequencing for mutations within the *IDH1* gene was performed using the primers specified by Setty and colleagues [16].

Statistical analysis

Statistical analysis was performed using IBM SPSS statistics 19. Hierarchical clustering was based on the degree of methylation. Average linkage was used with squared Euclidean distance as an interval measure. MannWhitney test was used to correlate *IDH1* mutation with *MGMT* methylation and determine the relationship between *MGMT* methylation, as a quantitative variable, and expression, as a qualitative variable (low versus high expression). Fractional methylation of a promoter region was calculated by averaging methylation of all CpG sites in that region. The correlation of individual CpG site methylation with *MGMT* mRNA expression level was

calculated using Spearman's rank correlation. Chi-square test was used to determine the repartition between *IDH1* mutation and glioma classification.

Results

Determination of *MGMT* gene promoter methylation by pyrosequencing

To identify the DNA methylation region or sites in *MGMT* gene that could represent *MGMT* overall methylation status and further define the region that significantly correlate with *MGMT* mRNA expression, the entire sequence of *MGMT* gene was examined. A total of 98 CpG dinucleotides in the promoter and the first exon region were identified, which span 762 bp in length and start from -452 bp to +308 bp relative to the transcription start site (TSS), of which 57 CpG dinucleotides were upstream and 30 were downstream of the TSS. Firstly, we designed pyrosequencing primers for the 98 CpG dinucleotides and tested these primers using SW480 cell line DNA as the template, which was reported to be *MGMT* gene highly methylated cell line. To further validate the feasibility of these primers on human samples, DNA from glioma patients was used in this established system. The results showed that this system works well for human tissues, and the representative spectrum is

shown in Fig. 1a and the sequencing primers are shown in Table S1.

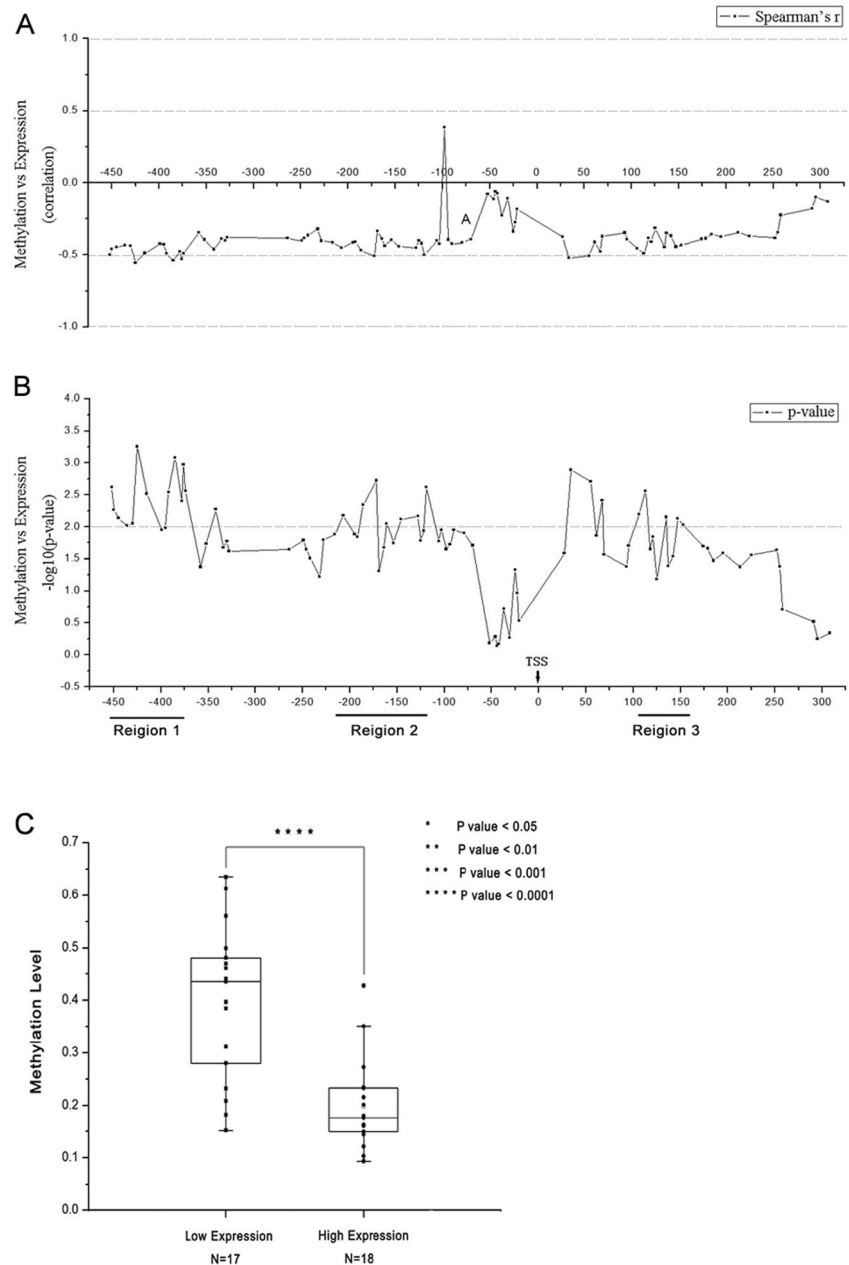
The methylation status of 42 glioma patients was then determined by the established pyrosequencing system. In total, 98 CpG sites were analyzed, 11 CpG sites located in -300, -63, +5, +11, +16, +38, +47, +75, +209, +242, and +271 showed uniformly methylated in all samples, and, therefore, the data mining in this study has excluded these 11 sites. Hierarchical clustering of the methylation profiles of the 87 CpG sites showed strong stratification of two distinct patient groups. The first group included 13 patients (32.5 %) with considerable methylation at almost all of the CpG sites tested (average range, 25–53 %). The second group included the 27

other patients (67.5 %) that had a lower global methylation level (average range, 7–20 %) (Fig. 1b).

Correlation between overall methylation and mRNA expression of *MGMT* gene

We selected *ACTB* as the reference gene and determined the *MGMT* mRNA expression in 37 glioma samples. According to the median value of all tested samples, the patients were divided into two groups: high expression group and low expression group. *MGMT* expression and its overall methylation (mean value) were considered as qualitative and quantitative variables, respectively. The

Fig. 2 CpG methylation of the *MGMT* promoter region and *MGMT* gene expression in gliomas. **a** Spearman's rank correlations between gene expression and methylation of the 87 CpG methylation in the promoter region spanning TSS. **b** *p* values of associations between 87 CpG sites methylation and *MGMT* gene expression. The dotted lines in **b** correspond to the threshold of 0.01. The graph at the bottom indicates the three CpG regions. **c** The squares and dots represent the average methylation levels of 28 individual CpG sites for each patient. The *MGMT* gene expression and methylation levels had extremely significant differences, $p < 0.0001$



two variables were analyzed using the Mann-Whitney test, p value of one-tailed test <0.001 , suggesting that the overall *MGMT* methylation significantly correlated with its mRNA expression (Fig. 1c).

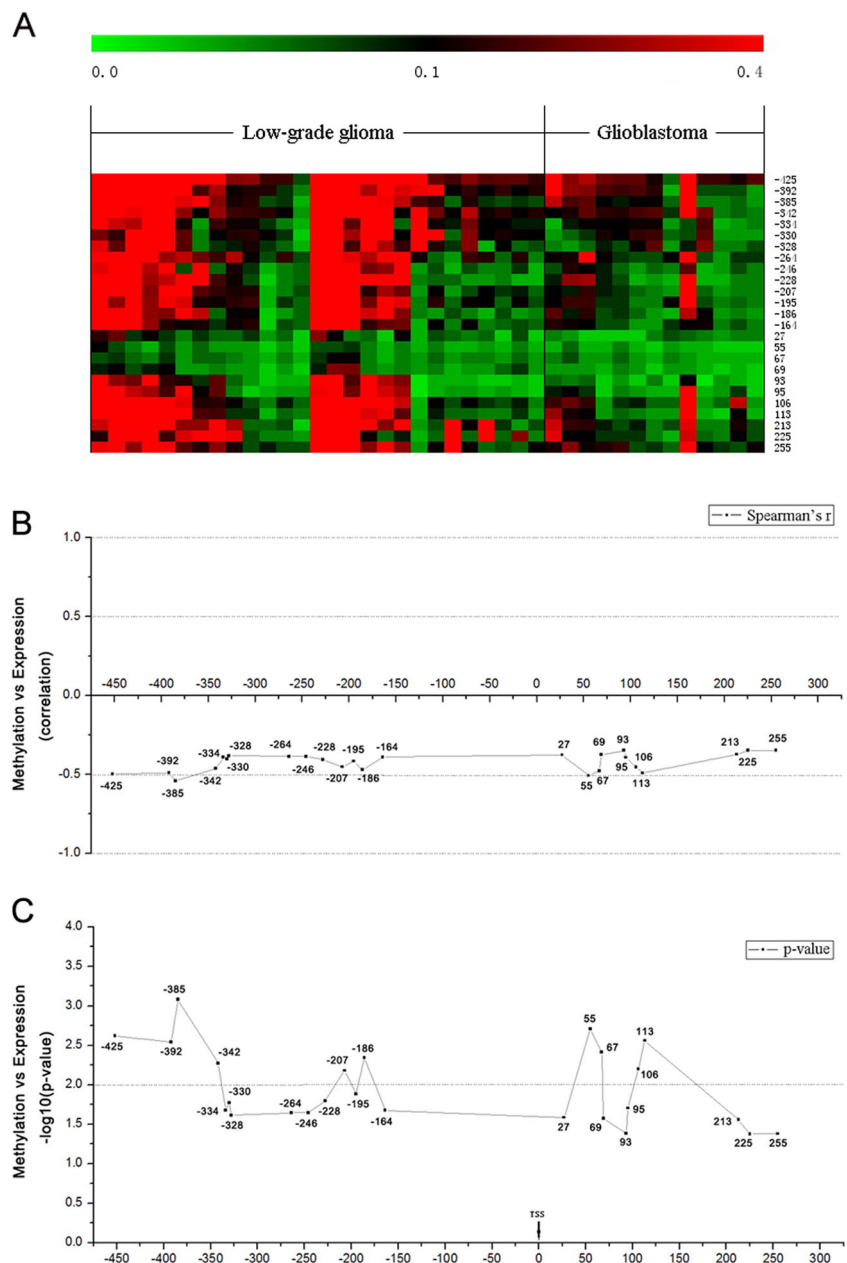
Correlation between specific CpG island methylation and gene expression

To further identify the specific CpG dinucleotides that correlated best with *MGMT* mRNA expression, the association of methylation status of each CpG dinucleotides and *MGMT* expression was separately analyzed. By using Spearman's rank correlation, a total of 28 individual CpG dinucleotides

were identified that significantly correlated with *MGMT* gene expression ($p < 0.01$), (Fig. 2a, b). Meanwhile, we also analyzed the association of the average methylation of these 28 CpG dinucleotides and *MGMT* expression by Mann-Whitney test. As shown in Fig. 2c, these 28 dinucleotides correlated significantly with *MGMT* gene expression ($p < 0.0001$). (Fig. 2c).

To define a region that could facilitate the future clinical detection of *MGMT* methylation, we further examined all the CpG sites in three regions (CpGs at -452 to -374 , -216 to -119 , and 106 to 153) and correlated the methylation and gene expression using Spearman's rank. All three CpG regions significantly correlated with mRNA expression ($p < 0.01$).

Fig. 3 **a** 87 CpG sites between WHO II, III grade gliomas and WHO IV patients were analyzed by the t test. In the clustering figure, 25 methylation sites had significant differences, $p < 0.05$. CpG methylation of the *MGMT* promoter region and *MGMT* gene expression in gliomas. **b** Spearman's rank correlations between gene expression and methylation of the 25 CpG sites methylation in the promoter region spanning TSS. **c** p values of associations between 25 CpG sites methylation and *MGMT* gene expression. The dotted line in **c** corresponds to the threshold of 0.01



Association between *MGMT* methylation and glioma grades

Among the 42 glioma patients, 13 were GBM (WHO IV) and 27 were other grades gliomas (WHO II, III). In order to find potential biomarkers that will be useful in glioma classification, we focused on the CpG sites methylation related to GBM tumor grade and low-grade gliomas. The data was therefore divided into two groups: GBM patients and low-grade glioma patients, and analyzed with the *t* test. Twenty-five methylation sites had significant differences between the two groups ($p < 0.05$). The average level of methylation for relatively low-grade glioma patients was 23.1 %, but for GBM patients, it was 14.4 % (Fig. 3a). Eleven of these sites were downstream and 14 were upstream of the TSS.

Using Spearman's rank correlation, a total of 10 individual CpG sites spanning the promoter were significantly correlated with *MGMT* gene expression ($p < 0.01$). The 10 individual CpG sites correlated with *MGMT* gene expression and glioma classification (Fig. 3b, c).

IDH1 mutation significantly correlated with *MGMT* hypermethylation

A total of 40 glioma patients were retrospectively analyzed by pyrosequencing to reveal *IDH1* gene mutation status. Of these, 20 samples had *IDH1* mutation, and 20 had intact *IDH1* (Table 1). Mann-Whitney test showed a significant correlation between *IDH1* mutation and *MGMT* hypermethylation (p value of one-tailed test = 0.012) (Fig. 4a). There was no significant correlation between *IDH1* mutation and *MGMT* gene expression ($p = 0.957$) (Fig. 4b).

Discussion

Epigenetic silencing of *MGMT* promoter methylation has been associated with decreased risk and longer survival in patients with glioma who receive alkylating agent. A meta-analysis including 573 patients in eight cohort studies showed that the aberrant methylation of *MGMT* was associated with a longer overall survival (OS) in glioma patients. Further, subgroup analysis based on ethnicity indicated that there was an association between the aberrant methylation of *MGMT* and disease free survival (DFS) in glioma patients among both Asian and Caucasian populations (Asians, HR = 1.40, 95 % CI = 0.03 ~ 2.78, $p = 0.046$; Caucasians, HR = 1.89, 95 % CI = 0.28 ~ 3.50, $p = 0.021$, respectively). However, *MGMT* methylation and OS were found to not be correlated in Asian populations [17, 18], suggesting that the predictive value of *MGMT* methylation might be varied in different genetic background; another possibility for this contradictory result may lie in the position of CpG islands detected are of different importance for the silencing of the *MGMT* gene in different populations. Thus, identifying the most reliable CpGs dinucleotides of *MGMT* gene methylation in Chinese population is very crucial for personalized tumor therapy.

In this study, we identified three specific regions covering 28 CpG sites of *MGMT* gene that have significant correlation with its expression and further proved that hypermethylation of CpG sites in *MGMT* gene promoter is associated with *IDH1* mutation in Chinese glioma patients. Everhard et al. examined 52 CpG sites of *MGMT* gene in 54 patients with GBM and 24 non-tumoral brain tissues. These patients were then divided into three groups: unmethylated group, intermediate group, and methylated group. They found that six isolated CpG sites (CpGs -228, -186, 95, 113, 135, 137) and two CpG regions (-186 to -172 and 93 to 153) were significantly correlated with *MGMT* gene expression [19]. Malley et al. examined 98

Table 1 Sample characteristics of 42 glioma patients included in this study and frequency of *MGMT* methylation and *IDH1* mutation

WHO grade	<i>IDH1</i> mutated Number (incidence %)	<i>IDH1</i> wild type Number (incidence %)	Mean methylation of all CpGs (%)	Male	Female	Age (years) Range (median)
AI	0 (0 %)	2 (5 %)	24.2	2	0	27–32 (29.5)
AII	2 (5 %)	4 (10 %)	19.7	4	2	26–51 (32)
OAI	6 (15 %)	0 (0 %)	28.4	3	3	28–59 (34)
AIII	4 (10 %)	6 (15 %)	20.3	6	4	27–60 (42)
OAI	3 (7.5 %)	0 (0 %)	34.6	1	2	36–63 (60)
pGBM IV	0 (0 %)	8 (20 %)	12.4	6	2	18–56 (42)
sGBM IV	5 (12.5 %)	0 (0 %)	20.9	4	1	29–66 (54)
Total	20 (50 %)	20 (50 %)	20.7	26	14	18–66 (40)

AI pilocytic astrocytoma, AII grade II diffuse astrocytoma, AIII grade III anaplastic astrocytoma, OII grade II oligodendroglioma, OIII grade III anaplastic oligodendroglioma, OAI grade II oligoastrocytoma, OAI grade III anaplastic oligoastrocytoma, pGBM primary GBM, sGBM secondary GBM

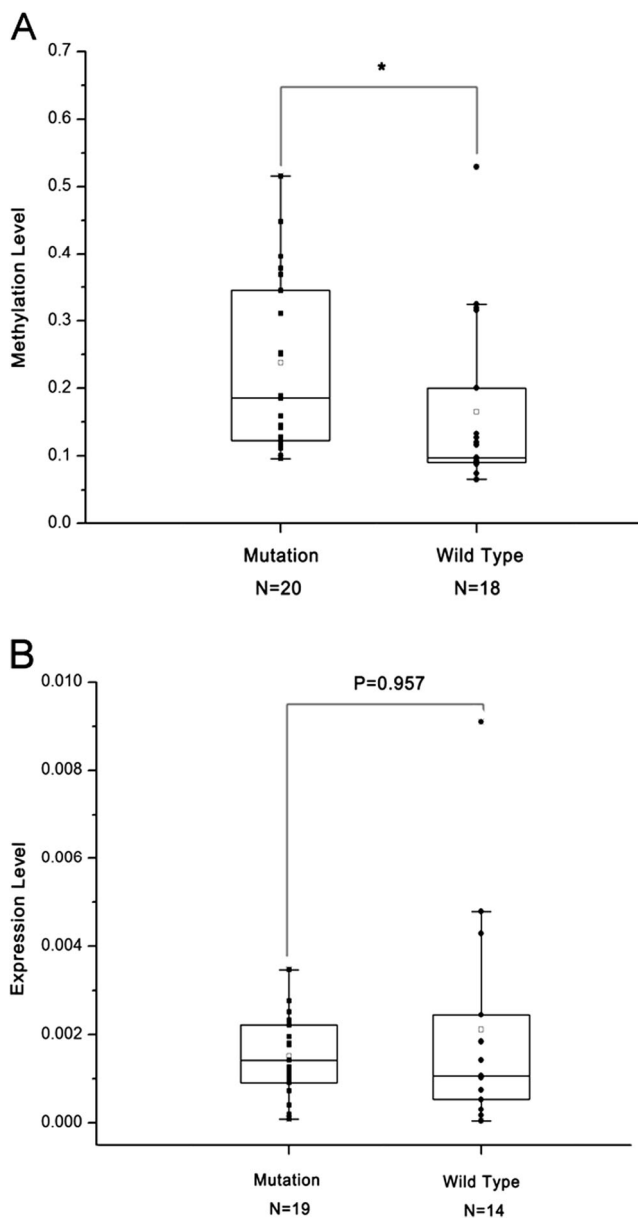


Fig. 4 Box plot of *MGMT* gene methylation levels and *IDH1* mutation. **a** The squares and dots represent the average methylation levels of 87 CpG sites for each patient. The *IDH1* mutation between *MGMT* gene hypermethylation and hypomethylation had significant differences, $p < 0.05$. **b** The squares and dots represent the expression level for each patient. The *IDH1* mutation and *MGMT* gene expression levels had no significant correlation

CpG sites in 22 patients with GBM, 13 glioma cell lines, and six normal brain tissue and identified two differentially methylated regions (DMR): DMR1 (CpG25-50) and DMR2 (CpG73-90). These regions were highly correlated with *MGMT* promoter activity [5]. Shah et al. used bisulfite sequencing to analyze methylation levels of 97 CpG sites in 70 patients. All the CpG sites were clustered into three regions: R1 and R2 were upstream, while R3 was downstream of TSS. They also correlated individual CpG site methylation

patterns to mRNA expression, protein expression, and progression-free survival (PFS) and found that seven CpG sites were significantly correlated with protein expression and PFS [20]. In our study, the 28 CpG sites fall under either DMR1 or DMR2 and are similar to the Everhard and Shah studies. Our results showed that the methylation sites of *MGMT* gene in Chinese glioma patients were similar to European and American glioma patients.

IDH mutation is the cause of CpG island methylator phenotype (CIMP) and leads to the CIMP phenotype by stably reshaping the epigenome. This remodeling involves modulating patterns of methylation on a genome-wide scale, changing transcriptional programs, and altering the differentiation state [21–25]. In our study, there was a significant correlation between *IDH1* mutation and *MGMT* hypermethylation, which was consistent with previous reports [26]. However, we did not find any correlation between *IDH1* mutation and *MGMT* gene expression. *IDH1* mutation is believed to often occur in low-grade gliomas [27, 28], which was seen in our study as well. However, the data is statistically not significant, and we believe that with a larger sample size, this data will be statistically significant. Methylation of the *MGMT* gene promoter and *IDH1* mutation were shown to be independent prognostic markers for survival of GBM patients [6, 29, 30]. Recently, several studies have demonstrated that the combination of *IDH1* mutation and *MGMT* methylation status was significantly better than *IDH1* or *MGMT* alone in predicting survival of glioma patients [31–33].

McDonald et al. found that the c.-56C>T (rs16906252) SNP in the promoter was associated with the presence of *MGMT* methylation in de novo GBM. Furthermore, carriers of the variant T allele demonstrated a clear survival benefit [34]. However, in our research, this SNP was not found in Chinese glioma patients.

There are many methods to detect *MGMT* gene methylation. Methylation-specific PCR (MSP) is a simple and widely used method. However, it is a qualitative method, which sometimes produces false-positive results. Bisulfite genomic sequencing (BSP) is an accurate method. The greatest disadvantage of this method is its cumbersome operation. Pyrosequencing is a new and reliable technique to detect *MGMT* methylation levels and can provide quantitative information at each individual CpG [35, 36]. The principle of this method is “Sequencing by Synthesis.” Pyrosequencing is a reliable technique to detect *MGMT* methylation levels and was the primary technique to analyze individual CpGs of *MGMT* gene in our study.

A significant limitation in this study is in the absence of a validation cohort of these identified CpG islands. The survival information for these patients was not available, which greatly restricted us to correlate the methylation of the newly identified CpGs sites and patient outcome. The second limitation of this study is the small sample size of clinical cohort, especially

the even small number of the subtype of gliomas; thus, we have difficulty to further analyze the association of the methylation and specific subgroup tumors. A larger cohort validation will greatly strengthen the current result.

Acknowledgments This work was supported by the National Natural Science Foundation of China (31271353) and the Program for New Century Excellent Talents in University (NCET121048).

Compliance with ethical standards

Conflicts of interest None

References

- Louis DN et al. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol.* 2007;114:97–109.
- Wen PY et al. Malignant gliomas in adults. *N Engl J Med.* 2008;359:492–507.
- Cankovic M et al. The role of MGMT testing in clinical practice: a report of the association for molecular pathology. *J Mol Diagn.* 2013;15:539–55.
- Xu-Welliver M et al. Degradation of the alkylated form of the DNA repair protein, O(6)-alkylguanine-DNA alkyltransferase. *Carcinogenesis.* 2002;23:823–30.
- Malley DS et al. A distinct region of the MGMT CpG island critical for transcriptional regulation is preferentially methylated in glioblastoma cells and xenografts. *Acta Neuropathol.* 2011;121:651–61.
- Hegi ME et al. MGMT gene silencing and benefit from temozolomide in glioblastoma. *N Engl J Med.* 2005;352:997–1003.
- Skorpen F et al. The methylation status of the gene for O6-methylguanine-DNA methyltransferase in human Mer+ and Mer- cells. *Carcinogenesis.* 1995;16:1857–63.
- Hegi ME et al. Correlation of O6-methylguanine methyltransferase (MGMT) promoter methylation with clinical outcomes in glioblastoma and clinical strategies to modulate MGMT activity. *J Clin Oncol Off J Am Soc Clin Oncol.* 2008;26:4189–99.
- Everhard S et al. MGMT methylation: a marker of response to temozolomide in low-grade gliomas. *Ann Neurol.* 2006;60:740–3.
- Stupp R et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol.* 2009;10:459–66.
- Felsberg J et al. Promoter methylation and expression of MGMT and the DNA mismatch repair genes MLH1, MSH2, MSH6 and PMS2 in paired primary and recurrent glioblastomas. *Int J Cancer.* 2011;129:659–70.
- Maxwell JA et al. Quantitative analysis of O6-alkylguanine-DNA alkyltransferase in malignant glioma. *Mol Cancer Ther.* 2006;5:2531–9.
- Nakagawachi T et al. Silencing effect of CpG island hypermethylation and histone modifications on O6-methylguanine-DNA methyltransferase (MGMT) gene expression in human cancer. *Oncogene.* 2003;22:8835–44.
- Mikeska T et al. Optimization of quantitative MGMT promoter methylation analysis using pyrosequencing and combined bisulfite restriction analysis. *J Mol Diagn.* 2007;9:368–81.
- Livak KJ et al. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) method. *Methods.* 2001;25:402–8.
- Setty P et al. A pyrosequencing-based assay for the rapid detection of IDH1 mutations in clinical samples. *J Mol Diagn.* 2010;12:750–6.
- Dong X et al. Correlation of promoter methylation in the MGMT Gene with glioma risk and prognosis: a meta-analysis. *Mol Neurobiol.* 2014;52:1–9.
- Tang K et al. Clinical correlation of MGMT protein expression and promoter methylation in Chinese glioblastoma patients. *Med Oncol.* 2012;29:1292–6.
- Everhard S et al. Identification of regions correlating MGMT promoter methylation and gene expression in glioblastomas. *Neuro-Oncology.* 2009;11:348–56.
- Shah N et al. Comprehensive analysis of MGMT promoter methylation: correlation with MGMT expression and clinical response in GBM. *PLoS One.* 2011;6:e16146.
- Watanabe T et al. IDH1 mutations are early events in the development of astrocytomas and oligodendrogliomas. *Am J Pathol.* 2009;174:1149–53.
- Jin G et al. Mutant IDH1 is required for IDH1 mutated tumor cell growth. *Oncotarget.* 2012;3:774–82.
- Leu S et al. IDH/MGMT-driven molecular classification of low-grade glioma is a strong predictor for long-term survival. *Neuro-Oncology.* 2013;15:469–79.
- Deimling A et al. The next generation of glioma biomarkers: MGMT methylation, BRAF fusions and IDH1 mutations. *Brain Pathol.* 2011;21:74–87.
- Turcan S et al. IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. *Nature.* 2012;483:479–83.
- Mulholland S et al. MGMT CpG island is invariably methylated in adult astrocytic and oligodendroglial tumors with IDH1 or IDH2 mutations. *Int J Cancer.* 2012;131:1104–13.
- Weller M et al. MGMT promoter methylation in malignant gliomas: ready for personalized medicine? *Nat Rev Neurol.* 2012;6:39–51.
- Weller M et al. Isocitrate dehydrogenase mutations: a challenge to traditional views on the genesis and malignant progression of gliomas. *Glia.* 2011;59:1200–4.
- Weller M et al. Molecular predictors of progression-free and overall survival in patients with newly diagnosed glioblastoma: a prospective translational study of the German Glioma Network. *J Clin Oncol Off J Am Soc Clin Oncol.* 2009;27:5743–50.
- Boots-Sprenger SH et al. Significance of complete 1p/19q co-deletion, IDH1 mutation and MGMT promoter methylation in gliomas: use with caution. *Modn Pathol: Off J U S Can Acad Pathol.* 2013;26:922–9.
- Minniti G et al. IDH1 mutation and MGMT methylation status predict survival in patients with anaplastic astrocytoma treated with temozolomide-based chemoradiotherapy. *J Neuro-Oncol.* 2014;118:377–83.
- Phillips HS et al. Molecular subclasses of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. *Cancer Cell.* 2006;9:157–73.
- Verhaak RG et al. Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell.* 2010;17:98–110.
- McDonald KL et al. The T genotype of the MGMT C>T (rs16906252) enhancer single-nucleotide polymorphism (SNP) is associated with promoter methylation and longer survival in glioblastoma patients. *Eur J Cancer.* 2013;49:360–8.
- Lof-Ohlin ZM et al. Pyrosequencing assays to study promoter CpG site methylation of the O6-MGMT, hMLH1, p14ARF, p16INK4a, RASSF1A, and APC1A genes. *Oncol Rep.* 2009;21:721–9.
- Stupp R et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med.* 2005;352:987–96.