·Basic Research·

Detection of O^6-methylguanine-DNA methyltransferase promoter methylation in chemotherapy for glioma

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[**Abstract**] **Background and Objective**: Epigenetic silencing of the DNA repair gene, O^6-methylguanine-DNA methyltransferase (MGMT), is associated with the therapeutic response to methylating agents. This study was to assess the value of detecting the promoter methylation of MGMT gene in chemotherapy for glioma. **Methods**: Methylation-specific PCR (MSP) was employed to detect MGMT promoter CpG island methylation in 39 samples of glioma taken from surgery. Western blot and immunohistochemistry were used to detect protein expression. MTT were employed to detect the sensitivity of two glioma cell lines to alkylating agents, ACNU and TMZ. The Kaplan-Meier curve was adopted to estimate the overall survival according to the methylation status of the MGMT promoter. **Results**: Methylation of MGMT promoter CpG island was detectable in 46.2% of glioma tissues, but not in any normal tissues. The expression rate of MGMT protein was 61.5%. The status of MGMT methylation status was association with the protein level of MGMT ($P<0.05$). The MGMT gene was demethylated in glioma cell line SHG-44 following 5-Aza-CdR treatment; the expression of MGMT protein was restored and the resistance of SHG44 cells to alkylating agents was reversed. The overall survival was higher in patients with methylated MGMT promoter than in those with unmethylated MGMT promoter ($P<0.05$). **Conclusions**: The status of MGMT promoter CpG island methylation is closely correlated to MGMT protein expression and sensitivity of cells to alkylating agents in glioma. Detection of the methylated sequences of MGMT may be used as a predictive factor for the treatment of giloma.

Key words: glioma, promoter CpG island, hypermethyaltion, DNA repair, O^6-methylguanine-DNA methyltransferase (MGMT)

Malignant glioma is one of the most refractory cancers. The prognosis for such a cancer has not been significantly improved in last decades mainly due to drug resistance, although its therapeutic strategies have been advanced from simple resection to the combination of multiple treatments, including surgery, radiotherapy and chemotherapy. It has been confirmed that O^6-methylguaine-DNA methyltransferase (MGMT) is capable of rapidly repairing DNA damage caused by alkylating agents, which in turn results in the cellular resistance to alkylating agents.[1,2] Gliomas often has drug resistance mainly due to expression of MGMT. Thus, the predictive and personalized clinical trials based on inhibition of MGMT with combination of alkylating agents are

being carried out.[3-5] But the detection of MGMT for diagnosis is being challenged, because the methods and sample collection are difficult. A number of progresses have been recently made in gene methylation related epigenetic silencing of MGMT, and MGMT methylation is closely related to sensitivity to alkylating agents.[6] The detection of gene methylation may be one of the molecular markers for diagnosis and for predicting sensitivity to alkylating agents. This study was to evaluate the significance for MGMT methylation in the sensitivity to alkylating agents for gliomas.

Materials and Methods

Patients and samples. Thirty-nine glioma tumor samples were collected from April, 2003 to December, 2007 in the First Affiliated hospital, PLA General Hospital. All samples were preserved in liquid nitrogen immediately after resection. They were all primary tumors, of which 11 were astrocytomas, five oligodendrogliomas, 10 oligoastrocytomas, 13 glioblastoma multiforme (GBM). Fourteen cases were grade Ⅰ - Ⅱ, and 25 were grade Ⅲ - Ⅳ according to WHO CNS tumor classification and grade. Eighteen cases were male and twenty-one were female. Age ranged 18 to 72 year, median age was 37 years. All the patients received resection, radiation and alkylating agents, such as ACNU treatments. Follow-up information was updated every three months. Control samples were obtained from six cerebral traumas and cortex fistulizations.

Demethylation of glioma cell lines with 5-Aza-CdR . Glioma cell lines SHG-44, U251 were bought from Shanghai Cell bank, Chinese Academy of Science. SHG-44 was cultured in RPMI-1640 (Sigma) and U251 was in high glucose DMEM (Gibco). Fetal bovine serum (FBS) (10%) and double antibiotics were added both medium. The cell lines were incubated in 37℃, 5% CO2, 100% humidity incubator. SHG-44 and U251 cells were cultured as routine, 5umol/L 5-Aza-CdR was added to the medium for 6 days, and the medium with the same concentrations was changed on day 1, 3, 5, and then was switched to routine culture for another 7 days, and finally the drug sensitivity was tested for these cells. Genomic DNA and protein were prepared. Untreated cells were used as controls.

MTT assay. Reagents preparation: ACNU (Sankyo Co., Ltd., Hiratsuka Plant, JP) 5mg/ml was prepared in PBS, temozolomide (Sigma) was dissolved in hydrotrop DMSO and diluted to 5mg/mL in PBS. MTT solution (Amersco) was prepared at 5mg/ml in PBS, filtered through a 0.22 μ m filter, then stored in -20℃。

The cells were suspended to $1x10^5$/ml with culture medium, and 190ul of cells per well were added to a 96-well plate and incubate for 24 hours prior to treatments. Ten microliter of vehicles, ACUN or TMZ were added to each well at seven different concentrations for 72 h, and each treatment was tripled. Cell viability was measured by MTT. Survival rate of the cells=A_{570} experiment/ A_{570} control x 100% . The half maximal inhibitory concentration (IC_{50}) was obtained. The experiments were repeated three times and the mean values were obtained for statistical analysis.

DNA extraction. Genomic DNA was isolated from the frozen tumor sections and cell lines by DNA extraction kit (Promega) as described in the protocol. Agarose gel electrophoresis and ultraviolet spectrophotometer were used for DNA purity and concentration measurement, respectively.

Methylation specific PCR (MSP). DNA modification kit (EZ DNA Methylation-goldTM Kit) was used to modify the bisulfite of the genome DNA. The unmethylated cytosines was converted into uracil (U), which was detected as thymine (T) by following PCR, whereas the methylated cytosines (C) remained unchanged. The MGMT promoter containing -20 to exon 1 was amplified using methylated primers or unmethylated primers. The primers as described previously[6] were synthesized by Aoke Biology CO. LTD, Beijing. The sequences for the methylated MGMT primers were:
5-TTTCGACGTTCGTAGGTTTTCGC-3 (forward),
5-GCACTCTTCCGAAAACGAAACG-3 (reverse). The sequences for the unmethylated MGMT primers were:

5-TTTGTGTTTTGATGTTTGTAGGTTTTT GT-3 (forward), 5-AACTCCACACTCTTCCAAAAACAAAACA-3 (reverse). The annealing temperature was 55℃ and 63℃, respectively.

DNA amplification was performed using Hotstart Taq DNA polymerase (TaKaRa) under the following condition: 95℃ 5min; 95℃ 45s, annealing temperature 45s, 72℃ 60s, 35 cycles; extension 72℃ for 5min. DNA from human PBL or PBL treated with DNA methylase (MSSsI, New England Biolabs) were used as the unmethylated or methylated positive controls. Distilled water instead of DNA was set as negative control. PCR products were separated by 3% agarose electrophoresis and fluoresced by ethidium bromide. The results were showed exposing to ultraviolet light.

Immnohistochemical staining. Tissues were formalin-fixed and paraffin-embedded. DAB was used to show the immunohistochemical staining for MGMT (mouse monoclonal antibody to human, Zhongshan Goldenbridge Biotechnology CO, LTD, Beijing), staining nuclei and cytoplasm. The positive staining was brown and stronger than the background, otherwise was negative. Positive staining of less than 5% cell was regarded negative, ≥ 5% and <10% was regarded weakly positive, ≥ 10% and <30% was positive, and ≥ 30% was strong positive.

Statistical analysis. Statistics was analyzed by SPSS 13.0 for Windows software. Multiple-sample means were compared by one-way analysis of variance (ANOVA). Comparison of means of two groups was performed by one-sample t test. Comparisons of pre- and post-treatment were performed by paired-sample t test. Kaplan-Merier survival curve and log-rank test were employed to analyze the status of gene methylation and survival. $p<0.05$ was regarded as significantly different.

Results

Correlation of methylation status of MGMT promoter to its protein expression levels in glioma tissues. MSP results are shown in Fig 1. If there was an unmethylated band (U), but not a methylated band (M), then the MGMT promoter was unmethylated in these tissues. The MGMT promoter was considered methylated if there were both U and M bands.

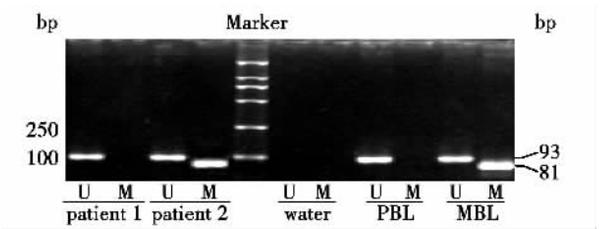

Figure 1 Methylation-specific PCR analysis of MGMT promoter CpG island methylation in glioma tissues

PBL: peripheral blood lymphocytes; MPBL: methylated PBL; U: unmethylated; M: methylated.

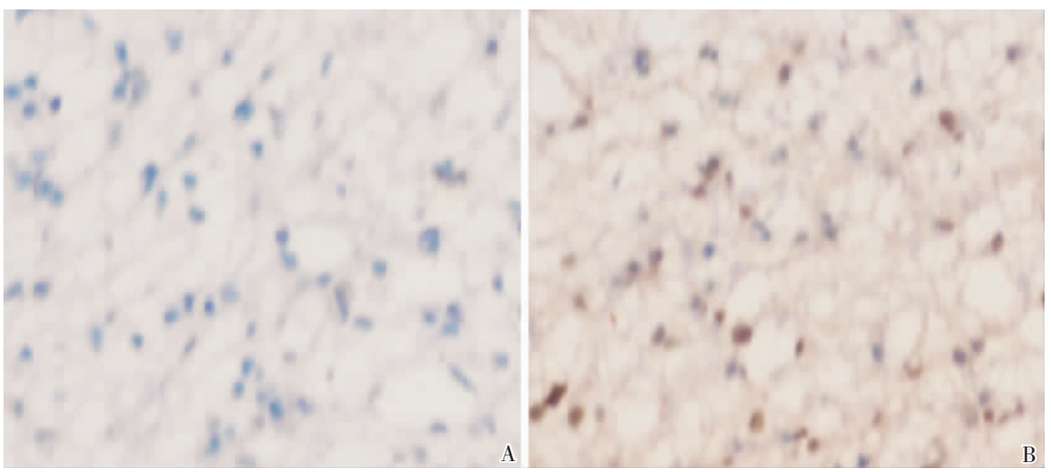

Figure 2 Expression of MGMT in glioma tissues detected by immunohistochemistry (DAB ×200)

A: negative expression of MGMT in the nucleolus and cytoplasm; B: positive expression of MGMT (brown) in the nucleolus and cytoplasm.

Methylation of the MGMT promoter CpG island was detected in 46.2% (18/39) glioma samples whereas unmethylation of MGMT promoter was found in six normal controls. As shown in Table 1, MGMT protein was positive in 61.5% (25/39) glioma samples detected by immunohistochemical staining. Interestingly, among eighteen cases with methylated MGMT promoter, the protein levels of MGMT were negative in 14 cases, weekly positive in three cases, and positive in one case. However, only one case was negative for MGMT protein among 21 cases that were unmethylated in the MGMT promoter. MGMT promoter methylation was closely related to MGMT protein expression in glioma tumor detected by Spearmman test ($p<0.05$, Table 1). MGMT promoter methylation was not associated with gender, age, tumor size, pathological type and stage ($p>0.05$).

Table 1 The relationship between the status of MGMT promoter methylation and the expression of MGMT protein in gliomas tissue

MGMT promoter	MGMT protein expression (case)		Total [cases (%)]	Spearman
	Positive	Negative		
Methylation	4	14	18 (46.2)	0.031
Unmethylation	20	1	21 (53.8)	

The status of MGMT methylation, MGMT protein expression and sensitivity to alkylating agents. The CpG island of MGMT promoter in SHG-44, a glioma cell line, was hypermethylated and its protein was undetectable by Western blot. However, MGMT gene was totally demethylated in SHG-44 cells after the treatment with 5umol/L 5-Aza-CdR, and MGMT protein was detected. MGMT protein was overexpressed in U251, another gliomia cell line whose MGMT promoter is unmethylated. MGMT protein expression was not altered in U251 cells after being treated with 5-Aza-CdR ($p>0.05$, Fig 3, 4).

Results of sensitivity test of SHG-44 and U251 to alkylating agents are shown in Fig 5. IC_{50} of ACNU and TMZ for SHG-44 cells was 30ug/mL and 11ug/mL, respectively, whereas IC_{50} of ACNU and TMZ for U251 cells was

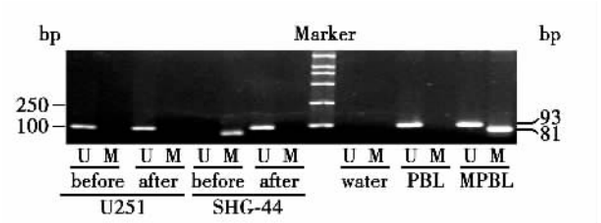

Figure 3 Changes in the status of MGMT mehylation of glioma cell lines after 5-Aza-CdR treatment

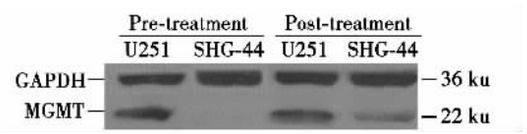

Figure 4 Expression of MGMT protein in glioma cell lines after 5-Aza-CdR treatment by western blot

93.6ug/mL and 25ug/mL, indicating that SHG-44 cells were more sensitive to alkylating agents than U251 cells. Moreover, the sensitivity of SHG-44 to alkylating agents was reversed after 5-Aza-CdR treatment, and IC_{50} of ACNU and TMZ was increased by 2.5 and 3.1 times, respectively, when SHG-44 cells were treated with 5-Aza-CdR. However, the sensitivity of U251 to ACNU and TMZ did not change significantly after 5-Aza-CdR treatment ($p>0.05$).

The relationship of MGMT methylation and surival. Thirty-nine gliomas patients received surgery, radiotherapy and alkylating agent therapy. The relationship between the status of MGMT methylation and survival was estimated by Kaplan-Merier survival analysis and log-rank test. The median survival of patients with methylated MGMT was 26 months, while that of those with unmethylated MGMT was 16 months ($p<0.05$, Fig 6).

Disscussion

Chemotherapy plays a critical role in the combination of multiple treatments for gliomas, and alkylating agents are particularly important for gliomas. Chloroethylnutrimustines (CENUs) and TMZ are the first choice for gliomas, but the individual response varies. Drug resistance is the

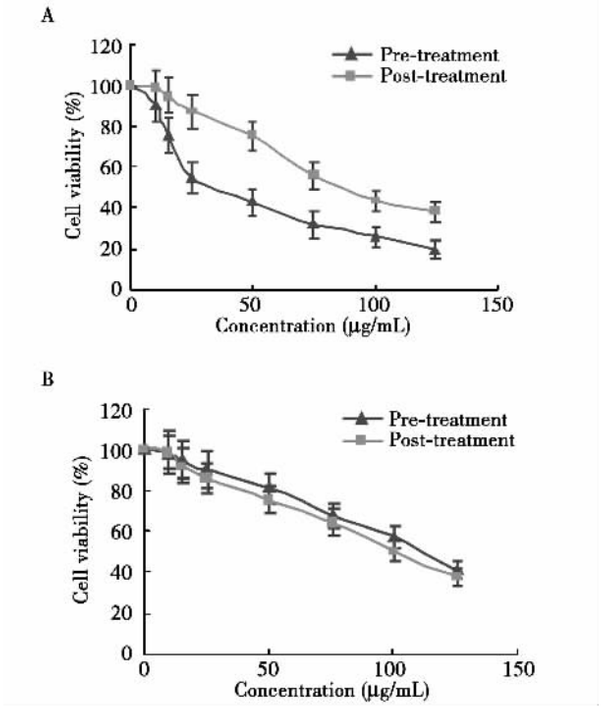

Figure 5 Drug sensitivity of SHG-44 and U251 towards alkylating agents before and after 5-Aza-CdR treatment
A: SHG-44 cells; B: U251 cells

major reason of the failure of chemotherapy for gliomas. It is urgent to predict and to solve the problem of drug resistance of chemotherapy in gliomas.

MGMT, a DNA repair protein, removes mutagenic and cytotoxic alkyl group from O^6 position of guanine, an important site of DNA alkylation. Thus, the damaged guanine is repaired and cell is protected against alkylating radicals, which is the main cause for drug resistance. MGMT protein is unique in DNA repairing and reverses the damage directly. It can immediately transfer O^6 AG alkyl group to an internal cysteine residual in the active site of MGMT, thereby restoring the guanine in damaged DNA. This is a so-called suicide reaction. The methylated MGMT protein is inactivated and released from DNA and degraded through uibiquitin, and such process is irreversible even by demethylation. It is well-documented that the expression of MGMT in glioma tumors obviously affects the response of tumor cells to alkylating agents and shortens the survival of patients.[5-7]

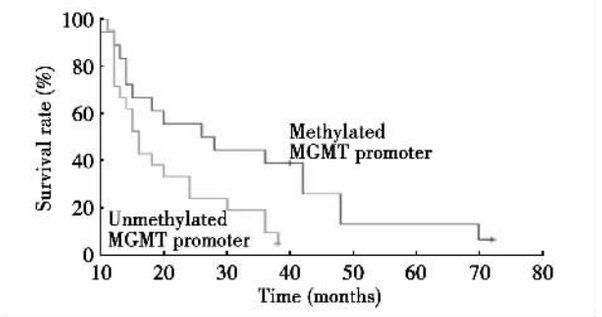

Figure 6 Overall survival of 39 patients with glioblastoma according to MGMT promoter methylation status

Recently, great progress has been made in epigenetic silencing of MGMT gene by promoter methylation. On one hand, silencing of MGMT gene can cause accumulation of promutagenic lesions of oncogenes, which subsequently trigger and promote carcinogenesis. On the other hand, hypermethylation of MGMT gene can increase the sensitivity of tumor cell to alkylating agents.[7,8]

A variety of MGMT inhibitors are being investigated to combine with alkylating agents to explore a predictive, individual regimen. But diagnostic methods for MGMT are difficult in technology and tissue derivation. Researchers are trying to detect the protein level or the activity of MGMT to evaluate the sensitivity to chemotherapies. But there are many factors that could affect the results. The expression of MGMT is prone to be induced by alkylating agents, radiotherapy, and glucorticoidl.[8] Immunohistochemistry and enzyme activity could be affected by normal tissues and tumor infiltrating lymphocytes. Measurement of gene methylation status by MSP is more reliable and simpler than testing protein expression, because only tumor tissues have methylation alles, as a result, contamination with non-cancerous tissues would not influence the results. Promoter methylation is a stable marker to predict the cellular capacity to induce MGMT. Thus MGMT gene methylation has an important role in early diagnosis of glioma and in predicting resistance of glioma to alkylating agents.

MGMT gene methylation has been reported in many human cancer, such as lung cancer, esophageal cancer, colon cancer, cervical cancer, and so on.[6,9,10] MGMT gene methylation has

been rarely revealed in normal tissues. Consistent with other reports, our results also showed that MGMT promoter methylation was detected in 46.2% of glioma tissues but not in normal controls, suggesting that MGMT methylation is a common molecular event in the development of glioma. Kaplan-Merier survival analysis suggests that patients with MGMT methylation had longer survival than those with unmethylated MGMT. All patients in the study received resection, radiotherapy and ACNU therapy. The survival difference appears to be related to the status of MGMT methylation. MGMT methylation regulates the MGMT expression and affects the sensitivity of glioma cells to alkylating agents, as shown in this study. This is supported by Hegi et al.[7] Collectively, the status of MGMT methylation plays an important role in increasing response of cancer cells to alkylating agents and in predicting the prognosis of glioma.

In summary, the methylation status of MGMT promoter may be a valuable marker for prognosis and for predicting resistance to alkylating agents in patients with glioma.[9-11]

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