Original Article
The change of MGMT gene expression in glioma patients was affected by methylation regulation and in the treatment of alkylation agent

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Abstract: Glioma is a common malignant tumor in the brain that severely threatens public health. DNA methylation is a manifestation of epigenetics and the MGMT gene correlates with cell metabolism. Previous studies showed the involvement of MGMT methylation in the malignancy of various tumors. This study focused on the role of the MGMT methylation level and its role in the treatment of glioma by alkylating agents. Human neuroglioma cell line SHG-44 was treated with 5, 10, and 20 μM 5-Aza-CdR, followed by a qRT-PCR analysis of the MGMT mRNA expression level. Pyrophosphate sequencing was performed to analyze the methylation level of the MGMT gene. A total of 89 neuroglioma patients in our hospital from Jan 2014 to Feb 2017 were recruited for measuring the methylation level in the promoter region of the MGMT gene using the pyrophosphate sequencing approach. Gene methylation levels were compared at 1, 3, 6, and 12 months after drug treatment (temozolomide and nimustine). Treatment with 5-Aza-CdR gradually increased MGMT mRNA expression in a dose-dependent manner, and the MGMT mRNA level was negatively correlated with the methylation level. MGMT gene methylation showed statistical significance among patients across age, smoking, and drinking history. Those patients with more advanced tumor stages presented with higher methylation levels (P<0.05). Glioma patients presented a decreased trend of MGMT methylation levels with an elongated treatment duration. MGMG expression is under the regulation of methylation in glioma cells and is correlated with the efficacy of alkylating drugs.

Keywords: DNA methylation, glioma, MGMT gene, alkylating agents

Introduction

Among various primary brain tumors, neuroglioma is the most prevalent form, with a relatively higher incidence and mortality. In the US, around 13,000 people are newly diagnosed with glioma each year [1], severely threatening public health. Current common treatment methods include surgery in combination with chemotherapy, but these two methods frequently cannot achieve a satisfactory effect due to biological diversity and the chemotherapy insensitivity of glioma cells. The MGMT gene is involved with DNA repair and in the resistance to alkylating drugs of glioma cells [2, 3]. Epigenetics, including DNA methylation, histone covalent modification, and chromosomal remodeling are closely correlated with tumor genesis and progression [4], and an increasing number of studies have focused on the effect of DNA methylation abnormalities on tumor occurrence, working to develop reliable biomarkers for the early diagnosis and treatment of tumors [5]. Previous studies have demonstrated a correlation between MGMT protein expression and the methylation level of the gene promoter region, which usually shows about a 30%-60% methylation level [6, 7]. More specifically, the methylation levels of the promoter regions are 20%-30% for WHO grade I tumors, 60%-80% for grade II, 40%-45% for grade III, and 20%-45% for grade IV tumor patients. Therefore, the MGMT methylation level benefits the clinical staging of glioma in addition to helping individualize treatment approaches.

As a proteinase for DNA damage repair, MGMT is of critical importance in the post-damage repair of DNA, and alkylating agents are a common drug for treating tumors. The CpG methyla-
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The hypermethylation of the MGMT promoter plays crucial roles in the development of temozolomide resistance [9]. However, most approaches for methylation level assays are not precise enough. Pyrophosphate sequencing is more precise compared to the normal sequencing approach. This study thus employed pyrophosphate sequencing for examining DNA methylation levels of the promoter region, for analyzing the methylation level in tumors with different malignant stages. Meanwhile, we also analyzed the effect of alkylation agents on methylation levels, to provide more effective evidence for clinical diagnosis and treatment.

Materials and methods

Major equipment and reagent

We obtained the following: Human neuroglioma cell line SHG-44 (Beinuo Biotech, China). Whole genome extraction kit (Invitrogen, US). Hydrosulfite epigenetic modification kit (Qiagen, Germany); PCR amplification kit (Qiagen, Germany); Pyrophosphate sequencing analyzing reagent (Qiagen, Germany); Pyromark Q96 ID sequencer (Qiagen, Germany). Trizol reagent for RNA extraction (Invitrogen, U.S.); Real-time fluorescent RT-PCR test kit (TaKaRa, Japan); Gel imaging system and model ViiA7 fluorescent quantitative PCR cycler (ABI, U.S.). RT-PCR test kit (Toyobo, China); 5-Aza-CdR (Sigma, U.S.).

Patient information

A retrospective study was performed on 89 brain glioblastoma patients (53 males and 36 females, between 13 and 80 years old) who were recruited from the First Hospital of Jilin University (Changchun, Jilin, China) between January 2014 and February 2017. The general information of the patients was gathered and recorded through telephone follow-up. The diagnostic criteria followed the WHO typing criteria of the central nervous system established in 2007 [10]. The inclusion criteria were: i) the patients were treated with conventional therapies consisting of maximal surgical resection, followed by radiotherapy and/or chemotherapy; ii) the patients received follow-up and were >18 years old. Patients who received radiotherapy or chemotherapy before admission or who died from non-glioma-related diseases were excluded from this study. All patients received surgery in conjunction with chemotherapy using temozolomide and/or nimustine. Using the WHO staging criteria, we classified 35, 21, and 33 cases into grades II, III, and IV.

This study was approved by the ethics committee of the First Hospital of Jilin University (Changchun, Jilin, China) and all the enrolled subjects signed an informed written consent.

Culture and 5-Aza-CdR treatment for human glioma cell line SHG-44

The human neuroglioma cell line SHG-44 was kept in an RPMI1640 medium with 5% CO₂. 5-Aza-CdR was dissolved in DMSO and was added into the culture medium at 5, 10, and 20 µM concentrations. The control group received DMSO treatment, and the culture medium was changed every 24 h. Each treatment group was tested in triplicate for 72 h, and the cells were digested by trypsin for collection. Following the instructions of the qRT-PCR test kit (see protocols in the next section), the mRNA expression level of the human neuroglioma cell line SHG-44 was measured using agarose gel electrophoresis of the amplified products.

mRNA extraction and real-time, fluorescence-based quantitative PCR

Liquid nitrogen was used to homogenize the cancer tissue samples, and RNA was extracted using Trizol reagent. The RNA purity and concentration were determined by spectrometry. The RNA samples were then loaded onto 1% agarose gel electrophoresis to determine their integrity. 1 µg mRNA was used for cDNA reverse transcription. In brief, mRNA was mixed with oligoDT at 65°C in a warm bath for 5 min, followed by 4°C cold centrifugation. A 5XPrimerscript™ buffer was mixed with Primerscript™ RTase and dNTP, for 37°C amplification on a PCR cycler for 5 min. Reverse transcriptase was added into the reaction system for a 42°C, 60 min incubation, and a 70°C, 10 min incubation. cDNA prepared by reverse transcription was used in the PCR system, consisting of 2 µl SYBRGreen, 0.1 µl forward primers, 0.1 µl reverse primer, 0.1 µl dNTP, 1 µl cDNA, 0.2 µl Taq, and 6.5 µl ddH₂O (total volume = 10 µl). The amplification conditions were: 95°C denaturing for 5 min, followed by 40 cycles each consisting of 95°C 15 sec, 60°C 60 sec. PCR was performed on a ViiA7.
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Table 1. The methylation level of the MGMT gene and clinical parameters

<table>
<thead>
<tr>
<th>Clinical index</th>
<th>N</th>
<th>MGMT methylation level</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60 years</td>
<td>45</td>
<td>45.13±12.24</td>
<td>0.320008</td>
</tr>
<tr>
<td>≥60 years</td>
<td>44</td>
<td>47.53±10.29</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>42</td>
<td>43.21±8.09</td>
<td>0.133224</td>
</tr>
<tr>
<td>No</td>
<td>47</td>
<td>46.01±9.21</td>
<td></td>
</tr>
<tr>
<td>Drinking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>38</td>
<td>51.43±9.87</td>
<td>0.283787</td>
</tr>
<tr>
<td>No</td>
<td>51</td>
<td>49.32±8.54</td>
<td></td>
</tr>
<tr>
<td>WHO grade</td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>II</td>
<td>35</td>
<td>44.32±7.34</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>21</td>
<td>48.17±5.41</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>33</td>
<td>53.21±6.78</td>
<td></td>
</tr>
</tbody>
</table>

DNA extraction and whole genome DNA hydro-sulfite modification

A whole genome extraction kit was used to extract DNA from the cancerous and adjacent tissues following the kit’s instructions. UV spectrometry was used to measure the DNA content and purity. Those samples with an A260/A280 ratio between 1.7 and 1.9 were used for further assays. 1 μg DNA was used for hydro-sulfite modification, following the instructions of the test kit. Sss I methylase was used for processing, using those human genomic DNA transformed by bisulfite sodium as a positive control. The DNA with modifications and purification was kept at -20°C for the subsequent assays.

Pyrophosphate sequencing for the MGMT gene methylation levels of the cancerous and adjacent non-cancerous tissues

DNA after bisulfite modification and purification was used for amplifying the MGMT gene by PCR following the kit’s instructions. A targeted fragment was amplified and separated by electrophoresis to obtain single and obvious bands for further assays. Biotin-labelled PCR products were mixed with streptavidin-loaded microbeads. Pre-treatment in a vacuum was performed to separate the single strands with biotin labels and the unlabeled strands. Single stranded DNA was mixed with the MGMT sequencing primer (5'-ATGGGTACACC TGACT GAC-3'). After mixing, the methylation status of the MGMT gene was measured by a Pyromark Q96 ID pyrophosphate sequencer. The average methylation rate was calculated from all loci.

Statistical methods

SPSS 13.0 software was used for the statistical analysis of all data. The clinical and biological characteristics of the patients were expressed as the means ± SD. The comparisons were performed using Fisher’s exact test. The enumeration data were analyzed using a chi-squared test. The Pearson method was employed for a correlation analysis between the methylation level and mRNA. Statistical significance was defined when P<0.05.

Results

General clinical information

We analyzed the patients’ ages and smoking/drinking histories. The results showed no significant differences in MGMT gene methylation levels among the patients with various ages, or different smoking/drinking histories (P>0.05). Across tumors with different malignancies and MGMT methylation levels, however, the methylation level was elevated with advanced malignancy (P<0.05, Table 1).

MGMT expression mediates the methylation level of the promoter region

After the 5-Aza-CdR treatment, the cells were extracted to measure the mRNA level of MGMT genes by qRT-PCR (Figure 1). The DNA methylation assay is shown in Figure 2. With an elevated 5-Aza-CdR concentration, MGMT expression was gradually increased (P<0.05).

The Pearson correlation approach was used to analyze the relationship between DNA methylation levels and mRNA expression of the MGMT gene in cancer tissues (Table 2). The results...
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Figure 1. The MGMT mRNA level of SHG-44 after treatment using different concentrations of 5-Aza-CdR. Total RNA was isolated from SHG-44 cells after treatment with different concentrations of 5-Aza-CdR followed by measuring the MGMT mRNA level using quantitative RT-PCR. Compared with MGMT in the control group, **P<0.01.

Figure 2. MGMT methylation level of SHG-44 cells after 5-Aza-CdR treatment. After treatment with different concentrations of 5-Aza-CdR, the MGMT methylation level in the SHG-44 cells was measured by pyrophosphate sequencing. **, P<0.05 compared to the control group.

showed a significantly negative correlation between these two factors (R = -0.46, P<0.01). The MGMT expression level was gradually decreased with a higher DNA methylation level, indicating that the DNA methylation level of tissue MGMT might affect E-cadherin protein expression.

The mRNA and methylation levels of cancer tissues and adjacent glioma tissues

A statistical analysis of pyrophosphate sequencing and qRT-PCR found significantly lower

Table 2. MGMT mRNA and methylation level I SHG-44 cells after 5-Aza-CdR treatment (mean±SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>Methylation level</th>
<th>mRNA relative expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>57.51±8.97</td>
<td>1.00±0.30</td>
</tr>
<tr>
<td>5 μM</td>
<td>52.43±8.98</td>
<td>2.60±0.27</td>
</tr>
<tr>
<td>10 μM</td>
<td>48.43±7.98</td>
<td>7.60±1.32</td>
</tr>
<tr>
<td>20 μM</td>
<td>42.43±5.98</td>
<td>13.00±2.14</td>
</tr>
</tbody>
</table>

MGMT mRNA levels in cancer tissues compared to adjacent tissues (P<0.05), but the methylation levels in the cancer tissue was significantly higher than it was in the adjacent tissues (60.21% vs 23.12%, P<0.05, Figure 3). With a more advanced malignancy, the methylation rate was gradually decreased, but the mRNA expression was enhanced.

Methylation level of 10 CpG sites of MGMT promoter after drug treatment

The pyrophosphate sequencing approach was employed to examine the methylation levels of 10 CpG loci, as shown in Figure 4. Drug treatment changed the MGMT methylation level. Further analysis of methylation levels across different loci revealed a decreased methylation level but consistent patterns across different malignant stages. Meanwhile, the amplitudes of the methylation level changes were different at specific loci (Figure 4).

Discussion

Previous studies showed a significant correlation between promoter methylation and gene down-regulation of MGMT in glioma patients [11]. The results of this study showed a negative correlation between DNA methylation level and mRNA expression, as CpG island methylation of the promoter region regulates mRNA expression, which has also been obtained from most studies [12, 13].

Different assays of DNA methylation can lead to different results [14]. The application of a DNA methylation assay with high accuracy and efficacy is thus of critical importance for data interpretation. With the continuous advancement of DNA methylation assays, traditional approaches, including methylation-specific PCR (MSP), have a relatively higher cost and a longer duration, plus a smaller sample size.
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Figure 3. mRNA and methylation levels of cancer/adjacent tissues of glioma patients. DNA or RNA was isolated from the cancerous or adjacent non-cancerous tissues of glioma patients followed by analysis of the MGMT methylation levels by pyrophosphate sequencing (A) or MTMG mRNA level by quantitative RT-PCR (B). **, P<0.05 compared to the control group.

Figure 4. Methylation levels after drug treatment. DNA was isolated from the cancer tissues of glioma patients before and after treatment followed by an analysis of the methylation levels of 10 CpG loci by the pyrophosphate sequencing approach.

compared to bisulfite sequencing PCR (BSP), which can detect methylation levels in a real-time quantitative manner, making the results more reliable. Pyrophosphate sequencing has now become the gold standard of DNA methylation assays. The results of this study showed
that the mRNA expression of the MGMT gene was under the regulation of methylation, which is consistent with previous studies [13, 14]. Temozolomide is a newly discovered alkylating reagent that has brought new insights for glioma treatment [15]. Temozolomide induces about 5% DNA methylation at the O6-methyl group of purine, which is believed to play an important role in cytotoxicity. MGMT can remove the O6-methyl group of purine, thus suppressing the cytotoxicity of Temozolomide [16-18]. There are 97 CpG loci within the MGMT promoter region, but not every methylation site regulates MGMT expression, not to mention the two different methylation domains [19, 20]. Therefore, this study followed the work of Michal Bienkowski et al., and found an alternation of the MGMT methylation level after drug treatment. Further analyses of methylation across different loci found decreasing trends of methylation levels across sites with elongated treatment times and consistent trends at all malignant grades. Meanwhile, the amplitude of the methylation level change was different across loci, as locus 2 and 3 showed hypo-methylation compared to those loci before the drug treatment. These two CpG loci thus may become important targets for clinical treatment.

In clinical studies, however, not every glioma patient is suitable for surgery, and there are lots of patients who must seek alternative treatments other than surgery, making individualized therapy of critical importance for those patients. The pyrophosphate sequencing approach for the MGMT methylation assay had a critical guidance role for clinical staging. However, due to the lack of a unified standard for methylation level or test protocol [21-23], further clinical studies are warranted.

**Conclusion**

MGMT expression is closely correlated with promoter methylation, and it plays important roles in the occurrence and progression of neoglioma. Those patients with more advanced tumor stages show even higher methylation levels. Drug treatment can decrease the MGMT methylation level in a time-dependent manner. Therefore, assays for the analysis of MGMT methylation levels might provide guidance on the efficacy of drug treatment.

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**Disclosure of conflict of interest**

None.

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