Is the absolute value of $O^6$-methylguanine-DNA methyltransferase gene messenger RNA a prognostic factor, and does it predict the results of treatment of glioblastoma with temozolomide?

Clinical article

SATOSHI TANAKA, M.D., M.Sc.,1 JIRO AKIMOTO, M.D., M.Sc.,2 YOSHIKATA NARITA, M.D., M.Sc.,3 HIDEHiro OKA, M.D., M.Sc.,4 AND TAKASHI TASHIRO, M.D., M.Sc.5

1Department of Neuro-Oncology and Neurosurgery, Tokyo Nishi Tokushukai Hospital; 2Department of Neurosurgery, Tokyo Medical University Hospital; 3Neurosurgery Division, National Cancer Center Hospital, Tokyo; 4Department of Neurosurgery, Kitasato University School of Medicine, Sagamihara; and 5Department of Neurosurgery, Shioya Hospital, International University of Health and Welfare, Yaita, Japan

Object. Methylation of $O^6$-methylguanine-DNA methyltransferase (MGMT) has been reported to be a good prognostic factor for patients with glioblastoma multiforme (GBM). To determine whether the absolute value of MGMT messenger RNA (mRNA) might be a prognostic factor and useful for predicting the therapeutic effectiveness of temozolomide, especially with regard to GBMs, the authors measured the absolute value of MGMT mRNA in gliomas by using real-time reverse-transcription polymerase chain reaction (RT-PCR).

Methods. MGMT mRNA was measured in 140 newly diagnosed gliomas by real-time RT-PCR using the TaqMan probe. Among 73 GBMs, 45 had been initially treated with temozolomide and radiation.

Results. The mean MGMT mRNA value was significantly lower in oligodendroglial tumors than in other tumors. In the 73 GBMs, a significant prognostic factor for progression-free survival was fewer than 1000 copies/μgRNA of MGMT mRNA (p = 0.0150). Of 45 patients with GBMs that had been treated with temozolomide and radiation, progression-free survival was significantly longer for those whose GMB had fewer than 1000 copies/μgRNA of MGMT mRNA than for those whose GBM had more than 1000 copies/μgRNA (p = 0.0090). In 32 patients with GBMs treated by temozolomide and radiation whose age was younger than 75 years and whose Karnofsky Performance Scale score was more than 70, progression-free and overall survival times were longer for those with GBMs of fewer than 5000 copies/μgRNA of MGMT mRNA than for those with GBMs of more than 5000 copies/μgRNA (p = 0.0365 and p = 0.0312).

Conclusions. MGMT mRNA might be useful as a prognostic factor and for predicting the results of therapy for GBMs treated by temozolomide. New individual adjuvant therapy based on the results of MGMT mRNA quantitation has been proposed.

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**Key Words** • MGMT • glioblastoma • real-time RT-PCR • mRNA • temozolomide • oncology

Abbreviations used in this paper: ACNU = 1-(4-amino-2-methyl-5-pyrimidynyl) methyl-3-(2-chloroethyl)-3-nitrosourea hydrochloride; cDNA = complementary DNA; GADPH = glyceraldehyde-3-phosphate dehydrogenase; G-CIMP = glioma-CpG island methylator phenotype; MGMT = $O^6$-methylguanine-DNA methyltransferase; mRNA = messenger RNA; RT-PCR = reverse-transcription polymerase chain reaction.
Quantitation of MGMT mRNA in gliomas

procarbazine, and temozolomide. We previously reported that quantitation of MGMT messenger RNA (mRNA) was an excellent method for predicting the effectiveness of ACNU in glioma therapy and that individual adjuvant therapy based on the absolute value of MGMT mRNA was effective for glioma therapy.

MGMT is inactivated by methylation of the promoter domain DNA. MGMT that has been methylated in promoter DNA is not transcribed, and MGMT mRNA cannot be generated. Because temozolomide is an alkylating agent, MGMT DNA methylation causes resistance to not only ACNU but also temozolomide. Methylation of MGMT has been reported to be a good prognostic factor for GBM. However, there is no clinical evidence regarding whether MGMT methylation can predict the results of temozolomide therapy for GBM.

We previously measured the absolute value of MGMT mRNA of gliomas by using real-time reverse-transcription polymerase chain reaction (RT-PCR). In the study reported here, especially with regard to GBMs, we investigated whether the absolute value of MGMT mRNA could be a prognostic factor and predict the results of therapy with temozolomide.

Methods

Patient Population

Included in the study were 140 patients with newly diagnosed gliomas treated at the 7 institutes participating in the Tokyo Consortium for Brain Tumor Treatment. Among these 140 patients, 96 received treatment at Tokyo Medical University Hospital, Tokyo; 19 at Kitasato University Hospital, Sagamihara; 10 at Kitasato University Medical Center, Kitamoto; 10 at National Cancer Center Hospital, Tokyo; 2 at Shioya Hospital, International University of Health and Welfare, Yaita; 2 at Kawasaki Hospital, Hitachiota; and 1 at Tokyo Women’s Medical University Hospital, Tokyo, Japan. Other than 10 patients at the National Cancer Center Hospital, 130 were consecutively enrolled. Histologically, 18 tumors were low-grade astrocytomas, 25 were oligodendrogliomas, 24 were anaplastic astrocytomas, and 73 were GBMs. Among the 73 patients with GBM, 45 received treatment after temozolomide was approved in Japan in 2006 and initially received temozolomide and radiation after surgery according to the Stupp protocol. Since 2005, a total of 100 tumors were obtained fresh after resection, and the 40 tumors obtained before 2005 had been frozen. Patients received treatment from 1999 through 2013, and MGMT mRNA quantitation was performed from 2005 through 2013.

All patients provided written informed consent for quantitation of MGMT mRNA in tumor samples. MGMT mRNA quantitation by real-time RT-PCR was approved by the Ethics Committee at Tokyo Medical University in 2005, at Kitasato University in 2002, and at the International University of Health and Welfare in 2012.

Real-Time RT-PCR

The 100 freshly obtained tumor samples were about 5 mm in diameter; were obtained immediately after resection, after the removal of necrotic tissues by direct visualization of trained surgeons; and were stored at 4°C in QIAGEN RNAlater TissueProtect Tubes (Ambion, Inc). Frozen samples for retrospective analysis had been stored at −70°C. All samples were anonymously collected and tested within about 2 days by Special Reference Laboratory Co. Ltd., Hino, Japan.

Total RNA was extracted from about 10 mg of fresh or frozen tissues by the guanidinium thiocyanate-phenol-chloroform extraction method by using Isogen (Wako Junyaku) and was collected from the precipitate in ethanol. The complementary DNA (cDNA) was synthesized from 1 μg total RNA with a random primer (Invitrogen), 40 U M-MLV Reverse Transcriptase (Invitrogen), 0.5 mM dNTP (Takara Bio), 24 U RNase inhibitor (Takara Bio), 10 μM DTT (Sigma Genosys), and 5× reverse transcriptase buffer, and incubated at 37°C for 60 minutes and then stored at −20°C until use. The real-time PCR reaction mixture was prepared by using a TaqMan Universal Master Mix (Applied Biosystems), 120 nM of each primer, 200 nM of probe (5'-CGA GTG GGA GCA ATG AGA-3'), and 2.5 μL of each cDNA sample. The conditions for PCR were denaturation at 95°C for 10 minutes and 50 cycles at 95°C for 30 seconds, 60°C for 40 seconds, and 72°C for 30 seconds, with a real-time PCR system (ABI PRISM 7700 Sequence Detection System, Applied Biosystems). For use as a quantitative internal control, we monitored the expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Standard curves for MGMT and GAPDH mRNA were generated by using 10-fold serially diluted standard plasmid clones into which MGMT or GAPDH PCR products had been inserted as a template; the expression level of each mRNA was calculated from the standard curve. For more accurate quantitation, the MGMT mRNA expression level of each sample was normalized by the expression of the GAPDH gene.

Statistical Analyses

The effectiveness of adjuvant therapy was evaluated at least 2 months after the beginning of therapy according to the response criteria of Macdonald et al. The overall survival and progression-free survival periods were also investigated, and the durations of median survival and median progression-free survival were calculated. We statistically analyzed some independent prognostic factors regarding the effectiveness of therapy, such as patient age, sex, Karnofsky Performance Scale score, eloquence of the tumor, tumor resection rate, effectiveness of the initial therapy, and the absolute value of MGMT mRNA in a multivariate regression analysis. Multivariate regression analysis was used to identify which of the variables described above predicted the effectiveness of therapy. A Kaplan-Meier analysis was used to estimate survival times, and the log-rank (Mantel-Cox) test was used
for binary variables. The Cox proportional hazard model and the Wald test were used for continuous variables. All reported p values are 2-tailed. Statistical significance was set at a level of $\alpha = 0.05$.

Results

Quantitation of MGMT mRNA in Gliomas

The absolute values of MGMT mRNA normalized to the level of GAPDH in 140 gliomas are summarized in Table 1. The absolute value of MGMT mRNA in 140 gliomas was $7.22 \times 10^3 \pm 10.17 \times 10^3$ copies/μgRNA (mean ± SD). Although the mean values for low-grade gliomas, anaplastic astrocytomas, and GBMs did not significantly differ from those of the other 3 groups ($p = 0.0782$, $0.3846$, and $0.3953$, by Student t-test, respectively), the mean absolute value for anaplastic oligodendrogial tumors ($3.74 \times 10^3 \pm 3.87 \times 10^3$ copies/μgRNA) was significantly lower than mean absolute values for other tumors ($p = 0.0300$ by Student t-test).

Quantitation of MGMT mRNA in GBMs

Prognostic factors were analyzed for 73 patients with GBM for which the absolute value of MGMT mRNA was measured. The results are summarized in Table 2. According to single-variant analysis, significant prognostic factors for longer progression-free survival were tumor location in a noneloquent area, fewer than 1000 copies/μgRNA of MGMT mRNA ($p = 0.0150$ by log-rank test), higher surgical resection rate, and good response to initial adjuvant therapy. According to multivariate regression analysis, significant prognostic factors for longer progression-free survival were fewer than 1000 copies/μgRNA of MGMT mRNA (p = 0.0365 by log-rank test). Progression-free survival was significantly longer for patients with fewer than 1000 copies/μgRNA of MGMT mRNA (p = 0.0274 and p = 0.0322 by log-rank test).

Figure 2 shows the Kaplan-Meier survival curves for progression-free and overall survival times for the 32 GBM patients who did not have inherently bad prognostic factors, who were younger than 75 years of age, and whose Karnofsky Performance Scale score was more than 70. Progression-free and overall survival times were significantly longer for patients with fewer than 5000 copies/μgRNA of MGMT mRNA than for those with 5000 or more copies/μgRNA of MGMT mRNA (p = 0.0365 and p = 0.0312 by log-rank test). According to single-variant and multivariate regression analyses, significant prognostic factors for longer progression-free survival were fewer than 5000 copies/μgRNA of MGMT mRNA and higher surgical reduction rate. Single-variant regression analysis showed these 2 factors and the effectiveness of initial treatment were significant factors that indicate a better prognosis.

The same analyses for prognostic factors were performed for 45 patients with GBM that had initially been treated with temozolomide and radiation by the Stupp protocol. Progression-free survival was significantly longer for the 14 patients who received temozolomide for a GBM with fewer than 1000 copies/μgRNA of MGMT mRNA than for the 31 patients who received temozolomide for a GBM with more than 1000 copies/μgRNA of MGMT mRNA ($p = 0.0090$ by log-rank test) (Table 3). According to single- and multivariate regression analyses, significant factors that indicate a better prognosis were tumor location in a noneloquent area and fewer than 1000 copies/μgRNA of MGMT mRNA. According to single-variant analysis, significant prognostic factors for longer overall survival were younger patient age, tumor location in a noneloquent area, and the effectiveness of initial adjuvant therapy. According to multivariate regression analysis, a good response to initial adjuvant therapy alone was a significant prognostic factor for longer overall survival time. Neither the absolute value of MGMT mRNA nor fewer than 1000 copies/μgRNA of MGMT mRNA was a significant prognostic factor for overall survival time. The Kaplan-Meier survival curves for the patients with fewer than 1000 copies/μgRNA of MGMT mRNA and for those with 10,000 or more copies/μgRNA of MGMT mRNA for progression-free survival and overall survival times are shown in Fig. 1. Progression-free and overall survival times were significantly longer for patients with fewer than 1000 copies/μgRNA of MGMT mRNA than for patients with 10,000 or more copies/μgRNA of MGMT mRNA ($p = 0.0274$ and p = 0.0322 by log-rank test).

Figure 2 shows the Kaplan-Meier survival curves for progression-free and overall survival times for the 32 GBM patients who did not have inherently bad prognostic factors, who were younger than 75 years of age, and whose Karnofsky Performance Scale score was more than 70. Progression-free and overall survival times were significantly longer for patients with fewer than 5000 copies/μgRNA of MGMT mRNA than for those with 5000 or more copies/μgRNA of MGMT mRNA (p = 0.0365 and p = 0.0312 by log-rank test). According to single-variant and multivariate regression analyses, significant prognostic factors for longer progression-free survival were fewer than 5000 copies/μgRNA of MGMT mRNA and higher surgical reduction rate. Single-variant regression analysis showed these 2 factors and the effectiveness of initial treatment were significant factors that indicate a better prognosis.

### Table 1: Absolute values of MGMT mRNA of gliomas quantitated by real-time RT-PCR with TaqMan probe

<table>
<thead>
<tr>
<th>Glioma Type</th>
<th>No. of Tumors</th>
<th>Mean Patient Age (yrs ± SD)</th>
<th>Median PFS (mos)</th>
<th>Median Survival (mos)</th>
<th>MGMT mRNA (mean ± SD copies/μgRNA)</th>
<th>p Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>astrocytoma</td>
<td>18</td>
<td>35.6 ± 13.7</td>
<td>35</td>
<td>43</td>
<td>10,406 ± 7997</td>
<td>0.0782</td>
</tr>
<tr>
<td>oligodendrogial</td>
<td>25</td>
<td>45.8 ± 11.5</td>
<td>21</td>
<td>26</td>
<td>3739 ± 3874</td>
<td>0.0300</td>
</tr>
<tr>
<td>anaplastic astrocytoma</td>
<td>24</td>
<td>48.8 ± 15.9</td>
<td>20</td>
<td>24</td>
<td>7776 ± 10,552</td>
<td>0.3846</td>
</tr>
<tr>
<td>GBM</td>
<td>73</td>
<td>56.0 ± 15.2</td>
<td>8</td>
<td>13</td>
<td>7437 ± 11,581</td>
<td>0.3953</td>
</tr>
<tr>
<td>all gliomas</td>
<td>140</td>
<td>50.3 ± 16.2</td>
<td>13</td>
<td>20</td>
<td>7216 ± 10,172</td>
<td>not applicable</td>
</tr>
</tbody>
</table>

* Boldface indicates statistical significance at p < 0.05. PFS = progression-free survival.
† Student t-test.
adjuvant therapy to also be significant prognostic factors for overall survival. Among these 32 patients, no direct tumor regression occurred after temozolomide and radiation for any GBM with 5000 or more copies/μgRNA of MGMT mRNA.

**Discussion**

As noted earlier, MGMT is inactivated by hypermethylation of its DNA promoter domain. The existence of glioma-CpG island methylator phenotype (G-CIMP) is noteworthy. Patients with G-CIMP tumors are younger at the time of diagnosis and experience significantly better outcomes than those with non–G-CIMP tumors. On molecular and clinical grounds, G-CIMP has been identified as a distinct subset of human gliomas. Previously, methylation-specific PCR was used to detect methylation. Although Hegi et al. have described methods of quantitative methylation-specific PCR for assessment of MGMT promoter methylation, methylation-specific PCR is not essentially quantitative and, therefore, is not appropriate for identifying the indications and protocols for temozolomide therapy. The relationship between absolute MGMT mRNA and MGMT methylation status has been reported by some authors. Kreth et al. compared MGMT mRNA expression by real-time quantitative RT-PCR to the results of methylation-specific PCR and sequencing. They documented that the degree of MGMT mRNA expression was highly correlated with the MGMT promoter methylation status; however, for 12 glioblastoma patients, findings were discordant. Patients with methylated tumors with high MGMT mRNA expression had significantly shorter survival times than did those with tumors with low transcriptional activity. Conversely, patients with nonmethylated tumors with low MGMT mRNA expression had longer survival times than their counterparts. These results suggest that MGMT mRNA expression is a more precise prognostic factor than the result of methylation-specific PCR.

Pyrosequencing has recently been used for the quantitative detection of methylation. The MGMT methylation status as measured by pyrosequencing has been reported to be a prognostic factor for GBM treated by temozolomide plus radiation, but clear standards for the use of pyrosequencing to determine MGMT methylation have not yet been established. Everhard et al. reported methylation status concordance of 85% between pyrosequencing and RNA expression by real-time RT-PCR with the SYBR Green method. They recommended that if transcriptional repression is the key mechanism for the higher chemosensitivity of MGMT-methylated tumors, a substantial rate of discordance warrants caution when deciding on a therapeutic strategy based on MGMT methylation status alone.

The absolute value of MGMT mRNA is the result of the methylation status of promoter DNA of MGMT. The absolute value of MGMT mRNA seems to coincide more with enzyme activity than with DNA methylation status. In contrast, an analysis at the protein level of MGMT20 determined that immunohistochemistry shows a high specificity and is also not quantitative. Although mRNA levels measured by RT-PCR may not always correlate with enzyme activity, MGMT activity itself seems to be more strongly correlated with clinical resistance to nitrosoureas than to mRNA expression. The association between MGMT expression and its activity was investigated about 20 years ago. Recently, micro-RNA has been reported to regulate MGMT protein expression. Indeed, miR-181d downregulates MGMT mRNA and protein expression. These reports did not show that the pyrosequencing method corresponded more with protein expression than did our mRNA quantitation. Because the quantitation of MGMT activity is complicated and would require a long time for clinical use, mRNA expression has been examined instead. Real-time RT-PCR is a simple, rapid, and clinically applicable method for evaluating the resistance to the alkylating agents described in this report.

**TABLE 2: Statistical analyses of prognostic factors of 73 patients with glioblastomas**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
<th>PFS Rate</th>
<th>Overall Survival Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Single Variant</td>
<td>Multivariant</td>
</tr>
<tr>
<td>patient age (yrs), mean ± SD</td>
<td>55.9 ± 15.2</td>
<td>0.1084†</td>
<td>0.0061†</td>
</tr>
<tr>
<td>patient sex (M/F)</td>
<td>47.26</td>
<td>0.5311†</td>
<td>0.3914†</td>
</tr>
<tr>
<td>KPS score (± SD)</td>
<td>73.0 ± 14.0</td>
<td>0.4663†</td>
<td>0.2758†</td>
</tr>
<tr>
<td>tumor in noneloquent area</td>
<td>50.7%</td>
<td><strong>0.0261‡</strong></td>
<td>0.4525†</td>
</tr>
<tr>
<td>MGMT mRNA value (copies/μgRNA ± SD)</td>
<td>7437 ± 11,581</td>
<td>0.6366†</td>
<td>0.8188†</td>
</tr>
<tr>
<td>MGMT mRNA &lt;1000 copies/μgRNA</td>
<td>21.9%</td>
<td><strong>0.0150‡</strong></td>
<td><strong>0.0466‡</strong></td>
</tr>
<tr>
<td>rate of surgical reduction (%)</td>
<td>80.3 ± 24.6</td>
<td><strong>0.0207‡</strong></td>
<td>0.6233†</td>
</tr>
<tr>
<td>use of temozolomide</td>
<td>61.6%</td>
<td>0.2895‡</td>
<td>0.9644‡</td>
</tr>
<tr>
<td>use of ACNU</td>
<td>33.3%</td>
<td>0.7485‡</td>
<td>0.7796‡</td>
</tr>
<tr>
<td>use of interferon-β</td>
<td>34.7%</td>
<td>0.6602‡</td>
<td>0.1329‡</td>
</tr>
<tr>
<td>effect of initial therapy (CR + PR)</td>
<td>35.7%</td>
<td><strong>0.0398‡</strong></td>
<td><strong>0.0310‡</strong></td>
</tr>
</tbody>
</table>

* Boldface indicates statistical significance at p < 0.05. CR = complete response; KPS = Karnofsky Performance Scale; PR = partial response (more than 50% tumor reduction).
† Wald test.
‡ Log-rank (Mantel-Cox) test.
TABLE 3: Statistical analysis of prognostic factors of 45 patients with glioblastomas initially treated with temozolomide and radiation

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
<th>MGMT mRNA (copies/μgRNA)</th>
<th>PFS Rate</th>
<th>Overall Survival Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt;1000</td>
<td>≥1000</td>
<td>p Value</td>
</tr>
<tr>
<td>no.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>patient age (yrs), mean ± SD</td>
<td>58.5 ± 13.9</td>
<td>56.1 ± 10.8</td>
<td>59.5 ± 15.0</td>
<td>0.4595†</td>
</tr>
<tr>
<td>patient sex (M/F)</td>
<td>30:15</td>
<td>8:6</td>
<td>22.9</td>
<td>0.4968§</td>
</tr>
<tr>
<td>KPS score, mean ± SD</td>
<td>72.9 ± 12.4</td>
<td>73.6 ± 12.3</td>
<td>72.6 ± 12.4</td>
<td>0.8094†</td>
</tr>
<tr>
<td>tumor in noneloquent area (%)</td>
<td>48.9%</td>
<td>50.0%</td>
<td>48.4%</td>
<td>&gt;0.9999§</td>
</tr>
<tr>
<td>MGMT mRNA value (copies/μgRNA ± SD)</td>
<td>6014 ± 12630</td>
<td>209 ± 331</td>
<td>8635 ± 14472</td>
<td>0.0390†</td>
</tr>
<tr>
<td>surgical reduction rate (%), mean ± SD</td>
<td>87.6 ± 18.5</td>
<td>82.9 ± 25.5</td>
<td>89.7 ± 13.8</td>
<td>0.2626†</td>
</tr>
<tr>
<td>effect of initial therapy (CR + PR)</td>
<td>33.3%</td>
<td>42.9%</td>
<td>29.4%</td>
<td>0.6466§</td>
</tr>
<tr>
<td>median PFS (mos)</td>
<td>7</td>
<td>14</td>
<td>7</td>
<td>0.0090¶</td>
</tr>
<tr>
<td>median survival (mos)</td>
<td>13</td>
<td>21</td>
<td>10</td>
<td>0.0910¶</td>
</tr>
<tr>
<td>1-yr survival rate</td>
<td>59.5%</td>
<td>76.9%</td>
<td>50.4%</td>
<td></td>
</tr>
<tr>
<td>2-yr survival rate</td>
<td>40.5%</td>
<td>58.6%</td>
<td>26.2%</td>
<td></td>
</tr>
</tbody>
</table>

* Boldface indicates statistical significance at p < 0.05.
† Student t-test.
‡ Wald test.
§ Fisher exact probability test.
¶ Log-rank (Mantel-Cox) test.
Quantitation of MGMT mRNA in gliomas

**Fig. 1.** Kaplan-Meier survival curves for patients who received temozolomide and radiation therapy for GBMs with fewer than 1000 copies/μgRNA of MGMT mRNA and 10,000 or more copies/μgRNA of MGMT mRNA for progression-free survival (PFS, left) and overall survival (OS, right). For patients with tumors with fewer than 1000 copies/μgRNA of MGMT mRNA, both PFS and OS were significantly longer than for patients with tumors with 10,000 or more copies/μgRNA of MGMT mRNA (p = 0.0274 by log-rank test and p = 0.0322 by log-rank test).

**Fig. 2.** Kaplan-Meier survival curves for patients younger than 75 years and with a Karnofsky Performance Scale score of more than 70, who received temozolomide and radiation therapy for GBM with fewer than 5000 copies/μgRNA of MGMT mRNA or with 5000 or more copies/μgRNA of MGMT mRNA for progression-free survival (PFS, left) and overall survival (OS, right). For the patients with GBMs with fewer than 5000 copies/μgRNA of MGMT mRNA, both PFS and OS were significantly longer than they were for patients with 5000 or more copies/μgRNA of MGMT mRNA (p = 0.0365 by log-rank test and p = 0.0312 by log-rank test).
Although we indicated that we had no potential conflict of interest to disclose in this study, with regard to real-time RT-PCR, together with the Special Reference Laboratory Co., Ltd, we developed a quantitative assay of MGMT mRNA that uses a TaqMan probe. PCR was once considered to be unsuitable for quantitation. However, because it is easy, rapid, and sensitive, quantitation by PCR has been attempted. At present, real-time PCR seems to be the most sensitive and rapid method for the quantitation of DNA and RNA. We previously used SYBR Green I dye, which binds to double-stranded DNA and provides a fluorescent signal. This approach is simpler and more sensitive because many fluorescent labels, instead of just one molecule, are incorporated into an amplified fragment. However, the disadvantage of a fluorescent dye is that both specific and nonspecific products generate a signal. In the study reported here, an originally designed TaqMan probe was used for absolute quantitation by real-time PCR. In most studies of real-time PCR for diagnostic purposes, fluorescently labeled probes have been used to detect amplified products. Although conventional real-time RT-PCR gives only a relative value, double normalization with standard cDNA of MGMT and an internal control (GAPDH) in our method has made it possible to measure the absolute value of mRNA. Recently, a digital RT-PCR method has been developed. Although digital PCR has seemed to be more precise than real-time RT-PCR, it seemed to be not suitable for quantitation of MGMT mRNA because the range over which results were distributed was too wide. At present, our quantitative real-time PCR is thought to be the most suitable method for the quantification of MGMT mRNA.

In our analysis, the mean absolute value of MGMT mRNA in GBMs did not significantly differ from values in other gliomas. The mean value in low-grade gliomas also did not significantly differ from values in other gliomas. It seems that MGMT status is not correlated to the degree of malignancy. However, the value in anaplastic oligodendrogial tumors was significantly lower than values in other gliomas. Anaplastic oligodendrogialmomas, which often present chromosomal 1p19q loss of heterozygosity, are more chemosensitive than other malignant gliomas. In gliomas, 3 molecular markers have recently been the subject of extensive studies: 1p19q loss of heterozygosity, methylation of MGMT promoter, and mutations of isocitrate dehydrogenase I and II. A low expression level of MGMT mRNA, as well as 1p19q loss of heterozygosity, might contribute to the high chemosensitivity of anaplastic oligodendrogial tumors.

Conversely, in all 73 GBMs examined in this study, the MGMT mRNA value was a significant prognostic factor for longer progression-free survival but not for overall survival. In Japan, multimodal therapy, MGMT modification and consumption by chemotherapy using alkylating agents, and enthusiastic treatment for recurrent tumors seems to explain, at least in part, why the MGMT mRNA value at the first operation was not a significant prognostic factor of GBM for overall survival. MGMT is a suicide enzyme, and temozolomide, interferon-β, and platinum compounds consume the MGMT molecule. This fact is one of the reasons why the Stupp protocol of continuous administration of low-dose temozolomide at the first adjuvant therapy is significantly effective. In Japan, the rate of surgical resection of gliomas is generally high and treatment is vigorous, even for recurrent tumors. This finding might be related to the association between the results of molecular biological examinations and the prognosis.

The made-to-order treatment of malignant tumors based on genetic screening is attracting increasing attention. We started individual adjuvant therapy based on the results of RT-PCR for malignant gliomas. We had detected MGMT mRNA in operative specimens by RT-PCR. Patients with tumors with low expression of MGMT received treatment with ACNU, and those with tumors with high expression of MGMT received mainly platinum compounds such as cis-platinum. Since 2001, we have performed individual adjuvant therapy based on the results of quantitative real-time RT-PCR. For 90 gliomas, for which 103 individual adjuvant therapies were performed, excellent results were reported. The effectiveness of adjuvant therapy for all gliomas was 51.1%, and the 2-year survival rate for patients with GBM was 53.3%.

Although our study has many limitations (for example, an almost retrospective design, a heterogeneous glioma group, a limited number of patients in subgroups, and multiple treatment paradigms within tumor groups), the measurement of MGMT mRNA seems to be useful for predicting the sensitivity to chemotherapy with temozolomide and for planning both the initial and maintenance chemotherapy schedules. Our results suggest that the absolute value of MGMT mRNA in GBMs can predict the effectiveness of treatment with temozolomide; this finding supports the notion of a new individual adjuvant therapy based on the results of MGMT mRNA measurement. For patients whose tumors contain a small amount of MGMT mRNA (fewer than 1000 copies/µgRNA), relatively longer progression-free survival and overall survival times can be expected. For patients whose tumors contain 1000–5000 copies/µgRNA of MGMT mRNA, use of bevacizumab with temozolomide should be considered. Bevacizumab is reportedly more effective for MGMT-methylated than for -nonmethylated tumors. An unpublished Phase III study also reported that a course of bevacizumab with temozolomide and radiation prolonged progression-free survival times for patients with GBM. For patients whose tumors contain 5000–10,000 copies/µgRNA of MGMT mRNA, interferon-β should be used with temozolomide and radiation because interferon-β downregulates MGMT expression and seems to be useful for MGMT-nonmethylated tumors. For patients whose tumors contain more than 10,000 copies/µgRNA of MGMT mRNA, interferon-β and bevacizumab with radiation without temozolomide should be used. In this new setting of individual adjuvant therapy, we might be able to prolong the survival times of patients with GBM, for whom prognosis is otherwise extremely poor.

Conclusions

The prognosis for patients with GBMs with fewer
Quantification of MGMT mRNA in gliomas

than 1000 copies/µgRNA of MGMT mRNA can be good. For tumors with 10,000 or more copies/µgRNA MGMT mRNA, temozolomide may not be effective. These results suggest that MGMT mRNA might be useful as a prognostic factor and for predicting the results of therapy for GBMs treated by temozolomide. A large-scale prospective randomized study will disclose the relationship between MGMT mRNA and prognosis of GBM treated with temozolomide.

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Disclosure

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

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Address correspondence to: Satoshi Tanaka, M.D., M.Sc., Department of Neuro-Oncology and Neurosurgery, Tokyo Nishi Tokushukai Hospital, Matsubara-cho 3-1-1, Akishima, Tokyo 196-0003, Japan. email: stanaka-nsu@umin.net.