SPANDIDOS PUBLICATIONS

MOLECULAR AND CLINICAL ONCOLOGY 7: 1107-1111, 2017

Effect of O^6-methylguanine-DNA methyltransferase methylation in medulloblastoma

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Received March 8, 2017; Accepted July 4, 2017

DOI: 10.3892/mco.2017.1431

Abstract. Medulloblastoma is a highly malignant brain tumor that predominately affects children and requires multimodal treatment, including chemotherapy with alkylating agents. O^6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme that plays an important role in tumor resistance to alkylating agents. Recent studies demonstrated that *MGMT* promoter methylation suppresses the expression of *MGMT* and is associated with favorable outcomes of malignant glioma patients. However, the *MGMT* methylation status and its prognostic impact on medulloblastoma have not been fully elucidated to date. The objective of the present study was to investigate the association between *MGMT* status and clinical outcomes of pediatric medulloblastoma patients. The records of 15 patients with medulloblastoma treated at our institution were reviewed, and the methylation status of 18 CpG sites in the *MGMT* promoter region was determined using bisulfite sequencing analysis. A larger number of methylated CpG sites was identified in 9 patients with complete remission (median, 5 sites; range, 2-9 sites) compared with that in 6 patients with relapse (median, 2 sites, range, 1-4 sites; P=0.041). These results suggest that a higher number of methylated CpG sites in the *MGMT* promoter region are associated with a favorable outcome of medulloblastoma.

Introduction

Medulloblastoma is a highly malignant brain tumor that predominately affects children. The standard treatment of medulloblastoma is surgery followed by radiotherapy and chemotherapy using alkylating agents (1-3).

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Key words: alkylating antineoplastic agents, antineoplastic agents, brain neoplasms, medulloblastoma, methylguanine-DNA methyltransferase

O^6-methylguanine-DNA methyltransferase (MGMT) is a DNA repair enzyme that plays an important role in tumor resistance to alkylating agents. MGMT removes alkyl adducts from the O^6-position of guanine by inactivating itself. As O^6-alkylated guanine leads to double-strand breaks and base mispairing, which eventually induces cell apoptosis, MGMT protects normal cells as well as tumor cells from alkylating agents (4). Expression of *MGMT* is suppressed by methylation of CpG islands in the promoter region (4-6). It was previously demonstrated that a greater methylation status of the *MGMT* promoter region is associated with favorable outcomes in adult and pediatric patients with glioblastoma treated with alkylating agents, such as temozolomide (7,8). However, only a limited number of studies have investigated *MGMT* status in medulloblastoma and its effect on disease outcome (5,9-11).

The aim of the present study was to determine the methylation status of CpG sites in the *MGMT* promoter region in tumor cells obtained from medulloblastoma patients and evaluate the association between *MGMT* status and clinical outcome.

Patients and methods

Patients. The records of pediatric patients with medulloblastoma treated at Juntendo University Hospital (Tokyo, Japan) between 1995 and 2012 were reviewed. Patients who underwent institutional standard treatment for medulloblastoma (initial tumor removal, craniospinal irradiation and chemotherapy) and who were observed for at least 36 months after diagnosis, or who experienced relapse of the disease after initiation of chemotherapy were included in the study. Patients were excluded if their treatment regimen deviated significantly from the standard treatment, such as omission of radiotherapy, or underwent biopsy as the only surgical intervention. Relevant clinical information, including current disease status, was obtained from hospital charts. The study was approved by the Juntendo University Ethics Committee, and written informed consent was obtained from all the patients and/or their legal guardians.

Analysis of MGMT status. Tumor tissues obtained at the first surgery for tumor removal were used for analysis. Genomic DNA was extracted from paraffin-embedded samples with deparaffinization solution and the QIAamp DNA FFPE Tissue kit (Qiagen, Hilden, Germany). DNA from each

cccg<u>cg</u>[1]cccc tagaa<u>cg</u>[2]ctt tg<u>cg</u>[3]tcc<u>cg</u>[4]a <u>cg</u>[5]cc<u>cg</u>[6]cagg tcct<u>cg</u>[7]<u>cg</u>[8]gt
g<u>cg</u>[9]cac<u>cg</u>[10]tt tg<u>cg</u>[11]acttgg tgagtgtctg ggt<u>cg</u>[12]cct<u>cg</u>[13] ctcc<u>cg</u>[14]gaag
agtg<u>cg</u>[15]gagc tctccct<u>cg</u>[16]g ga<u>cg</u>[17]gtggca gcct<u>cg</u>[18]agtg gtcctgcagg

Figure 1. Sequence alignment of the O^6-methylguanine-DNA methyltransferase promoter region. The CpG sites analyzed in this study are underlined and numbered from 1 to 18. CpG1 is located +95 bp from the transcriptional start site (TSS), and CpG18 is located +225 bp from the TSS. Sequence data were submitted to the GenBank database under accession number NC 000010.11.

sample (300 mg) was treated with sodium bisulfite using the Cells-to-CpG Bisulfite Conversion kit (Applied Biosystems, Foster City, CA, USA).

The direct sequence method was used to analyze bisulfite-treated DNA. *MGMT* promoter primers were designed to cover 18 CpG sites (chr10:129467232-129467363 GenBank) by Methyl Primer Express software v1.0 (Applied Biosystems) (Fig. 1). Two polymerase chain reaction (PCR) products were made, namely product 1 (99 bp) and product 2 (89 bp). Product 1 primers were as follows: Forward, GGATATGTT GGGATAGTTYG; and reverse, ACCCAAACACTCACC AAAT. Product 2 primers were as follows: Forward, ATT TGGTGAGTGTTTGGG; and reverse, ACRCCTACAAAA CCACTC. PCR was performed by a two-step approach using AmpliTaqGold 360 Master Mix (Applied Biosystems). PCR products were sequenced on an ABI 3130 Genetic Analyzer (Applied Biosystems) with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were analyzed with SeqScape software v3.0 (Applied Biosystems). Genomic data were based on the GRCh38/hg38 assembly from the University of California Santa Cruz Genome Browser (http://genome.ucsc.edu/), accessed December 2013.

Statistical analysis. Differences between groups were analyzed by Mann-Whitney U-test using GraphPad Prism 4 software (San Diego, CA). P-values <0.05 were considered to indicate statistically significant differences.

Results

Characteristics and clinical outcomes of the patients. A total of 22 patients with available tumor tissue and clinical data were identified. Of those, 7 patients were excluded due to the following reasons: 3 were followed up for <36 months after the diagnosis with no disease recurrence; 1 did not receive radiotherapy due to being aged <24 months at diagnosis; 2 received biopsy only as the initial surgical intervention due to massive dissemination; and 1 experienced tumor relapse prior to the initiation of radiotherapy and chemotherapy.

A total of 15 patients were finally included in the present study (Table I). The median age at diagnosis was 9 years (range, 2-15 years) and the median follow-up period was 41 months (range, 13-193 months). In all the patients, the tumor was located in the fourth ventricle at the midline of the cerebellum. The patients received multiple courses of chemotherapy consisting of ifosfamide, cisplatin and etoposide (n=8); cisplatin, vincristine and cyclophosphamide (n=3); or ifosfamide, cisplatin, etoposide, vincristine and cyclophosphamide (n=4). Following first-line treatment, 9 patients achieved complete remission and 6 patients relapsed.

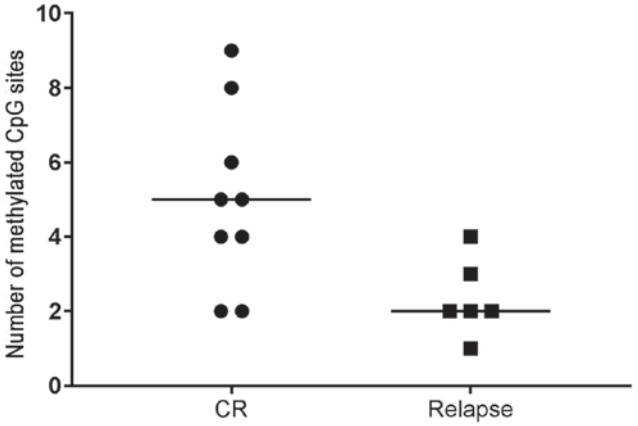

Figure 2. Number of methylated CpG sites. The number of methylated CpG sites in patients in complete remission (CR) was significantly higher compared with that in patients who relapsed (P=0.043).

Analysis of MGMT status. The methylation status of 18 CpG sites of the *MGMT* promoter region is shown in Table II. CpG sites with unmethylated cytosine are displayed as thymine in the final sequences and are indicated as 'T', whereas CpG sites with methylated cytosine are indicated as 'C'. Certain samples displayed mixed cytosine and thymine signals and are indicated as 'Y'. Among 270 CpG sites analyzed, 59 (21.9%) were methylated and 170 (63.0%) were unmethylated, whereas mixed signals were observed in 41 sites (15.2%). A higher number of methylated CpG sites was observed in patients with complete remission compared with that in patients who relapsed (P=0.041) (Fig. 2).

Discussion

There was variability in the *MGMT* status among medulloblastoma tumor samples, and an association was observed between more extensive *MGMT* promoter methylation and favorable clinical outcome of medulloblastoma. Previous studies on the effect of *MGMT* status on the treatment of medulloblastoma yielded conflicting results. Neben *et al* reported that high levels of *MGMT* expression were associated with unfavorable survival outcome using microarray-based screening of 35 medulloblastomas (10). Bobola *et al* observed that *MGMT* expression is a major determinant of carmustine and temozolomide sensitivity in medulloblastoma cell lines (12). However, Faoro *et al* reported no association between *MGMT* mRNA expression and progression-free or overall survival of medulloblastoma patients (5).

These differences in the study results may be caused in part by heterogeneity of chemotherapy regimens among

Table I. Patient characteristics and clinical outcomes.

Patient	Age at diagnosis (years)	Sex	Pathological classification	Dissemination	Surgery	Outcome after first-line treatment
1	11	Female	Classic	-	GTR	CR
2	7	Female	Anaplastic	-	STR	CR
3	5	Female	Classic	-	STR	CR
4	5	Male	Desmoplastic	-	STR	CR
5	4	Male	Nodular	+	GTR	CR
6	10	Female	Anaplastic	+	GTR	CR
7	11	Male	Classic	-	STR	CR
8	12	Male	Anaplastic	-	STR	CR
9	9	Male	Classic	-	STR	CR
10	10	Male	Classic	-	STR	Relapse
11	2	Male	Anaplastic	-	STR	Relapse
12	7	Male	Classic	-	STR	Relapse
13	15	Female	Anaplastic	+	STR	Relapse
14	11	Male	Anaplastic	+	GTR	Relapse
15	8	Male	Classic	-	GTR	Relapse

GTR, gross total resection; STR, subtotal resection; CR, complete remission.

Table II. Methylation status of CpG sites in the *MGMT* promoter region.

		CpG site																	
Patient	Outcome	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1	CR	T	Y	T	T	T	Y	C	Y	Y	Y	Y	C	C	C	T	T	T	T
2	CR	T	T	T	Y	C	T	Y	T	T	Y	T	T	T	T	T	T	T	C
3	CR	T	T	T	C	T	C	Y	T	T	Y	T	T	T	T	C	C	Y	C
4	CR	T	C	C	T	T	T	C	T	T	C	Y	T	T	T	T	T	T	T
5	CR	T	T	C	T	T	T	T	T	T	C	T	Y	T	C	T	C	C	T
6	CR	T	C	T	Y	T	T	T	Y	Y	C	Y	T	T	T	T	T	T	T
7	CR	T	T	T	T	T	C	C	C	C	T	Y	T	T	C	T	T	T	C
8	CR	C	T	C	T	T	C	C	C	C	T	T	T	T	C	T	C	Y	C
9	CR	C	C	C	T	C	C	T	T	T	C	T	T	T	T	T	C	C	T
10	Relapse	T	Y	T	Y	T	T	Y	T	Y	Y	Y	T	T	T	C	T	T	Y
11	Relapse	T	T	C	T	T	Y	Y	T	Y	Y	T	T	T	T	T	T	Y	C
12	Relapse	T	T	C	T	T	C	T	T	T	C	T	T	C	T	T	Y	T	T
13	Relapse	T	T	C	T	T	T	T	C	T	C	T	T	T	T	T	T	T	T
14	Relapse	C	T	T	T	T	T	T	C	T	T	T	T	T	T	T	T	T	T
15	Relapse	T	C	T	Y	Y	Y	T	Y	Y	Y	Y	T	T	Y	T	C	T	T

MGMT, O^6-methylguanine-DNA methyltransferase gene; CR, complete remission; C, methylated site; T, unmethylated site; Y, undefined site (mixed signal).

studies. In the study by Neben *et al*, the patients were treated with lomustine, cisplatin and vincristine (10). In the study by Faoro *et al*, the patients were treated as reported in the randomized trial HIT'91, which consisted of two chemotherapy arms: One treated with procarbazine, ifosfamide, etoposide, high-dose methotrexate, cisplatin and cytarabine, and the other with cisplatin, lomustine and vincristine. Recently, several genes were found to play important roles in the pharmacokinetics or pharmacodynamics of chemotherapy agents, such as the role of polymorphism of reduced folate carrier 1 and methylenetetetrahydrofolate reductase in high-dose methotrexate treatment (13). Therefore, the effect of *MGMT* status on the survival of patients with

medulloblastoma may vary with different combinations of chemotherapy agents.

Another factor that may cause conflicting results among studies is the complexity of determining *MGMT* status. For example, a study using commercially available anti-MGMT antibodies to determine MGMT expression reported major interobserver variability (9,14). Furthermore, as *MGMT* promoter methylation is inversely correlated with *MGMT* expression (5,6), methylation-specific PCR (MSP) with bisulfate-treated DNA has been widely used to analyze *MGMT* promoter methylation status (7-9). However, MSP is only able to detect a limited number of methylated CpG sites in the primer region, and recent studies report that the region commonly investigated by MSP does not cover CpG sites that are most highly associated with expression of *MGMT*, and that MSP may not be well-suited for predicting the prognosis of patients with glioblastoma (15,16). Direct sequencing and pyrosequencing are alternative methods for quantitatively analyzing the methylation status of *MGMT*. As pyrosequencing is effective for high-throughput screening, but is quite costly, the direct sequence method was used to determine the methylation status of selected CpG sites in the present study.

The *MGMT* promoter contains a 762-bp CpG island with 98 CpG sites, with certain CpG regions reflecting *MGMT* expression better than others. Everhard *et al* reported CpG sites at +95, +113, +135 and +137 bp from the transcriptional start site (TSS) (CpG 1, 3, 7 and 8 in our study, respectively) and high concordance between methylation and expression of *MGMT* in their analysis of 53 CpG sites in 54 glioblastoma samples (15). Malley *et al* reported that individual or multiple consecutive methylation of CpG sites at +153, +185, +195 and +213 bp from the TSS (CpG 11, 14, 15 and 17 in our study, respectively) attenuated the activity of the *MGMT* promoter in their study of 98 CpG sites in xenografted glioblastoma samples and cell lines (6). Although we were unable to determine whether methylation at specific CpG sites was more closely associated with prognosis than others, due to our limited sample size, our results suggest that the overall CpG methylation profile of the targeted region in the present study is associated with the outcome of medulloblastoma.

Recent rapid advances in genetic techniques currently allow the subdivision of medulloblastoma into four molecular subgroups with distinct demographics, clinical presentations and clinical outcomes (17,18). Unfortunately, the cases were not classified into molecular subgroups at the time of diagnosis. However, Von Bueren *et al* reported similar MGMT expression levels in the WNT and SHH groups, that are higher compared with those of group 3 and group 4, although there is wide variation even within groups (11). Considering that the prognosis of WNT group patients is better compared with that of SHH group patients, the *MGMT* methylation status may be an independent factor affecting the prognosis of medulloblastoma.

The results of the present study should be interpreted with caution. First, the methylation status of *MGMT* was assessed in primary tumors resected prior to initiation of radiotherapy and chemotherapy; however, radiotherapy may affect *MGMT* methylation status and upregulate expression of the gene (19). Chemotherapy may also affect the *MGMT* status of the tumors; thus, relapsed tumors may exhibit different methylation profiles. Temozolomide is an alkylating agent that is increasingly used for relapsed medulloblastoma (20-22). Although a significant correlation between *MGMT* methylation status and temozolomide sensitivity has been confirmed in glioblastoma (7,8), sensitivity to temozolomide in relapsed medulloblastoma may not be predicted from the *MGMT* status of primarily resected tumor samples. Second, the small sample size prevented further analysis of other clinical factors that may be associated with patient outcome, such as molecular subtypes, pathological characteristics, dissemination status and chemotherapy regimens.

In conclusion, there was variability in the methylation status of the *MGMT* promoter region among tumor samples from pediatric medulloblastoma patients using the direct sequencing method. Our results indicate that a larger number of methylated CpG sites in the *MGMT* promoter region is associated with a favorable outcome of medulloblastoma.

Acknowledgements

The present study was supported by the Graduate School Research Program of Juntendo University. The authors would like to thank everyone who helped conduct this research at Juntendo University and Juntendo University Hospital.

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