Effect of O⁶-methylguanaine-DNA methyltransferase methylation in medulloblastoma

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Abstract. Medulloblastoma is a highly malignant brain tumor that predominately affects children and requires multimodal treatment, including chemotherapy with alkylating agents. O⁶-methylguanaine-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).

O⁶-methylguanaine-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 O⁶-position of guanine by inactivating itself. As O⁶-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
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, GGA TAT GTT GGG ATA GTT YG; and reverse, ACC CAA ACA CTC ACC AAA T. Product 2 primers were as follows: Forward, ATT TGG TGA GTG TTT GGG; and reverse, ACR CCT ACA AAA CCA CTC. 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.

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=0.043) (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
In the study by Neben et al, the patients were treated with lomustine, cisplatin and vincristine. 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 methylenetetrahydrofolate reductase in high-dose methotrexate treatment. 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 Buuren 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.

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References


