Molecular characteristics of pediatric non-ependymal, non-pilocytic gliomas associated with resistance to temozolomide

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Abstract. Temozolomide constitutes current standard of care for adult patients with high-grade gliomas. However, results for pediatric gliomas are rather disappointing. In order to investigate the molecular differences between pediatric and adult gliomas that could affect sensitivity to temozolomide, we studied 23 pediatric non-ependymal, non-pilocytic gliomas in comparison to 59 consecutive adult gliomas for the expression of O6-methylguanine methyltransferase (MGMT) and the DNA mismatch repair protein, mutS homolog 6 (MSH6) by immunohistochemistry, as well as for the presence or absence of promoter methylation of the MGMT gene by methylation-specific PCR. The expression of MGMT in pediatric gliomas was significantly higher than in adult gliomas, as shown by immunohistochemistry (p=0.00004). This association was conserved if statistical analysis was carried out only in astrocytic tumors (diffuse astrocytoma, anaplastic astrocytoma and glioblastoma, p=0.00007), or in oligodendroglial tumors (oligodendroglioma and anaplastic oligodendroglioma, p=0.020). Although methylation-specific PCR was successfully performed only in 15 pediatric gliomas, it also showed a trend toward less frequent methylation in pediatric as opposed to adult gliomas (p=0.242). MSH6 was almost equally expressed in pediatric and adult gliomas. Pediatric gliomas appear to have a distinct molecular profile associated with resistance to temozolomide. Higher expression of MGMT and a trend toward less frequent methylation of the promoter region of MGMT gene may partly account for relative resistance to temozolomide in pediatric gliomas as compared to adult gliomas.

Introduction

Temozolomide, an oral methylating agent, has better activity and less toxicity than conventional agents, including nitrosoureas in the treatment of gliomas (1,2). Indeed, concomitant and adjuvant temozolomide has improved progression-free survival and overall survival compared to radiotherapy alone in adult patients with newly diagnosed supratentorial glioblastoma, and constitutes current standard of care for those patients (3,4). Nowadays, the majority of clinical trials for gliomas comprises protocols, including temozolomide. However, the efficacy of temozolomide is virtually based on reports on adult gliomas, and results for pediatric gliomas are rather disappointing. Verschuur et al treated 20 children with recurrent high-grade gliomas, including 11 grade III and 8 grade IV gliomas. Progression-free survival at 6 months was 18 and 12.5% for each grade (5). Those figures are less favorably compared to those reported in adult studies (1,2). Ruggiero et al and Lashford et al also reported lower response rate to temozolomide in recurrent pediatric, high-grade gliomas as compared to adult gliomas (6,7). Moreover, in a previous phase II trial (ACNS0126) for newly diagnosed pediatric high-grade gliomas, chemoradiotherapy with temozolomide only provided comparable results to the historical control with BCNU (8).

Temozolomide rapidly hydrolyzed into 5-amino-1H-imidazole-4-carboxamide and methyl diazonium ion at physiological pH via 5-[(1z)-3-Methyl-1-en-1-yl]-1H-imidazole-4-carboxamide. The cytotoxicity of temozolomide is caused by methyl diazonium ion (9,10). The methylating activity of the methyl diazonium ion is exerted on the N7-position (70%), N1-position (10%) and O6-position of guanine (9%) (11). However, methylation of the O6-guanine is, in most cases, repaired by the base excision repair system in cancer cells. However, methylation of the O6-guanine leads to a mispaired thymine (GT-mismatch) in DNA reproduction (12), and finally leads to DNA strand break through the futile mismatch repair cycle (13). Therefore, a defect in the mismatch repair system as well as the increased expression of O6-methylguanine methyltransferase (MGMT), the enzyme that removes alkyl group from O6-guanine, appear to be associated with resistance to temozolomide in gliomas. Indeed, the expression of the mismatch repair protein, mutS homolog 6 (MSH6), is lost in 41% of recurrent glioblastomas in comparison to 0% of pre-treatment glioblastomas, and loss
of MSH6 may be associated with progression during temozolo-
mide treatment (14).

Recent studies suggest that pediatric gliomas have unique
 genetic characteristic and/or expression profiles associated
 with particular signaling pathways as compared to adult tumors
 (15). We therefore examined molecular factors that could affect
 sensitivity to temozolomide in pediatric gliomas in comparison
 to adult gliomas. We studied the expression of MGMT and the
 expression of the DNA mismatch repair protein, MSH6, by
 immunostaining, as well as the presence or absence of promoter
 methylation of the MGMT gene by methylation-specific PCR.

Materials and methods

Tumor samples. Formalin-fixed, paraffin-embedded tissues of
 non-ependymal, non-pilocytic gliomas from 23 pediatric
 (≤20 years of age) patients were collected from the tissue
 bank at the Department of Neurosurgery, Keio University,
 Tokyo, Japan. All were intracranial tumors and histologically
 proven. For comparison, 59 consecutive cases of adult glioma
 newly diagnosed between 2005 and 2009 were also analyzed.
 Pathological classifications were made according to the World
 Health Organization (WHO) criteria (16). No cases had any
 prior adjuvant therapy (radiation or chemotherapy).

Immunohistochemistry of MGMT and MSH6. Immunohisto-
chemistry was performed on the formalin-fixed, paraffin-embedded
 sections. Microwave antigen retrieval was carried out in 10 mM sodium citrate buffer of pH 6.0 (MGMT)
 or of pH 7.0 (MSH6) (14,17). Sections were incubated with
 mouse monoclonal antibody against MGMT (clone MT 3.1;
 Dako, Glostrup, Denmark) or MSH6 (clone 44; BD Biosciences,
 San Jose, CA, USA). Vascular endothelial cells and brain metas-
tasis of colon adenocarcinoma were used as the positive control
 for MGMT and MSH6, respectively. The staining indices were
 analyzed in a total of >1,000 tumor cells on more than three
 areas showing typical appearance of each tumor.

Methylation-specific PCR for MGMT gene. Tumor DNA was
 extracted from a microdissected piece of the formalin-fixed,
 paraffin-embedded sections. Tissue microdissection was
 performed referring to the pathological findings of consecutive
 sections that were stained with H&E or mouse monoclonal anti-
 Ki-67 antibody (MIB-1; Dako) to select areas showing typical
 appearance of each tumor. Bisulfate reaction and subsequent
 PCR were performed using the EZ DNA Methylation-Direct™
 kit (Zymo Research Corp., Orange, CA, USA). PCR was
 performed with primers specific for methylated or unmethyl-
 ated DNAs (Sigma-Aldrich Japan, Inc.) and Taq polymerase
 HotStart Version (Takara Taq™ HotStart Version; Takara
 Bio, Inc., Shiga, Japan), and the annealing temperature was
 decreased from 65 to 58˚C with 35 cycles in total (18-23). The
 PCR products were separated on a 4% MetaPhor Agarose gel
 (Lonza, Rockland, ME, USA). Control DNAs for the methyl-
 ated, unmethylated and wild-type sequence were obtained in the
 Chemicon CpG WIZ MGMT Amplification kit (Temecula,
 CA, USA).

Statistical analysis. The F-test and Student’s t-test were used to
 compare staining indices of MGMT or MSH6, and χ² test was
 used to compare frequency of the methylation of the MGMT
 promoter between pediatric and adult astrocytic gliomas.

Results

Patient characteristics. Clinical data of 23 patients with pedi-
 atric non-ependymal, non-pilocytic gliomas in comparison to
 59 patients with newly diagnosed adult gliomas are given in
 Table I. There were 13 males and 10 females with ages ranging
 from 1 to 20 years (median, 10). The pediatric tumors were
 classified as diffuse astrocytoma in 11, anaplastic astrocytoma
 in 6, glioblastoma in 3, oligodendroglioma in 2 and anaplastic
 oligodendroglioma in 1 patient. Ten tumors were located in the
 cerebrum, 9 in the brain stem, 2 in the thalamus and 2 in other
 locations (1 optic nerve and 1 pineal lesion).

Immunohistochemical analysis of MGMT and MSH6. Immu-
 nohistochemistry of MGMT and MSH6 was performed on 21 and 12
 (due to lack of tissues) pediatric gliomas for
 Table I. Patient characteristics.

<table>
<thead>
<tr>
<th>Variable (histology)</th>
<th>No. of patients (pediatric/adult)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glioblastoma</td>
<td>3/24</td>
</tr>
<tr>
<td>Anaplastic astrocytoma</td>
<td>6/7</td>
</tr>
<tr>
<td>Diffuse astrocytoma</td>
<td>11/7</td>
</tr>
<tr>
<td>Anaplastic oligodendroglioma</td>
<td>1/7</td>
</tr>
<tr>
<td>Oligodendroglioma</td>
<td>2/12</td>
</tr>
<tr>
<td>Oligoastrocytoma</td>
<td>0/2</td>
</tr>
<tr>
<td>Age (years) ≤20</td>
<td>23</td>
</tr>
<tr>
<td>&gt;20</td>
<td>59</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>13/35</td>
</tr>
<tr>
<td>Female</td>
<td>10/24</td>
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</tbody>
</table>

This association was also found if the statistical analysis was
 limited to include only astrocytic gliomas (diffuse astrocy-
toma, anaplastic astrocytoma and glioblastoma, p=0.00007)
 or to include only astrocytic gliomas of grade 2 and 3 (diffuse
 astrocytoma and anaplastic astrocytoma, p=0.0001) in order
to eliminate the influence of number in the expression of
astrocytomas between the pediatric and adult groups. Moreover, a
difference in the expression of MGMT was also significantly
noted in oligodendroglial tumors (p=0.020). MSH6 was
almost equally expressed in pediatric and adult gliomas, and
glioblastomas showed a trend toward higher expression than
the other lower grade gliomas in both the pediatric (p=0.520)
and adult (p=0.017) groups (Table II).

Methylation-specific PCR. Methylation-specific PCR of
the promoter region of the MGMT gene was successfully

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**Table I. Patient characteristics.**

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<td>Female</td>
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</tbody>
</table>
performed in 15 pediatric gliomas (Fig. 2), and showed a trend toward less frequent methylation in the pediatric gliomas as opposed to the adult gliomas (p=0.242; Figs. 3 and 4). This association was also found if statistical analysis was carried out only in astrocytic gliomas (n=315; glioblastoma (GM) =2, anaplastic astrocytoma (AA) =5, diffuse astrocytoma (DA) =7 and oligodendroglioma (OD) =1).

Table II. Immunohistochemical analysis for MGMT and MSH6.

<table>
<thead>
<tr>
<th>Histology</th>
<th>MGMT average (%)</th>
<th>MSH6 average (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(no. of pediatric/</td>
<td>(no. of pediatric/</td>
</tr>
<tr>
<td></td>
<td>no. of adult)</td>
<td>no. of adult)</td>
</tr>
<tr>
<td>GM</td>
<td>41.2 (11.0)</td>
<td>49.7 (35.7)</td>
</tr>
<tr>
<td></td>
<td>3/24</td>
<td>2/14</td>
</tr>
<tr>
<td>AA</td>
<td>39.2 (6.47)</td>
<td>0.7 (7.4)</td>
</tr>
<tr>
<td></td>
<td>5/7</td>
<td>4/2</td>
</tr>
<tr>
<td>DA</td>
<td>64.2 (20.0)</td>
<td>5.22 (8.9)</td>
</tr>
<tr>
<td></td>
<td>10/7</td>
<td>5/3</td>
</tr>
<tr>
<td>AO</td>
<td>97.8 (4.83)</td>
<td>N/M (0)</td>
</tr>
<tr>
<td></td>
<td>1/7</td>
<td>0/1</td>
</tr>
<tr>
<td>OD</td>
<td>7.7 (4.72)</td>
<td>0 (0)</td>
</tr>
<tr>
<td></td>
<td>2/12</td>
<td>1/2</td>
</tr>
<tr>
<td>OA</td>
<td>N/M (7.7)</td>
<td>N/M (15.7)</td>
</tr>
<tr>
<td></td>
<td>0/2</td>
<td>0/2</td>
</tr>
</tbody>
</table>

GM, glioblastoma; AA, anaplastic astrocytoma; DA, diffuse astrocytoma; AO, anaplastic oligodendroglioma; OD, oligodendroglioma; OA, oligoastrocytoma; N/M, no material.

Discussion
The effectiveness of temozolomide, alone or combination with radiation and/or other agents, in pediatric gliomas remains
uncertain (24). Regardless of the number of patients, to date, there have been no reports showing clinical results supporting the efficacy of temozolomide. Although studies on MGMT in pediatric gliomas are sparse, there have been some recent studies. Pollack et al. detected the overexpression (more than 25% positivity) of MGMT in 25 out of 68 (37%) pediatric high-grade gliomas by immunohistochemistry (26). Schlosser et al. studied the methylation status of the MGMT promoter in 24 pediatric high-grade gliomas by the quantitative method, and found that MGMT methylation was associated with an elevated event-free survival only if methylation was over 14% (27). However, none of these studies compared the MGMT status between pediatric and adult gliomas. To our knowledge, this is the first study showing that the expression of MGMT is significantly higher in pediatric astrocytic tumors as well as oligodendrogial tumors than in their adult counterparts. Although methylation-specific PCR was successfully performed only in 15 pediatric gliomas, our study also shows a trend toward less frequent methylation in pediatric, as opposed to adult gliomas. The number of cases was small, however, the present findings may partly explain the inferior outcome of temozolomide therapy in pediatric gliomas, as opposed to adult ones.

The difference in MGMT expression between pediatric and adult gliomas could be due to the fact that pediatric gliomas are associated with distinct molecular characteristics compared to adult tumors. Indeed, in the present series of pediatric gliomas, a single copy gain of chromosome 1q was frequently found (50% in 20 tumors) as opposed to adult gliomas (2.5% in 170 tumors) (15). Moreover, codeletion of 1p/19q, the genetic hallmark of oligodendrogliomas in adults, was not observed in the 3 oligodendrogliomas included in the present study (15).

Loss of expression and mutation of the mismatch repair gene, MSH6, may be associated with glioblastoma progression during temozolomide treatment (14,25). In the present study, however, there was no difference in MSH6 immunohistochemistry between pediatric and adult gliomas. Pollack et al. found microsatellite instability only in 3 of 68 pediatric malignant gliomas, and suggested that mismatch repair deficiency is an uncommon mechanism of alkylator resistance in those tumors (26). Although the number of cases was small, our study, similar to the one by Pollack et al., does not show any evidence suggesting the association of MSH6 status with the difference in sensitivity to temozolomide between pediatric and adult gliomas.

The present results suggest that the higher expression of MGMT may be associated with relative resistance to temozolomide in pediatric gliomas as compared to adult gliomas. Therefore, to improve the effectiveness of temozolomide chemotherapy in pediatric gliomas, the depletion of MGMT with guanine analogues (e.g., O6-benzylguanine and interferon β) is a theoretically reasonable treatment strategy (28,29). Inhibition of the self repair of N7-methylguanine with PARP inhibitor (e.g., BSI-201) may also be practical as a different approach (30). Further elucidation of the molecular characteristics of pediatric gliomas is important for the effective treatment and better outcome of the patients with those tumors.

In conclusion, non-ependymal, non-pilocytic, pediatric gliomas are associated with higher expression of MGMT, and with a trend toward the less frequent methylation of the MGMT promoter, as opposed to adult gliomas. However, MSH6 was equally expressed in pediatric and adult gliomas. Although the number of cases was small, these results suggest that the difference in MGMT status partly accounts for the different sensitivity to temozolomide in pediatric gliomas as compared to adult gliomas. Moreover, together with our previous publication (15), these data clearly suggest that pediatric gliomas have distinct genetic characteristics from their adult counterparts.

Acknowledgements

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References


