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Expression of O\(^6\)-methylguanine DNA methyltransferase (MGMT) and immunohistochemical analysis of 12 pineal parenchymal tumors.

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Running title: MGMT expression in pineal parenchymal tumors.

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Abstract

Pineal parenchymal tumors (PPTs) are rare neoplasms which occupy less than 1% of primary central nervous system tumors. Because of their rare incidence, the previous reports on PPTs are limited in number and the useful molecular markers for deciding the histological grading and even selecting chemotherapy are undetermined. In this study, we conducted immunohistochemical analysis of 12 PPT specimens, especially for expression of O\(^6\)-methylguanine DNA methyltransferase (MGMT) to assess whether temozolomide (TMZ) could serve as a possible alternative therapy for PPTs. We analyzed 12 PPTs consisted of 3 pineocytomas, 6 pineal parenchymal tumors of intermediate differentiation (PPTIDs), and 3 pineoblastomas. Immunohistochemical analysis was performed using antibody against MGMT, synaptophysin, neurofilament protein (NF), p53, and NeuN. Immunohistochemically, 11 out of 12 cases were positive for MGMT. The mean MIB-1 labeling index was less than 1% in pineocytoma, 3.5% in PPTID, and 10.5% in pineoblastoma. All 12 cases were positive for synaptophysin and 11 cases except 1 PPTID case showed positive for NF. Nuclear staining of NeuN was negative in all cases although cytoplasmic stain of NeuN was observed in 5 cases. No case was positive for p53. Eleven out of 12 cases of PPTs demonstrated MGMT expression, suggesting the chemoresistancy to TMZ treatment. This is the first report showing MGMT expression in PPTs. In addition, MIB-1 labeling index correlated with WHO grade, although the immunoreactivity of synaptophysin, NF, NeuN, and p53 did not correlate with the histological grade. (240 words)

Keywords: pinealoma, immunohistochemistry, MGMT, temozolomide, MIB-1
Introduction

Pineal parenchymal tumors (PPTs) are rare neoplasms arising from the pineal gland, and account for less than 1% of primary central nervous system (CNS) tumors \(^1\). According to the latest edition of the World Health Organization (WHO) Classification of CNS Tumors \(^2\), PPTs are subdivided into pineocytoma, pineal parenchymal tumor of intermediate differentiation (PPTID) and pineoblasoma, which correspond to Grade I, Grade II or III and Grade IV, respectively. Except for pineocytoma, PPTs are potentially aggressive tumors and sometimes demonstrate craniospinal seeding \(^3\). The biological characteristics of PPTs are still to be understood; however, the histopathological studies for PPTs which indicate prognostic factor or predictive factor are limited because of the rare incidence of PPT. Moreover, the therapeutic modality to high grade PPTs have yet to be standardized; these range from localized radiotherapy to cranio-spinal radiotherapy, radical resection and multidrug chemotherapy \(^4\). Temozolomide (TMZ) is an oral alkylating agent which is widely used in the treatment of glioblastoma. Recently, TMZ has been applied to other neuro-oncological tumors including pituitary tumor, melanomas, neuroendocrine tumors and metastatic tumors \(^5-10\). \(\text{O}^6\)-methylguanine DNA methyltransferase (MGMT) is a DNA repair protein that reverses alkylation at the \(\text{O}^6\) position of guanine and counteracts the anti-neoplastic effect of TMZ. As previous studies showed an inverse correlation between MGMT expression and responsiveness to TMZ \(^11-13\), the examination of MGMT expression is important to predict the response to TMZ therapy. PPTs could be one of the potential therapeutic targets for TMZ; however, no data is available about MGMT expression in PPTs so far.
In this study, we evaluated the expression of MGMT in 12 cases of PPT to assess whether TMZ could be one of the possible therapeutic agents for PPTs. In addition, to clarify the state of differentiation or biological characteristics of each grades of PPTs, we also evaluated the immunohistochemical molecular expression of PPTs including MIB-1 labeling index as a proliferation marker, and the major prognostic markers of PPTs reported in previously \textsuperscript{14,15} such as Neurofilament (NF); the most famous tumor suppressor, p53; and NeuN, the neuronal marker.
Materials and Methods

Between 1992 and 2011, 12 patients were diagnosed as PPT in our faculty. The microscopic slides, paraffin-embedded tissue and clinical information of these patients were collected retrospectively. For histological examinations, formalin-fixed, paraffin-embedded tissue was sectioned to 3 μm, and stained using hematoxylin and eosin (H&E). We reassessed the histological diagnoses according to the latest WHO classification. The differentiation between PPTID Grade II and PPTID Grade III was defined according to morphologic and immunohistochemical features described in the previous report [Jouvet, 2000 #18]. Mitoses were counted in 10 randomly selected high power fields (HPFs, x400) and expressed as the number of mitoses per 10 HPFs as previously described [Jouvet, 2000 #18].

Immunohistochemistry was performed according to standard procedures. Briefly, tissue sections of 5 μm were deparaffinized. Antigen retrieval was carried out using pressure cooking (in citrate buffer for 3 min). Endogenous peroxidase activity was blocked by incubating sections in 3% hydrogen peroxide for 5 min. After blocking, tissue sections were incubated with the primary antibodies listed in Table 1. The EnVision kit from Dako (Denmark) was used to detect the staining. The evaluation of staining of each antibody was scored as 0 (no staining), 1 (<1/3 of cells positive), 2 (between 1/3 and 2/3 of cells positive), and 3 (>2/3 cells positive). The MIB-1 index was calculated in the field of maximal activity as percentage of positive cells.
Results

Histological examination

Characteristics of the patients and immunohistochemical results are summarized in Table 2. The patients consisted of 3 males and 9 females with a median age of 53.5 years (range, 24-71). Three cases were classified as pineocytoma (Cases 1-3), six as PPTID (Cases 4-9), and three as pineoblastoma (Cases 10-12).

Pineocytomas demonstrated low to moderate cellularity and large pineocytomatous rosettes with abundant cell processes. The cells were uniform and had round-to-oval nuclei (Fig. 1a). PPTIDs showed moderate to high cellularity and rosette-like formation but lacked conspicuous pineocytomatous rosettes. The nuclear atypia was mild to moderate (Fig. 2a). There were no mitotic figures or necroses in pineocytomas and PPTIDs. Pineoblastomas were composed of highly cellular, patternless sheets of small undifferentiated cells. The cells showed high nuclear cytoplasmic ratio and hyperchromatic nuclei (Fig. 3a). Mitoses were seen in 2 pineoblastoma cases, which corresponded to 3 and 1 /10 HPFs, respectively, and one case demonstrated necrosis. The histological appearances of the other 9 cases are indicated in the Supplementary figure.

Immunohistochemistry

MGMT expression was detected in almost all cases except 1 pineocytoma (Fig. 1b, 2b, 3b and Supplementary figures 2c, 2f, 2i, 2l, 2o, 2r, 2u, 2x, 2A); in particular, all PPTIDs and pineoblastomas showed maximum staining intensity. The mean MIB-1
labeling index was less than 1 % (range: 0) in pineocytoma, 3.5 % (range: 0.7 - 6.4 %) in PPTID, and 10.5 % (range: 8.4 - 12.7 %) in pineoblastoma (Figs. 2e, 3e and Supplementary figures 1b, 2b, 2e, 2h, 2k, 2n, 2q, 2t, 2w, 2z). Synaptophysin was positive in all PPTs (Fig. 1e, Supplementary figures 1c, 1e), and NF staining was present in almost all cases except the 1 PPTID case (Figs. 1c, 2c, 3c). There was no difference in NF-positivity between pineocytomas and the other types of PPTs. Nuclear staining of NeuN was not observed in any cases (Figs. 1d, 2d, 3d), although 4 PPTIDs and 1 pineoblastoma showed cytoplasmic staining in a dot-like manner (Figs. 2d). p53 immunolabeling was negative in all PPTs (Supplementary figures 1b, 1d, 1f).
Discussion

The prognosis of pineocytomas is considered to be favorable and the 5-year survival rate is 86 to 100 \% \cite{16,17}, while PPTIDs and pineoblastomas sometimes show poor prognosis. In fact, the reported five-year survival of PPTIDs and pineoblastoma is 39 to 74 \% \cite{17} and 58 \% \cite{3}, respectively. The therapeutic spectrum includes surgery, localized or craniospinal radiotherapy and multidrug chemotherapy such as etoposide, cisplatinum, hydroxyurea, carboplatinum, cyclophosphamide, vinblastine and ifosfamide \cite{4,18}; however, an evident standardized therapeutic protocol for PPTIDs and pineoblastoma has not been established.

TMZ, a widely used alkylating agent in the treatment of glioblastoma, could be a possible candidate of therapeutic agents for PPTs, because this drug is oral-available and effectively delivered into the tumors through the blood-brain-barrier. In fact, it has been applied for the treatment of aggressive pituitary adenoma \cite{9,10} as well as melanomas, neuroendocrine tumors and metastatic tumors \cite{5-8}. Previous reports demonstrated 13 to 54 \% of pituitary tumors show low MGMT expression \cite{19-21}[Fevre-Montange, 2011 #64], and low MGMT expression has been shown to correlate with TMZ response \cite{21,22}. In this study, we have performed the immunohistochemical evaluation for MGMT expression because the assessment of MGMT expression as a predictive factor for responsiveness to TMZ has been proposed in various types of the tumor \cite{23-25}. As a result, 11 out of 12 PPTs in our series showed positivity for MGMT. Furthermore, all PPTIDs and pineoblastomas demonstrated maximum staining intensity of MGMT; therefore, our results may indicate the
insusceptibility of TMZ treatment for high grade PPTs, although the fact that the one case of pineocytoma was negative for MGMT might indicate the presence of TMZ-sensitive cases among the low grade pineal tumors such as PPTIDs and pineocytomas. We didn’t examine the methylation status of MGMT gene promoter because all our samples were paraffin-embedded material. The methylation status should be examined by using fresh frozen samples in the future. In addition, we have to consider the possibility that the treatment-resistant mechanism of MGMT expression can’t be applied to PPTs.

Here we also evaluated other immunohistochemical markers, synaptophysin, NF, p53, NeuN, and MIB-1 to specify the neuronal differentiation and biological characteristics among each grades of PPTs. Because PPTs comprise a spectrum of increasing malignancy, and thus it is sometimes difficult to obtain the proper diagnosis for each tumor grades. Moreover, even though the WHO defined PPTIDs as grade II or III, more definite grading criteria have yet to be established. The Recent immunohistochemical studies of pineal parenchymal tumors are summarized in Table 3. Our result of the mean MIB-1 labeling index was consistent with the previous studies which reported the mean MIB-1 labeling indices as 0.27 - 3.9 % in pineocytoma, 2.7 - 10.1 % in PPTID, and 6.49 – 36.4 % in pineoblastoma\textsuperscript{14,15,26}. We failed to obtain the positive staining of p53 in immunohistochemistry as shown in previous report\textsuperscript{27}. Meanwhile, our staining result of NF was not relevant to previous reports. Jouvet et al reported that PPTIDs and pineoblastomas were less positive for NF, and positive immunostaining for NF was associated with a better survival\textsuperscript{1}. In this study, immunoreactivity of NF was observed in all types of PPTs not associated with the histological grade, although we have to consider the limited number of the cases in our
study.

We also analyzed the expression of NeuN, which is a neuron-specific nuclear protein widely used in neurodevelopmental research and histopathologic diagnosis. Immunohistochemically, NeuN was not detected in the nucleus of any PPTs, while some PPTIDs and pineoblastomas demonstrated cytoplasmic staining of NeuN in a dot-like manner (Fig. 2d). Although some previous reports suggest the cytoplasmic expression of NeuN, the functional significance has not been determined.

In conclusion, our immunohistochemical result of MGMT expression in the majority of PPTs let us endeavor to find the effective chemotherapeutic agents for high grade PPTs other than TMZ. In addition, the MIB-1 labeling index will help to reach to proper WHO grade of PPTs.
**Figure Legends**

**Fig. 1**  A representative case of pineocytoma. (Case 2)

a. Pineocytoma shows low to moderate cellularity and multiple pineocytomatous rosettes which are composed of abundant tumor cell processes. The nuclei of tumor cells are moderate-sized, and nucleoli are conspicuous. There are no mitotic figures or necroses. b. Some tumor cells show weak positivity for O\(^6\)-methylguanine DNA methyltransferase (MGMT). c. Neurofilament protein (NF) highlights a few cell processes, although the majority of tumor cells were negative for NF. d. Tumor cells were negative for NeuN. e. Synaptophysin is strong positive in cytoplasm of tumor cells or pineocytomatous rosettes. a-e: x400

**Fig. 2**  A representative case of pineal parenchymal tumor of intermediate differentiation (PPTID). (Case 4)

a. PPTID is more cellular than pineocytoma and less cellular than pineoblastoma. The rosette-like formations were found, while the conspicuous pineocytomatous rosettes were lacking. Nuclei are smaller and denser than those in pineocytoma, and nuclear:cytoplasmic ratio is higher. There are no mitotic figures or necroses. b. Almost all tumor cells demonstrate intense positivity for MGMT. c. Some tumor cells show positivity for NF. d. Nuclear staining of NeuN is negative; however, cytoplasmic staining is observed in a dot-like manner. e. MIB-1 labeling index is 3.7 %. a-e: x400

**Fig. 3**  A representative case of pineoblastoma. (Case 10)
a. Pineoblastoma is composed of highly cellular, densely packed small cells, which is reminiscent of medulloblastoma. The tumor cells are highly nuclear: cytoplasmic ratio, hyperchromatic nuclei. b. The majority of tumor cells show positivity for MGMT. c. A few tumor cells are positive for NF. d. Tumor cells are negative for NeuN. e. MIB-1 labeling index is 12.7%. a-e: x400
References


whole-brain radiotherapy in patients with brain metastases from solid tumors. 

*Onkologie.* 2007; **30**: 361-6.


Figure 2

Case 4
<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Type</th>
<th>Dilution</th>
<th>Antigen retrieval</th>
<th>Company</th>
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<tr>
<td>synaptophysin</td>
<td>SY38</td>
<td>mouse</td>
<td>1:100</td>
<td>Pressure cook (PC)</td>
<td>Dako</td>
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<tr>
<td>Neurofilament</td>
<td>2F11</td>
<td>mouse</td>
<td>1:50</td>
<td>none</td>
<td>Dako</td>
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<td>NeuN</td>
<td>A60</td>
<td>mouse</td>
<td>1:100</td>
<td>PC</td>
<td>CHEMICON</td>
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<tr>
<td>p53</td>
<td>DO-7</td>
<td>mouse</td>
<td>1:100</td>
<td>PC</td>
<td>Dako</td>
</tr>
<tr>
<td>Ki-67</td>
<td>MIB-1</td>
<td>mouse</td>
<td>1:100</td>
<td>PC</td>
<td>Dako</td>
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<tr>
<td>MGMT</td>
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<td>1:200</td>
<td>PC</td>
<td>CHEMICON</td>
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<td>Case No.</td>
<td>Age (yr)</td>
<td>Sex</td>
<td>Histology</td>
<td>WHO grade</td>
<td>Mitotic index (/HPF)</td>
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<tr>
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<td>PPTID</td>
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<tr>
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<td>F</td>
<td>PPTID</td>
<td>III</td>
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<td>Pineoblastoma</td>
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<td>12</td>
<td>49</td>
<td>M</td>
<td>Pineoblastoma</td>
<td>IV</td>
<td>1</td>
</tr>
</tbody>
</table>

PPTID: Pineal parenchymal tumor of intermediated differentiation
MGMT: O^6^-methylguanine DNA methyltransferase, NF: neurofilament protein
Table 3. Recent immunohistochemical studies of pineal parenchymal tumors

<table>
<thead>
<tr>
<th>Authors</th>
<th>Histology</th>
<th>Total (N)</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Numoto R (1994)</td>
<td>5 PCs, 2 mixed PC/PBs and 4 PBs</td>
<td>11</td>
<td>The MIB-1 labeling index correlated with histological malignancy. NF and synaptophysin positivity correlated with seeding potentials.</td>
</tr>
<tr>
<td>Tsumanuma I et al (1995)</td>
<td>5 PCs and 4 PBs</td>
<td>9</td>
<td>All cases were immunonegative for p53.</td>
</tr>
<tr>
<td>Tsumanuma I et al (1999)</td>
<td>4 PCs, 4 PCs with anaplasia and 5 PBs</td>
<td>13</td>
<td>The mean MIB-1 index of PBs was significantly higher than that of other types of PPT. The mean MIB-1 index of NF-positive cases was significantly lower than that of negative cases.</td>
</tr>
<tr>
<td>Jouvet A et al (2000)</td>
<td>11 PCs, 39 PPTIDs and 16 PBs</td>
<td>66</td>
<td>A number of mitoses greater than 6 and the presence of necrosis were associated with a poorer outcome, while positive immunostaining for NF was associated with a better survival.</td>
</tr>
<tr>
<td>Fauchon F et al (2000)</td>
<td>19 PCs, 28 PPTIDs and 29 PBs</td>
<td>76</td>
<td>The findings of the report of Jouvet A et al were confirmed.</td>
</tr>
<tr>
<td>Rickert CH et al (2001)</td>
<td>3 PCs, 3 PPTIDs and 3 PBs</td>
<td>9</td>
<td>The average MIB-1 indices were 1.3% for PCs, 10.1% for PPTIDs, and 27.2% for PBs.</td>
</tr>
<tr>
<td>Yamane Y et al (2002)</td>
<td>4 PCs, 5 PPTIDs and 14 PBs</td>
<td>23</td>
<td>The NF showed significant differences of reactivity among PC, PPTID and PB.</td>
</tr>
<tr>
<td>Arivazhagan A et al (2008)</td>
<td>6 PCs, 10 PPTIDs, 17 PBs</td>
<td>33</td>
<td>The mean MIB-1 labeling index in PCs, PPTIDs and PBs were 1.58, 16.1 and 23.52. NF immunoreactivity indicated better prognosis.</td>
</tr>
<tr>
<td>Marcol W et al (2009)</td>
<td>15 PCs, 1 PPTIDs and 11 PBs</td>
<td>27</td>
<td>The immunoreactivity of tubulin and NSE was correlated with patients' survival type while Bcl-2, p53 and nestin correlated negatively with survival time.</td>
</tr>
<tr>
<td>Fèvre-Montange M (2011)</td>
<td>2 PC, 27 PPTID and 4 PB</td>
<td>33</td>
<td>The number of mitosis and the mean MIB-1 labeling index were significantly different among tumor grades. The mean MIB-1 labeling index in PCs, PPTID grade II, PPTID grade III and PB were 0, 5.2, 11.2 and 36.4.</td>
</tr>
</tbody>
</table>

NSE: Neuron specific enolase