High incidence of low O\textsuperscript{6}-methylguanine DNA methyltransferase (MGMT) expression in invasive macroadenomas of Cushing’s disease

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A short running title: Low MGMT expression in Crooke cell adenoma

Total word count: 4078 words

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Abstract

Context: Crooke’s cell adenoma (CCA), characterized by massive Crooke’s hyaline change in corticotroph adenoma, causes a rare subtype of Cushing’s disease. In contrast to ordinary corticotroph adenomas, CCAs are generally aggressive and present as invasive macroadenomas, which are refractory to both surgery and radiotherapy and have a high recurrence rate. Moreover, some patients with CCA present with distant or cranio-spinal metastases. Currently, there are no effective standard therapies for CCA.

Objective: We report a patient with Crooke’s cell carcinoma who presented with local invasion and liver metastases, that was refractory to conventional therapeutic modalities including transsphenoidal surgery, radiosurgery, medications, and hepatic transcatheter arterial embolization. After all these treatments failed, patient had monthly temozolomide administrations, resulting in gradual clinical improvement and biochemical data that was consistent with tumor shrinkage. In glioblastoma, low O\(^6\)-methylguanine DNA methyltransferase (MGMT) expression is associated with epigenetic gene silencing and predicts better response to temozolomide.

Methods: We thus investigated MGMT expression immunohistochemically in 7 CCAs (5 invasive macroadenomas and 2 invasive microadenomas) and 17 ordinary-type adenomas (OTAs) (3 non-invasive macroadenomas, 12 non-invasive microadenomas, and 2 invasive microadenomas) from patients with Cushing’s disease.

Results: In 7 CCAs, all 5 invasive macroadenomas exhibited low MGMT expression, defined as <5% nuclear MGMT staining. In 17 OTAs, only 1 adenoma showed low MGMT expression.

Conclusion: In Cushing’s disease, invasive macroadenomas including CCA usually have low MGMT expression. Temozolomide thus may be a new therapeutic option for invasive macroadenomas such as CCA particularly when conventional treatments are ineffective.
Introduction

Crooke’s cell adenoma (CCA), a histologic variant type of ACTH-producing adenoma, is characterized by Crooke’s hyaline change in most corticotroph adenoma cells. Although CCAs are a rare subtype of Cushing’s disease with estimated prevalence of 4.4-14% in all corticotroph adenomas, CCAs are generally aggressive presenting as invasive macroadenomas, and resistant to surgery and radiotherapy with high recurrence rate (1-3). Corticotroph carcinomas developed in preexisting CCA are not unusual and have been reported in the literatures (1,4).

Pituitary carcinomas are defined as pituitary tumors with cranio-spinal and/or systemic metastases. Pituitary carcinomas are rare, making up 0.1-0.2% of all pituitary tumors. The majority of carcinomas arise from corticotroph adenomas or prolactinomas. Pituitary carcinomas usually do not respond well to radiation therapy or systemic chemotherapy. Consequently, most patients die within 1 year after the diagnosis of pituitary carcinoma (reviewed in 5,6).

Lim et al. first reported the successful use of temozolomide in a 72-year-old man with a prolactin-producing pituitary carcinoma (7). They observed a decrease in serum prolactin levels with clinical improvement and significant tumor shrinkage. Since then, there have been several case reports showing the beneficial antitumor effects of temozolomide in the treatment of invasive pituitary tumors including pituitary carcinomas (8-11) and a case of Nelson’s syndrome (12). Recently, Mohammed et al. reported two cases of CCA treated with temozolomide (13).

Here, we describe a case of metastatic Crooke’s cell carcinoma, which was refractory to any conventional treatment. Temozolomide administration successfully controlled ACTH hypersecretion and tumor growth. Previous studies reported that patients with glioblastoma containing O\(^6\)-methylguanine DNA methyltransferase (MGMT) methylation of the gene promoter had a better response to temozolomide than those without methylated MGMT promoter (reviewed in 14). MGMT gene promoter hypermethylation has been associated with epigenetic inactivation of the gene and the subsequent loss of the MGMT protein, which plays an important role in the repair of DNA damage induced by alkylating agents such as temozolomide (reviewed in 15). These observations suggest that...
low MGMT expression in CCAs may predict a better response to temozolomide treatment. Thus, we examined immunohistochemical expression of MGMT in CCAs as well as other corticotroph tumors from Cushing’s disease patients.

Case Report

In 1998, a 46-year-old woman was diagnosed with Cushing’s disease. Transsphenoidal surgery (TSS) was performed, however the infiltrating tumor remained in left cavernous sinus. She underwent gamma-knife radiosurgery twice in 2000 and 2002, and a second TSS in 2003, without any significant clinical improvement.

In January 2004, the patient was referred to Toranomon hospital for further evaluation. Laboratory data showed elevated plasma ACTH (182 pg/ml at 0800, reference range 9-52 pg/ml) and serum cortisol (43.1 μg/dl, reference range 4.5-21 μg/dl), with loss of their diurnal rhythm, and increased urinary free cortisol excretion (230.7 μg/day). A third TSS was performed in March 2004, but the patient remained hypercortisolemia. Medical therapies such as cabergoline, thiazolidinedione (pioglitazone), and octreotide failed to reduce ACTH and cortisol levels or prevent tumor invasion of the left cavernous sinus. Additional CyberKnife radiosurgery was undertaken in February 2006, without significant tumor shrinkage. Figure 1 shows the patient’s blood ACTH and cortisol concentration over the course of treatment since 2004.

Hematoxylin and eosin-staining showed that the resected tumor cells were polygonal with moderate nuclear pleomorphism, hyperchromaticity, and nucleolar prominence. The vast majority of cells exhibited Crooke’s hyaline change characterized by a prominent hyaline cytoplasmic ring that displaces the basophilic granules of the corticotroph cell (Fig. 2A, a). The hyaline material was strongly positive for cytokeratin (CAM 5.2) staining (Fig. 2A, b). The ACTH reactivity was apparent only in the peripheral rim of the cytoplasm (Fig. 2A, c). The Ki-67 labeling index was approximately 3%. Scattered neoplastic cells showed weak nuclear p53 immunoreactivity. Electron microscopy showed that large rounded tumor cells were packed with the dense masses of cytokeratin filaments having ovoid or somewhat irregular nucleus harboring one or more prominent nucleolus. So-called
macronucleoli, known to occur in aggressive corticotroph tumors, were also noted. Moreover secretory granules, being 200-350 nm in diameter, were quite numerous but they were invariably displaced to the cell periphery by cytokeratin filamentous masses or remained trapped within the Golgi region (Fig. 2A, d).

In April 2005, multiple liver metastases were found by computed tomography (CT) scan (Fig. 2B, a). Histopathology of a hepatic biopsy confirmed metastases of pituitary tumor having Crooke’s hyaline change (Fig. 2C, a and b). Hepatic transcatheter arterial embolization was repeated 3 times, resulting in approximately one-third decrease in tumor size.

Persistent hypercortisolemia resulted in multiple clinical complications, including diabetes requiring insulin therapy, severe hypertension, massive lower leg edema with cutaneous ulcer and cellulitis, brain infarction, and spinal compression fractures due to osteoporosis. Metyrapone treatment could temporally control her serum cortisol at approximately 20 μg/dl. In April 2005, metyrapone therapy was substituted to mitotane at a daily dose of 2-3 g. After April 2007, cortisol level fell below 10 μg/dl, probably due to the adrenolytic effect of mitotane. On the other hand, plasma ACTH concentrations rose to a peak of 15,000 pg/ml at that time, suggesting that the mitotane treatment induced Nelson’s syndrome. Mitotane was discontinued. Pituitary MRI showed the tumor extending into the left sphenoid sinus (Fig. 2D, a).

In July 2007, the patient started temozolomide 200 mg (150 mg/m²) daily for 5 days in monthly cycles. Then the daily dose was increased to 260 mg (190 mg/m²) from the second cycle. We adopted the standard schedule of temozolomide treatment widely used for malignant glioma at a dose of 150-200 mg/m² once daily for Days 1-5 of a 28-day cycle (16). Three months after treatment initiation, plasma ACTH fell to 1,008 pg/ml. In parallel with reduced ACTH, serial MRI scans have shown a reduction in pituitary tumor volume in the left sphenoid sinus (Fig. 2D, b). Liver metastases were hardly detectable in abdominal CT after the 6 cycles of treatment (Fig. 2B, b). Her clinical features of Cushing’s disease gradually improved, so medications for her hypertension, diabetes, and edema were discontinued. After one year, the monthly temozolomide treatment was reduced to every other month at a dose of 200 mg per day for 5 days. In a recent visit in June 2009, her ACTH and cortisol were
found to be 20.8 pg/ml and <0.2 μg/dl, respectively. Since bimonthly temozolomide treatment during the second year effectively kept favorable response, we decided to decrease further the frequency of temozolomide treatment to trimonthly from the third year. Currently, we plan to continue the trimonthly treatment as a maintenance therapy to prevent relapse because there are two case reports describing recurrence of pituitary carcinoma after discontinuation of temozolomide treatment (9,13). She has continued to take dexamethasone to prevent adrenal insufficiency. Throughout her temozolomide treatments, the patient tolerated the medication well, without significant nausea, vomiting, myelosuppression, or other adverse events.

Materials and Methods

Patients and Tumor Pathology

We reviewed all tumor samples from the 103 patients with clinically active Cushing’s disease who underwent TSS between Jan 2004 and Feb 2009. The samples were studied by routine histological methods such as hematoxylin and eosin-staining, and immunohistochemical staining using antibodies for anterior pituitary hormones (ACTH, GH, PRL, TSH, FSH, LH) and cytokeratin (CAM 5.2) as previously described (17,18). Samples were classified as CCA whenever the percentage of Crooke’s hyaline change of tumor cells was greater than 50%, and remaining tumor samples were considered as ordinary-type adenomas (OTAs) (1). The samples were reviewed by two pathologists (N.I. and T.S.) independently, and differences were resolved by consensus in each equivocal case. Seven tumor samples were identified as CCA from 103 samples, and they were compared with randomly selected 17 tumor samples of OTAs obtained from 2007 to 2008. Informed consent was obtained from all patients of whom we assessed MGMT expression for investigational purposes, and tissue collection was approved by Institutional Review Board of Toranomon hospital. Tumor size and invasiveness were defined on the basis of preoperative radiological findings and operative findings. Microadenomas were defined as tumors less than 10 mm in maximal diameter whereas macroadenomas were larger than 10 mm. Tumor invasion was defined by evidence of bony
destruction and/or tumor within the sphenoid and/or cavernous sinuses and/or into brain that was confirmed at the time of surgery. Patients were considered as surgical remission on the basis of the postoperative serum cortisol levels less than 3 µg/dl (19).

**MGMT Immunoexpression and Analysis**

Sections of formalin-fixed, paraffin-embedded specimens of tumors underwent heat-induced antigen retrieval for 40 minutes at 95°C in Target Retrieval Solution (Dako, Glostrup, Denmark). Sections were incubated overnight at 4°C with a mouse monoclonal anti-MGMT antibody (clone MT3.1; Novus Biologicals, Littleton, CO) at 1:300 dilution. The slides were stained using an EnVision+System-HRP Kit (Dako). Immunohistochemical reactions were developed with diaminobenzidine, and slides were counterstained with hematoxylin. The level of MGMT protein expression was defined semiquantitatively by visual impression according to the fraction of positive nuclear staining and was scored as low (few endothelial cells immunostained and <5% of tumor cells immunostained), moderate (5% to 20% of tumor cells immunostained), or high (>20% of tumor cells immunostained). Nonneoplastic cells (vascular endothelial cells and lymphocytes) served as an internal positive control in all tissue sections. The results of the MGMT immunostaining were scored separately by two independent pathologists (N.I. and T.S.) who were blinded to the subtype of the tumors. In case of disparate scoring, the results were discussed by 3 of us (N.I., T.S., A.T.) using multiheaded microscope and obtained a final consensus in each equivocal case.

**Results**

**Patient Characteristics**

The clinical and morphologic characteristics of the tumors examined are summarized in Table 1. The tumor from temozolomide-responsive patient in the case report is shown as case 1. In 7 CCAs, 5 were macroadenomas, and 2 were microadenomas. All 7 CCAs exhibited carvernous sinus invasion, and 4 cases were from re-operations. Two cases of macroadenoma failed to achieve surgical remission. In 17 OTAs, 3 were macroadenomas, and 14 were microadenomas. Two microadenomas
had cavernous sinus invasion, and 4 were from re-operated cases. All 17 cases of OTAs achieved surgical remission.

**Immunohistochemical Analysis of MGMT**

In a surgical specimen of normal pituitary tissue adjacent to pituitary tumor, more than half of the cell nuclei were immunostained using a specific monoclonal antibody against MGMT (Fig. 3A). Results of immunohistochemical expression of MGMT in corticotroph tumors are referenced in Table 1. Moderate or high expression of MGMT was found in 18/24 (75%) of the tumors, whereas MGMT expression was low in 6/24 (25%) of the tumors. The tumor from temozolomide-responsive patient (case 1) showed low MGMT expression although vascular endothelial cells were positively-stained with anti-MGMT antibody (Fig. 3B). Importantly, all 5 invasive CCA macroadenomas exhibited low MGMT expression (case 1-5). Thus, among the 6 tumors, which showed low MGMT staining, 5 were CCAs. Among 17 OTAs, only 1 adenoma (case 8) showed low MGMT expression.

**Discussion**

We described a case of Cushing’s disease due to an ACTH-secreting invasive pituitary carcinoma that metastasized to the liver 7 years after the initial diagnosis. Both pituitary tumor and metastatic lesions showed similar histologic features that were compatible with CCA. George et al. reported 36 cases of CCAs in which they defined tumor as CCA when more than 50% of the adenoma cells exhibited Crooke’s hyaline change (1). They also found that 81% of the CCAs were macroadenomas and 72% were invasive. Moreover 60% of 25 follow-up cases developed recurrent tumor and 24% had multiple recurrences. Three of these 25 patients died of tumor: One due to multiple local recurrences and 2 from pituitary carcinoma. They concluded that these tumors are innately aggressive and represent a distinct clinicopathologic entity that should be separated from ordinary corticotroph adenomas, although the latter usually contain Crooke’s hyaline change to some extent. CCA is also classified as a variant histologic entity of ACTH-producing adenoma in 2004 WHO classification of tumors of endocrine organs (20). Other several case reports (2-4, 13) and our
own experience with cases of CCA confirm the aggressive clinical features observed in CCA.

Pituitary carcinoma is rapidly progressive, and usually refractory to current available therapies including classic chemotherapeutic agents (21). In contrast, the alkylating compound, temozolomide, which was originally developed for the treatment of malignant gliomas, was recently used as a new alternative approach to resistant pituitary tumors (7-13). A phase II clinical trial entitled “Temozolomide in Treating Patients With Invasive Pituitary Tumors” currently is underway in the USA (22). In comparison to many other chemotherapeutic agents, temozolomide is well tolerated, although some patients may develop mild to moderate nausea, vomiting, and fatigue. Myelosuppression also can occur in a minority of patients. Our patient did not show any chemotherapy-related complications although she received prophylactic treatment with a serotonin 5-HT3 receptor antagonist and sulfamethoxazole/trimethoprim to prevent nausea and Pneumocystis carinii pneumonia, respectively.

Epigenetic hypermethylation of the CpG-island in the MGMT promoter results in loss of the MGMT protein that blocks the repair of genetic mistakes, thereby possibly contributing to malignant transformation of the cell. Paradoxically, such tumors cannot repair DNA damage induced by alkylating agents such as temozolomide (15). In patients with glioblastoma, low MGMT levels, as determined by immunohistochemistry or promoter methylation analysis such as methylation-specific PCR, have been associated with improved survival and benefit from temozolomide in many studies (reviewed in 14). Like temozolomide-responsive gliomas, there are several recent case reports describing absent MGMT expression in the temozolomide-responsive pituitary tumors and MGMT expression in the temozolomide-resistant pituitary tumors (11,23). Consistent with these observations, our case showed low MGMT expression. Large scale multi-institutional clinical trials including pathological MGMT expression analysis will be necessary to prove the relationship of MGMT status to the extent of temozolomide response.

Several studies have evaluated MGMT expression status by immunohistochemistry and/or methylation-specific PCR in pituitary adenomas (11,24,25). However, to our knowledge, this is the first report of immunohistochemical analysis of MGMT expression in corticotroph tumors from patients with Cushing’s disease, in which a large number of tumor samples was classified according to
histologic types. Previous studies have not found significant differences in MGMT expression between invasive and non-invasive pituitary adenomas, or between recurrent and non-recurrent adenomas in their examined materials (11,25). These results are different from our finding that low MGMT expression was more common in invasive macroadenomas, including CCA, compared to microadenomas in Cushing’s disease. The difference may be due to different histological samples used in each study since the previous studies were mainly performed in non-functioning pituitary adenomas whereas our study focused on CCAs. Indeed, the issue we actually want to address is how to manage patients with CCAs refractory to conventional therapeutic modalities. Our observations suggest that such patients have tumors that usually have low MGMT expression, and thus may be sensitive to temozolomide chemotherapy. It would have been of interest to know the methylation status of the promoter in CCAs, as well.

Direct evaluation of MGMT protein expression using immunohistochemistry is the most feasible technique to estimate MGMT expression in tumor samples. Pituitary tumors are composed of relatively homogenous tumor cells with few non-neoplastic cells, providing favorable features for the reliable assessment of MGMT expression by immunohistochemistry. However, immunohistochemical stains may be prone to false negative staining for a variety of reasons, including differences in antibodies, preservation, fixation, or processing. Given the potential clinical significance of negative MGMT immunostaining, we have used endothelial cells as a reliable internal positive control since they are scattered throughout all pituitary tumors (11). Standardization of techniques to assess MGMT status will be beneficial to allow more accurate predictions of the clinical outcome of treatment with temozolomide in a patient with refractory pituitary tumor.

In summary, low MGMT expression appears to be common in invasive macroadenomas of Cushing’s disease, including CCA. Our findings suggest that MGMT status could be a predictive marker to identify patients who are likely to respond to treatment with temozolomide. The favorable outcome of our present case supports the view that when conventional therapies fail, temozolomide may be administered to patients with invasive and aggressive CCA if their tumors have low MGMT expression. It also appears that this promising drug can reduce both tumor size and circulating ACTH levels substantially. Further studies will be necessary to substantiate the efficacy of this medication in
a larger sample of patients with CCA who are refractory to standard therapies.

Declaration of interest: The authors declare that there is no conflict of interest.

Funding: This work was supported by Okinaka Memorial Institute for Medical Research, Tokyo, Japan.

Acknowledgment

We are grateful for critical review and editing of the manuscript by Paul M Yen, M.D., Department of Medicine, Johns Hopkins Bayview Medical Center, Baltimore, MD, USA. We also thank Professors Kalman Kovacs, M.D., and Eva Horvath, Ph.D., Department of Laboratory Medicine and Pathobiology, St. Michael’s Hospital, Toronto, Canada, for their kind help in making the diagnosis of Crooke’s cell adenoma by electron microscopy.

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**Figure legends**

**Figure 1**
Changes of plasma ACTH and serum cortisol levels over the course of treatment since 2004.
TSS, transsphenoidal surgery; TAE, hepatic transcatheater arterial embolization

**Figure 2**
Histopathologic and radiologic features of the temozolomide-responded patient.
A, Histology of Crooke’s cell adenoma. The Crooke’s cell adenoma characterized by a prominent hyalinized cytoplasm (hematoxylin and eosin-staining, magnification, X 400) (a). An inset micrograph in (a) is the expansion image. Crooke’s hyalinization resulting from the accumulation of a ring of cytokeratin filaments around the nucleus (CAM 5.2 immunostaing, magnification X 400) (b). ACTH-positive granules are displaced to the peripheral rim of the cytoplasm (ACTH immunostaing, magnification, X 400) (c). Electron microscopy shows the accumulation of massive intermediate filaments (d).

B, Contrast-enhanced abdominal CT when the multiple liver metastases were found (a) and 6 months after temozolomide therapy (b). After TAE and temozolomide therapy, multiple metastases (black arrows) were hardly detectable, and marked reduction in size of bilateral adrenal hyperplasia (white arrows) was apparent.

C, Histology of liver metastases. Metastatic cells display Crooke’s hyaline change structurally similar to the cells found in the pituitary tumor. Images of hematoxylin and eosin-staining (a) and ACTH immunostaing (b) (magnification, X 200).
D, Coronal gadolinium-enhanced T1-weighted MRI before (a) and 18 months after (b) temozolomide therapy. Note the marked reduction in the size of the invasive tumor following temozolomide therapy.

**Figure 3**

A, MGMT staining of normal pituitary (magnification, X 200). More than half of the cell nuclei were immunostained.

B-D, Representative microscopy of semiquantitative assessment of MGMT immunoexpression in ACTH-producing pituitary adenomas according to the percentage of stained tumor cells: (B) low (case 1 of Table 1), <5% of tumor cells immunostained (stained endothelial cells [arrow] served as a positive control); (C) moderate (case 9 of Table 1), from 5% to 20% of tumor cells immunostained; and (D) high (case 12 of Table 1), >20% of tumor cells immunostained (magnification, X 100 and X 400 for insets).
Mitotane 2-3 g/day
Metyrapone 1-1.5 g/day
dexamethasone 0.5→1 mg/day

3rd TSS TAETAE
dexamethasone 0.5→1 mg/day
Temozolomide

3rd radiosurgery

Cortisol (µg/dl)
ACTH (pg/ml)
Table 1. Summary of patient characteristics

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<th>Age at diagnosis</th>
<th>Size</th>
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<th>Prior TSS</th>
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<th>0800-serum cortisol (μg/dl)</th>
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CCA, Crooke's cell adenoma; OTA, ordinary-type adenoma; MGMT, low (L), moderate (M), high (H); F, female; M, male

Size, macroadenoma (M), microadenoma (m); TSS, transsphenoidal surgery

# Preoperative medication could influence preoperative values of plasma ACTH, serum cortisol, and urinary free cortisol.