MGMT promoter methylation and immunoexpression in aggressive pituitary adenomas and carcinomas

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Abstract MGMT promoter hypermethylation of aggressive pituitary adenomas and pituitary carcinomas and low protein expression are implicated in improved response to treatment with temozolomide (TMZ). The aim of the present study was to investigate MGMT promoter methylation and immunoexpression in an aggressive subset of pituitary adenomas and carcinomas. Our material consisted of 12 silent subtype 3 (SS3) adenomas, 10 primary carcinomas, and 4 disseminated metastases. Two different tissue samples of 7 of the 12 SS3 adenomas and all carcinomas were analyzed for MGMT promoter methylation and immunohistochemical expression of MGMT. Immunoexpression was assessed semi-quantitatively as a percentage of immunoreactive nuclei. Overall 33% of carcinomas exhibited homogenous MGMT methylation in tumor and metastatic specimens. Low immunohistochemical MGMT expression was noted in 50% of carcinomas. Overall, 42% of the SS3 adenomas exhibited MGMT promoter methylation. MGMT immunostaining was predominantly negative (92%), with homogenous immunostaining results across different samples. Whereas all the methylated SS3 adenomas had low MGMT immunoreactivity, five unmethylated adenomas exhibited absent/low MGMT expression. There was no relationship between methylation status and MGMT immunoexpression was not apparent. MGMT methylation and low immunohistochemical expression seen in a subset of carcinomas and SS3 adenomas, suggesting that a subset of tumors may respond to treatment with TMZ. Heterogeneous MGMT methylation status in SS3 adenomas and the lack of concordance between methylation and immunohistochemical expression of MGMT suggest complex regulatory mechanisms, highlighting the need for improved methods in the research on a correlation between MGMT changes and response to TMZ.

Keywords MGMT immunoexpression · MGMT methylation · Pituitary carcinoma · Silent subtype 3 pituitary adenoma · Temozolomide susceptibility

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Introduction

Pituitary neoplasms are typically benign, slow-growing lesions. Only a subset behaves more aggressively and necessitates protracted treatment, including repeat surgery and adjuvant therapy, often with little success. Among more aggressive adenomas, the uncommon SS3 variant, a form of plurihormonal adenoma, is particularly so, tending to invade and recur, often necessitating multimodality treatment. Pituitary carcinomas, although extremely rare, pose a particular therapeutic challenge since treatment options for such multiply recurring, metastasizing pituitary tumors are limited.

Recent reports of the successful use of temozolomide (TMZ) as a therapeutic agent in the treatment of aggressive pituitary tumors refractory to other treatment modalities are promising [1–10]. Response to TMZ therapy is known to be closely related to expression levels of O-6-methylguanine-DNA methyltransferase (MGMT), a DNA repair enzyme [11, 12]. MGMT interrupts the therapeutic action of TMZ by removing the O6-guanine alkyl groups induced by treatment, thus repairing TMZ-induced DNA damage and counteracting its therapeutic effect. As such, low-level MGMT immunoexpression is correlated with TMZ response. Low MGMT levels are also associated with a longer disease-free and overall survival in gliomas [11, 12]. Although several recent reports of aggressive pituitary tumors successfully treated with TMZ have shown absent or low immunohistochemical expression of MGMT to be a factor, some patients whose tumors exhibited higher MGT levels also experienced clinical improvement and tumor reduction following TMZ treatment [13, 14].

MGMT gene promoter methylation is associated with suppressed MGMT protein levels [15]. Thus, it is thought to be one of the proposed mechanisms underlying absent or low MGMT expression. Indeed, MGMT methylation has been correlated with improved response to TMZ and longer patient survival [12]. MGMT promoter methylation attenuates tumoral binding of transcription factors and initiation of replication [16–19], resulting in absent or decreased levels of MGMT protein expression. Improved success of TMZ therapy has been well documented in glioblastoma (GBM) exhibiting MGMT promoter methylation [20]. Hegi et al. found MGMT methylation to be a favorable prognostic indicator in TMZ-treated patients with GBMs exhibiting promoter hypermethylation, survival being prolonged by 55% [12]. Preusser et al. also found MGMT promoter methylation status to be associated with longer patient survival [21]. Interestingly, MGMT promoter methylation status is also associated with higher tumor grade and invasiveness as well as poor prognosis in most human neoplasms studied. These include GBM, various sarcomas, non-small cell lung carcinoma, carcinomas of bladder, cervix, prostate and salivary gland, as well as other tumors including pituitary neoplasms [3, 22–29]. In cell culture, Nakasu et al. recently found loss of MGMT expression to be correlated to anaplastic transformation of astrocytomas [30]. Whether MGMT promoter methylation status and MGMT immunohistochemical expression are correlated remains unclear, with several studies reporting a positive association and other finding a lack of correlation [13, 14]. In one study of GBMs, MGMT methylation was associated with prolonged patient survival, but no such relationship with MGMT immunohistochemical expression was observed [21]. Similar observations were noted with respect to non-small cell lung carcinomas [31]. A recent report by Hamilton et al.

found heterogeneous MGMT promoter methylation within the same GBM, when using formalin fixed paraffin embedded (FFPE) tumor tissue [32]. This raised questions about the reliability of current methods for the detection of MGMT methylation and treatment planning.

The aim of the present study was to investigate (a) MGMT promoter methylation status in SS3 adenomas and pituitary carcinoma, (b) immunohistochemical expression of MGMT in these two aggressive and therapy-resistant tumor groups, (c) the potential utility of TMZ as a therapeutic agent, (d) the correlation of MGMT immunoexpression and promoter methylation in terms of tumor homogeneity, and (e) examine MGMT methylation status and immunoexpression in matched primary and metastatic pituitary carcinomas.

Materials and methods

Our material consisted of 12 SS3 pituitary adenomas and 10 pituitary carcinomas. All tumors were identified in the Mayo Clinic Anatomic Pathology Database archives, Rochester, MN. All patients had undergone transsphenoidal resection of the primary between the years 1988 to 1994. The SS3 adenomas consisted of a subset of 12 adenomas in which ample tissue was available for methylation analysis, from our recent clinicopathological study [33]. Specimens were 10% neutral formalin-fixed, routinely processed, paraffin-embedded, and cut at 5 μm. Routine stains applied included hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and the Gomori reticulin method. Immunohistochemical characterization of adenohypophysial hormone content was performed using the streptavidin–biotin-peroxidase complex method seeking growth hormone (GH), prolactin (PRL), adrenocorticotropin (ACTH), thyroid stimulating hormone (TSH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), as well as the alpha subunit of glycoprotein hormones. All SS3 adenomas had been ultrastructurally diagnosed [33]. Distant metastases were histologically documented in all carcinomas. MGMT immunodetection utilized a mouse monoclonal antibody (MT 3.1, Thermofisher, Fremont, CA; dilution 1:50). Antibody sources, clonality and dilutions as well as control methods have been previously reported.

Patient and tumor characteristics for both the SS3 pituitary adenomas and carcinomas were retrieved from our previously reported studies [33, 34]. As previously reported, all tumors had been studied for MGMT immunoexpression [34, 35]. Seven of the 12 SS3 adenomas and 6 of the 10 carcinomas had sufficient tissue for determination of intratumoral heterogeneity, MGMT immunoexpression and of MGMT promoter methylation. Additionally, in 6 of the 10 carcinomas, both the primary tumor and the metastasis were available for IHC immunohistochemical

Table 1 MGMT promoter methylation and immunohistochemical staining in pituitary carcinoma

Number	Methylation	MGMT IHC score	MGMT (%N)	Site
1	M	4	50% weak	Skull met
	M		50% weak	Skull met
				Skull met
2	UM	0	0%	Pituitary
	UM		0%	Pituitary
			0%	Left cheek
			1%	Soft tissue
			8%	Tubes and ovaries
3[a]	UM	4	80%	R jugular LN
	UM		90%	Triceps mm
			70%	Lymph node
4[a]	UM	2	10%	Pituitary
	UM		10%	Brain, tentorial
	M	1	5%	Pituitary
5	M	1	5%	Pituitary
6	F, F	3	5%	Liver
			35%	Liver
			40%	Liver
7	UM	0		Pituitary
	UM		0%	Right neck
8	UM	0	0%	Lymph node
	UM	0	0%	Lymph node
9	UM, UM	3	45%	Pituitary
			30%	Foramen magnum
			30%	Spinal Sbarachnoid
10	M	4	60%	Pituitary
	M	4	60%	Pituitary
			60%	Pituitary
			70%	Buccalmucosa

[a] These specimens consisted of two different sites primary and/or metastasis

study. In one carcinoma case, both the primary tumor and a brain metastasis were quantitatively sufficient for methylation analysis. In yet another carcinoma, methylation was analyzed in two different metastases (jugular lymph node and triceps muscle) (Table 1).

Microscopy

MGMT immunoreactivity was evaluated microscopically under 10 high-powered fields (×200) by three observers (FS, BWS, and KK). Areas of optimal immunoreactivity with highest tumoral and vascular MGMT staining and minimal background reactivity were considered representative and used to estimate percentage immunopositivity. MGMT immunostaining was assessed as a percent immunopositive tumoral nuclei; cytoplasmic immunoreactivity was regarded as non-specific. Percentages registered were <10%, 10–25%, 25–50%, 50–75%, or 75%.

Methylation-Specific Multiplex Ligation-Dependent Probe Amplification (MS-MLPA) for *MGMT*

The MS-MLPA probe mix used (ME011; MRC Holland, Amsterdam, the Netherlands) includes 21 probe sequences containing a *Hha*I recognition site, which yields information about the methylation status of the target sequences. In addition, eight control probe sequences are used which are not influenced by the methylation-sensitive restriction enzyme *Hha*I digestion. The probes containing a *Hha*I recognition site should only generate a signal if the DNA target is methylated and cannot be digested. The probe mix has been developed to detect CpG island methylation of six mismatch repair genes and includes three specific probes for semiquantitative hypermethylation detection of the *MGMT* promoter region. This MS-MLPA assay for *MGMT* promoter hypermethylation of gliomas was performed as described earlier by Jeuken et al. with minor modifications [36]. For fragment analysis, PCR products were separated

by capillary gel electrophoresis (ABI PRISM 3130xl, Applied Biosystems, Foster City, CA, USA) and quantified using the GeneMarker software version 1.7 (SoftGenetics, State College, PA, USA).

MS-MLPA data analysis

The MS-MLPA results were normalized by dividing the peak height of each *MGMT* probe signal by the mean peak height of the eight control fragments within the same sample. To estimate the fraction of methylated *MGMT* promoter DNA, normalized values of each *MGMT* probe of digested DNA samples were divided by normalized values of corresponding undigested DNAs. Methylation analyses were performed in duplicate and the average ratios of both experiments for each probe were calculated. For the promoter hypermethylation detection of *MGMT*, we used the $MGMT_2$ probe from the probe mix only. This probe is located near the widely used CpG island region for *MGMT* promoter hypermethylation detection by Methylation-Specific PCR (MS-PCR), and hypermethylation of this region has been found to be an independent predictor for the response to TMZ in gliomas [12, 20, 37, 38]. Beside that, the $MGMT_2$ probe had the best assay reproducibility in our hands (Spearman's rho 0.658, $P < 0.001$) in comparison with the two other *MGMT* probes ($MGMT_1$ probe Spearman's rho 0.448, $P = 0.032$ and $MGMT_3$ probe Spearman's rho 0.431, $P = 0.040$). When the *MGMT* digested/*MGMT* undigested ratio of probe $MGMT_2$ is below 0.30, the *MGMT* promoter fragment is considered non-methylated as described earlier by Worsham et al. and Chen et al. for their MS-MLPA assay [39, 40].

Statistical analysis methods

To investigate correlations between MGMT immunoexpression and promoter methylation status as well as potential associations between patient age, sex, tumor recurrence and invasiveness, statistical analyses applied test included Pearson correlation, Student's *t*-test, and Fisher's exact test in the SPSS statistical program.

Results

Clinical data

Carcinomas

Based on availability of sufficient tissue for methylation analysis, 10 of 30 carcinomas were included in the study. Pituitary carcinoma diagnosis was made upon histologic documentation of metastases involving a variety of sites,

including brain, tentorium, spinal cord, vertebrae, jugular lymph nodes, oral submucosa, buccal mucosa, liver, ovaries, sternocleidomastoid and triceps muscles, and the femur. Average time to diagnosis of metastatic sites was 6.7 years (median, 5; range, 1–16 years). The hormonal immunophenotype of the carcinomas included 5 PRL, 3 ACTH, 1 GH, and 1 TSH-producing tumor. Further clinicopathologic data regarding these cases may be found in Lau et al. [34].

Silent Subtype III

The 12 SS3 adenomas studied included tumors of eight male and four female patients (mean age, 35 years; median, 34; range, 17–74). Three of the 12 SS3 adenomas studied presented as "particularly aggressive" tumors based on the large size ($\geq$4 cm) and at least one of the following parameters: multiple pituitary surgeries, external beam radiation therapy, and persistence of tumor despite multimodality treatment. One patient had multiple endocrine neoplasia type 1 (MEN1), presenting with pituitary tumor, hyperparathyroidism, and multiple neuroendocrine pancreatic tumors. Symptoms upon presentation of the SS3 adenomas included mass effect as well as endocrine dysfunction (Table 2). Hypopituitarism, including adrenal insufficiency (3/12) and hypogonadism (5/12), was found in 6 of 12 patients (Table 2). As expected, no preoperative diabetes insipidus was noted. One incidentally discovered SS3 adenoma was included in the study. Further information regarding this series is summarized in a previously published study of our series of SS3 adenomas. [33].

Measuring greater than 2 cm, all 12 SS3 pituitary adenomas were macroadenomas. Intraoperatively and/or by neuroimaging (MRI/CT) invasion of dura, cavernous sinus and/or sphenoid sinus was evident in seven cases. A complete transsphenoidal resection was achieved in eight instances. Postoperatively, 1 patient had a CSF leak, which was managed successfully. Reversal of hypopituitarism was noted in 3 patients, but 4 patients developed postoperative hypopituitarism. At 5 years follow-up, 2 patients were found to have recurrent tumor, which necessitated repeat surgery after a latency of 4 years in both instances. Of the remaining 10 patients, 6 had persistent tumor, necessitating adjuvant radiation and/or medical therapy. Radiation therapy was administered in 6 patients, one of which was treated prophylactically to prevent tumor recurrence.

Molecular data

Carcinomas

In 2 of 10 cases, MGMT promoter methylation was investigated in two different specimens, i.e. from different

Table 2 Clinical characteristics of silent subtype 3 adenomas in the present study

Case	MGMT results	MGMT methylation status	Sex	Age	Invasion	Symptoms	Hypogonadism	Adrenal Insufficiency	Reversal of hypopituitarism	Tumor recurrence or persistence	Time to recurrence
1	Neg	UM	F	36	Yes	VF defect, hypogondaism, elevated PRL, headache, galactorhea					
2	Neg	M	M	64	Yes	VF defect, hypogondaism	Yes		Yes	Per	
3	Neg	M	M	18		Incidental				Per	
4	Neg	UM / UM	M	17		VF defects, hypogonadism, elevated PRL, decreased cortisol and TSH, headaches, decreased growth		Yes		Per	
5	Neg	UM / M	M	44	Yes	Headaches, CN palsy				No rec	
6[a]	Neg	UM / UM	F	41		Unknown	Yes	Yes	Yes	Rec	4 years
7	Neg	UM / UM	F	24	Yes	Galactorea				Rec	4 years
8	Neg	UM	M	30		VF defects				No rec	
9	Neg	UM	M	30		Coarsening of features, thyroidomegaly, large acra, hyperhydrosis	Yes	Yes	Yes	No rec	
10[a]	Neg	UM / M	M	30	Yes	VF defect, hypogonadism, headaches, seizure	Yes		No	Per	
11[a]	10%	M / UM	M	31	Yes	Coarsening of features, VF defect, headaches, large acra and hyperhydrosis	Yes		No	Per	
12	20%	UM / UM	F	48	Yes	VF defects					

MEN1, *re* recurrent, *Per* persistence, *M* male, *F* female, *UM* unmethylated, *M* methylated

[a] Particularly aggressive case

Table 3 MGMT promoter methylation association with immuno-histochemical (IHC) staining

Promoter methylation status	Low IHC	High IHC
M	1	2
UM	4	2

M methylated, *UM* unmethhylated

metastatic sites where available. Fully 9 of the 10 specimens exhibited homogenous tumoral methylation (Table 2). Three of the nine (33%) pituitary carcinomas demonstrated MGMT promoter methylation; six tumors were unmethylated and one yielded no results. In two cases, methylation status was identical in primary tumors and their respective metastases both being unmethylated.

MGMT immunoexpression was low (<10%) in five specimens and high, defined as >10% in five carcinomas (range 0–90%). Similar to the observed promoter methylation homogeneity, MGMT immunostaining strengths (low vs. high) were consistent between different sections of the same specimen studied. In only one instance, liver metastases exhibited variable MGMT immunoreactivity (5% in one liver metastasis and 35% in two others) (Table 1). In two cases wherein ample tissue was available, MGMT immunostaining was consistent in the primary pituitary carcinoma and/or their distant metastases (Table 1). Promoter methylation was present in two cases with strong MGMT immunostaining. In addition, four of the unmethylated carcinomas demonstrated weak MGMT immunoreactivity. Thus, the Chi-squared test revealed no statistically significant correlation between methylation status and MGMT immunostaining (Table 3).

SS3 pituitary adenomas

Overall, MGMT promoter methylation was observed in 2 of 12 (16.7%) of SS3 adenomas, 6 adenomas exhibiting an unmethylated promoter, and 3 showing methylation heterogeneity in samples of the same tumor (Table 2). Inclusive of the three adenomas exhibiting heterogeneous methylation, fully 42% of SS3 pituitary adenomas showed promoter methylation. In 7 of the 12 tumors studied, two different paraffin embedded samples were available for promoter methylation analysis. Of these, three exhibited heterogeneous methylation status (Table 2). One of the three clinically more aggressive SS3 pituitary adenomas demonstrated lack of promoter methylation, whereas the other two showed heterogeneous methylation status.

MGMT immunohistochemical staining was negative/low in 11/12 SS3 adenomas, 10 being fully negative. In one tumor, the MGMT immunostaining index was 20%, a value considered high. All three of the clinically aggressive SS3 adenomas displayed absent/low MGMT immunoreactivity. Immunohistochemical staining being low in five of the

methylated and six of the unmethylated tumors, there was no association between the two parameters.

Correlations

By Fisher's exact test, no correlation was found between methylation status and gender, but there was a preponderance of females among methylated carcinomas (3 of 4) and among unmethylated SS3 adenomas (4 of 5). Immunoexpression of MGMT did not differ significantly between males and females, although of the four tumors with higher MGMT immunopositivity, three were in female patients. No correlation was found between patient age and methylation status in either tumor type.

With respect to endocrine activity of the tumors, there was no preponderance of MGMT promoter methylation in PRL versus ACTH carcinomas. Similarly, SS3 pituitary adenomas showed variable promoter methylation and MGMT staining across their various hormone immunoprofiles.

In the one case, both the primary pituitary carcinoma and its brain metastasis showed identical MGMT promoter methylation status (unmethylated).

Discussion

The present study resulted in several key findings. These included (a) MGMT promoter methylation occurs in a significant subset of pituitary carcinomas and SS3 adenomas, (b) among carcinomas, MGMT promoter methylation status in primary lesions and their respective metastases, carcinomas demonstrate an identical methylation status, (c) MGMT promoter methylation status is homogenous in paraffin-embedded tissue of carcinomas, but not in SS3 pituitary adenomas, (d) methylation status is not concordant with MGMT immunoexpression, (e) MGMT immunoreactivity is primarily negative in most SS3 adenomas.

Epigenetic silencing of MGMT via methylation of its promoter is associated with higher tumor grade and with prognosis in several human neoplasms [3, 22–29]. Accordingly, we report MGMT promoter methylation in 33% of pituitary carcinomas and in 42% of SS3 adenomas. Our results indicate higher rates of MGMT immunonegativity than those reported by Bello et al. who found 22% of 23 non-functioning adenomas and 25% of 12 functioning adenomas to exhibit MGMT promoter methylation. Whether the methylated tumors were particularly aggressive or not was not stated, but the differences observed by us may be due to the aggressive nature of the subset of adenomas included in our study. In keeping with our findings, Raverot et al. recently reported MGMT methylation in 2 of 7 (43%) of aggressive pituitary adenoma/carcinomas examined [14]. On the other hand, McCormack

et al. found MGMT promoter methylation in only 4 of 46 (9%) of nonfunctioning pituitary adenomas studied; no data regarding aggressive tumoral behavior and its relation to MGMT methylation was available [41].

Our results are also in keeping with most studies showing MGMT epigenetic silencing in higher grade and more aggressive tumors arising outside the pituitary gland [22, 23]. Piperi et al. reported MGMT promoter methylation in 59% of the 17 GBMs studied, and that a correlation existed between methylation status and patient survival, thus suggesting methylation is a prognostic indicator [22]. Similarly, Kim et al. observed MGMT promoter hypermethylation in 34% of the 63 soft tissue sarcomas, there being a strong correlation with tumor stage, grade, and aggressive behavior [23]. We have shown considerable MGMT promoter methylation in at least 50% of SS3 adenomas, tumors behaving more aggressively than other pituitary adenoma subtypes. This is entirely in keeping with the role of MGMT in DNA damage repair, tumor development and progression pathways [42]. Lastly, the recent finding of MGMT promoter methylation in 33% of a series of carcinomas investigated is in accordance with prior studies of human carcinomas [43].

Our results are promising as MGMT promoter methylation status has been shown to be a positive prognostic indicator in TMZ therapy of glioblastomas [12]. It is of note, however, that a subset of these tumors not methylated do respond to TMZ therapy, an observation challenging the notion that MGMT promoter methylation as an indication for TMZ treatment. Indeed, two recent studies of MGMT promoter methylation and TMZ response in eight aggressive adenomas and pituitary carcinomas found no association between promoter methylation status and TMZ response [13, 14]. Thus, our findings must be interpreted with caution. The validity of MGMT promoter methylation in the selection of patients for TMZ treatment remains controversial and an active area of research.

Frequently proposed explanations for this observed disparity are two-fold. Firstly, heterogeneous MGMT methylation within tumors confounds sampling of tissue for methylation determination. Secondly, technical limitations of promoter methylation analysis in formalin-fixed, paraffin-embedded tissue since DNA changes may be induced by tissue processing [32]. The recent study of Hamilton et al. compared MGMT promoter methylation status in frozen and paraffin-embedded tumor tissue using three different tumor samples obtained by stereotactic biopsy. The authors found MGMT methylation status to be heterogeneous in one paraffin-embedded tissue using MS-PCR, whereas frozen tumor tissue yielded homogenous methylation status regardless of biopsy site [32]. Furthermore, 4 of the 12 GBMs studied showed inconsistent promoter methylation status when comparing frozen and paraffin-embedded tumor samples. These findings are in keeping with the report of [44], demonstrating inconsistent MGMT promoter methylation status in 2 of 14 tumors examined when both frozen and formalin-fixed, paraffin-embedded tissue from different tumor sites were assessed. Our report of heterogeneous intra-tumoral MGMT methylation status in 3 of the 12 formalin-fixed, paraffin-embedded SS3 adenomas is in keeping with these various reports, and underscores the need to develop a uniform, reliable MGMT methylation detection technique. The answer may lie in utilization of frozen tissue. Indeed, it is reassuring that frozen samples yield consistent results in many studies of GBMs. Corroborating the findings of Hamilton et al., Cao et al. observed consistent methylation results in frozen biopsy samples from 37 sites in 12 GBMs [45]. Grasbon-Frodl et al. also found homogenous inter-sample methylation status using frozen tissue from 25 GBMs [46]; intra-sample methylation was heterogeneous in only one instance. As well, Parkinson et al. showed predominantly intra-tumoral homogeneity in 7 GBMs examined, with 2 MGMT methylation sites exhibiting variable methylation status in frozen samples [47]. Thus, it appears that frozen tumor tissue yields more homogenous MGMT promoter methylation results than formalin-fixed, paraffin-embedded tumor samples. To our knowledge, no such investigation of pituitary tumors has been undertaken.

Our finding of heterogeneous MGMT methylation in a number of pituitary tumors studied highlights the need for multi-sampling of tumor tissue. The present study found that in 3 of the 16 pituitary carcinoma/adenomas (19%) wherein two tumor samples were assessed, promoter methylation was heterogeneous; all three were SS3 adenomas. Whereas MGMT promoter methylation heterogeneity in GBM has been reported by several investigators, only one such study focused upon pituitary tumors, namely nonfunctioning adenomas [41]. Our findings are in keeping with those of McCormack et al. who found four methylated non-functioning pituitary adenomas all formalin-fixed and paraffin-embedded to be heterogeneously so [41]. Furthermore, we draw attention to technical limitations of the MSP method that may underlie false negative results. For example, Kitange et al. reported the presence of nearly 100 potential MGMT methylation CpG dinucleotide sites, of which the MSP-PCR method detects only those near the transcription initiation site [48]. This finding underscores the need for more sensitive methylation detection techniques. A cell culture investigation of pituitary adenoma cell lines response to treatment with TMZ showed differential therapeutic effect in the three cell lines examined [49]. It will be important to determine MGMT methylation status and immunohistochemical expression in such cell lines to establish whether a relationship exists between TMZ efficacy and MGMT methylation and/or protein

status. It is possible that the mechanism of TMZ response in pituitary tumors is different from that seen in GBMs, and more detailed cellular and clinical studies are needed to elucidate on the pathways via which TMZ elicits its therapeutic effects in pituitary tumors.

In addition to its potential value as a therapeutic indicator and prognostic marker, MGMT methylation status is implicated in tumorigenesis. To investigate the role of MGMT methylation in tumor metastasis, we examined methylation status in primary pituitary carcinomas and their respective metastasis to several tissues, finding for the first time that MGMT methylation status is consistent in matched primary and disseminated metastases. Our findings are concordant with those of Wu et al., who recently observed consistent MGMT methylation status between primary and metastatic lung carcinomas [43]. Although primary and metastatic tumors are shown to be heterogeneous in their molecular profiles [43, 50, 51] we found that MGMT methylation (2 of 2 cases) and immunoexpression (4 of 5 cases) were concordant in the investigated primary carcinomas and their metastases. To our knowledge, only one other study has examined primary and metastatic pituitary carcinoma in terms of molecular profiles [52]. Zahedi et al. found that an ACTH primary carcinoma and its metastases differed in their clonal composition. The metastases demonstrated a loss-to-retention pattern at two distinct loci (IFNA and D22S156) compared to the primary pituitary carcinoma [52]. Clearly, further molecular characterization of primary and metastatic pituitary carcinomas will shed light on the mechanisms involved in pituitary tumorigenesis, progression, and metastasis.

Immunohistochemical detection of MGMT has been explored as a rapid means of predicting response to treatment with TMZ in patients with GBMs as well as small numbers of pituitary adenomas and carcinomas. We report absent or low (<10%) MGMT immunoexpression in 5 of 10 pituitary carcinomas and in 8 of 12 SS3 adenomas, figures considerably higher than those reported in two other series of pituitary adenomas. Previously, our group found MGMT immunonegativity in 78% of the 23 SS3 adenomas studied [35]. We believe our frequent finding of particularly low-level MGMT expression compared to others is a reflection of the more aggressive nature of SS3 adenomas comprising the bulk of tumors in our study [3, 41, 53]. Indeed, several reports have found particularly low levels of MGMT immunostaining in more aggressive tumors [43].

Widhalm et al. [53] found absent or low MGMT immunoreactivity to be present in 50% of pituitary tumors re-growing following surgical removal whereas only 24% of tumors not undergoing postoperative growth had low MGMT immunoexpression. Similarly, Takeshita et al. found absent/low MGMT immunopositivity in all 7 Crooke's cell adenomas as studied compared to 25% of

conventional ACTH-secreting pituitary adenomas. Crooke cell pituitary adenomas are a particularly aggressive subset of corticotrophic tumors presenting with Cushing disease [3]. It is of note that lower MGMT immunoexpression was correlated with shorter time to re-operation among re-growing tumors. On the other hand, such an association was not found by McCormack et al. in their investigation of MGMT immunoexpression in 88 pituitary adenomas; the authors found 13% of all tumors to show low MGMT immunoexpression but no association with tumor recurrence or invasiveness. It was noted, however, that PRL adenomas showed significantly lower MGMT immunoreactivity than other adenoma subtypes, a finding in keeping with their more aggressive behavior [43].

In summary, our finding of more frequent low-level MGMT immunoreactivity in aggressive and metastatic pituitary tumors is in accordance with most reports, and suggests fundamental molecular differences between indolent and aggressive tumors. Whether MGMT immunoexpression levels are causal or secondary to tumor progression remains unclear and requires further investigation. Regardless, low MGMT immunoreactivity is suggestive of a successful response to TMZ treatment. Although case reports and small series of TMZ-treated pituitary tumors have shown an association between TMZ response and low-level MGMT immunoreactivity, this is not always the case [1–10]. At the time of the present study, we retrieved 12 reported cases and a series of 8 patients with aggressive pituitary neoplasms where MGMT staining and TMZ treatment results were reported. Of these cases, 5 had significant MGMT immunoreactivity, but a positive response to TMZ treatment as determined by tumor shrinkage and clinical improvement [5]. In one instance, an oncocytic gonadotroph adenoma showing 30–50% MGMT immunoreactivity underwent shrinkage with clinical improvement after treatment [4]. On the other hand, a GH-secreting pituitary tumor showing strong MGMT was associated with a lack of response to TMZ therapy [41].

Preusser et al. [21] reported significant inter-observer variability as a factor affecting validity of MGMT immunoexpression estimates as a prognostic factor in their study of 146 GBM patients treated with TMZ. In addition, they reported a lack of correlation between MGMT methylation status and immunoreactivity [21]. Our study also found that methylation status is not closely associated with MGMT immunoexpression; 2 of the 3 methylated carcinomas showed strong MGMT immunopositivity and 6 unmethylated SS3 adenomas showed absent or low-level MGMT immunopositivity. The discordance between MGMT methylation status and protein immunoexpression may be due either to technical factors outlined or to as yet unknown regulatory molecular mechanisms. For example, NF-kappa-B was recently found to regulate MGMT expression

independent of MGMT promoter methylation status [54]. In addition, p53 regulates MGMT protein expression in human astrocytic glioma cells regardless of its methylation status [55]. A clear understanding of MGMT regulatory mechanisms is essential in order to optimize the use of temozolomide as a therapeutic agent.

That MGMT methylation is not closely correlated with its protein expression as detected by immunohistochemistry is not surprising given the outlined issues regarding technical considerations, including tissue sampling and fixation, as well as the sensitivity of MSP in the detection of MGMT methylation. There may be yet unknown MGMT methylation and/or expression regulatory mechanisms that result in its variable expression profiles. The issue of the utility of MGMT immunohistochemical expression as a predictive factor for treatment with TMZ remains an active area of research.

In summary, the present study is the first to show concordant methylation status in the one case of the primary and metastatic pituitary carcinoma examined. Whether MGMT plays a direct role in tumor metastasis remains to be elucidated.

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