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RESEARCH ARTICLE

Anti-O6-Methylguanine-Methyltransferase (MGMT) Immunohistochemistry in Glioblastoma Multiforme: Observer Variability and Lack of Association with Patient Survival Impede Its Use as Clinical Biomarker*

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Abstract

Silencing of O6-methylguanine-DNA methyltransferase (MGMT) protein expression because of *MGMT* gene promoter hypermethylation is considered to be associated with postoperative chemoradiotherapy benefits in glioblastoma multiforme (GBM) patients. The objective of this study was to clarify the usability of MGMT immunohistochemistry (IHC) as a clinical biomarker.

We immunostained a tissue microarray containing biopsy samples of 164 GBM patients from the European Organization for Research and Treatment of Cancer and the National Cancer Institute of Canada (EORTC/NCIC) trial 26981/22981 using two commercial anti-MGMT antibodies (clones MT3.1 and MT23.2). Immunostaining results were semiquantitatively evaluated by four observers from three neuropathological laboratories using a predefined algorithm. We analyzed (i) inter- and intraobserver agreement on MGMT expression (kappa statistics); (ii) correlation of MGMT expression with *MGMT* promoter methylation status (kappa statistics); and (iii) correlation of MGMT expression with patient outcome (log-rank test). Interobserver agreement on MGMT expression varied from slight to almost perfect, whereas intraobserver agreement ranged from substantial to almost perfect. MGMT expression showed poor to moderate correlation with *MGMT* promoter methylation status. We found no significant association of MGMT expression with patient outcome. In our hands, observer variability as well as lack of association with the *MGMT* promoter methylation status and patient survival impeded the use of anti-MGMT immunohistochemistry as a clinical biomarker for routine diagnostic purposes.

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common malignant type of primary brain tumor (33). A recent prospective multicenter study conducted through the collaboration of the European Organization for Research and Treatment of Cancer (EORTC) and the National Cancer Institute of Canada (NCIC) (EORTC/NCIC trial 26981/22981) showed that the addition of temozolomide to radiotherapy for newly diagnosed GBM results in a clinically meaningful and statistically significant survival benefit with minimal toxicity (52). Consequently, postoperative combined radio–chemotherapy followed by adjuvant chemotherapy with temozolomide is currently considered as standard adjuvant therapy for GBM patients. In a translational study conducted in parallel to the EORTC/NCIC trial 26981/22981, a strong correlation of the methylation status of the O6-methylguanine-methyltransferase

(*MGMT*) gene promoter with temozolomide treatment effect and outcome was shown (26). *MGMT* promoter methylation results in transcriptional silencing, and therefore, inhibition of expression of MGMT, a DNA repair protein that removes methyl groups from the O6-position of guanine, thus counteracting the effect of alkylating chemotherapy (17, 49, 55).

In the study by Hegi *et al*, *MGMT* promoter methylation status was assessed by methylation-specific polymerase chain reaction (MSP) (26). However, MSP is a relatively complex and time-consuming method not often available in the local treatment centers. In addition, the formalin fixation and paraffin embedding of tumor tissues deteriorates the DNA quality in the tissue, which may lead to failure of amplification by MSP, particularly in small samples (eg, stereotactic biopsies). MGMT protein can be visualized immunohistochemically, and commercial anti-MGMT antibodies are available. There are several potential advantages of immunohistochemistry (IHC) as compared with MSP. IHC is a commonly used and reliable method in diagnostic histopathology and is available in most laboratories. Furthermore, IHC works on formalin-fixed and paraffin-embedded tissue and is less expensive than MSP. Several studies have reported significant associations of immunohistochemically assessed MGMT expression with patient outcome in glioma (2, 7, 12, 44, 48). A study by Friedman *et al* in 1998 indicated that pretherapy analysis of MGMT protein expression in malignant gliomas may help to identify patients in whom tumors are resistant to temozolomide (19). Some more recent studies on small patient series reported similar findings. Anda *et al* reported in a study on 18 patients that glioblastomas with strong immunohistochemical MGMT staining may show more resistance to alkylating chemotherapy (2). Chinot *et al* found in a study on 29 glioblastoma patients that MGMT expression correlated with response to temozolomide (12). Brell *et al* reported a correlation between MGMT protein expression and survival in patients with anaplastic gliomas who had received alkylating chemotherapy (7). Similar results were reported for pediatric patients with malignant gliomas (48). Therefore, there is broad interest in the clinical use of MGMT immunostaining in this tumor type. However, the clinical usability of MGMT IHC has never been systematically studied so far. Essential prerequisites for use of anti-MGMT staining in the diagnostic setting are high observer agreement (analytical performance) and reproducible association with treatment response and patient outcome (clinical performance) (24).

The objective of the present study was to test whether MGMT IHC in GBM can be used as a clinical biomarker in the routine setting. To this end, we systematically assessed for the first time whether the analytical and clinical performances of MGMT immunostaining are adequate for routine diagnostic purposes.

MATERIALS AND METHODS

Patients

A total of 164 biopsies of glioblastoma patients were available for this study. All the cases belonged to a previously published glioblastoma cohort of 573 cases that have been prospectively recruited in a multicenter approach by collaboration of the EORTC and the NCIC (EORTC/NCIC trial 26981/22981) (26, 52). For our study, we extracted the data on *MGMT* promoter methylation status and patient outcome from the database of EORTC/NCIC trial 26981/22981.

In our total study cohort of 164 cases, the median age was 54 years (age range 25–70 years). Eighty-one cases (49.4%) had been randomized to the "radiotherapy only" arm and 83 (50.6%) cases to the "radiotherapy plus temozolomide" arm. Median follow-up time was 28 months (range 0–39 months). In 122 out of 164 (74.4%) cases, methylation status of the *MGMT* promoter as assessed by MSP was available from the database of the EORTC/NCIC trial 26981/22981. Of these 122 cases, 59 (48.4%) had a methylated *MGMT* promoter and 63 (51.6%) had an unmethylated *MGMT* promoter.

All patients provided written informed consent for molecular studies of their tumor, and the protocol was approved by the ethics committee at each study center.

Tissue microarray (TMA)

TMA (Figure 1) was constructed from paraffin blocks of glioblastoma specimens from 164 patients for which paraffin blocks comprising compact tumor tissue of adequate surface and 5 mm depth were available. The tissue array was constructed by retrieving tissue core biopsies of 0.6 mm diameter from selected tumor regions of the donor paraffin blocks and precisely arraying them on a new recipient block using an arrayer instrument (34). We included one tissue core per patient on the TMA. The representative area of the tumor on the block was chosen based on the respective hematoxylin- and eosin-stained tissue section. All TMA cores were selected from tumor areas that were histologically representative for the entire tumor. These same blocks had been used for central pathology review and for assessment of the *MGMT* promoter methylation status by MSP (26, 52).

Immunohistochemistry

TMA sections were immunostained using two different commercially available anti-MGMT antibodies, namely clones MT3.1 (Dako, Copenhagen, Denmark) and MT23.2 (Zymed laboratories, Carlsbad, CA, USA). The TMA sections were deparaffinized with xylene for 30 minutes and rehydrated in decreasing concentrations of ethanol. The primary antibodies were used at final dilutions of 0.2 µg/mL (MT23.2) and 1.4 µg/mL (MT3.1), respectively, for an incubation period of 15 minutes at room temperature. Antibody binding was demonstrated with the DAKO-catalyzed signal amplification horseradish peroxidase system® (Glostrup, Denmark), which was used according to the manufacturer's protocol. Immunoreactivity was visualized with 3′3′-diaminobenzidine as the chromogen. All sections were counterstained with hematoxylin. Negative controls were carried out by omission of the respective primary antibody. In addition, tumor sections from cases with or without previously demonstrated MGMT expression were stained in parallel as positive and negative controls for the staining reaction.

Analysis

Evaluation of anti-MGMT immunohistochemistry was performed by four observers from three neuropathology laboratories (JAH, JF, MP and RCJ). As a first step, an evaluation algorithm was

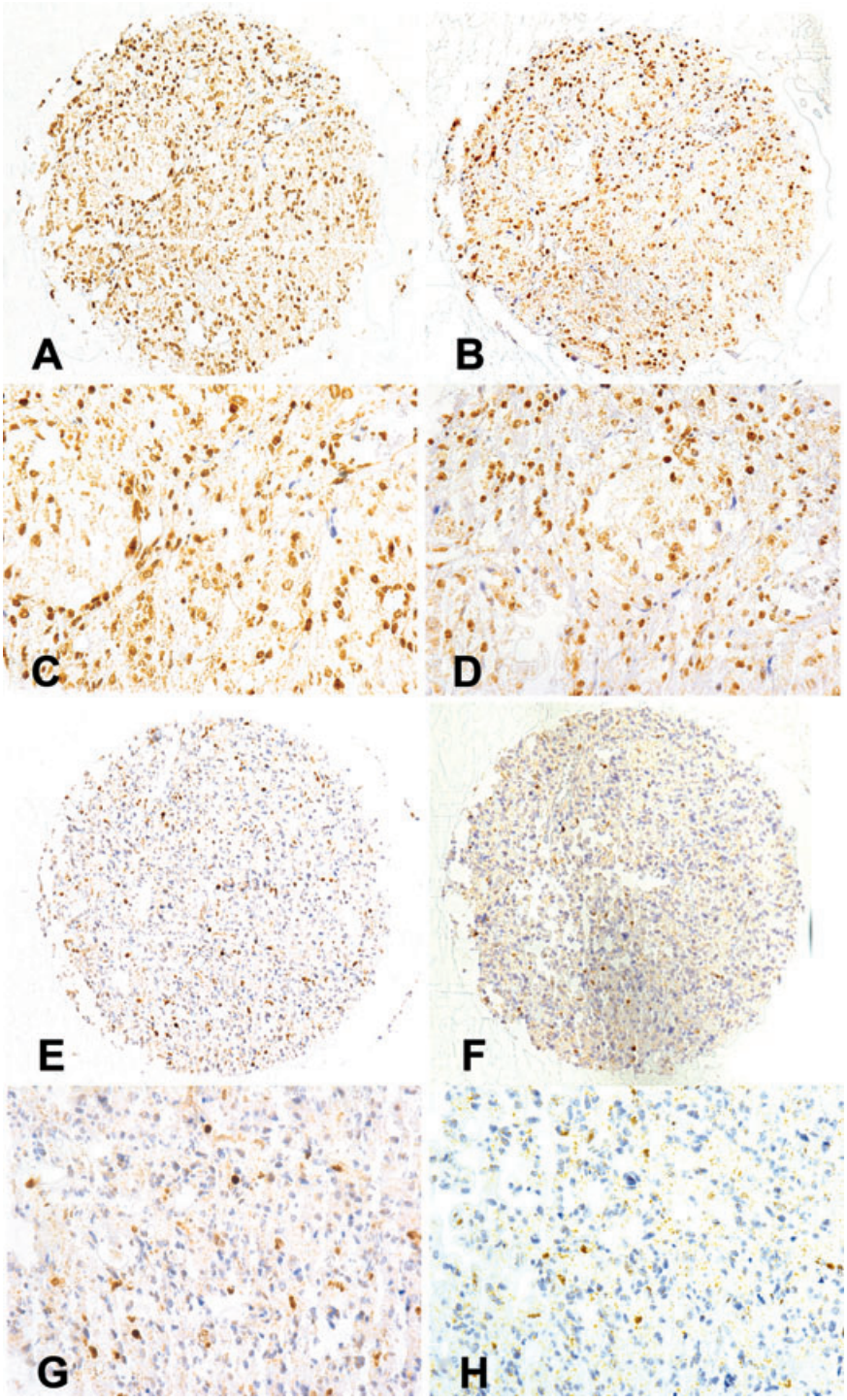


Figure 1. *Immunohistochemical visualization of O6-methylguanine-methyltransferase (MGMT) protein expression on a tissue microarray (TMA) using two different primary antibodies (clones MT 3.1 and MT23.2).* **A–D.** Sections of the same TMA tissue core stained with the two anti-MGMT antibodies [antibody MT3.1 (**A,C**) and antibody MT23.2 (**B,D**); magnification: ×10 objective lens (**A,B**) and ×40 objective lens (**C,D**)]. Both antibodies (clones MT3.1 and MT23.2) show immunolabeling of the majority of tumor cell nuclei. **E–H.** Sections of another TMA tissue core stained with antibodies MT3.1 and MT23.2 [antibody MT3.1 (**E,G**) and antibody MT23.2 (**F,H**); magnification: ×10 objective lens (**E,F**) and ×40 objective lens (**G,H**)]. Both antibodies (clones MT3.1 and MT23.2) show immunolabeling of a minority of tumor cell nuclei.

circulated to all observers. After critical review and agreement on the algorithm by all observers (Table 1), assessment of both anti-MGMT immunostained TMA sections (MT3.1 and MT23.2 antibodies) was performed. All observers reviewed the same TMA stained slides. Each observer evaluated both sections independently and was blinded to clinical data and *MGMT* promoter methylation status. For analysis of intraobserver agreement, each observer independently re-evaluated both sections at least 3 weeks (range 3 to 6 weeks) after the first assessment. At reassessment, all observers were blinded to the results from the original first evaluation as well as to all clinical data and *MGMT* promoter methylation status as assessed by MSP.

Table 1. Sequential questionnaire for evaluation of immunohistochemically visualized MGMT expression. For each biopsy sample (Figure 1) on both tissue microarray sections (one immunostained with MT3.1 antibody, one immunostained with MT23.2 antibody), each observer had to sequentially answer four questions as outlined in Table 1. For a given case, the "no" answer to question 1 terminated the evaluation. Semiquantitative assessment of MGMT expression in tumor cells was performed in two steps (questions 2a and 2b in Table 1). Abbreviation: MGMT = O6-methylguanine-methyltransferase.

Question number	Question	Possible answers
1	Is assessable tumor tissue present?	No [no tumor tissue present/necrosis/too small (<30% of section)]
		Yes
2a	Is the tumor tissue MGMT positive?	No
		Yes
2b	How many tumor cells are positive?	Few (<10% of tumor cell nuclei)
		Some (10%–50% of tumor cell nuclei)
		Many (>50% of tumor cell nuclei)
3	Are there endothelial cells showing MGMT immunoreactivity?	No
		Yes (only unequivocal endothelial cells)
4	Are there hematogenous cells showing MGMT immunoreactivity?	No
		Yes (only unequivocal hematogenous cells; eg, lymphocytes)

Statistics

The statistical software packages SAS® (SAS Institute Inc., Cary, NC, USA) and SPSS® (SPSS Inc., Chicago, IL, USA) were used for statistical calculations. A two-tailed significance level of 5% was assumed.

Observer agreement

For each question of the evaluation algorithm (Table 1), Cohen's kappa and Cohen's weighted kappa were used to measure inter- and intraobserver agreement of MGMT IHC assessment. Kappa values were interpreted as follows: <0.2, poor observer agreement; 0.2–0.4, slight observer agreement; 0.4–0.6, moderate observer agreement; 0.6–0.8, substantial observer agreement; 0.8–1, almost perfect observer agreement (27). A two-way ANOVA model was employed to compare differences between staining methods.

Correlation of MGMT IHC results with MSP results

Cohen's kappa was used to measure agreement between *MGMT* promoter methylation status (methylated or unmethylated) and immunohistochemically evaluated MGMT expression in tumor cells (semiquantitative MGMT values as assessed by question 2). For this purpose, we categorized the immunohistochemical MGMT values in three different ways: MGMT negative ("no" MGMT immunoreactive tumor cells) vs. MGMT positive ("few," "some" or "many" MGMT immunoreactive tumor cells); low ("no" or "few" MGMT immunoreactive tumor cells) vs. high ("some" or "many" MGMT immunoreactive tumor cells) MGMT expression; and finally, MGMT expression in <50% of tumor cells ("no," "few" or "some" immunoreactive tumor cells) vs. MGMT expression in ≥50% of tumor cells ("many" immunoreactive tumor cells).

Survival analysis

Overall survival was defined from the day of randomization until death of the patient. Patients not reported dead or lost to follow-up were censored on the date of last visit. Survival probabilities were computed according to the Kaplan–Meier method. Log-rank test was used to assess the prognostic effect of MGMT expression and *MGMT* promoter methylation status on overall survival. No correction for multiple testing was done as the statistical tests were performed for demonstration purposes only.

In order to explore the potential associations of the immunohistochemically evaluated MGMT expression in tumor cells (semiquantitative MGMT values as assessed by question 2) with patient survival, a four-group comparison of MGMT expressions "no" vs. "few" vs. "some" vs. "many" MGMT immunoreactive tumor cells was tested (log-rank test). In addition, in order to account for the semiquantitative nature of MGMT expressions, a linear trend across the four ordered MGMT categories was tested (log-rank test).

RESULTS

Immunohistochemistry

Both anti-MGMT antibodies (MT3.1 and MT23.2 antibodies) used in this study showed nuclear immunlabeling of variable extent and intensity (Figure 1). In addition to tumor cells, endothelial cells of tumor vasculature and tumor infiltrating lymphocytes (hematogenous cells) also showed labeling in a fraction of cases.

Interobserver agreement on MGMT IHC

Evaluation of both immunostained TMA sections (MT3.1 and MT23.2 antibodies) was performed at two different timepoints. For each antibody, timepoint and all six observer pairs (four observers = six pairs), kappa values were calculated as a measure of interobserver agreement. For the assessment of the sections, all observers used the same algorithm consisting of four predefined questions (Table 1). Ranges of kappa values for interobserver agreement are illustrated in Figure 2.

- Question 1: Is assessable tumor tissue present?

Kappa values calculated for both antibodies and for both timepoints showed variable interobserver agreement ranging from slight to almost perfect agreement (see Figure 2A).

- Question 2: A. Is the tumor tissue MGMT positive (yes/no)? B. If positive, how many tumor cells are immunolabeled [few (<10%)/some (11%-50%)/many (>50%)]?

As the assessment of the amount of positive tumor cells is a semiquantitative evaluation, weighted kappa calculation was used. Weighted kappa values calculated for both timepoints showed similar results (Figure 2B): The interobserver agreement was sig-

Figure 2. *Illustration of kappa values for interobserver agreement on immunohistochemically visualized O6-methylguanine-methyltransferase protein expression in tissue microarray.* (For a detailed description, refer to Results section.) Note that for question 2 (**B**), there are two kappa value ties at timepoint 2 with MT3.1 antibody. Therefore, only four instead of the expected six symbols (four observers = six observer pairs) are shown. For question 4 (**D**), less than six kappa values are shown for each antibody and timepoint because some observers showed no variation in an assessment run (ie, always voted solely "yes" or "no"). In such cases, no kappa values were computed.

nificantly higher on MT23.2 stained sections as compared with MT3.1 stained sections ($P = 0.021$). However, kappa values for both antibodies and both timepoints showed broad ranges (MT3.1: poor to substantial agreement; MT23.2: moderate to almost perfect agreement). Therefore, the degree of interobserver agreement seems to be associated with the type of anti-MGMT antibody.

- Question 3: Are there endothelial cells showing MGMT immunoreactivity (yes/no)?

Kappa calculation for both timepoints and both antibodies showed a similar, rather broad spectrum of interobserver agreement, ranging from poor to moderate (Figure 2C).

- Question 4: Are there hematogenous cells showing MGMT immunoreactivity (yes/no)?

Kappa calculation for both timepoints and both antibodies showed only poor or slight interobserver agreement (Figure 2D).

Intraobserver agreement on MGMT IHC

To test intraobserver agreement, evaluation of both immunostained TMA sections (MT3.1 and MT23.2 antibodies) was performed twice by each observer at two different timepoints (time interval of 3 to 6 weeks). For each observer, kappa values were calculated between results of the first and second assessments. Ranges of kappa values for intraobserver agreement are illustrated in Figure 3.

- Question 1: Is assessable tumor tissue present?

Kappa calculation for both antibodies showed substantial or almost perfect intraobserver agreement (Figure 3). One observer reached perfect intraobserver agreement.

- Question 2: A. Is the tumor tissue MGMT positive (yes/no)? B. If positive, how many tumor cells are immunolabeled [few (<10%)/some (11%-50%)/many (>50%)]?

Kappa calculation for both antibodies showed substantial or almost perfect intraobserver agreement (Figure 3).

- Question 3: Are there endothelial cells showing MGMT immunoreactivity (yes/no)?

Kappa calculation showed slight (MT3.1) or moderate (MT23.2) to almost perfect intraobserver agreement (Figure 3).

- Question 4: Are there hematogenous cells showing MGMT immunoreactivity (yes/no)?

Kappa calculation showed poor to substantial (MT3.1) and slight to almost perfect (MT23.2) intraobserver agreement (Figure 3).

Correlation of MGMT MSP results with MGMT IHC results

For a detailed evaluation of the agreement between *MGMT* MSP (methylated or unmethylated) and MGMT IHC, we used the data of semiquantitative MGMT expression in tumor cells (MGMT values as assessed by question 2). We evaluated agreement using kappa analysis. For this purpose, we categorized the immunohistochemical MGMT values in three different ways (see also Materials and Methods):

(i) MGMT negative vs. MGMT positive (Figure 4A): We found poor agreement between MSP and MGMT IHC for both antibodies (MT3.1 and MT23.2) and both timepoints of immunohistochemical MGMT evaluation.

(ii) Low vs. high MGMT expression (Figure 4B): We found poor to slight (MT3.1) or poor to moderate (MT23.2) agreement between MSP and MGMT IHC for both timepoints of immunohistochemical MGMT evaluation.

(iii) MGMT expression in <50% of tumor cells vs. MGMT expression in ≥50% of tumor cells (Figure 4C): For immunohistochemical MGMT evaluation utilizing the MT3.1 antibody, we found poor to slight agreement with MSP at timepoint 1 and poor agreement at timepoint 2. For the MT23.2 antibody, we found slight to moderate agreement with MSP results at both timepoints of immunohistochemical MGMT evaluation.

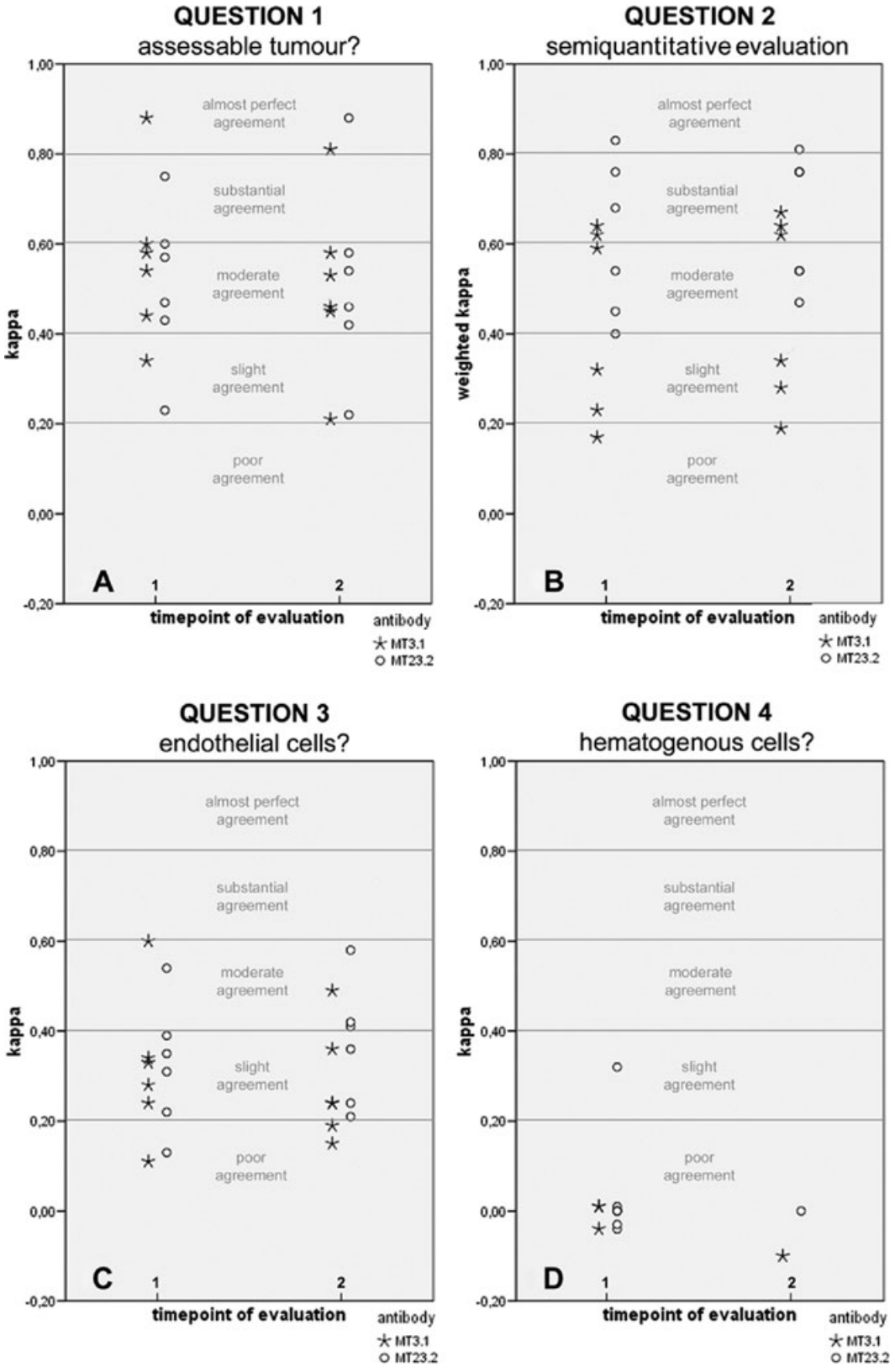
Survival analysis

We explored the potential impact of immunohistochemical MGMT expression in tumor cells (question 2; see also Materials and Methods) on patient survival on the basis of 16 assessments (four observers, two stainings, two timepoints; see also Table 2). None of the 16 assessments had a significant association of MGMT expression with patient outcome (Table 2). In contrast, presence of *MGMT* promoter methylation showed significant correlation with favorable patient survival ($P = 0.0001$, Figure 5), as reported previously in the larger original patient series (26).

DISCUSSION

An association of *MGMT* promoter methylation status and clinical outcome of glioblastoma patients has been shown in independent investigations (14, 18, 25, 26, 28, 29, 35, 39, 47). Based on these findings, *MGMT* promoter methylation is considered a promising molecular factor predictive for chemotherapy response and longer survival in glioblastoma.

Some groups have also reported a prognostic significance of immunohistochemically assessed MGMT expression in glioblastoma (Table 3) (2, 11, 12, 44). Therefore, the use of anti-MGMT IHC for diagnostic purposes is debated (2–4, 7, 11–13, 19, 21, 28, 30, 31, 36, 40, 41, 44, 48, 50). As a prerequisite for the routine diagnostic use of MGMT IHC in daily patient management, testing of its clinical usability is required (11, 40, 44, 51). In our study, we tested the clinical usability of MGMT IHC by analysis of observer agreement, its correlation with *MGMT* promoter methylation and its association with patient outcome. We used glioblastoma specimens from the patient cohort of the prospective



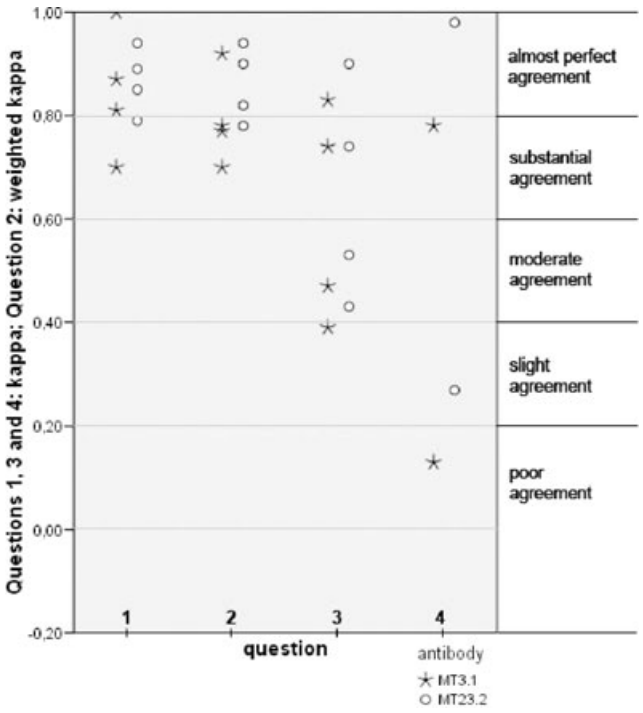


Figure 3. *Illustration of kappa values for intraobserver agreement on immunohistochemically visualized O6-methylguanine-methyltransferase protein expression in tissue microarray.* (For a detailed description, refer to Results section.) For question 4, less than four kappa values are shown for each antibody because some observers showed no variation in an assessment run (ie, always voted solely "yes" or "no"). In such cases, no kappa value was computed.

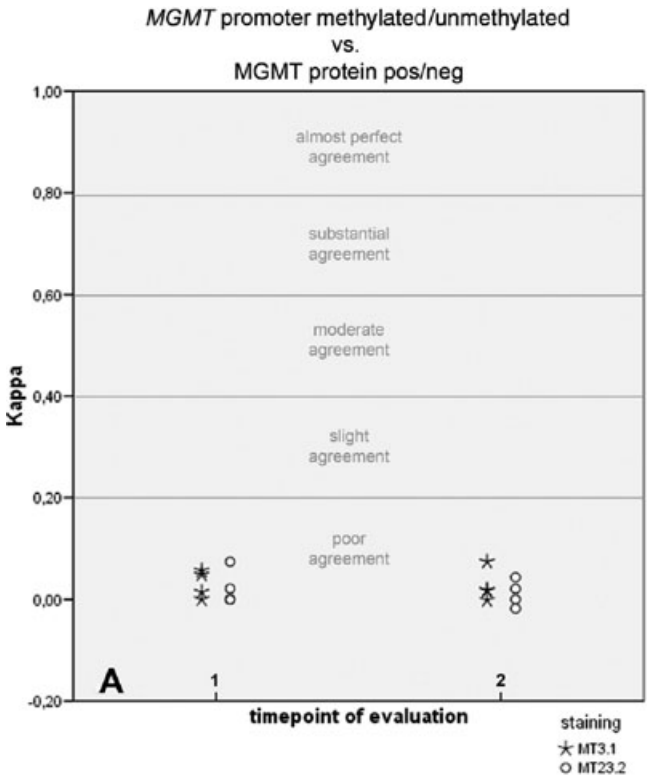
randomized EORTC/NCIC trial 26981/22981 (26, 52). We found variable observer agreement on immunohistochemical MGMT protein expression, insufficient correlation with *MGMT* promoter methylation status and no correlation with patient survival. Therefore, in our study, MGMT IHC does not prove to be a clinically usable tool in the diagnostic assessment of glioblastoma.

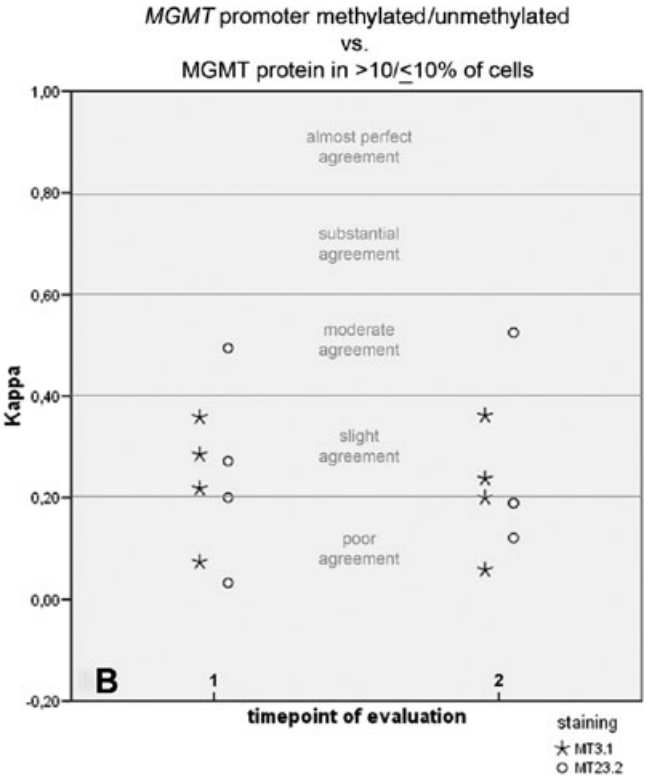
Previously published studies on glioblastoma (2, 11, 12, 44) and other types of diffuse glioma (7, 11, 48) reported a significant association of immunohistochemically assessed MGMT expression and patient survival (see also Table 3). In glioblastoma, this association was confirmed neither by two other investigations in small retrospective series (9, 50) nor by our present study in a large prospective patient cohort. Possible explanations for the discrepancy of findings are sample sets composed of heterogeneous glioma types or small patient numbers in some of the studies (Table 3) (11). Other explanations may be methodological differences (eg, different pretreatment of sections prior to immunostaining; see Table 3). However, we used the same primary monoclonal antibodies that have been used in most of the previous studies in gliomas so far (Table 3).

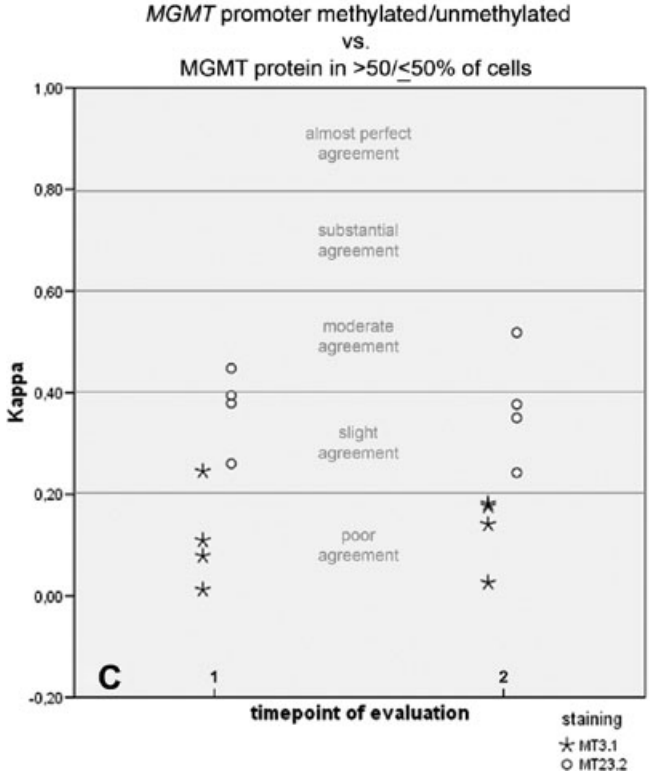
Furthermore, different cutoff levels of semiquantitative MGMT assessment by immunostaining could be the cause for a significant association with patient outcome in some of the studies (11). In our study, we tested the potential association of MGMT expression with patient survival based on 16 immunohistochemical MGMT assessments using an overall group comparison of all four semiquantitative categories. In addition, a linear trend across the four ordered MGMT categories was also tested. In none of the cases was significance achieved. Therefore, evidence prevails that immunohistochemically detectable MGMT expression is not firmly associated with patient outcome. A possible explanation for this finding is that MGMT protein can be upregulated, and this upregulation may be induced by glucocorticoids, chemotherapy and radiotherapy. Thus, the expression of the protein at the time of diagnosis might not reflect the expression of the protein during therapy (1, 5, 20, 23, 37, 38, 51, 54).

In contrast to all but one (11) of the previous studies, we used TMA for immunohistochemical assessment of MGMT protein expression. The TMA contained all samples of the series in a single paraffin block allowing MGMT immunostaining of the whole series on one section, thus allowing for homogeneous staining conditions for the whole sample set, although differences in tissue handling prior to the TMA production (eg, tissue fixation, storage conditions) may introduce some heterogeneity. Furthermore, the TMA is particularly useful for inter- and intraobserver comparison of semiquantitative MGMT assessments, because all observers perform their assessments on the same restricted tissue area (15, 16, 32, 43). Under these conditions, intraobserver agreement was generally high, whereas interobserver agreement was highly variable. Possible explanations for the high interobserver variability could be interobserver differences in cutoff definition for intensity of the immunostaining signal (ie, differences in perception whether a given nucleus is stained darkly enough to be considered as positive) or interobserver differences in discrimination between specific immunostaining signal and background staining. Furthermore, high variability at identification of nonneoplastic cell elements within the tumor tissue (eg, endothelial cells, reactive astrocytes, microglial cells/macrophages and tumor-infiltrating lymphocytes) may contribute to poor observer agreement. High intraobserver agreement may indicate that joint training of observers (eg, discussion of cases with discrepant results) could increase interobserver agreement. However, the results of immunohistochemical MGMT expression analysis did not reach a substantial correlation with *MGMT* promoter methylation status or a significant association with patient survival for any of the observers. Therefore, improvement of interobserver agreement on

Figure 4. *Illustration of kappa values for correlation between immunohistochemically visualized O6-methylguanine-methyltransferase (MGMT) protein expression and* MGMT *gene promoter status as assessed by methylation-specific PCR.* Immunohistochemical MGMT values were categorized in three different ways (for a detailed description, refer to Results section): MGMT negative vs. MGMT positive (**A**), low vs. high MGMT expression (cutoff 10%; **B**), MGMT expression in <50% of tumor cells vs. MGMT expression in ≥50% of tumor cells (**C**). Note that in **A**, there is one kappa value tie for the MT3.1 antibody at both timepoints, respectively, and one tie for the MT23.2 antibody at timepoint 1. In **B**, there is one kappa value tie at timepoint 2 with the MT23.2 antibody.







immunohistochemical MGMT expression by means of joint training is unlikely to provide additional analytical benefit.

Correlating semiquantitatively assessed MGMT expression with *MGMT* promoter methylation, we only found poor to moderate correlation. The best agreement (slight to moderate agreement) between MSP and MGMT IHC results was achieved using MT23.2 antibody and a cutoff of 50% immunolabeled cells for categorization of immunohistochemically evaluated MGMT expression. However, kappa values indicating substantial agreement between MGMT IHC and MSP were not achieved in any case. In our study, a potential methodological problem leading to insufficient correlation between MGMT IHC and MSP could be that the small tissue fraction viewed on the TMA may not be representative for the entire tumor in a given case. However, the TMA cores used in our study were specifically selected from tumor areas that were histologically classified as representative for the entire tumor. Moreover, a recently published paper systematically analyzed intratumoral distribution of MGMT promoter methylation and MGMT expression in anaplastic astrocytoma and glioblastoma (22). They found equal or highly similar MGMT expression in tissue samples taken from different sites of each individual tumor, thus clearly illustrating intratumoral homogeneity of immunohistochemical MGMT expression. However, major intratumoral regional variation in MGMT immunostaining would constitute another strong argument against the reliability of the immunohistochemical MGMT assessment for diagnostic purposes. Furthermore, in four previous studies, a comparably poor correlation between MGMT IHC and MSP was found (22, 31, 40, 50). Therefore, there is increasing evidence that immunohistochemically assessed MGMT expression is a poor indicator of *MGMT* promoter methylation status in glioblastoma. As discussed previously, a possible explanation for this finding is MGMT protein expression in entrapped pre-existing, reactive or infiltrating cells (31, 44, 50, 51). MGMT expression has been shown in nonneoplastic endothelial cells, astrocytes, oligodendroglial cells and inflammatory cells (44, 50). Furthermore, thus far unknown control mechanisms of MGMT protein expression at transcriptional, posttranscriptional or translational level may exist (6, 8, 22, 57).

To conclude, in our study, observer variability and lack of association with *MGMT* promoter methylation status and with patient

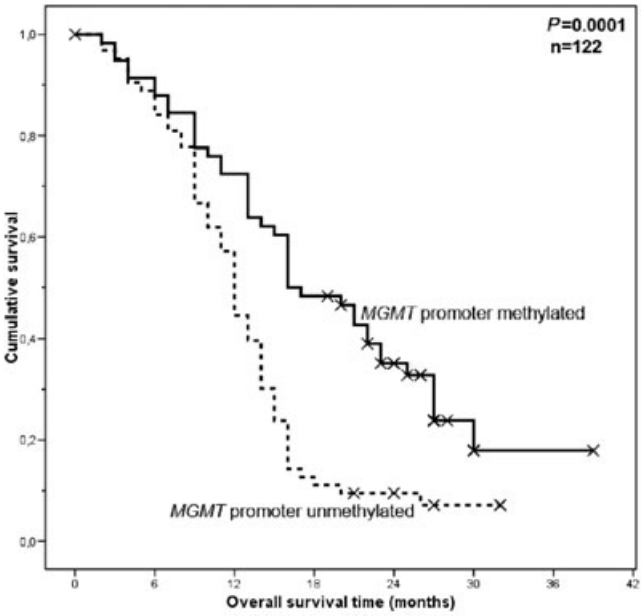


Figure 5. Kaplan–Meier curve showing a significant association of *MGMT* promoter methylation status with patient overall survival.

Table 2. Summary of results of survival analyses for all immunohistochemical assessments of MGMT expression in tumor cells (semiquantitative MGMT values as assessed by question 2, Table 1). The following situations were tested for all 16 assessments (four observers, two MGMT stainings, two timepoints): (i) MGMT qualitative—all four semiquantitative categories of MGMT expression ("no," "few," "some," "many" MGMT immunoreactive tumor cells) were entered into log-rank test as separate variables; (ii) MGMT linear trend—a linear trend across the four ordered MGMT categories ("no," "few," "some," "many" MGMT immunoreactive tumor cells) was tested using the log-rank test. We found no significant association of MGMT expression with patient outcome in any case. Abbreviation: MGMT = O6-methylguanine-methyltransferase.

Assessment	Observer	Antibody	Timepoint	*P*-values: MGMT qualitative	*P*-values: MGMT linear trend
1	1	MT3.1	1	0.66	0.45
2	1	MT3.1	2	0.98	0.66
3	2	MT3.1	1	0.78	0.62
4	2	MT3.1	2	0.80	0.66
5	3	MT3.1	1	0.53	0.30
6	3	MT3.1	2	0.19	0.11
7	4	MT3.1	1	0.28	0.08
8	4	MT3.1	2	0.30	0.14
9	1	MT23.2	1	0.62	0.36
10	1	MT23.2	2	0.62	0.37
11	2	MT23.2	1	0.63	0.38
12	2	MT23.2	2	0.42	0.17
13	3	MT23.2	1	0.19	0.10
14	3	MT23.2	2	0.18	0.14
15	4	MT23.2	1	0.43	0.20
16	4	MT23.2	2	0.37	0.36

Table 3. Summary of publications that have analyzed MGMT expression in glioblastoma by means of immunohistochemistry. Abbreviations: AA = anaplastic astrocytoma; AB = antibody; AOA = anaplastic oligoastrocytoma; AR-10 = antigen retrieval 10; DA = diffuse astrocytoma; EDTA = ethylenediaminetetraacetic acid; GBM = glioblastoma multiforme; HGG = high grade glioma; MC = monoclonal; MIN = minutes; n.a. = not applicable; n.s. = not specified; O = oligodendroglioma; OA = oligoastrocytoma; o.n. = overnight; pAA = pediatric anaplastic astrocytoma; PBS = phosphate buffered saline; pGBM = pediatric glioblastoma multiforme; pHGG = pediatric high-grade glioma; prGBM = primary glioblastoma multiforme; rGBM = relapsed glioblastoma multiforme; TEC = Tris-ethylenediamine tetraacetic acid-citrate.

Reference number	Study cohort	MGMT AB clone (clonality)	MGMT AB supplier	Pretreatment, AB concentration, AB incubation time	Mode of evaluation of immunohistochemical MGMT expression	Categories used for semiquantitative evaluation of MGMT expression*	Observer agreement on MGMT expression	Correlation of MGMT expression with MGMT promoter methylation status	Cutoffs used for correlation of MGMT expression with patient survival	Significant correlation of MGMT expression with patient survival
2	18 GBM	MT3.1 (mc)	Neomarkers, CA, USA	15 minutes microwave heating in citrate buffer (ph 6), 1:50, o.n.	Semiquantitative	0, ≤19%, 20%–49%, ≥50%	Not done	Not done	20%	Yes
3	5 DA, 11 O, 6 AO, 12 AA/GBM	MT3.1 (mc)	Chemicon, CA, USA	Microwave heating in citrate buffer (ph 6), 1:200, o.n.	Semiquantitative	0, <20%, 20%–40%, >40%	Not done	Not done		Not done
4	47 AA, 99 GBM	3B8 (mc)	n.s.	Treatment with 0.1% Triton X-l00 in PBS, 37 μg/mL, 60 minutes	Densitometry		Not done	Not done	60 000 molecules/nucleus	Yes
8	21 O, 5 OA, 15 GBM	MT3.1 (mc)	Neomarkers, CA, USA	Microwave heating in TEC buffer (ph 7.8) for 36 minutes, 1:100, 120 minutes	Semiquantitative	≤10%, 10%–50%, >50%	Not done	Not done		Not done
9	54 GBM	MT3.1 (mc)	Chemicon, CA, USA	15 minutes incubation in citrate buffer (ph 6), 1:50, o.n.	Semiquantitative	<20%, >20%	Not done	Not done	20%	No
11	36 DA, 51 AA, 75 pGBM, 19 rGBM	MT3.1 (mc)	Chemicon, CA, USA	20 minutes microwave boiling in 0.05% citraconic anhydride (ph 7.4)	Quantitative		Not done	Not done	5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%	Yes
12	25 GBM	MT3.1 (mc)	Chemicon, CA, USA	n.s., 1:100, 60 minutes	Semiquantitative	<35%, >35%	Not done	Not done	35%	Yes
13	2 AA 1GBM	3B8 (mc)	n.s.	n.s.	n.s.		Not done	Not done		Not done
19	5 AA 33 GBM	MT3.1 (mc)	n.s.	Microwave heating in antigen retrieval buffer for 10 minutes, 5 μg/mL, o.n.	Semiquantitative	<20%, >20%	Not done	Not done		Not done
21	71 DA, 40 O 39 AA 259 GBM	MT3.1 (mc)	Chemicon, CA, USA	n.s.	Semiquantitative	<5%, >5%	Not done	Not done		Not done
22	6 AA, 17 GBM	MT3.1 (mc)	Dako, Copenhagen, Denmark	Heating in steamer, 1:500, 15 minutes	Semiquantitative	<10%, 10%–50%, >50%	Not done	No		Not done
30	24 AA, 40 GBM	3B8 (mc)	n.s.	Treatment with 0.1% Triton X-l00 in PBS, 37 μg/mL, 60 minutes	Densitometry		Not done	Not done	60 000 molecules/nucleus	Yes
31	2 A, 2 O, 4 OA, 1 AA, 5 AO, 5 AOA, 14 GBM	n.s.	Chemicon, Hampshire, UK	13 minutes microwave heating in sodium citrate, 1:1600, n.s.	n. s.		Not done	Poor		Not done

Table 3. *Continued.*

Reference number	Study cohort	MGMT AB clone (clonality)	MGMT AB supplier	Pretreatment, AB concentration, AB incubation time	Mode of evaluation of immunohistochemical MGMT expression	Categories used for semiquantitative evaluation of MGMT expression*	Observer agreement on MGMT expression	Correlation of MGMT expression with MGMT promoter methylation status	Cutoffs used for correlation of MGMT expression with patient survival	Significant correlation of MGMT expression with patient survival
36	3 DA, 15 AA, 8 GBM	Polyclonal	n.s.	n.s.	Semiquantitative	<33%, 34%–66%, ≥67%	Not done	Poor		Not done
40	1 DA, 1 XA, 3 AO, 5 AA, 22 GBM	MT3.1 (mc)	n.s.	Heating in AR-10 buffer for 10 minutes, 4 µg/mL, o.n.	Semiquantitative	<20%, >20%	Not done	Poor		Not done
41	48 HGG	MT3.1 (mc)	Chemicon, CA, USA	Heating in AR-10 buffer for 10 minutes, 5 µg/mL, o.n.	Quantitative		Not done	Not done		Not done
44	69 HGG	MT3.1 (mc)	Neomarkers, CA, USA	Autoclave heating in citrate buffer (ph 6) for 10 minutes, 1:60, o.n.	Semiquantitative	<10%, >10%	Not done	Not done	10%	Yes
46	3 A, 14 AA, 17 GBM	MT3.1 (mc)	Lab Vision, CA, USA	0.3% H_2O_2, 0.1% sodium azide for 20 minutes in PBS, 4 µg/mL, n.s.	n.s.		Not done	Not done		Not done
48	8 pHGG, 31 pAA, 33 pGBM	MT23.2 (mc)	Zymed, CA, USA	20 minutes heating in 10 mmol citrate buffer (ph 6)	Semiquantitative	Little or no expression, scattered positive cells, comparable to normal brain, overexpression	Not done	Not done	No, little or scattered positive cells vs. comparable to normal brain or over expression	Yes
50	50 GBM	MT3.1 (mc)	Neomarkers, CA, USA	Steam disodium ethylene diamine-tetraacetate (ph 8), 1:50, 30 minutes	Semiquantitative	0, <10%, 10%–50%, >50%	Not done	No	Neg vs. pos	No
57	10 A, 9 AA, 16 GBM	Polyclonal	n.s.	3× microwave heating for 10 minutes in EDTA buffer (ph 8), 1:500, o.n.	Quantitative		Not done	Not done		Not done
Present study	164 GBM	MT3.1 (mc)	Dako, Copenhagen, Denmark	1.4 µg/mL, 15 minutes	Semiquantitative	0, <10%, 10%–50%, >50%	Variable	Poor to moderate	Neg vs. pos, 10%, 50%	No
		MT23.2 (mc)	Zymed, CA, USA	0.2 µg/mL, 15 minutes						

*Percent signifies fraction of immunoreactive cells.

survival impede the use of MGMT IHC as a clinically useful biomarker for routine diagnostic purposes and clinical decision making. It remains to be seen whether novel anti-MGMT antibodies directed against other epitopes are associated with better clinical and analytical performances.

Analysis of *MGMT* promoter methylation status (MSP-PCR, real-time MSP-PCR, multiplex ligation-dependent probe amplification, pyrosequencing, methylation-sensitive high resolution melting) in glioblastoma remain promising tools for diagnostic purposes (10, 31, 42, 45, 46, 53, 56). However, the clinical and analytical performance of these molecular methods remain to be validated in prospective trials and by means of interlaboratory comparisons (28, 40, 51).

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