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Posttreatment Effect of *MGMT* Methylation Level on Glioblastoma Survival

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Abstract

The DNA repair protein O6-methylguanine-DNA methyltransferase (*MGMT*) removes temozolomide-induced alkylation, thereby preventing DNA damage and cytotoxicity. We investigated the prognostic effect of different *MGMT* methylation levels on overall and progression-free survival in 327 patients with primary glioblastoma undergoing standard treatment. We obtained *MGMT* methylation level in 4 CpG sites using pyrosequencing. The association between *MGMT* methylation level and survival was investigated using Cox proportional hazards model and an extension to detect time-varying effects. We found an association between *MGMT* methylation level and overall survival (OS) from around 9 months after the diagnosis, with no association between *MGMT* methylation level and OS before that. For patients surviving at least 9 months even small increases in *MGMT* methylation level are significantly beneficial (HR = 0.97, 95% CI [0.96, 0.98]). The predictive ability of *MGMT* methylation level on OS from 9 months after diagnosis has a Harrel's *C* of 66%. We conclude that the *MGMT* methylation level is strongly associated with survival only for patients surviving beyond 9 months with considerable effects for levels much lower than previously reported. Prognostic evaluation of cut-points of *MGMT* methylation levels and of CpG island site selection should take the time-varying effect on overall survival into account.

Key Words: Epigenetic marker prognostic, Glioblastoma, O6-methylguanine-DNA methyltransferase (*MGMT*), Survival, Time-varying effect.

INTRODUCTION

Standard treatment for patients with glioblastoma (GBM) consists of surgery followed by radiotherapy (RT) with concomitant and adjuvant treatment with the alkylating agent temozolomide (TMZ) based on the EORTC/NCIC clinical trial published by Stupp et al (1). It has been shown that patients whose tumors carried a methylated O6-methylguanine-DNA methyltransferase (*MGMT*) promoter benefited more from treatment with TMZ (2). Therefore, determination of the *MGMT* methylation status is recommended for GBM patients in the latest guidelines from the European Society for Medical Oncology (ESMO) and the European Association of Neuro-Oncology (EANO), especially for elderly patients, before choosing treatment (3, 4). Recommendations for elderly patients are based on studies showing that elderly patients with a *MGMT*-methylated GBM should be treated with TMZ or combined RT and TMZ, while patients whose tumors lack *MGMT* promoter methylation should receive RT alone or combined RT and TMZ (5, 6). This is in agreement with a recent study by Perry et al (7) investigating short-course RT in combination with TMZ in patients aged 70 years or older. The study showed that patients with a methylated *MGMT* promoter treated with RT + TMZ had a median overall survival (OS) of 13.5 months compared with 7.7 months in patients who received RT alone. For patients with an unmethylated *MGMT* promoter, median OS was 10.0 (RT + TMZ) and 7.9 (RT) months. These results emphasize the importance of implementing *MGMT* promoter methylation testing in the clinical setting along with an establishment of robust and reproducible methods.

An important challenge in determining *MGMT* methylation status is to identify a clinically relevant cut-point to classify patients with an *MGMT* methylated or unmethylated tumor, which was addressed in a review by Wick et al (8). Although both lower (9) and higher cut-points (10, 11) have been reported, a mean percentage of methylated alleles ~8%–10%, usually calculated as mean value across the investigated CpG sites, appears to be most commonly used in published studies using quantitative assays (12–19). However, only few studies report a reason for choosing the given cut-point (13, 19–21). A meta-analysis on the association between *MGMT* methylation status and OS suggests that patients with *MGMT* methylation level above the cut-point have better OS than

patients with lower *MGMT* methylation level ([22]). Brigliadori et al considered 3 categories of *MGMT* methylation levels 0%–9%, 10%–29%, and ≥30%, and they reported that patients with *MGMT* methylation level ≥30% had the best median OS, whereas median OS did not differ between patients with 0%–9% and 10%–29% methylation levels. The authors conclude that *MGMT* methylation status is only predictive in patients with tumors methylated 30% or more ([11]). Examination of the Kaplan-Meier curves in these and other studies indicate that the curves are almost identical during the first 9–12 months from diagnosis for any choice of cut-point of *MGMT* methylation level; only after this period, the prognostically beneficial effect of *MGMT* methylation status becomes evident ([2], [9], [19–21]). This strongly indicates a time-varying effect which, to our knowledge, has not been reported previously.

The aims of the present study were to investigate whether the association between *MGMT* methylation level and OS is modified by time after diagnosis, and to systematically evaluate the prognostic effect of different *MGMT* methylation levels on patient OS and progression-free survival (PFS) when stratifying on time after diagnosis.

MATERIALS AND METHODS

Patients

A total of 508 patients with primary GBM diagnosed in the Region of Southern Denmark between 2005 and 2014 were identified based on a report from the Danish Cancer Register. All patients were inhabitants in the Region of Southern Denmark at the time of diagnosis and no patients had received treatment before surgery. A total of 236 patients were diagnosed between 2005 and 2009, and they have been thoroughly described in previous publications ([23–27]). The remaining patients (n = 272) were diagnosed between 2010 and 2014. In 327 patients, a sufficient amount of viable tissue allowed retrospective determination of the *MGMT* promoter methylation level using pyrosequencing. To avoid potential bias from different treatment regimens, only patients treated according to the Stupp regimen (RT 60 Gy in 30 fractions, followed by concomitant and 6 cycles of adjuvant TMZ) were included in the statistical evaluations, leaving 226 patients for the final prognostic analyses (Supplementary Data Fig. S1).

Pathology

All GBMs were classified according to the World Health Organization guidelines 2016. *MGMT* promoter methylation level was identified using pyrosequencing (Therascreen *MGMT* Pyro Kit, Qiagen; Ref. 971061, Hilden, Germany) as described by the manufacturer. Briefly, DNA was purified from 10-µm paraffin slides using the GeneRead DNA FFPE Kit (Qiagen), bisulfite converted with EpiTect Plus DNA Bisulfite Kit (Qiagen) and *MGMT* pyrosequencing was performed according to the kit instructions. *MGMT* methylation percentages at 4 CpG sites were measured. The 4 sites are located in exon 1 of the human *MGMT* gene corresponding to chromosome sequence chr10: 131, 265, 519, 131, 265, 522, 131, 265, 526, and 131, 265, 536, respectively (GRCh37/hg19). These 4 sites

are referred to as CpGs 76–79 in the literature ([28], [29]). IDH1 status was investigated by immunohistochemistry as described previously ([25], [30]).

Ethics

The study was approved by the Local Committee on Health Research Ethics (ProjektID. S2DO9Oo8O) and the Danish Data Protection Agency (J.nr. 2015-41-4320). The use of tissue was not prohibited by any patient according to the Danish Tissue Application Register.

Statistics

All analyses were performed using the mean percentage of methylated alleles across the 4 CpG sites representing *MGMT* activity. The Wilcoxon rank sum test was used to compare mean *MGMT* methylation level between men and women and between patients with *IDH* mutated and wild-type tumors, and Cox proportional hazards models were used to compare OS between men and women. OS was defined as time of diagnosis until death or date of censoring (January 1, 2017), and PFS was defined as time of diagnosis until clinical or radiological progression, death, or date of censoring (January 1, 2017). Cause of death was due to tumor progression in all patients; this was verified in the medical journals and in the Danish Cause of Death Register. Survival functions were illustrated by Kaplan Meier curves for different cut-points of *MGMT* methylation percentages. Aalen's additive hazard models were used to assess time-invariance of the effect of *MGMT* methylation level on OS and on PFS ([31], [32]). We used Cox proportional hazard models to estimate separate effects of *MGMT* methylation level on OS during the first 9 months after diagnosis and on OS beyond 9 months' survival. The time-stratified Cox modeling was based empirically on the time-invariance analysis using the Aalen model. All Cox models were adjusted for age at diagnosis and ECOG performance status. Because it has previously been reported that the extent of resection is not prognostic in these patients, and that only 2% of the patients have an *IDH* mutation ([25]), these variables were not included as confounders in the analyses. Assumptions on proportional hazards were verified using Schoenfeld residuals tests. To measure the prognostic performance of the Cox models, the concordance between *MGMT* methylation level and OS before and after 9 months from diagnosis, as well as the concordance between *MGMT* methylation level and PFS were assessed using Harrel's *C* and Gönen-Heller concordance measures ([33], [34]). Supplementary analyses on OS and PFS were conducted stratifying on age younger or older than 70 years.

RESULTS

The patient population included 92 females and 134 males with a median age of 62 years at the time of diagnosis (Supplementary Data Table S1). The majority of the patients underwent surgical removal (surgery 98%; biopsy only 2%) and the patients were generally in a good performance status. A total of 202 patients (89.4%) died during follow up. The median OS was 17.8 months, and 68.1% of the patients were alive
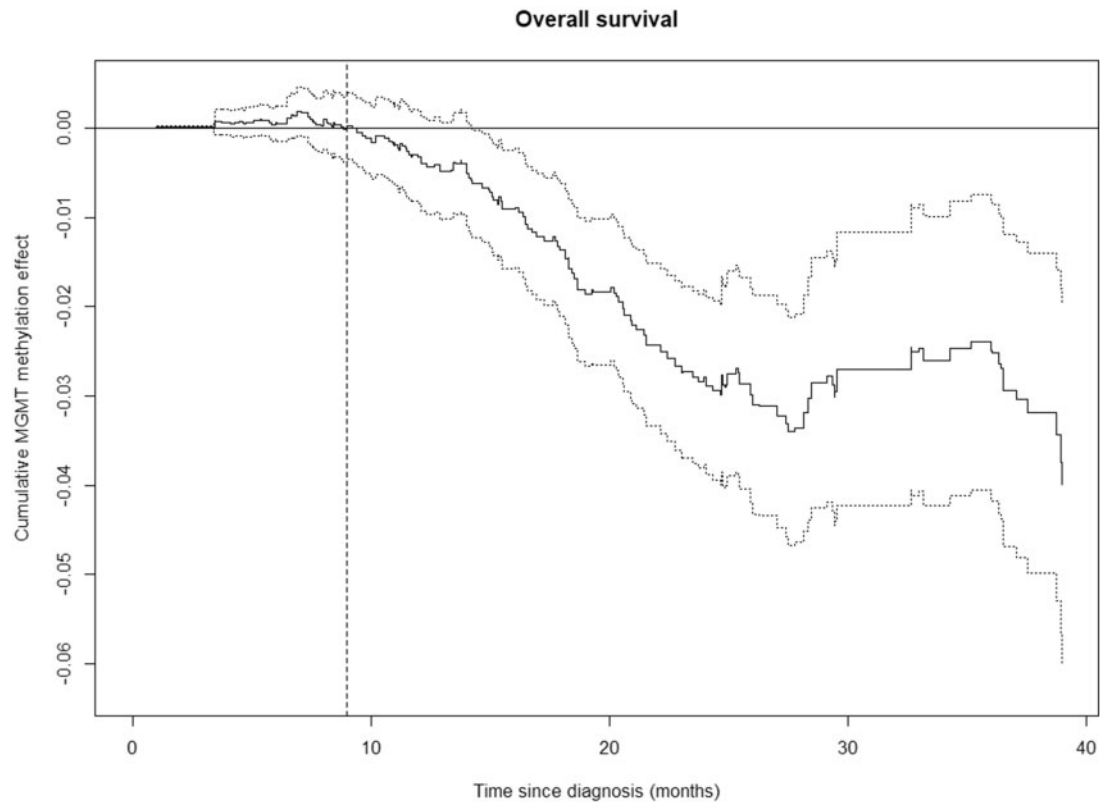
Overall survival

FIGURE 1. Time-varying effects of *MGMT* methylation level on OS (solid line) with 95% confidence bands (dotted lines) according to Aalen's additive hazard model, n = 226. There is no cumulative effect of the *MGMT* methylation level during the first 9 months after diagnosis, indicated by the horizontal curve. After 9 months, the *MGMT* level becomes associated with OS, indicated by the curve sloping away from zero with 95% confidence bands below zero. After ~24 months, the curve levels out suggesting a weaker influence of the *MGMT* methylation level on OS from that point, although the pattern is less clear due to the wide confidence bands.

1 year after the diagnosis. There was no difference in OS (p = 0.10) or *MGMT* methylation level (p = 0.31) between male and female patients. Furthermore, there was no difference in OS between patients with *IDH*-mutated (mean OS 22.2 months) and those with *IDH*-wildtype (mean OS 18.5 months) GBM (p = 0.18) or *MGMT* methylation level (p = 0.86). No analyses stratifying on gender or *IDH* status were performed.

The possible time-varying effect on OS was investigated using Aalen's additive hazard model (Fig. 1). The figure suggests that *MGMT* methylation level has no effect on OS during the first ~9 months after the diagnosis, whereas a clear association between *MGMT* methylation level and OS occurs after ~9 months. After ~24 months, the influence of the *MGMT* methylation level on OS seems to decline. Figure 1 indicates that the main assumption on proportional hazards of the Cox model is not satisfied, but suggests a change in the hazard ratio (HR) after 9 months. By splitting the study period in 2 (i.e. before and after 9 months) and analyzing each period separately, the proportional hazard assumptions in each period are satisfied (Shoenfeld residual tests: p > 0.05). Similar patterns are seen for patients younger than 70 years, and for patients aged 70 years or more (Supplementary Data Fig. S2).

The time-varying effect of *MGMT* methylation level on PFS was investigated, indicating that the *MGMT* methylation level is associated with PFS. Unlike OS, the association between PFS and *MGMT* methylation level is present from the time of diagnosis until ~18 months after the diagnosis where the effect lessens (Fig. 2). Similar patterns are seen for patients younger than 70 years, and for patients older than 70 years (Supplementary Data Fig. S3).

The associations between different *MGMT* methylation cut-points and OS are illustrated by Kaplan-Meier survival curves (Fig. 3). The Kaplan-Meier curves confirm the time-varying effect of the *MGMT* methylation level on OS. During the first 9 months after diagnosis, the Kaplan-Meier curves for the different cut-points are very close, suggesting no differences in OS, whereas clear differences in OS for different *MGMT* methylation cut-points occur 9 months after the diagnosis. Patients with an *MGMT* methylation level below 2% have very poor OS, whereas OS increases markedly with each step of increase in cut-point, until the level reaches 15%, whereupon the increase in OS becomes less pronounced.

To explore the association between *MGMT* methylation level and OS further, we estimated the *MGMT* methylation level corresponding to the minimum mortality risk. For
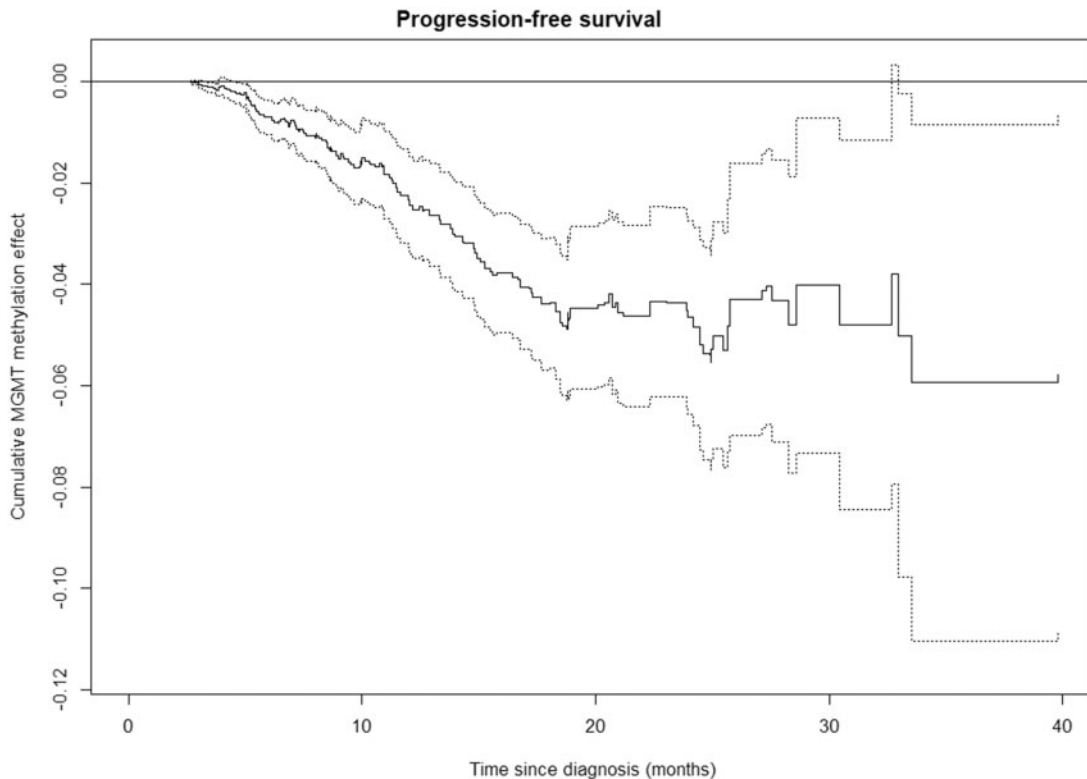
FIGURE 2. Time-varying effects of *MGMT* methylation level on progression-free survival (PFS) (solid line) with 95% confidence bands (dotted lines) according to Aalen's additive hazard model. The steady slope of the cumulative effect of *MGMT* methylation level from time of diagnosis suggests an immediate effect of *MGMT* methylation level on PFS. After ∼18 months, the cumulative effect of *MGMT* methylation level on PFS levels out with very wide confidence bands.

patients surviving 9 months or more from the time of diagnosis, we identified the *MGMT* methylation level associated with the longest OS as 38.4 (Supplementary Data Fig. S4). The risk of death for the remaining *MGMT* methylation levels was subsequently compared with the risk of death if the tumor had a *MGMT* methylation level of 38.4, showing that patients with a *MGMT* methylation level of ∼28 or less had a significant increased risk of dying compared with patients with a *MGMT* methylation level of 38.4 (Supplementary Data Fig. S4).

Only few patients (n = 5, 2%) had a methylation percentage <2%, whereas 109 patients (48%) had a methylation percentage below 10 (Table 1). For patients surviving 9 months or longer after diagnosis, the mortality hazard decreased by 3% for each percentage point increase in *MGMT* methylation level (HR = 0.97, 95% CI = 0.96, 0.98). For any choice of cut-point, the mortality hazard is reduced for patients with *MGMT* methylation level above the cut-point compared with patients with *MGMT* methylation level below the cut-point for patients surviving at least 9 months (Table 1). The OS during the first 9 months after diagnosis is not associated with *MGMT* methylation level (Table 1). The decrease of 3% in mortality hazard for each percentage point increase in *MGMT* methylation level applies also to patients younger than 70 years as well as patients aged 70 years or older (Supplementary Data Table S2). Generally, *MGMT* methylation has a

stronger effect in patients in the older age group than in younger patients (Supplementary Data Table S2).

The adequacies of the Cox regression models to make predictions of risks (i.e. the predictive abilities of the models) were examined using the Harrel's *C* and Gönen-Heller concordances. For patients surviving at least 9 months, the Harrel's *C* concordance between OS and *MGMT* methylation level (continuous) is 0.66, and the Gönen-Heller concordance is 0.62, indicating a fair predictive ability of the *MGMT* methylation level on OS (Table 2). Similar levels of concordance are observed for *MGMT* methylation cut-points at 5% or higher, while the concordance between OS and an *MGMT* methylation cut-point at 2% is very low (Harrel's *C* = 0.51, Gönen-Heller = 0.51; Table 2). For patients aged 70 years or older, the concordance between OS and *MGMT* methylation level is slightly higher (Supplementary Data Table S3).

When the effect of different cut-points of *MGMT* methylation level was investigated in relation to PFS, we found that the risk of tumor progression or death is reduced for patients with the higher *MGMT* methylation level. The risk of progression or death decreases by 3% for each percentage point the *MGMT* methylation level increases when *MGMT* methylation level is investigated as continuous (HR = 0.97, 95% CI = 0.96, 0.98) with Harrel's *C* concordance 0.64 and Gönen-Heller concordance 0.61 (Table 3). In patients aged 70 years of age or
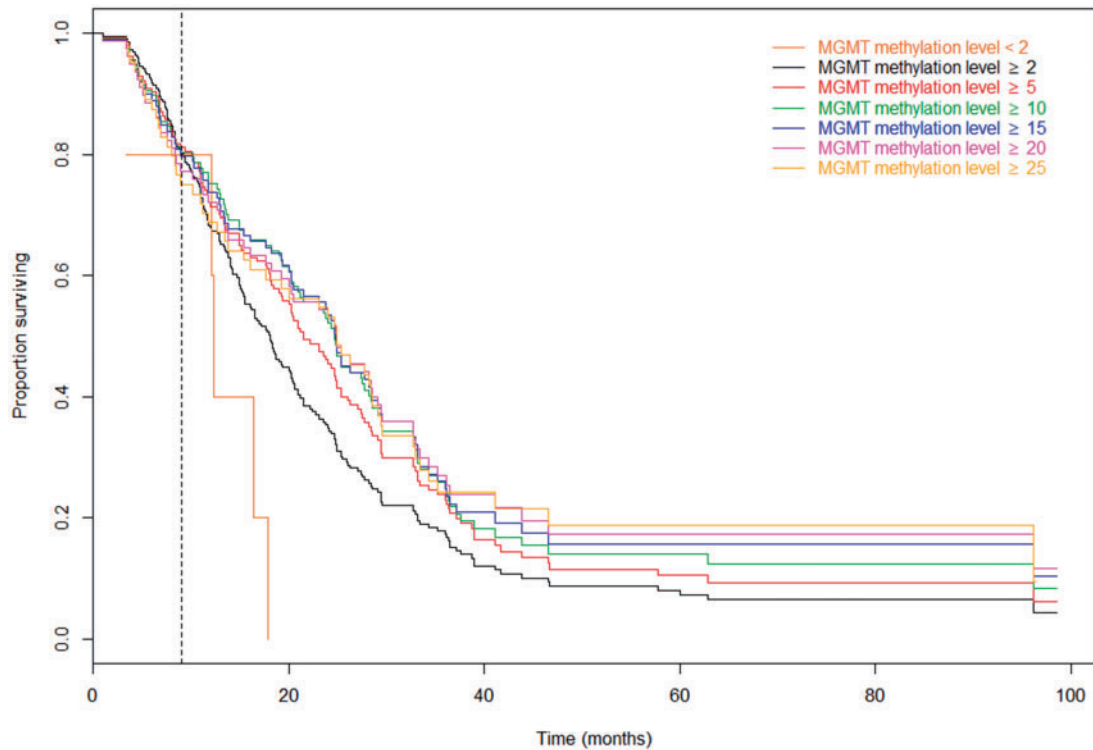
FIGURE 3. Kaplan-Meier overall survival curves for different *MGMT* methylation cut-points. All the curves coincide in the first 9 months, indicated by the vertical line. After 9 months, any increase in cut-point of *MGMT* methylation level increases the OS for patients above the cut-point. All curves are shown together to allow for comparisons of the curves during the first 9 months and later.

TABLE 1. Mortality Hazard Ratio According to Different Cut-Points of *MGMT* Methylation Level During the First 9 Months After Diagnosis and After 9 Months

MGMT Methylation level	N_{above}/N_{below}	Patient OS During the First 9 Months After Diagnosis		Patient OS From 9 Months After Diagnosis	
		HR	HR^a (95% CI)	HR	HR^a (95% CI)
Continuous	–	1.00	1.00 (0.98, 1.02)	0.97	0.97 (0.96, 0.98)***
2–100 versus 0–1	221/5	0.89	0.81 (0.11, 5.92)	0.29	0.30 (0.11, 0.82)*
5–100 versus 0–4	154/72	0.87	0.82 (0.44, 1.51)	0.32	0.30 (0.21, 0.43)***
10–100 versus 0–9	117/109	1.00	0.96 (0.54, 1.73)	0.36	0.36 (0.26, 0.49)***
15–100 versus 0–14	99/127	1.06	0.97 (0.54, 1.76)	0.40	0.38 (0.27, 0.53)***
20–100 versus 0–19	79/147	1.31	1.18 (0.65, 2.15)	0.41	0.38 (0.27, 0.55)***
25–100 versus 0–24	64/162	1.47	1.34 (0.73, 2.47)	0.45	0.42 (0.28, 0.62)***

Abbreviations: N_{above}, number of patients with *MGMT* methylation level above the cut-point; N_{below}, number of patients with *MGMT* methylation level below the cut-point; HR, mortality hazard ratio; CI, confidence interval; OS, overall survival.

^{a}Adjusted for age and performance status.

*p < 0.05, ***p < 0.001.

more, the concordance is slightly higher than in patients <70 years of age (Supplementary Data Table S4).

DISCUSSION

In the present study, we investigated the effect of *MGMT* methylation level on OS in a large group of GBM patients receiving the same treatment. Another strength is the availability of clinical patient data that allow for adjustment of age and performance status in the statistical analyses. As only a small minority (2%) of the patients underwent a biopsy, adjustment for extent of resection was prevented, although previous studies have shown that resection may influence both OS and PFS (35, 36). By conducting a simple yet effective time-stratifying analysis we obtained better knowledge on the true predictive value and survival effect across different levels of *MGMT*.

We did not find any difference either in OS or in *MGMT* methylation levels between male and female patients, which

has previously been reported in other studies (37, 38). Furthermore, we did not find any difference in OS between patients with *IDH*-mutated and those with *IDH*-wildtype GBMs. This is in contrast to a study from Yang et al (39) who reported that patients with *IDH*-mutated and *MGMT* methylated GBM had the best prognosis. Because of the limited patients with *IDH*-mutated GBMs (n = 4), it was not possible to perform a similar analysis in our population. In the present study, we only investigated the most common *IDH* mutation (IDH1R132H). It has previously been shown by Yan et al (40) that only a minority of patients with GBMs have an *IDH2* mutation, and we found it unlikely that this or other *IDH* mutations would lead to a significant change of our data.

We found a strong beneficial effect of higher methylation on PFS, whereas the effect of *MGMT* methylation level on OS is time-varying. During the first 9 months after diagnosis, the *MGMT* methylation level was not associated with OS, but after 9 months, there was a strong association between OS and increased *MGMT* methylation level. Similar time-varying effects were seen in patients younger than 70 years and in patients aged 70 or more. A possible reason for the delayed effect of *MGMT* methylation level on OS might be exposure to chemotherapy during the first 9 months after diagnosis.

The time-varying effect observed in the present study has not, to our knowledge, been reported previously. Although similar patterns can be seen in empirical Kaplan-Meier curves of previous studies, no attention has been paid to this finding. Hegi et al (2) were the first to identify and report that patients with a methylated *MGMT* promoter benefit more from treatment with TMZ. The Kaplan-Meier survival estimates used in their study suggest, in line with our results, that *MGMT* methylation may have little effect on OS during the first 9–10 months after diagnosis, and that the effect on OS begins 9–10 months after inclusion in the study, while the effect on PFS appears to begin shortly after diagnosis. It is noteworthy that these patterns are less pronounced in the Kaplan-Meier estimates from studies on elderly patients (6, 7, 16). In the study by Perry et al (7) only patients not suitable for long-course RT were included, and in the NOA-08 study (6), *MGMT* methylation levels were only obtained in 56% of the patients. It remains speculative whether these differences may explain the deviances from the results in the present study.

The association between *MGMT* methylation and OS and PFS has been analyzed in several studies using different *MGMT* methylation cut-points for initiating treatment (9–20) and detection methods (41–44). Brigliadori et al (11) compared mortality HRs and median OS between 2 different cut-points (*MGMT* methylation levels 10% and 30%), concluding that only the cut-point at 30% maintained the predictive value of *MGMT* promoter methylation. In contrast, Gurrieri et al (17) reported that although the three-class stratification has a prognostic impact, a cut-point at 9% may more reliably discriminate between methylated and unmethylated. Incorporation of time-varying effects of *MGMT* methylation level might influence the results in these studies and provide more detailed insight into the prognostic effect of different *MGMT* methylation cut-points on OS.

Several studies have aimed to identify biologically and clinically relevant minimal islands of differential CpG methylation within the *MGMT* promoter region (29). Malley et al (45) comprehensively determined the methylation status of 98 individual CpGs within the entire *MGMT* CpG island of 762 bp in 13 glioma cell lines and 22 patient-derived GBM xenografts using bisulfite converted DNA followed by pyrosequencing. Of importance, CpG methylation profiles were correlated with *MGMT* expression levels in GBM tumor cells from xenografts rather than primary tissues to rule out contributions from cells of nontumor origin, including vascular endothelial cells and microglia, with comparatively high *MGMT* expression as has been shown previously (46, 47). In concordance with the findings of Everhard et al, the study by Malley

TABLE 2. Concordance Between Patient Overall Survival Beyond 9 Months After Diagnosis and *MGMT* Methylation Level

MGMT Methylation Level	Harrell's C	Gönen-Heller
Continuous	0.66	0.62
2–100 versus 0–1	0.51	0.51
5–100 versus 0–4	0.62	0.61
10–100 versus 0–9	0.64	0.62
15–100 versus 0–14	0.62	0.61
20–100 versus 0–19	0.60	0.59
25–100 versus 0–24	0.58	0.58

TABLE 3. Patient Progression-Free Survival and *MGMT* Methylation Levels

MGMT Methylation Level	N_{above}/N_{below}	HR	HR[a] (95% CI)	Harrell's C	Gönen-Heller
Continuous	–	0.97	0.97 (0.96, 0.98)***	0.64	0.61
2–100 versus 0–1	221/5	0.33	0.32 (0.12, 0.78)*	0.51	0.51
5–100 versus 0–4	154/72	0.37	0.36 (0.26, 0.50)***	0.59	0.60
10–100 versus 0–9	117/109	0.42	0.40 (0.30, 0.54)***	0.61	0.60
15–100 versus 0–14	99/127	0.45	0.43 (0.32, 0.58)***	0.60	0.59
20–100 versus 0–19	79/147	0.46	0.44 (0.32, 0.61)***	0.59	0.58
25–100 versus 0–24	64/162	0.48	0.47 (0.34, 0.66)***	0.57	0.56

Abbreviations: N_{above}, number of patients with *MGMT* methylation level above the cut-point; N_{below}, number of patients with *MGMT* methylation level below the cut-point; HR, mortality/tumor progression hazard ratio; CI, confidence interval.
[a]Adjusted for age and performance level.
*p < 0.05, ***p < 0.001.

et al identified differential CpG methylation in a distinct region that is critical for regulation of *MGMT* transcription in GBM cells (45, 47). This commonly analyzed region, deemed sufficient for evaluation of *MGMT* methylation status in a clinical setting, encompasses CpGs 73–90, thus including CpGs 76–79, investigated in the present study using a commercial kit. In an in vitro approach to pinpoint the role of individual CpGs by C>T mutations, Malley et al (45) further identified CpGs 89 and 84–87 as potential transcriptional regulators. However, other factors may influence the expression of *MGMT*, as reviewed for example by Cabrini et al (48). Moreover, several studies show evidence of subsets of patients with unmethylated *MGMT* promoter, but low or undetectable *MGMT* protein in the tumor cells (43, 45, 49). The influence of other factors emphasizes the complexity of analyzing and interpreting the importance of the *MGMT* promoter region.

Conclusion

The *MGMT* methylation level is strongly associated with OS, but only in patients surviving 9 months or longer. We find considerable effects of *MGMT* methylation status for cutpoints much lower than previously reported, and a time-varying effect of *MGMT* methylation levels on OS that should be considered. On the basis of our results, we strongly encourage reanalysis of previous data using the demonstrated time-stratifying approach. Furthermore, the time-varying effect should be taken into consideration in future studies investigating the prognostic effect of the *MGMT* methylation levels in patients with GBMs.

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