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MGMT PROMOTER METHYLATION IN PLASMA OF GLIOMA PATIENTS RECEIVING TEMOZOLOMIDE

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Running Title: MGMT methylation in serial glioma plasma samples

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Abstract

Promoter methylation of the O6–methylguanine-DNA methyltransferase (MGMT) gene plays a role in cellular response to alkylating agents. In the present study aimed to: i) evaluate the concordance between MGMT promoter methylation status in tumor tissue and plasma; ii) monitor MGMT promoter methylation status in plasma taken before and during temozolomide treatment; iii) explore the value of MGMT promoter methylation status in plasma as a prognostic/predictive biomarker in glioma patients.

We enrolled 58 patients with histologically confirmed glioma at different grades of malignancy. All patients underwent surgical resection and temozolomide treatment. Paraffin-embedded tumor tissue was available for 48 patients. Blood samples were collected from all patients before temozolomide treatment (baseline) and at each MRI examination for a 12-month period. MGMT promoter methylation status was assessed in both sample types by real time PCR with a specific probe.

The frequency of MGMT promoter methylation was 60.4% in tumor tissue and 41.38% in plasma. MGMT promoter methylation status was concordant in the two sample types (Kappa=0.75, 95% confidence interval [CI] 0.57-0.93; p-value <0.001). Overall and progression-free survival were longer in patients with methylated MGMT promoter. Mortality was higher in patients with unmethylated MGMT promoter, whether in tumor tissue (hazard ratio [HR]: 2.21; 95% CI 0.99-4.95) or plasma (HR: 2.19; 95% CI 1.02-4.68). Progression-free survival was shorter in patients with unmethylated MGMT promoter, whether in tissue (HR: 2.30; 95% CI 1.19-4.45) or plasma (HR: 1.77; 95% CI 0.95-3.30).

The cumulative incidence of unmethylated MGMT promoter in plasma at baseline was 58%, and reached virtually 100% at 12 months. In conclusion MGMT promoter methylation status in tumor tissue and plasma was highly concordant, and both were associated with longer survival, supporting the role of the detection of methylated MGMT promoter in predicting

treatment response. However we suggest caution in using plasma as a surrogate of tumor tissue due to possible false-negative results.

Keywords: Glioma, plasma DNA, MGMT, methylation, temozolomide

Introduction

Gliomas are the most common type of primary brain tumor in adults, with a standardized incidence in Europe of 6/100,000 person-years, and a peak of 18.5/100,000 person-years in people over 65 years of age [1]. Despite recent advances in the treatment of gliomas, such as surgical resection, radiotherapy, and chemotherapy with alkylating agents (e.g. temozolomide, nitrosoureas), treatment response varies considerably, and the prognosis of glioma patients remains poor [2-4].

Temozolomide treatment, in particular, causes DNA damage and cell death through the alkylation of the O6 position of guanine. The DNA damage is repaired by the O6–methylguanine-DNA methyltransferase (MGMT) protein, which is ubiquitously expressed in normal human tissue. Epigenetic silencing of the MGMT gene through methylation of promoter CpGs impairs DNA repair and has been associated with better treatment response, and longer survival in patients with high-grade glioma who received chemotherapy with alkylating agents [2-12]. Thus, MGMT promoter methylation status may be used as a predictive biomarker to identify patients with a higher probability of positive response to temozolomide treatment [4-14]. Conversely, few data are available on low-grade gliomas [15-16].

Recent studies have shown that MGMT promoter methylation status can be detected in both tumor tissue and circulating DNA from the serum/plasma of glioma patients [17-20]. The rate of concordance in MGMT promoter methylation status between the two sample types varies, but is consistent with studies on other cancer types (breast [21], renal [22], lung [23], colorectal [24]), and suggests that MGMT promoter methylation status in circulating DNA is a reliable tool to determine tumor methylation status.

Tissue samples of patients with glioblastoma who have undergone multiple surgical resections may show changes in MGMT promoter methylation status over time [25]; changes

have been described more frequently in MGMT methylated than in unmethylated cases [26]. Conversely, there are currently no studies investigating MGMT methylation status in the circulating DNA of glioma patients during treatment, or the clinical correlations of this status.

In the present study we aimed to: i) evaluate the agreement between MGMT methylation status in tumor tissue and plasma; ii) monitor MGMT methylation status in plasma before and during temozolomide treatment; iii) explore the value of MGMT promoter methylation status in plasma as a prognostic/predictive marker in glioma patients.

Material and Methods

Study population and biological samples

The study included patients who were referred to the Division of Neuro-Oncology of the Città della Salute e della Scienza Hospital in Turin, Italy between April 2008 and November 2011. Patients with a histologically confirmed diagnosis of glioma at different grades of malignancy, and a treatment protocol that included temozolomide and magnetic resonance imaging (MRI) examination every 3 months were invited to participate. In total 58 patients accepted to participate in the study.

The same paraffin-embedded tumor tissues used by the Neurological Ward for histological testing were used in the present analysis. Of the included patients 48 out of 58 had tumor tissue available for additional laboratory analyses. Blood samples were collected from all patients before temozolomide treatment (baseline) and at each subsequent MRI examination for a 12-month period.

All included patients signed an informed consent form for the DNA analysis of their tumor tissue and blood samples. The study protocol was approved by the local ethical committee.

Treatment regimens

Patients with glioblastoma received conventional radiotherapy with concomitant and adjuvant temozolomide treatment (1 cycle=150-200 mg/m2 daily for 5 days) every 28 days for up to 12 months, or until evidence of tumor progression or unacceptable toxicity. Patients with grade III gliomas received conventional radiotherapy and up to 12 cycles of adjuvant temozolomide treatment. Patients with grade II gliomas with a residual or progressive tumor after surgical resection received 12 cycles of temozolomide treatment. Some patients were treated with up to 12 cycles of dose-dense temozolomide (150mg/m2/daily 1 week ON-1 week OFF).

DNA extraction

Genomic DNA was extracted and purified from 3-5 (10 µm thick) sequential sections of paraffin embedded tumor tissue using the QIAamp® DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). Circulating DNA was extracted from plasma using the QIAamp DNA Blood Mini kit (Qiagen).

Methylation analysis

Extracted DNA underwent bisulfite modification using the Epitect Bisulfite Kit (Qiagen). Positive controls for methylated MGMT promoter (CpGenomeTM universal methylated DNA, Chemicon Co., Billerica, MA, USA) and unmethylated MGMT promoter (CpGenomeTM universal unmethylated DNA, Chemicon Co.) were included in each modification set.

After bisulfite modification, an end point PCR assay, using primers specific to either methylated or unmethylated DNA, was used to determine MGMT promoter methylation status. Primer sequences of MGMT were chosen according to published sequences [27, 28]

and were as follows for the unmethylated reaction: forward 5′-TTGTGTTTTGATGTTTGTAGGTTTTTGT-3′ and reverse 5′-AACTCCACACTCTTCCAAAAACAAAACA-3′; and for the methylated reaction: forward 5′-TTTCGACGTTCGTAGGTTTTCGC-3′ and reverse 5′-GCACTCTTCCGAAAACGAAACG-3′.

Each PCR reaction contained: 1 x Buffer, 2 mM MgCl2, 08 mM dNTPs, 500 nM of primers for methylated and unmethylated, 5 U Taq, 4 µl of and 10 µl of modified DNA for tumor tissue and plasma, respectively, and distilled water to obtain a final volume of 25 µl. The reaction was carried out under the following PCR conditions: 10 minutes at 95°C, 1 minute at 95°C, 1 minute at 59°C, 1 minute at 72°C for 45 cycles, and 10 minutes at 72°C. To increase the sensitivity, a second round of PCR assays with a specific probe for methylated DNA was performed using 1 µl of the first PCR product for tumor tissue, and 3 µl for plasma. The methylated probe was chosen according to the following published sequence, which targeted five CpG islands in the amplified region: FAM-CGCAAACGATACGCACCGCGA-BHQ1 [28].

Each PCR reaction contained: 1 x iQ Super Mix, 600 nM of primers, 200 nM of specific probe for MGMT promoter methylated, modified DNA and distilled water to obtain a final volume of 25 µl. The reaction was carried out in a Real Time thermocycler (iCycler, BioRad, Hercules, CA, USA) under the following PCR conditions: 1.30 minutes at 95°C, 15 seconds at 95°C, 1 minute at a primer-specific annealing temperature (60°C) for 50 cycles, and 10 minutes at 72°C. The universal methylated DNA CpGenome was used as a positive control. Negative controls were included in all reactions.

Statistical analysis

The clinical characteristics of patients at baseline were described by absolute and percentage frequencies according to tumor grade. To assess the agreement between MGMT promoter methylation status in tumor tissue and plasma, we used the Kappa-statistic. Overall survival was defined as survival between the time of blood sample collection at baseline and the date of death or the date of last follow-up. Progression-free survival was defined as survival between blood sample collection at baseline and the date of tumor progression or death.

We performed a Log-rank test to evaluate the equality of survivor functions according to MGMT promoter methylation status. Univariate and multivariate hazard ratios (HRs) of patient characteristics at baseline were estimated using Cox proportional hazard regression models. To explore the prognostic/predictive value of MGMT promoter methylation status, we considered two sets of variables that differed only in the sample type used to determine MGMT promoter methylation status: in model 1 we included MGMT promoter methylation status in tumor tissue, tumor grade and age; in model 2 we included MGMT promoter methylation status in plasma at baseline, tumor grade and age. As a sensitivity analysis, we assessed the concordance in MGMT promoter methylation status between tumor tissue and plasma, and evaluated overall and progression-free survival according to MGMT promoter methylation status in patients with high-grade gliomas (grade III glioma and glioblastoma) alone, and in patients with glioblastoma alone. To estimate HRs we performed Cox's proportional hazard regression in patients with high-grade gliomas alone and in glioblastoma alone. Because of the small sample size we evaluated only univariate estimates.

Results

The characteristics of the 58 patients are shown in Table 1. Paraffin-embedded tumor tissue was available for 48 out of 58 patients. We obtained an average of 129.5 ng/µL of DNA in tumor tissue (range 31.2-382.8 ng/µL) and 7.7 ng/µL of circulating DNA in plasma (range 5.6-11 ng/µL). We found similar amounts of circulating DNA in the 12 (20.7%) patients who underwent subtotal/total resection (7.3 ng/µL) and in the 46 (79.3%) patients with partial resection (7.0 ng/µL). Methylated MGMT promoter was found in the tumor tissue of 29 out of 48 (60.4%) patients with tumor tissue available, and in plasma at baseline of 24 out of 58 (41.4%) patients.

MGMT promoter methylation status was concordant in the tumor tissue and plasma at baseline of 42 out of 48 patients (87.5%, Kappa=0.75, 95% CI 0.57-0.93) (Table 2). When the analysis was performed for high-grade gliomas alone, we obtained a 92.5% agreement and Kappa=0.85 (95% CI 0.69-1.00), while for glioblastomas alone we obtained 93.7% agreement and Kappa=0.87 (95% CI 0.70-1.00).

Overall survival was significantly longer for patients with methylated MGMT promoter in tumor tissue compared to those with unmethylated MGMT promoter in tumor tissue (p-value 0.046) (Fig. 1A). A similar trend was observed when we performed separate sensitivity analyses on high-grade gliomas alone (8 grade III gliomas and 32 grade glioblastomas), (p-value 0.142) (Fig. 1B) and on glioblastomas alone (p-value 0.198) (Fig.1C), though it was not statistically significant. We could not analyze low-grade gliomas separately due to the small sample size. When we considered MGMT promoter methylation status in plasma at baseline we found similar results for all patients (p-value 0.262) (Fig. 2A), for high-grade gliomas alone (11 grade III and 35 grade IV) (p-value 0.096) (Fig. 2B) and for glioblastomas alone (p-value 0.035) (Fig. 2C).

The adjusted mortality risk was higher in patients with unmethylated MGMT promoter in tumor tissue (adjusted HR: 2.21, 95% CI 0.99-4.95) and plasma at baseline (adjusted HR: 2.19, 95% CI 1.02-4.68) (Table 3). When we considered the unadjusted mortality risk in patients with high-grade gliomas and glioblastomas separately, the same trend was observed (tumor tissue HR: 1.83, 95% CI 0.81-4.16 and HR: 1.80, 95% CI 0.73-4.45 for high-grade gliomas and glioblastomas, respectively; plasma at baseline HR: 1.90, 95% CI 0.88-4.10 and HR: 2.51, 95% CI 1.04-6.07, for high-grade gliomas and glioblastomas, respectively).

Progression-free survival also was longer for all glioma patients with methylated MGMT promoter than for those with unmethylated MGMT promoter in tumor tissue (p-value 0.004) (Fig. 3A). Sensitivity analyses on high-grade glioma patients alone (p-value 0.022) (Fig. 3B) and glioblastoma patients alone (p-value 0.129) (Fig. 3C) confirmed this trend. MGMT promoter methylation status in plasma at baseline for all three groups of patients (Fig. 4A, B, C) showed similar results.

The risk of tumor progression was higher in patients with unmethylated MGMT promoter in tumor tissue (adjusted HR: 2.30, 95% CI 1.19-4.45) and plasma (adjusted HR: 1.77, 95% CI 0.95-3.30) (Table 4). When tumor progression was considered in patients with high-grade gliomas alone, it was higher in patients with unmethylated MGMT promoter in tumor tissue (unadjusted HR: 2.16, 95% CI 1.10-4.24) and in plasma at baseline (unadjusted HR: 1.61, 95% CI 0.86-3.02). The same trend was found for glioblastoma patients (HR: 1.79, 95% CI 0.84-3.81 and HR: 1.65, 95% CI 0.81-3.36 in tumor tissue and plasma at baseline, respectively).

Cumulative incidence of MGMT promoter unmethylation in plasma was 58% at baseline and reached 93.4% at 12 months. One patient was lost to follow-up, two died without showing MGMT promoter unmethylation, and at 12 months one patient with methylated

MGMT promoter was still alive (Fig. 5). The cumulative incidence of MGMT promoter unmethylation showed a similar pattern at 12 months when considering glioblastomas alone.

Discussion

MGMT promoter methylation is now considered a reliable predictive marker for survival in glioma patients, who are treated with alkylating agents, such as temozolomide especially in those with glioblastomas [7]. The association between hypermethylation of the MGMT gene promoter in tumor tissue and improved outcome in terms of progression-free and overall survival found in this study is significant, and is consistent with other studies [13,29,30]. Similarly to other cancers (i.e. colorectal [31], ovarian [32], prostate [33], lung [34] cancer), it has been suggested that the detection of MGMT hypermethylation in the circulating DNA of glioma patients represents a reliable tool to assess residual disease or disease progression after temozolomide treatment [17-20]. Moreover, the assessment of MGMT promoter methylation status in plasma could be useful when tumor tissue is of inadequate quantity or quality (stereotactic biopsy specimens), or when this tissue is simply unavailable (inoperable tumors). However, a systematic investigation of this molecular marker in the plasma of glioma patients has not yet been performed.

In accordance with the published literature [17-20] we found a good agreement between MGMT promoter methylation status in tumor tissue and plasma. The small discrepancy we found in some patients (6/48) could be explained by the presence of circulating DNA derived both from normal and tumor cells, or by the heterogeneity of tumors, for which genetically different clones may coexist and differ in their ability to shed DNA [35]. We did not include healthy controls, as the majority of the studies on this topic have reported methylated MGMT promoter in a very low percentage of controls [21, 22, 27, 36].

Our results suggest that patients treated with temozolomide who show methylated MGMT promoter in either tumor tissue or plasma have a longer overall survival and a better progression-free survival. We investigated the possibility that MGMT promoter methylation could affects the response to alkylating agents in all grades of gliomas, and we found a similar trend in all the methylated patients, regardless of tumor grade. This suggests that MGMT promoter methylation status could be a good predictor of response to temozolomide treatment for patients with all grades of gliomas. When we performed separate analyses on high-grade gliomas and glioblastomas, we observed results that were similar to those seen in the entire case-series. When we performed separate analyses on patients with grade II and grade III gliomas, we did not observe informative curves (data not shown) due to the small size of these subgroups. Studies focused on low-grade gliomas with adequate sample sizes are needed to confirm the benefit of MGMT promoter methylation that has been demonstrated in other studies in this subgroup of patients [16, 37, 38].

MGMT promoter methylation in tumor tissue was associated with survival and progression-free survival, which is in agreement with published literature [13,29,30]; unmethylated patients showed an increased risk of mortality and tumor progression. The estimated effect of MGMT promoter methylation in tumor tissue was more consistent than that in plasma; the HR of MGMT promoter methylation in tumor tissue did not change after adjusting for tumor grade and age. These results suggest that MGMT promoter methylation in tissue, but not in plasma, may be useful as an independent prognostic marker, which is in line with previous evidence [13].

With our data is not possible to draw final conclusions on methylation in plasma as a predictor of tumor response to alkylating agents. The absence of a strong association between MGMT promoter methylation in plasma and mortality could be in part due to the potential for false-negative detection in plasma. Nevertheless, our results indicate that the detection of

methylated MGMT promoter in plasma has similar impact on response to treatment with temozolomide as its detection in tumor tissue, leading to the suggestion that methylated MGMT promoter methylation in plasma is predictive of a better response to therapy, and thus could be used as a surrogate of methylation detection in tumor tissue. Conversely, unmethylated MGMT promoter in plasma at baseline had no predictive value. To address this, the use of other molecular markers or clinical features to predict response to treatment in glioma patients must be investigated. Several studies on methylation in the plasma of patients with different cancer types have reported conflicting results, indicating the need to combine different methylation markers to reach an appropriate sensitivity [35, 39]. In particular the detection of MGMT promoter methylation in the circulating DNA of patients with different cancers has been performed using a variety of methods, [e.g., methylation-specific PCR (MSP), quantitative MSP (QMSP), fluorescent MSP (F-MSP)], and this may be a reason for the variability of results [21, 22, 27, 36, 40]. In our study we used an established primer pair to evaluate MGMT methylation status, which is recommended in routine clinical settings [9]; moreover the average MGMT methylation in tissue was in line with published data. However, critical issues in analyzing circulating DNA in terms of isolation, quantification, origin, significance and functional meaning still persist. Sequencing and characterization could increase the level of knowledge on this topic, as reported in a recent review [39], but false-negative marker detection cannot be excluded.

In this study, the monitoring of MGMT promoter methylation in plasma showed that almost all patients with methylated MGMT promoter at baseline (i.e. before treatment) had unmethylated MGMT promoter within 12 months. A possible explanation, taking into account that methylated and unmethylated foci may coexist [41], is that tumor cells with MGMT promoter methylation are more sensitive to damage from temozolomide, which could promote a selection of unmethylated tumor clones that are more resistant to alkylating agents.

A failure to detect a methylated signal due to significant lack of tumor tissue shedding DNA in plasma is unlikely, considering that we obtained similar amounts of circulating DNA from the plasma of serial blood samples. Other unknown biological mechanisms could also contribute to the switch from methylated to unmethylated status. It is unlikely that the potential for false-negative detection of MGMT promoter methylation in plasma during treatment affected the overall trend of the cumulative incidence of unmethylation. Indeed, we did not find any contrary switch from unmethylated to methylated status. Recent studies on glioblastomas analyzed MGMT promoter methylation in paired tissue samples of original and recurrent tumors after radio-chemotherapy, and observed that a proportion (61.5%) of methylated tumors became unmethylated at tumor recurrence [26]. Conversely, Feldsberg et al [42] found that the pattern of MGMT promoter methylation remained unchanged from original tumor to recurrence, thus suggesting that testing for MGMT promoter methylation at recurrence is unnecessary due to the development of alternative resistance mechanisms. We could not perform an evaluation of the methylation status at tumor recurrence because no cases of recurrence were observed during the 12-month follow-up period. We did not find any association between histology and the probability of changing methylation status in plasma, likely due to the few cases of low-grade gliomas present in our case-series compared with the high proportion of grade III gliomas and glioblastomas.

The main limitation of our study is the small sample size, which hampered a meaningful statistical analysis by tumor grade. Nevertheless, to our knowledge, this is the first study evaluating MGMT promoter methylation status in plasma over time in glioma patients. Only one recent study on prostate cancer evaluated plasma at baseline and at two time points during chemotherapy [43]. Interestingly, these authors reported a trend in loss of methylation, which is in line with our findings.

In conclusion, this study confirms that the presence of MGMT promoter methylation in the plasma of glioma patients can reflect the methylation status in tumor tissue, and could represents a good surrogate when adequate tumor tissue is not available for analysis. However we suggest caution: when unmethylated MGMT promoter status in plasma is detected, the use of a panel of different markers to increase sensitivity is advisable. Moreover, we suggest that the analysis of MGMT promoter methylation in plasma could be useful to monitor the follow-up of methylated patients. This approach could help to identify when the tumor switches from methylated to unmethylated status, and thus the possible emergence of treatment resistance.

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Conflict of interests

The authors declare no conflict of interest.

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Fig. 1 Overall survival by MGMT promoter methylation status in tumor tissue in all patients (A), in high-grade glioma patients (B) and in glioblastoma patients (C)

Fig. 2 Overall survival by MGMT promoter methylation status in plasma at baseline in all patients (A), in high-grade glioma patients (B) and in glioblastoma patients (C)

Fig. 3 Progression-free survival by MGMT promoter methylation status in tumor tissue in all patients (A), in high-grade glioma patients (B) and in glioblastoma patients (C)

Fig. 4 Progression-free survival by MGMT promoter methylation status in plasma at baseline in all patients (A), in high-grade glioma patients (B) and in glioblastoma patients (C)

Fig. 5 Cumulative incidence of MGMT promoter unmethylation in plasma

Figure

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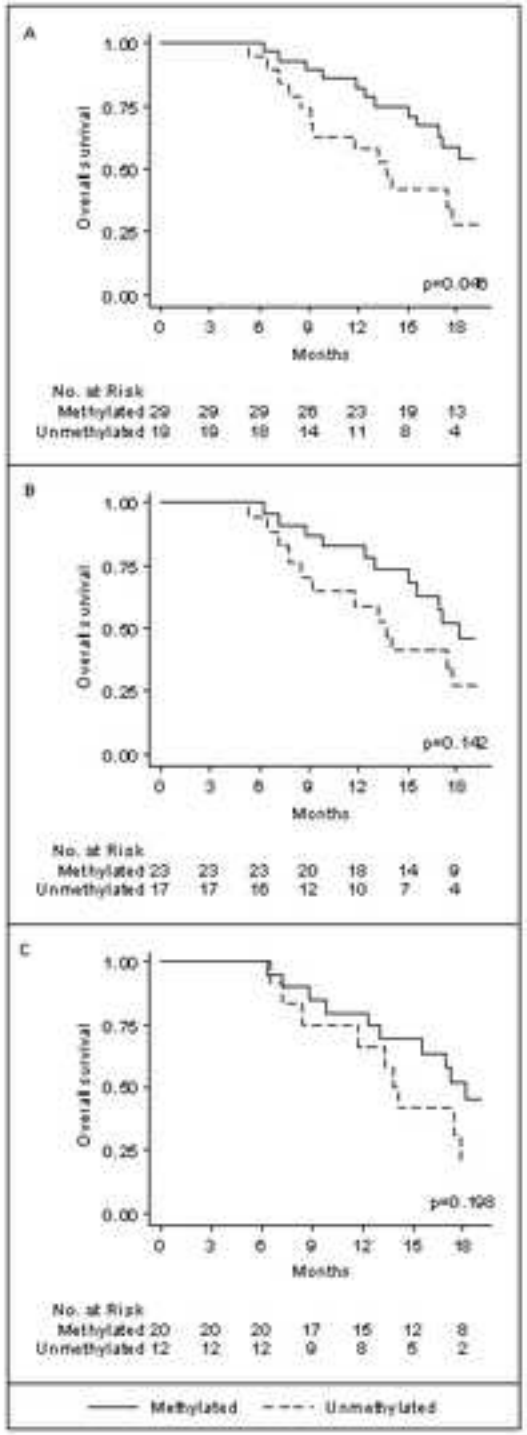

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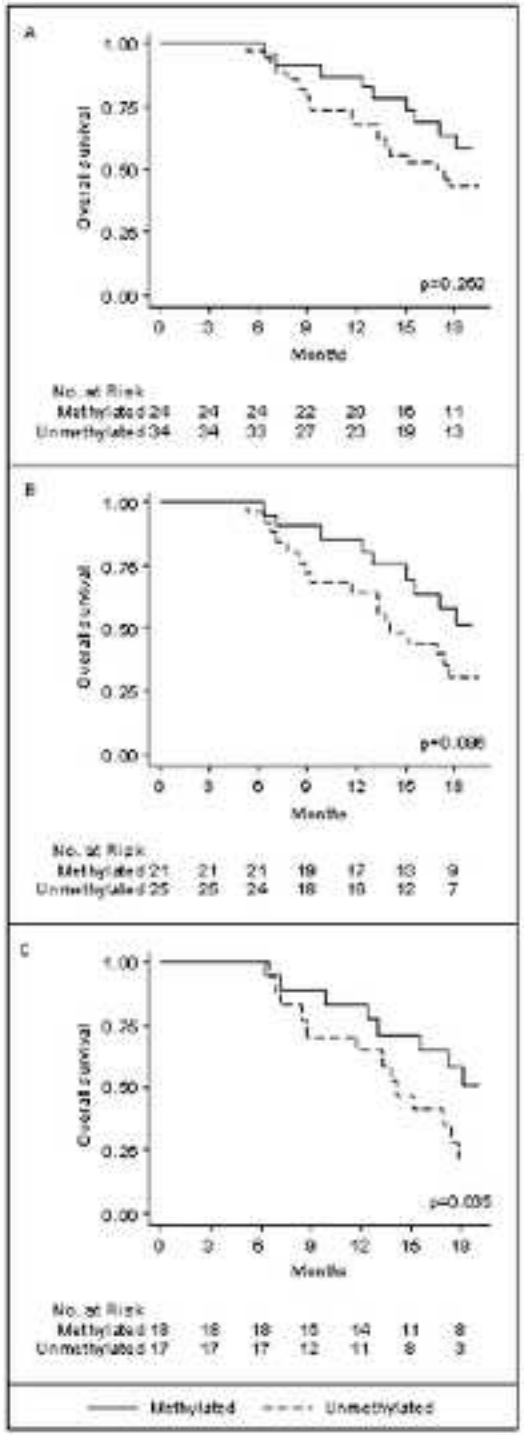

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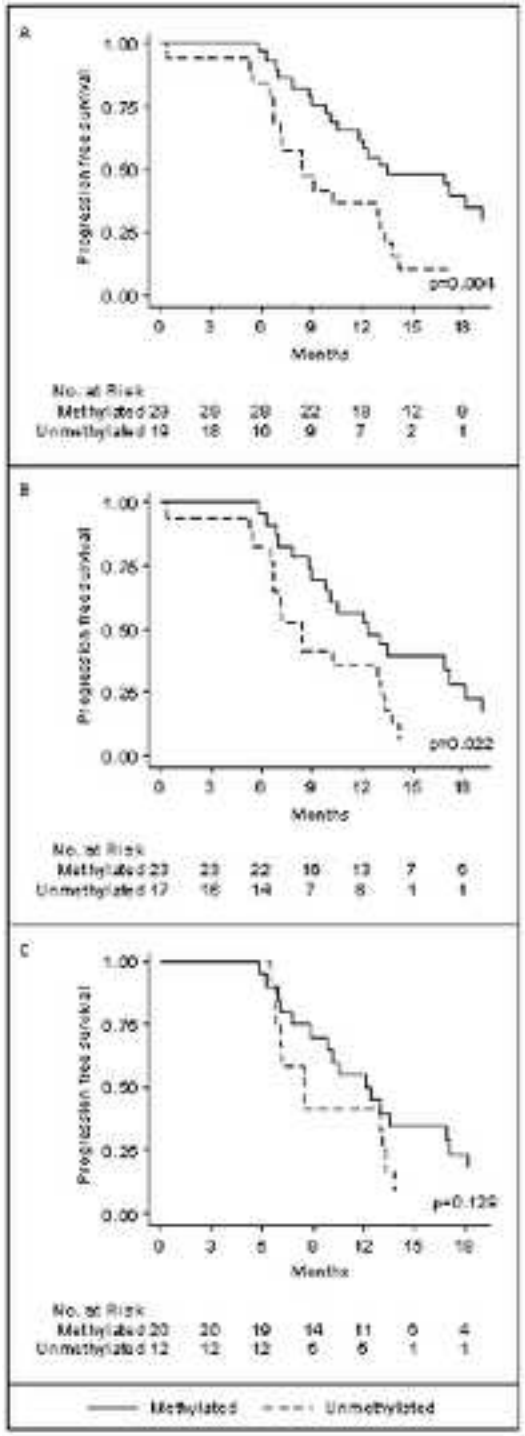

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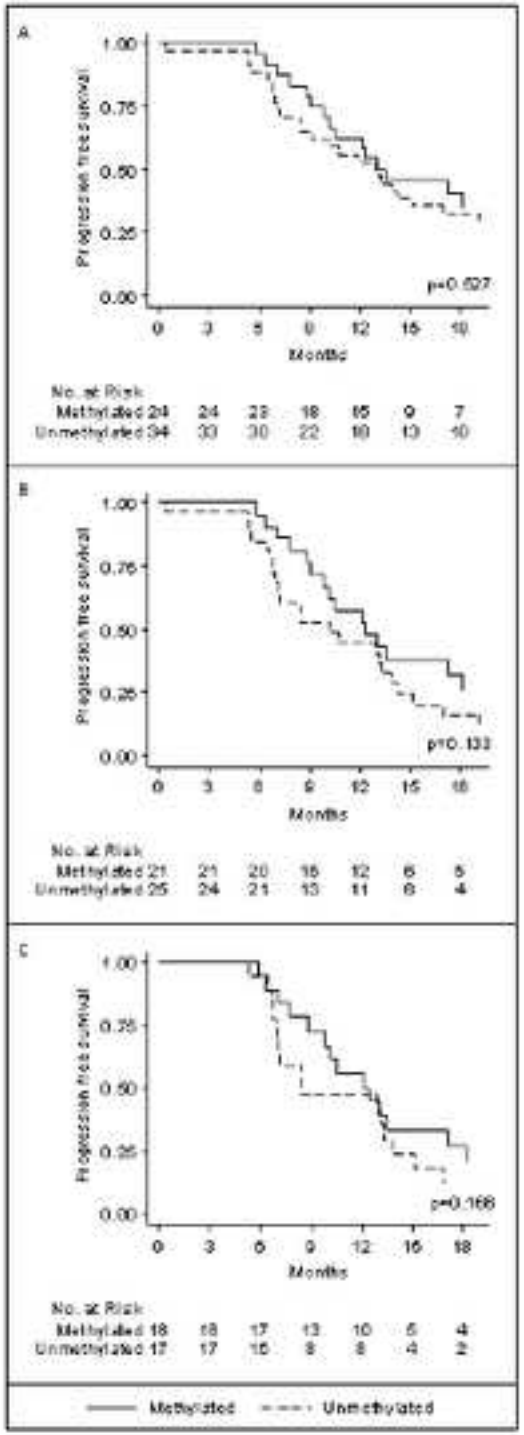

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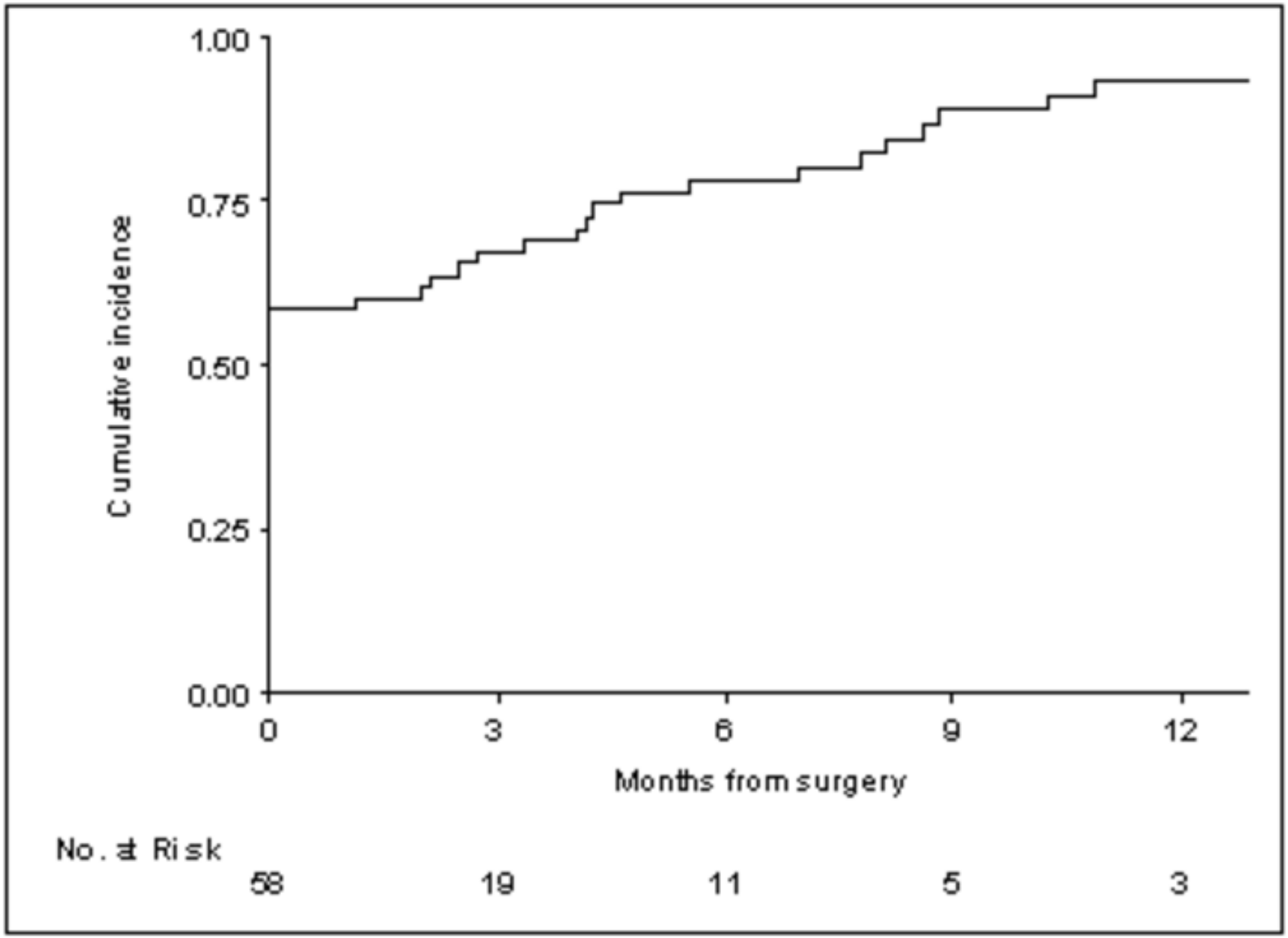

Table

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Table 1. Patients characteristics

	Grade							
	2*		3**		4		Total	
	No.	%	No.	%	No.	%	No.	%
Gender								
Male	8	66.67	7	63.64	23	65.71	38	65.52
Female	4	33.33	4	36.36	12	34.29	20	34.48
Age								
<60	9	75	7	63.64	11	31.43	27	46.55
>=60	3	25	4	36.36	24	68.57	31	53.45
Radical_surgery								
Biopsy/Partial resection	12	100	10	90.91	24	68.57	46	79.31
Radical resection	0	0	1	9.09	11	31.43	12	20.69
Tumor tissue status								
Methylated	6	75	3	37.5	20	62.5	29	60.4
Unmethylated	2	25	5	62.5	12	37.5	19	39.6
Missing	4	-	3	-	3	-	10	-
Plasma status								
Methylated	3	25	3	27.27	18	51.43	24	41.38
Unmethylated	9	75	8	72.73	17	48.57	34	58.62
Total	12	100	11	100	35	100	58	100

* Grade II : 2 Astrocytomas, 8 Oligoastrocytomas, 2 Oligodendrogliomas

** Grade III: 5 Anaplastic Astrocytomas, 2 Anaplastic Oligoastrocytomas, 4 Anaplastic Oligodendrogliomas

Table 2. Concordance of MGMT promoter methylation status in tumor tissue and in plasma

	Plasma				
	Methylated		**Unmethylated**		
	No.	%	No.	%	Total
Tumor tissue					
Methylated	23	79.3	6	20.7	29
Unmethylated	0	0.00	19	100.0	19
Total	23		25		48
Kappa = 0.75, 95%CI 0.57- 0.93					

Table 3 Unadjusted and adjusted effects of prognostic factors of mortality

	Unadjusted effect		**Model 1 ***		**Model 2 §**	
	Hazard ratio	95% CI	Hazard ratio	95% CI	Hazard ratio	95% CI
MGMT status tumor tissue						
Methylated	1		1			
Unmethylated	2.18	[0.99,4.80]	2.21	[0.99,4.95]		
MGMT status plasma						
Methylated	1				1	
Unmethylated	1.52	[0.73-3.18]			2.19	[1.02,4.68]
Grade						
2	1		1		1	
3-4	5.41	[1.29,22.78]	1.94	[0.44,8.60]	4.71	[1.05,21.06]
Age						
<=60	1		1		1	
>60	2.62	[1.21-5.65]	1.89	[0.80,4.50]	2.16	[0.97,4.82]

* Model 1: Methylation status in tumor tissue, tumor grade and age.

§ Model 2: Methylation status in plasma at baseline, tumor grade and age.

Table 4 Unadjusted and adjusted effects of prognostic factors of progression free survival

	Unadjusted effect		**Model 1 ***		**Model 2 §**	
	Hazard ratio	95% CI	Hazard ratio	95% CI	Hazard ratio	95% CI
MGMT status tumor tissue						
Methylated	1		1			
Unmethylated	2.55	[1.32,4.93]	2.30	[1.19,4.45]		
MGMT status plasma						
Methylated	1				1	
Unmethylated	1.22	[0.66-2.25]			1.77	[0.95,3.30]
Grade						
2	1		1		1	
3-4	10.24	[2.46,42.59]	5.24	[1.22,22.47]	9.58	[2.22,41.24]
Age						
<=60	1		1		1	
>60	2.42	[1.27,4.62]	1.26	[0.63,2.51]	1.67	[0.86,3.22]

* Model 1: Methylation status in tumor tissue, tumor grade and age.

§ Model 2: Methylation status in plasma at baseline, tumor grade and age.