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THE EFFECTS OF O^6-METHYL GUANINE DNA-METHYL TRANSFERASE PROMOTOR METHYLATION AND CpG1, CpG2, CpG3 AND CpG4 METHYLATION ON TREATMENT RESPONSE AND THEIR PROGNOSTIC SIGNIFICANCE IN PATIENTS WITH GLIOBLASTOMA

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ABSTRACT

This retrospective study examined the prognostic significance and treatment effect of promoter methylation of O^6- methyl guanine methyl transferase (MGMT) and meth-ylation of CpG1, CpG2, CpG3 and CpG4 in glioblastoma (GB) patients received postoperative radiotherapy (PORT), with or without adjuvant temozolomide (TMZ). One hundred patients with GB who received PORT with concomitant TMZ plus adjuvant TMZ or PORT alone, were included. The MGMT promoter methylation of CpG1, CpG2, CpG3 and CpG4 islands were examined. Overall, MGMT-methylation emerged as a significant prognostic factor for better overall survival (OS) and progression-free survival (PFS) [odds ratio (OR): 0.609, 95% confidence interval (95% CI): 0.395-0.939, $p = 0.02$; OR: 0.662, 95% CI: 0.430-1019, $p = 0.5$, respectively]. The methylation of each CpG1, CpG2, CpG3 and CpG4 islands was found to have no significant effects on OS and the methylation of each CpG1, CpG2 and CpG4 islands had no significant effect on PFS ($p < 0.05$ for all). On the other hand, the methylation of CpG3 had a positive prognostic effect on PFS (OR: 2.1, 95% CI: 0.99-4.67, $p = 0.04$). In the group that only received radiotherapy (RT), CpG1 and CpC3 methylations were found to have a positive prognostic significance in terms of PFS (OR: 266, 95% CI: 1.05-6.75, p -0.03 for CpG1; OR: 2.4, 95% CI: 1.01-5.92, $p = 0.04$ for CpG3). The MGMT promoter methylation represents an important biomarker for predicting response to therapy. Individual islands, particularly CpG3, deserves further investigation as a prognostic marker. Further studies need to be done with larger sample sizes to clarify the results.

Keywords: Glioblastoma (GB); O^6-Methyl guanine methyl transferase (MGMT) methylation; Radiotherapy (RT).

INTRODUCTION

Glioblastoma (GB) is not only the most prevalent primary tumor of the brain, but also has an aggressive clinical course with poor prognosis [1-3]. Standard management includes wide resection of the tumor and postoperative radiotherapy (PORT) with concomitant temozolomide (TMZ) followed by maintenance TMZ [4-6].

Although inclusion of TMZ in the treatment protocols was shown to improve survival in patients with GB, eventual progression occurs in approximately 70.0% of the subjects within 1 year [7]. Patients usually exhibit a variable response to TMZ [8], which induces apoptosis by damaging DNA through binding to alkyl groups responsible for DNA repair [9].

O^6-Methyl guanine methyl transferaseis (MGMT), a repair protein for cellular DNA that quickly reverses alkyl-ation and methylation of guanine at the O^6 position [9], neutralizing the cytotoxic effects of alkylating agents [10]. O^6-Methyl guanine methyl transferase is synthesized in all human tissues [11]. The majority of our current understanding of MGMT as a DNA repair protein is based on observations after exposure to alkylating agents [12]. The protective role of MGMT against cytotoxic effects of al-kylating agents has been shown in human cell line xenograft models and in MGMT transgenic mice [13,14]. The MGMT-mediated repair mechanism is unique and different from other DNA repair pathways in that it does

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not directly represent a component of the repair process and acts independently. It specifically separates the methyl group at the O^6 position, thereby repairing the nucleotide without any breaks in DNA fiber congruous with its natural form. It is referred to as a self-destruction (suicide) enzyme [11], as the enzyme is irreversibly inactivated due to the transfer of the alkyl group to the cysteine residue in the active part of MGMT. Therefore, the maintenance of activity of the enzyme is dependent upon new protein synthesis. When the process is saturated, the excess O^6-methyl guanine depletes MGMT [11]. Although the O^6 position of guanine is not a general target for alkylating agents, the consequently occurring pro-mutagenic lesions represent important inducers of cytotoxicity and apoptosis. If left unrepaired, this modified guanine may be paired with thymine, which activates the mismatch repair (MMR) pathway, instead of pairing with cytosine. Mismatch repair discards O^6-methyl guanine in the main strain, only focusing on the newly synthesized strain and repairing it. When the MMR pathway is attempting to repair this false pair, it enters into new cycles of synthesis and repair. The end result consists of double strain breaks in DNA, activating the apoptotic pathways and inducing cell death [14]. The increment in MGMT activity in tumor cells is related to the resistance to alkylating agents. Nevertheless, due to the promotor methylation in tumor cells, the epigenetic silence of MGMT results in low MGMT expression [15]. The MGMT is considered as a potential prognostic marker in patients with GB and it has also been shown to have a prognostic value in TMZ response [16]. In this study involving GB patients receiving adjuvant concomitant TMZ in addition to PORT and in patients receiving RT only, the effect of promotor methylation of MGMT on treatment response, its prognostic importance, as well as the effect of methylation of CpG1, CpG2, CpG3 and CpG4 were explored.

MATERIALS AND METHODS

Patient Groups and Study Design. In this retrospective study, 100 patients diagnosed with GB who received PORT with concomitant TMZ plus adjuvant TMZ or PORT alone at the Department of Radiation Oncology, Erciyes University Faculty of Medicine, Kayseri, Turkey, were included. The study protocol was approved by the local ethics committee. Clinical target volume (CTV) was determined by delineating a 1.5 to 2.0 cm margin to the peri-tumoral edema in magnetic resonance imaging (MR1) T2-weighted images prior to surgery. For the planned target volume (PTV), a 5 mm safety margin was also defined for the clinical target volume. During simulation and treatment, stabilizing thermoplastic head masks were used. The total planned dose was 60 Gray (Gy) delivered in 30 fractions, 5 days/week, with 2 Gy doses daily. In patients receiving TMZ, 75 mg/m2/day of TMZ was given 1 hour before RT and concomitantly with RT, followed by six additional courses of TMZ starting 4 weeks after completion of RT (150-200 mg/m2/day, day 1 to 5 in 28 day-cycles).

Preparation of the Samples. The paraffin-embedded tumor tissue samples obtained during surgery were retrieved from the archives of the Department of Pathology, Erciyes University, Faculty of Medicine, Kaysery, Turkey. The paraffin blocks were placed in a block-holder apparatus of the microtome. The superficial tissue layer was thinly-shaved to access the main tissue sample. Five sections, each 4 μm thick, were obtained and placed in 2 mL Eppendorf tubes.

Isolation and Preparation of Genomic DNA. In order to obtain genomic DNA from paraffin-embedded tissue sections, OIAamp DNA formalin-fixed, paraffin-embedded (FFPE) tissue kit (Qiagen GmbH, Hilden, Germany) was utilized in accordance with the manufacturer's instructions. The kit uses QIAampMinElute (Qiagen GmbH) spin columns for purification of high-quality DNA in small volumes. Epitect-Bisulfide kit was used for bisulfide conversion of genomic Qiagen DNA (Qiagen GmbH). After completion of bisulfide conversion, polymerase chain reaction (PCR) tubes were subjected to a short cen-trifugation, followed by purification of DNA through several steps. The PCR and PCR products were immobilized with streptavidinesepharose high performance beads in order to be analyzed through PyroMark Q24 (Qiagen GmbH). Then single-stranded DNA was prepared in PyroMark Q24 (Qiagen GmbB) for pyrosekans analysis. For pyrosequence analysis, main DNAs and their sequence primers are coupled together. PyroMark gold Q24 indicator is installed into Pyromark Q24 allowing obtainment of the analysis results (Qiagen GmbH).

Analyses of the Results. For the evaluation of the results, one control DNA sample was used for nine patients. Healthy donor blood was used as the control DNA sample. The methylation status of CpG1, CpG2, CpG3 and CpG4 islands were assessed. A thymine peak higher than 10.0% on any of the CpG islands was accepted as positive methylation (Figure 1).

Statistical Analyses. Data were analyzed by SPSS (Statistical Package for the Social Sciences) version 22 for Windows (IBM Inc., Armonk, NY, USA). Normality of the distribution was analyzed with one sample Kolmogorov-Smirnov test. For the comparison of categorical variables, the χ^2 test was used. Overall survival and time to progression were compared between patient groups with the Kaplan-Meier test (log-rank). Cox regression analysis was used to determine the independent variables for overall and progression-free survivals. Statistical significance was set at a p value of less than 0.05.

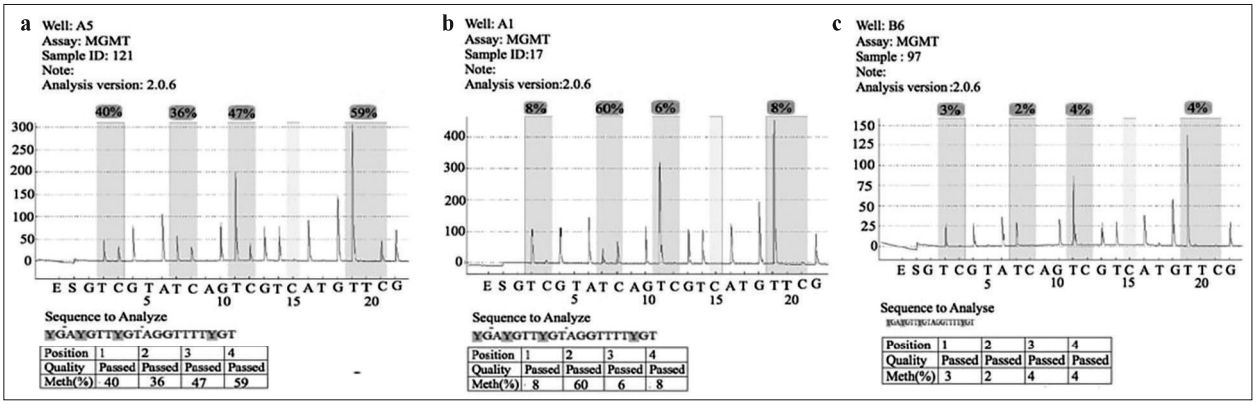

Figure 1. A positive case for MGMT-methylation (in the CpG1 island there was 40.0%, in the CpG2 island 36.0%, in the CpG3 island 47.0% and in the CpG4 island 59.0% positivity was detected) **(a)**. A positive case positive for MGMT-methylation (in the CpG2 island there was 60.0% positivity) **(b)**. A case with negative MGMT-methylation (in the CpG1 island there was 3.0%, in the CpG2 island 2.0%, in the CpG3 island 4.0% and in the CpG4 island 4.0% positivity was detected) **(c)**.

RESULTS

Median age was 53 years (18-73 years). DNA assessment of samples from patients yielded 52 patients (52.0%) with and 48 cases (48.0%) without promoter methylated MGMT (Table 1). Overall median, overall survival (OS) and progression free survival (PFS), were 10 and 6 months, respectively. Cox regression was used to evaluate the prognostic importance of MGMT-methylation, age, treatment and gender in terms of survival. Accordingly, MGMT-methylation emerged as a significant prognostic factor for OS and PFS [odds ratio (OR): 0.609, 95% confidence interval

Table 1. Patient characteristics.

Characteristics	All Patients		MGMT-Methylated		MGMT-Unmethylated	
	n	%	*n*	%	*n*	%
Gender:						
males	65	65.0	36	69.2	29	60.4
females	35	35.0	16	30.8	19	39.6
Age (years):						
<60	67	67.0	38	73.1	29	60.4
>60	33	33.0	14	26.9	19	39.6
Tumor location:						
frontal lobe	17	17.0	11	21.2	6	12.5
parietal lobe	25	25.0	13	25.0	12	25.0
temporal lobe	17	17.0	8	15.4	9	18.8
occipital lobe	3	3.0	1	1.9	2	4.2
basal ganglion	2	2.0	1	1.9	1	2.1
brain stem	2	2.0	1	1.9	1	2.1
cerebellum	5	5.0	1	1.9	4	8.3
multi-lobar	29	29.0	16	30.8	13	27.1
Number of tumors:						
single	96	96.0	49	94.2	47	97.9
multiple	4	4.0	3	5.8	1	2.1
Type of surgery:						
total resection	79	79.0	41	78.8	38	79.2
subtotal resection	17	17.0	7	13.5	10	20.8
biopsy	4	4.0	4	7.7	0	00.0
Treatment:						
RT+TMZ	55	55.0	23	44.2	32	66.7
RT only	45	45.0	29	55.8	16	33.3
Recurrence location:						
in the area	31	31.0	11	21.2	20	41.7
out of the area	37	37.0	21	40.4	16	33.3
in+out of area	14	14.0	9	17.3	5	10.4
Second series treatment	21	21.0	15	28.8	6	12.5
	2	2.0	1	1.9	1	2.1
	4	4.0	1	1.9	3	6.3
	73	73.0	24.0	67.3	38	79.2

MGMT: O^6-methyl guanine-methyl transferase; RT: radiotherapy; TMZ: temozolomide.

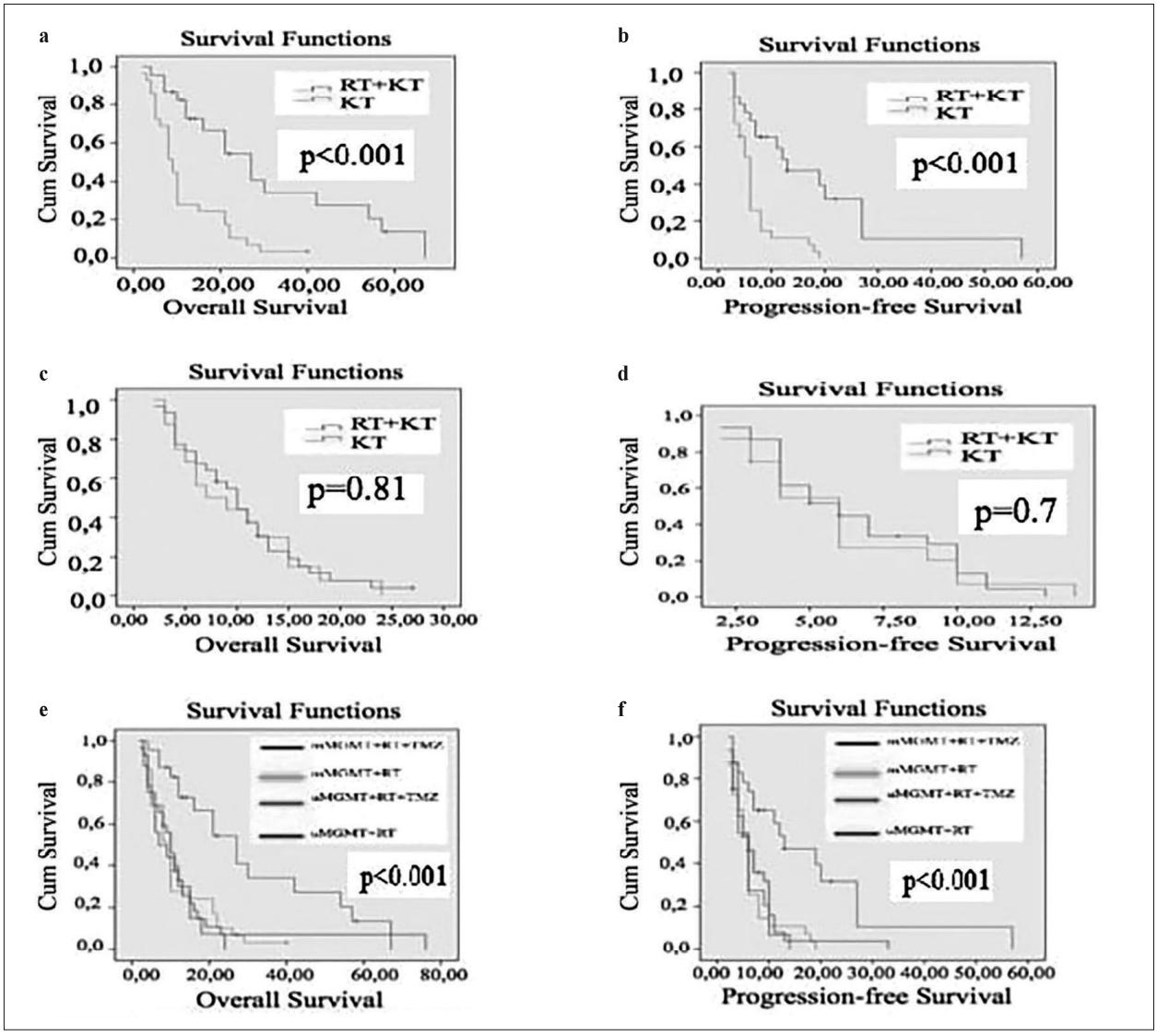

Figure 2. Overall survival curve for patients with MGMT-methylation receiving RT+TMZ and RT only **(a)**. Progression free survival curve for patients with MGMT-methylation receiving RT+TMZ and RT only **(b)**. The OS curve for patients without MGMT-methylation receiving RT+TMZ and RT only **(c)**. The PFS curve for patients without MGMT-methylation receiving RT+TMZ and RT only **(d)**. The OS curve for patients with and without MGMT-methylation receiving RT+TMZ and RT only **(e)**. The PFS curve for patients with and without MGMT-methylation receiving RT+TMZ and only RT **(f)**.

(95% CI): 0.395-0.939, $p = 0.02$; OR: 0.662, 95% CI: 0.430-0.1019, $p = 0.05$]. Inclusion of TMZ in the treatment protocol was also found to have prognostic significance in terms of OS and PFS (OR: 0.546, 95% CI: 0.353-0.939, $p = 0.006$; OR: 0.515, 95% CI: 0.332-0.789, $p = 0.03$). Compared to patients over 60 years of age, younger patients had a better OS, although the difference was insignificant for PFS (OR: 0.493, 95% CI: 0.313-0.777, $p = 0.002$; OR: 0.809, 95% CI: 0.521-1.254, $p = 0.3$). Gender was not a significant factor for prognosis (OR: 0.955, 95% CI: 0.618-1.476, $p = 0.8$; OR: 1.033, 95% CI: 0.665-1.605, $p = 0.8$) (Tables 2 and 3).

Of the 52 patients (52.0%) with MGMT-methylation, 23 (44.2%) received RT+TMZ, and 29 (55.8%) received RT alone (Table 1). Median age of the patients was 51 years (19-69 years). In patients with MGMT promotor methylation, median OS and PFS were 12 and 6 months, respectively. Overall and progression-free survival among patients with methylation who received RT plus TMZ or RT only were compared using Kaplan-Meier curves. The mean OS for the group receiving RT+TMZ was 27 months (95% CI: 16.91-37.08) *vs.* 9 months in the RT only group (95% CI: 6.75-11.25) ($p < 0.001$) (Figure 2). Furthermore, patients with MGMT-methylation receiving RT+TMZ had a 1-, 2- and 3-year OS of 82.0, 54.0 and 34.0%, respectively, whereas the corresponding figures among those who received RT only were 24.0, 10.0 and 3.0%.

Based on Cox regression model, addition of TMZ to RT was found to have prognostic significance in terms of OS and PFS in patients with MGMT-methylation (OR: 0.293, 95% CI: 0.146-0.587, p <0.001; OR: 0.285, 95 % CI: 0.142-0.575, p <0.001). A patient older than 60 years *vs.* younger than 60 was also found to represent a positive prognostic factor for OS, although not for PFS (OR: 0.524, 95% CI: 0.267-1.031, p = 0.05; OR: 0.872, 95% CI: 0.451-1.687, p = 0.6). Gender had no significant prognostic effects (OR: 1.066, 95% CI: 0.564-2.015, p = 0.8; OR: 1.3, 95% CI: 0.658-2.568, p = 0.4) (Tables 2 and 3).

Of the 48 cases (48%) without MGMT-methylation; 32 (66.7%) received RT+TMZ and 16 (33.3%) received RT alone (Table 1). Median age of the cases was 56 (19-73 years). Median OS and PFS were 10 and 6 months, respectively. The OS and PFS were determined using Kaplan-Meier estimates in the patients receiving either RT+TMZ or RT alone who had no MGMT-methylation. Those receiving RT+TMZ or RT alone, had a median OS of 10 and 7 months, respectively (p = 0.81). The PFS was 6 months in both of these groups (p = 0.71) (Figure 2). One-year and 2-year survival rates in those receiving RT+TMZ or RT alone were 38.0 and 3.0 *vs.* 30.0 and 0.0%, respectively. In these patients, addition of TMZ to RT had no prognostic effect in terms of OS and PFS (OR: 0.930, 95% CI: 0.469-1.745, p = 0.8; OR:0.9, 95% CI: 0.472-1.714, p = 0.7). A patient under the age of 60 years was found to have a positive prognostic influence on OS, with no effect on PFS (OR: 0.485, 95% CI: 0.256-0.920, p = 0.02; OR: 0.952, 95% CI: 0.516-1.756, p = 0.8). Gender had no effect on prognosis (OR: 0.999, 95% CI: 0.541-1.844, p = 0.9; OR: 1.04, 95% CI: 0.564-1.920, p = 0.8) (Tables 2 and 3).

A comparison of methylated *vs.* unmethylated MGMT patients receiving RT+TMZ in terms of OS and PFS showed a significant difference for the combination of methylated MGMT and RT+TMZ (p <0.001 for OS and PFS), while no such differences were observed between the other groups (Figure 2).

The effect of the methylation/unmethylation of CpG1, CpG2, CpG3 and CpG4 islands on OS and PFS were assessed by Kaplan-Meier analysis (log rank) in the RT+TMZ group. Methylation/unmethylation of CpG1, CpG2, or CpG3 islands did not have a significant effect on OS (p = 0.07 for CpG1 and CpG2, p = 0.3 for CpG3). In addition, methylation/unmethylation of CpG1, CpG2, or CpG3 islands had no significant effect on PFS (p = 0.3 for CpG1 and CpG2, p = 0.6 for CpG3). As the CpG4 islands was methylated in all cases, no comparison could be performed.

Similarly, the effect of methylation status (methylated/unmethylated) of CpG1, CpG2, CpG3 and CpG4 islands on OS and PFS was evaluated by Kaplan-Meier analysis (log rank) in groups receiving RT alone. The effect of methylation of CpG1, CpG2, and CpG3 islands on PFS was significant (p = 0.01 for CpG1; p = 0.02 for CpG2; p = 0.01 for CpG3). However, no significant effect was detected for CpG4 (p = 0.9) (Figure 3).

In the evaluation of the MGMT methylated group with 52 cases, within group adjustments were made and the effect of methylation/unmethylation status of CpG1, CpG2, CpG3 and CpG4 islands on OS and PFS were estimated by Kaplan-Meier analysis (log rank). Only methylation in CpG3 had a significant effect (p = 0.02) (Figure 3).

Prognostic factors were assessed using the Cox regression test. Among patients receiving RT+CT (chemotherapy), the methylation of CpG1, CpG2, CpG3 and CpG4 islands did not have a significant impact on OS and PFS (p = 0.1; p = 0.1; p = 0.4; the p value could not be

Table 2. Multivariate analysis according to risk factors for overall survival.

	All Patients			MGMT-Methylated			MGMT-Unmethylated		
Parameters	OR	95% CI	p Value	OR	95% CI	p Value	OR	95% CI	p Value
Age	0.493	0.313-0.777	0,0002	0.524	0.267-1.031	0.05	0.485	0.256-0.920	0.02
Gender (males/females)	0.955	0.618-1.476	0.8	1.066	0.564-2.015	0.8	0.999	0.541-1.844	0.9
TMZ treatment included	0.546	0.353-0.845	0.006	0.293	0.146-0.587	<0.0001	0.930	0.496-1.745	0.8
MGMT-methylation	0.609	0.395-0.939	0.02	–	–	–	–	–	–

MGMT: O^6-methyl guanine-methyl transferase; TMZ: temozolomide.

Table 3. Multivariate analysis according to risk factors for progression-free survival.

	All Patients			MGMT-Methylated			MGMT-Unmethylated		
Parameters	OR	95% CI	p Value	OR	95% CI	p Value	OR	95% CI	p Value
Age	0.809	0.521-1.254	0.3	0.872	0.451-1.687	0.6	0.952	0.516-1.756	0.8
Gender (males/females)	1.033	0.665-1.605	0.8	1.3	0.658-2.568	0.4	1.04	0.564-1.920	0.8
TMZ treatment included	0.515	0.332-0.798	0.03	0.285	0.142-0.575	<0.0001	0.90	0.472-1.714	0.7
MGMT-methylation	0.662	0.430-1.019	0.05	–	–	–	–	–	–

MGMT: O^6-methyl guanine-methyl transferase; TMZ: temozolomide.

evaluated for CpG4). The methylation of CpG2 and CpG4 had no significant effect on PFS, whereas the methylation of CpG1 and CpG3 was found to have a positive prognostic significance ($p = 0.03$ for CpG1; $p = 0.04$ for CpG3). The methylation of CpG3 island was found to have positive prognostic significance for PFS ($p = 0.05$) (Tables 4 and 5).

Among the overall group of 100 patients, the methylation of CpG1, CpG2, CpG3 and CpG4 was found to have no significant effects on OS, and the methylation of CpG1, CpG2 and CpG4 islands had no significant effect on PFS. The methylation of CpG3, on the other hand, had a positive prognostic effect on PFS ($p = 0.04$) (Tables 4 and 5).

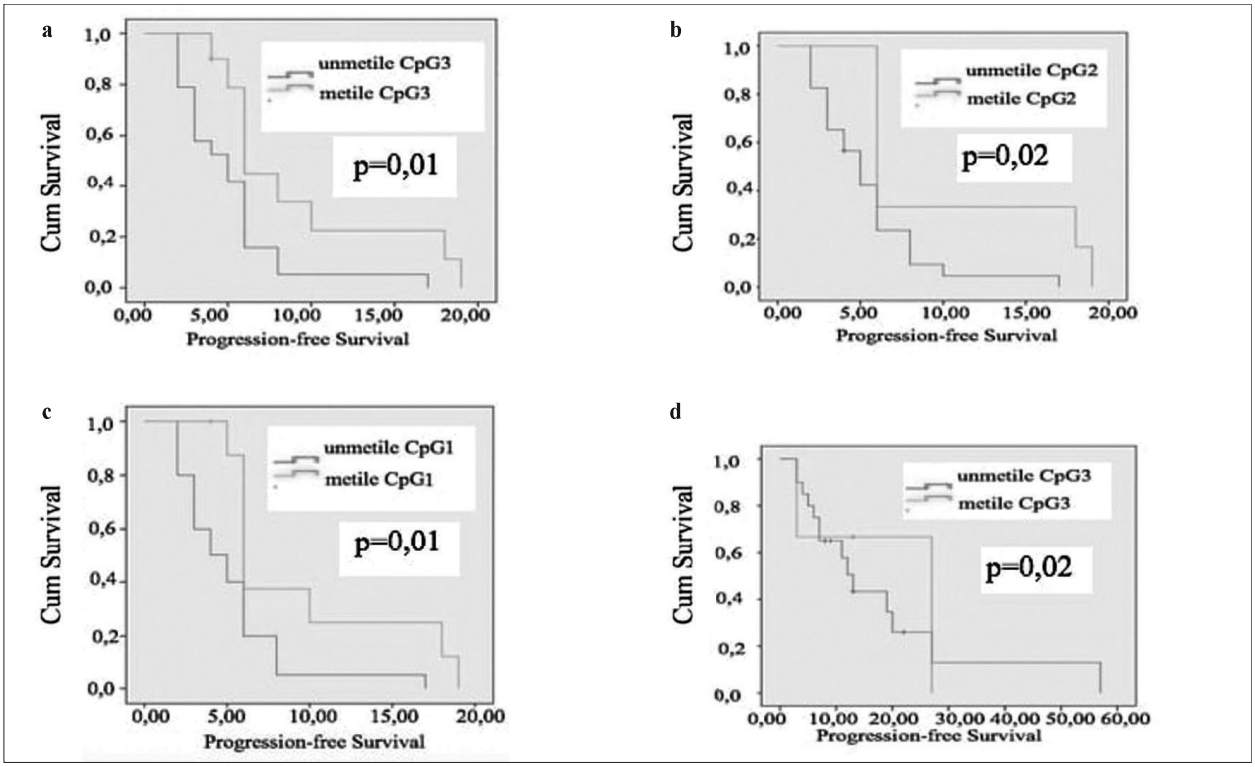

Figure 3. The PFS curve for patients receiving RT and positive for CpG3 island methylation **(a)**. The PFS curve for patients receiving RT and positive for CpG2 island methylation **(b)**. The PFS curve for patients with CpG1 island methylation and receiving RT **(c)**. The PFS curve for patients with CpG3 island methylation and receiving RT+TMZ and RT **(d)**.

Table 4. Multivariate analysis according to CpG island methylation status for overall survival.

	Methylated MGMT Patients Receiving RT+TMZ			Methylated MGMT Patients Receiving RT			Methylated MGMT Patients			All Patients		
	OR	95% CI	*p* Value	OR	95% CI	*p* Value	OR	95% CI	*p* Value	OR	95% CI	*p* Value
CpG1	0.243	0.044-1.332	0.07	2.077	0.867-4972	0.09	1.552	0.701-3.437	0.2	1.552	0.701-3.437	0.2
CpG2	0.243	0.044-1.332	0.07	1.490	0.598-3.713	0.3	1.121	0.498-2.523	0.7	1.121	0.498-2.523	0.71
CpG3	0.533	0.115-2.477	0.4	1.818	0.788-4.193	0.1	1.479	0.692-3.158	0.3	1.479	0.692-3.158	0.3
CpG4	–	–	–	0.325	0.041-2.603	0.3	0.325	0.041-2.603	0.2	0.325	0.041-2.603	0.2

MGMT: O^6-methyl guanine-methyl transferase; RT: radiotherapy; TMZ: temozolomide.

Table 5. Multivariate analysis according to CpG island methylation status for progression-free survival.

	Methylated MGMT Patients Receiving RT+TMZ			Methylated MGMT Patients Receiving RT			Methylated MGMT Patients			All Patients		
	OR	95% CI	*p* Value	OR	95% CI	*p* Value	OR	95% CI	*p* Value	OR	95% CI	*p* Value
CpG1	0.482	0.103-2.255	0.3	2.666	1.052-6.757	0.03	1.977	0.866-4.513	0.1	1.977	0.866-4.513	0.1
CpG2	0.482	0.103-2.255	0.3	2.838	0.958-8.404	0.05	1.931	0.779-4.791	0.1	1.931	0.779-4.791	0.1
CpG3	1.365	0.301-6.198	0.6	2.453	1.015-5.926	0.04	2.156	0.994-4.678	0.04	2.156	0.994-4.678	0.04
CpG4	–	–	–	–	–	–	1.099	0.147-8.212	0.9	1.099	0.147-8.212	0.9

MGMT: O^6-methyl guanine-methyl transferase; RT: radiotherapy; TMZ: temozolomide.

DISCUSSION

In the current study, OS and PFS for patients with methylation receiving RT+TMZ or RT alone was 27 and 9 months (OS) and 13 and 6 months (PFS), respectively, with a significant difference. On the other hand, no such differences could be detected for patients without methylation receiving RT+TMZ or RT alone as the corresponding figures were 10 and 7 months (OS) and 6 and 6 months (PFS), respectively.

Hegi *et al.* [16] analyzing 206 patients involved in the European Organisation for Research and Treatment of Cancer/National Cancer Institute of Canada (EORTC/NCIC) 26981-22981 trial found MGMT-methylation in 45.0% of the patients. The MGMT-methylation was a good prognostic factor regardless of treatment. Among patients with MGMT promotor methylation, RT+TMZ was associated with a significantly better median OS as compared to those receiving RT alone. On the other hand, among patients without MGMT promotor methylation, median OS was not significantly different between RT+TMZ and RT alone groups. However, these two groups differed significantly in terms of median PFS. In that study involving 206 patients, Cox regression and multivariate analyses were performed to assess the prognostic factors. In these analyses, MGMT promotor methylation was identified as an independent prognostic factor, while age and inclusion of TMZ in the treatment protocol did not emerge as having prognostic significance.

In the study by Dunn *et al.* [17] in which all patients were given RT and concomitant adjuvant TMZ, MGMT-methylation as well as the extent of the surgical resection were found to have independent prognostic significance with regard to improved OS and PFS, while age had no effect. Cao *et al.* [18], in their multi-center study, found that the combination of extensive surgical resection and MGMT promotor methylation was associated with prolonged survival, as shown by their multivariate analyses. In contrast, Tang *et al.* [19] found no effect of MGMT promotor methylation on OS and PFS. Esteller *et al.* [20] retrospectively analyzing 47 cases, administered whole brain RT and a variety of alkylating agents and observed better clinical outcomes in the presence of MGMT promoter methylation. Paz *et al.* [21] in their retrospective study, observed increased clinical response rates among patients with MGMT promotor methylation. Shah *et al.* [22] compared analytical methods for MGMT promotor methylation and administered RT and concomitant adjuvant TMZ to all their patients in a multi-center design. These researchers found a strong association between MGMT promotor methylation and disease-free survival; multivariate analyses also showed that age (<60 years) was a significant prognostic factor [22].

In the current study involving 100 patients, MGMT-methylation and inclusion of TMZ in the treatment protocol had a prognostic significance for both OS and PFS, while age (<60 years) had prognostic importance only for OS, and gender had no impact on prognosis. Among subjects with MGMT-methylation, addition of TMZ in the treatment was an independent prognostic factor for improved OS and PFS, and age (<60 years) had a positive prognostic effect only for OS; gender had no prognostic significance. In cases without MGMT-methylation, only age (<60 years) was a good independent prognostic factor for OS, whereas gender and inclusion of TMZ in the treatment protocol were not significant prognostic factors.

In a study by Binabaj *et al.* [23] although MGMT gene silencing was significantly associated OS for patients but no improvement of PFS was seen in methylated MGMT patients in a large-scale analysis. They investigated the relation of MGMT-methylation with OS and PFS in patients who underwent a comprehensive imaging evaluation. Their data revealed an association with better OS and PFS. They have also mentioned that further large-scale, prospective, controlled trials are needed to be done to find out the effects of combination and single targeted glioblastoma therapies in patients with MGMT methylated and unmeth-ylated in different age groups. At the same time, further studies need to be done to determine the best method of MGMT detection [23].

Previously, Karayan-Tapon *et al.* [24] compared five different methods for the determination of MGMT-methylation in a group of 81 patients. Of the 81 cases, 55 (67.9%) had MGMT-methylation. Univariate analyses revealed that age (<61 years) was a good prognostic factor. In all methods utilized to identify promotor MGMT-methylation, the presence of MGMT-methylation was associated with improved OS. However, among patients with MGMT promoter methylation, a significant association with OS and PFS was found with the pyrosequencing method. In multivariate analyses, age (<61 years) and particularly CpG4 methylation, emerged as strong prognostic factors. Furthermore, there was a significant correlation between CpG4 island methylation and OS and PFS.

In this study, among patients treated with RT+TMZ, methylation status of CpG1, CpG2, CpG3 and CpG4 islands had no effect on OS or PFS as examined by Kaplan-Meier analysis, while in the RT only group, methylation of CpG1, CpG2, CpG3 and CpG4 islands had significant effects on PFS. In the methylated group including 52 cases, methylation of the CpG3 island had a significant effect on PFS. With respect to prognostic factors, methylation of CpG1 and CpG3 islands had a positive prognostic effect on PFS. In the overall group of 100 patients, methylation of CpG3 island again was a good prognostic factor for PFS.

In the abovementioned study by Karayan-Tapon *et al.* [24], the superiority of five different methods for the determination of MGMT-methylation status were compared. Qualitative methyl-specific PCR (MSP), semi-qualitative methyl-specific PCR (SQ-MSP) and pyrosequencing methods were used to detect MGMT promotor methylation, while MGMT expression was evaluated through immuno-histochemistry (IHC) and quantitative real-time (RT-qPCR). The MGMT expression status was detected by IHC and RT-qPCR and no association was detected between OS and MGMT. When the MGMT-methylation status was evaluated using MSP, SQ-MSP and pyrosequencing, a significant association was observed between methylation status and OS. In that study [23], SQ-MSP and pyrosequencing methods proved to be more reliable than classical gel-based MSP used in studies by Hegi *et al.* [16] and Esteller *et al.* [20]. In our study, pyrosequencing was used for tissue samples fixed by formalin and embedded in paraffin.

In conclusion, MGMT promotor methylation represents an important biomarker influencing the response to RT and concomitant and adjuvant TMZ treatment as well as RT alone in GB patients. Currently, RT and concomitant TMZ followed by adjuvant TMZ, represents the standard approach until further treatments are defined. In patients without methylation, studies examining the molecular pathways to explore potential means for reduced resistance are warranted. Again, we believe further prospective studies with larger sample size are needed to improve clinical outcomes and to determine alternative therapeutic regimens and their clinical response rates.

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Declaration of Interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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