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Research article

MGMT promoter methylation in prognosis of patients with newly diagnosed IDH1 wildtype Glioblastoma

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ABSTRACT

The objective of this study was to analyse the prognosis values of O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation on the overall survival in patients with newly diagnosed isocitrate dehydrogenase 1 (IDH1) wild-type glioblastoma multiforme (GBM). A retrospective observational study was conducted on 108 IDH1 wild-type glioblastoma patients who underwent tumour resection at neurosurgical centres, including Hanoi Medical University Hospital and Viet Duc University Hospital, from October 2017 to December 2021. The MGMT status was assessed using methylation-specific Polymerase Chain Reaction (MSP). Multivariable Cox proportional hazards models and Kaplan-Meier survival analysis were performed. Of 108 patients, 79.6% had methylated MGMT. The median overall survival of patients with IDH1 wild-type GBM was 8.47 months (95%Confidence Interval (CI) =6.93-11.07). Patients with Methylated MGMT had a lower mortality risk (Hazard Ratio (HR) =0.63, 95%CI=0.41-0.98) than those without Methylated MGMT. Patients aged <55 had a lower risk of mortality (HR=0.55, 95%CI=0.35-0.85) compared to those aged >=55 years old. Having tumours on both sides of the hemisphere was associated with a higher risk of mortality (HR=3.18, 95%CI=1.68-6.00) compared to patients with tumours on the right side of the hemisphere. To conclude, our data underline the impact of the MGMT promoter methylation status in the treatment response of IDH1-wildtype GBM. In addition, age, tumour size and hemisphere were also significant prognostic factors in the overall survival of patients.

Keywords: Glioblastoma, MGMT, Prognostic factor, Survival prediction.

INTRODUCTION

Glioblastoma multiforme (GBM) is the most common malignant brain tumour, accounting for 14.7% of all primary tumours of the central nervous system and 47.7% of all malignant primary brain tumours [1]. The O6-methylguanine-DNA methyltransferase (MGMT) gene promoter region has been detected in 35-45% of patients with GBM (World Health Organization stage IV classification of GBM) [2]. The incidence of MGMT methylation has been predominantly observed in brain tumours, which have been associated with prolonged survival rates dependent on chemotherapy or radiation therapy [3-6]. In addition to its predictive role, studies on the methylation of the MGMT gene in patients with glioblastoma multiforme may also predict the response to temozolomide (TMZ) treatment. Patients without enhanced methylated MGMT gene respond less to TMZ. Thus, research on alternative treatment protocols by replacing TMZ with a

novel drug is warranted [7]. Therefore, MGMT's methylation status is crucial for clinical physicians in making treatment decisions and predicting patients' prognoses [3-6]. This study aimed to analyze the prognosis values of MGMT promoter methylation on the overall survival in patients with newly diagnosed IDH1 wild-type GBM.

MATERIALS AND METHODS

Study Design and Participants

A retrospective observational study was conducted on 108 patients who underwent surgical treatment at Viet Duc University Hospital and Hanoi Medical University Hospital, Hanoi, Vietnam, from 2017 to 2021. These patients were histopathologically confirmed to have GBM by neuropathologists with a minimum of 10 years of experience. Other criteria for patient selection include the following: 1) Patients with IDH1 wild-type GBM paraffin-embedded samples identified via Sanger sequencing; 2) Voluntary participation of the patient's family; 3) Corresponding standard tissue samples accompanying the study; 4) Absence of pathological features on the standard tissue of any other neoplasm, organ cancer other than GBM; and 5) Having complete research information. The exclusion criteria encompass individuals under the age of 18, those with a prior diagnosis of other forms of cancer, and individuals who have received radiotherapy or chemotherapy for alternative medical conditions. Tumour specimens that were insufficiently preserved or inadequately stored for clinical chemistry analysis were subsequently excluded from the study. The study conformed to the guidelines outlined in the Declaration of Helsinki (2013 revision) and received approval from the ethics committee of Hanoi Medical University (IRB-VN01. 001/IRB00003121/FWA 00004148). Before the commencement of the study, written informed consent was acquired from all patients involved or their respective family members/relatives.

Data Measurement

The patients were selected based on specific inclusion and exclusion criteria. The patients' epidemiological data and overall survival duration were obtained through a thorough examination of their medical records and in-depth interviews conducted with either the patients themselves or their family members. Additional research information was gathered from medical records and interviews with the patient's family. Clinical characteristics (symptoms, number of tumours, size, and location of tumours) and Glasgow coma scale (GCS) were collected. The specimens derived from the study participants were utilised to assess the methylation status within the promoter region of the MGMT gene via the Methylation-Specific Polymerase Chain Reaction (MSP). Subsequently, an examination was conducted to determine the relationship between the methylation status of the MGMT gene promoter and various clinical characteristics, para-clinical parameters, and survival outcomes.

Process of DNA Etraction from Tissue

Following the manufacturer's protocol, the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) was utilised to extract DNA from formalin-fixed paraffin-embedded (FFPE) tissue samples. The steps conducted included 1) paraffin removal, 2) tissue cell lysis and cell membrane disruption, 3) nuclear membrane lysis, 4) protein removal, 5) DNA precipitation, and 6) DNA wash and dissolution. The concentration and purity of the sample were determined by measuring the absorbance spectra at 260 nm and 280 nm using a NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific, MA, and USA). The purity of DNA samples measured at a range of 1.8-2.0 indicates adherence to the standard for conducting PCR amplification (**Mastercycler Pro S vapo.protect, Eppendorf, Germany**). The non-conforming samples were reacquired, and the DNA was re-extracted.

Bisulfite Conversion/Treatment and MGMT Methylation-Specific PCR (MSP) Analysis

The DNA extracted underwent bisulfite conversion using the EpiMark® Bisulfite Conversion Kit (NEB, MA, and USA). After undergoing bisulfite conversion, the DNA samples were subjected to amplification to detect methylated and unmethylated sequences of CpG within the promoter region of the MGMT gene at position 74-78. This was achieved using methylation-specific PCR (MSP) with specific primer pairs designed for this purpose:

Primers for methylated MGMT promoter sequence with amplification size of 81 bp [8].

- (5′-3′) F: TTTCGACGTTCGTAGGTTTTCGC
- (5′-3′) R: GCACTCTTCCGAAAACGAAACG

Primers for unmethylated MGMT promoter sequence with amplification size of 93 bp:

- (5′-3′) F: TTTGTGTTTTGATGTTTGTAGGTTTTTGT
- (5′-3′) R: AACTCCACACTCTTCCAAAAACAAAACA

The MSP was carried out in a total reaction volume of 25 μL, comprised of 1x polymerase chain reaction (PCR) buffer with:

- 5X EpiMark® Hot Start Taq Reaction Buffer (5ul);
- 0.2 μmol/L deoxyribonucleoside triphosphate (dNTP) mix;
- EpiMark® Hot Start Taq DNA Polymerase at a concentration of 0.025U/ul;
- 2ul of bisulphite converted template DNA (<1000ng); and
- 20 pmol/L of the specified forward (F) and reverse (R) primers.

The PCR was conducted utilising EpiMark ® Hot Start Taq DNA Polymerase (NEB, MA, USA) and following the specified amplification program: a 10-minute denaturation step at 95°C, subsequently followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 55°C for 30 seconds, extension at 68°C for 1 minute, and a final extension at 68°C for 5 minutes.

The EpiTect PCR Control DNA Set (Qiagen, Hilden, Germany), containing methylated and unmethylated DNA sequences, was incorporated as control samples in every reaction. Additionally,

negative controls lacking DNA were also included. The reactions were subsequently assessed using electrophoresis on a 3% agarose gel at a voltage of 100 V for 30 minutes. This was conducted to ascertain the existence of PCR products corresponding to methylated and unmethylated MGMT promoter sequences, which manifested in fragment lengths of 81 bp and 93 bp, respectively.

The gel is subjected to staining with ethidium bromide and subsequently observed under ultraviolet (UV) light for visualisation. The detection of a discernible M primer band in MGMT indicated a positive MGMT methylation status. In contrast, the lack of an M primer PCR product was construed as evidence of MGMT's negative methylation status.

Statistical Analysis

The data was inputted into the computer using the EpiData 3.1. software (EpiData Association, Odense, Denmark) and analysed using the Stata 16.0 software. Descriptive statistical tests with a 95% confidence interval were applied. The Chi-square test was used to compare proportions, while the student's t-test was used to compare means. The overall survival (OS) was calculated from the surgery date to the date of death or last follow-up, measured in months. The Kaplan-Meier survival curve was analysed using the log-rank test and subjected to multivariate analysis using the Cox proportional hazards model.

RESULTS

Among 108 patients, Table 1 shows their demographic and clinical characteristics. There were 79.6% having methylated MGMT. The mean age was 54.1 (SD=13.2) years old. Most of them were male (58.3%). The most common symptoms included headache (83.3%), motor or sense deficit (41.7%), nausea/vomiting (13.0%) and cognitive impairment (13.0%). The majority of patients had minor GCS (95.4%). Regarding tumours, most of them had one tumour (88.0%), and the major participants had tumours on the right (48.2%) or the left (46.3%) side. The most common location of the tumour was the frontal lobe (46.3%), followed by the temporal lobe (40.7%) and parietal lobe (27.8%). Statistical differences were found regarding gender, impaired consciousness, GCS groups, and hemisphere between un-methylated and methylated MGMT ($p<0.05$)

Figure 1: Kaplan–Meier curves of patients with IDH1 wild-type GBM —association of gene methylation MGMT

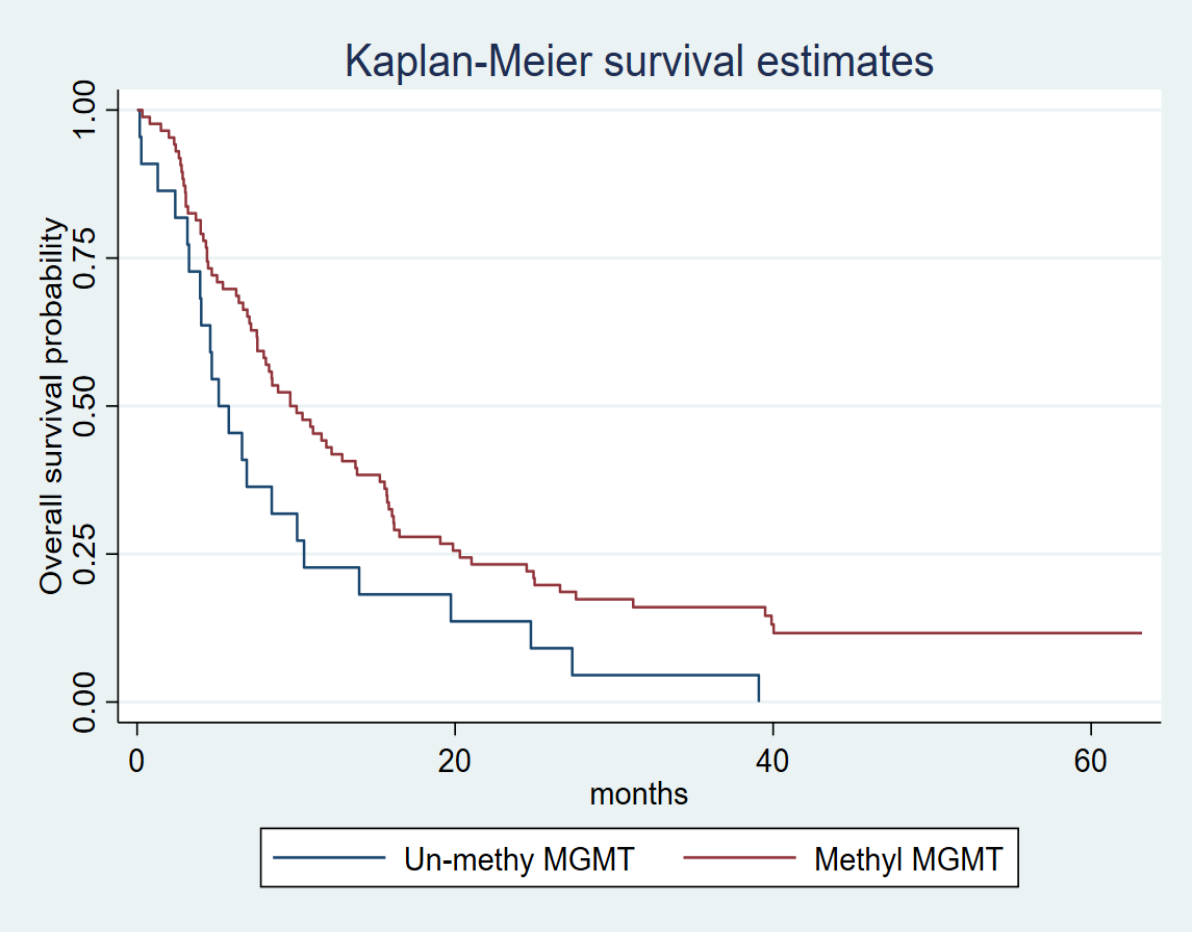

Table 1 shows MGMT promoter: O6-methylguanine–DNA methyl-transferase promoter; GCS: **Glasgow coma scale; S: Square of tumour; V: Volume of tumour;** OS: overall survival.

Table 2 shows that the median overall survival of patients with IDH1 wild-type GBM was 8.47 months (95%CI=6.93-11.07). Differences in the median overall survival time of patients were found among MGMT groups, age groups, GCS, hemisphere, and interaction between MGMT and tumour size ($p<0.05$) (Table 2, Figure 1 and Figure 2).

Table 1: Demographic and clinical characteristics of patients

Characteristics	Un-methylated MGMT	Methylated MGMT	Total	p-value
	N (%)	N (%)	N (%)	
Total	22 (20.4)	86 (79.6)	108 (100.0)	
Age at diagnosis, years, Mean (SD)	56.4 (13.4)	53.5 (13.2)	54.1 (13.2)	0.37
Gender, Male	17 (77.3)	46 (53.5)	63 (58.3)	0.04
Clinical features				
Headache	18 (81.8)	72 (83.7)	90 (83.3)	0.83
Seizure	1 (4.6)	6 (7.0)	7 (6.5)	0.68
Nausea/Vomiting	3 (13.6)	11 (12.8)	14 (13.0)	0.92
Cognitive impairment	2 (9.1)	12 (14.0)	14 (13.0)	0.55
Motor or sense deficit	7 (31.8)	38 (55.8)	45 (41.7)	0.29
Impaired consciousness	5 (22.7)	5 (5.8)	10 (9.3)	0.02
Visual deficit	0 (0.0)	5 (5.8)	5 (4.6)	0.25
Speech deficit	1 (4.6)	11 (12.8)	12 (11.1)	0.27
Other	6 (27.3)	9 (10.5)	15 (13.9)	0.04
Glasgow Coma Scale (GCS)				
Severe, GCS ≤ 8	1 (4.6)	0 (0.0)	1 (0.9)	0.04
Moderate, GCS 9–12	2 (9.1)	2 (2.3)	4 (3.7)	
Minor, GCS ≥ 13	19 (86.4)	84 (97.7)	103 (95.4)	
Number of tumours				
>1 tumor	4 (18.2)	9 (10.5)	13 (12.0)	0.32
One tumor	18 (81.8)	77 (89.5)	95 (88.0)	
Hemisphere				
Right	10 (45.5)	42 (48.8)	52 (48.2)	0.01
Left	8 (36.4)	42 (48.8)	50 (46.3)	
Both sides	4 (18.2)	2 (2.3)	6 (5.6)	
Location				
Frontal lobe	10 (45.5)	40 (46.5)	50 (46.3)	0.93
Temporal lobe	11 (50.0)	33 (38.4)	44 (40.7)	0.32
Parietal lobe	6 (27.3)	24 (27.9)	30 (27.8)	0.95
Occipital lobe	2 (9.1)	11 (12.8)	13 (12.0	0.63
Insular lobe	1 (4.6)	2 (2.3)	3 (2.8)	0.57
Basal nuclei	2 (9.1)	5 (5.8)	7 (6.5)	0.58
Corpus callosum	0 (0.0)	16 (18.6)	16 (14.8)	0.03
Thalamus	0 (0.0)	4 (4.7)	4 (3.7)	0.30
Ventricle	1 (4.6)	1 (1.2)	2 (1.9)	0.29
Cerebellum	1 (4.6)	1 (1.2)	2 (1.9)	0.29
Tumor max size, CMA				
S≥20 cm^2 or V≥60 cm^3	10 (45.5)	44 (51.2)	54 (50.0)	0.63
S<20 cm^2 and V<60 cm^3	12 (54.6)	42 (48.8)	54 (50.0)	

Table 2: Median overall survivor of patients with IDH1 wild-type GBM

Parameters		Median OS (months)	SE	95%CI		Log-rank	p (Log-rank)
				Lower limit	Upper limit		
Total		8.47	1.01	6.93	11.07		
MGMT	Un-Methyl	5.13	1.17	3.27	10.07	5.58	**0.018**
	Methyl	9.63	1.53	7.57	13.73		
Age group (years)	≥55	6.67	1.14	4.47	10.5	6.48	**0.010**
	<55	10.07	2.22	8.00	15.70		
Gender	Male	8.30	1.40	5.77	10.50	1.54	0.21
	Female	8.87	3.93	6.40	15.83		
Glasgow coma scale	Severe GCS <= 8	-	-	-	-	52.45	***<0.001**
	Moderate GCS 9–12	5.77	2.87	4.33	-		
	Minor GCS >= 13	8.50	1.31	7.07	11.90		
Number of tumours	> 1 tumor	3.70	5.23	2.40	16.03	0.299	0.584
	One tumor	8.50	1.11	7.07	11.07		
Hemisphere	Right	8.47	1.72	5.77	13.73	9.68	***0.008**
	Left	9.63	1.63	6.90	15.57		
	Both sides	3.17	1.27	1.30	-		
Tumor size	S≥20 cm^2 or V≥60 cm^3	8.30	1.10	6.90	11.90	0.03	0.870
	S<20 cm^2 and V<60 cm^3	8.50	2.04	5.03	12.90		
MGMT + Tumor size	Un-Methyl + S≥20 cm^2 or V≥60 cm^3	8.48	2.85	0.27	19.73	10.70	0.013
	Un-Methyl + S<20 cm^2 and V<60 cm^3	4.03	0.55	2.40	5.77		
	Methyl + S≥20 cm^2 or V≥60 cm^3	8.00	1.16	6.40	15.73		
	Methyl + S<20 cm^2 and V<60 cm^3	11.60	2.21	8.10	15.57		

MGMT promoter: O6-methylguanine–DNA methyl-transferase promoter; GCS: Glasgow coma scale; S: Square of tumour; V: Volume of tumour; OS: overall survival. SE: standard error. 95%CI: Confidence interval of 95%.

Figure 2: Kaplan–Meier curves of patients with IDH1 wild-type GBM —association with different factors: a) Glasgow coma scale; b) Age; c) Hemisphere; and 4) Interaction between gene methylation MGMT and tumour size

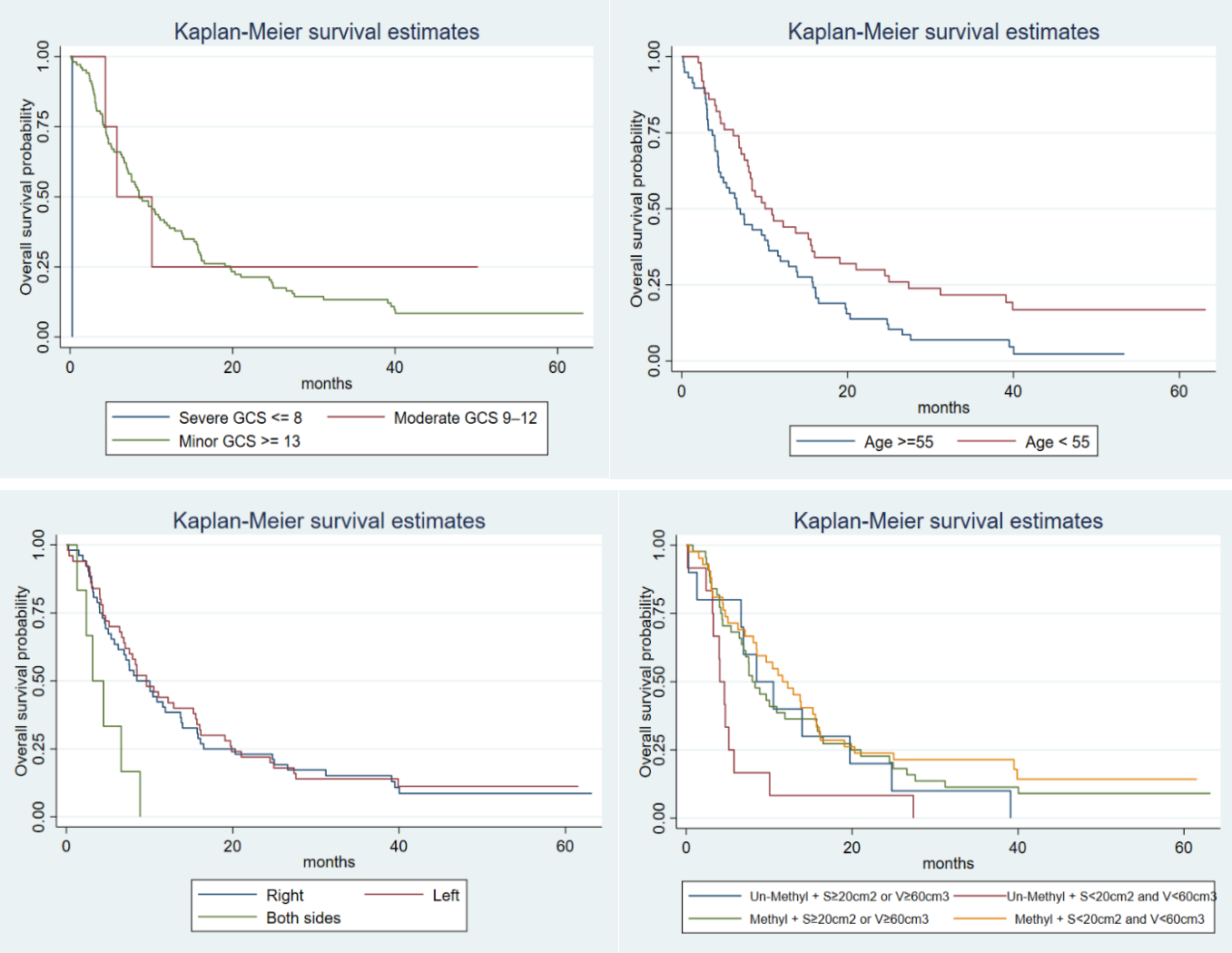

Table 3 indicates that after adjustments, patients with Methylated MGMT had a lower risk of mortality (HR=0.63, 95%CI=0.41-0.98) compared to those without Methylated MGMT. Patients aged <55 had a lower risk of mortality (HR=0.55, 95%CI=0.35-0.85) compared to those aged >=55 years old. Having tumours on both sides of the hemisphere was associated with a higher risk of mortality (HR=3.18, 95%CI=1.68-6.00) compared to patients with tumours on the right side of the hemisphere.

Table 3: Proportional Cox hazard model in the associations between outcomes and factors

Parameters	**HR**	**95%CI**		**p**
		Lower	Upper	
MGMT (Methyl vs. Un-Methyl-ref)	0.63	0.41	0.98	0.04
Age group (<55 vs. ≥55-ref)	0.55	0.35	0.85	<0.01
GCS score	0.87	0.67	1.13	0.29
Hemisphere (vs. Right)				
Left	1.06	0.68	1.65	0.79
Both sides	3.18	1.68	6.00	<0.01
Tumor size (S<20 cm2 vs. V<60 cm3 / S≥20 cm2 or V≥60 cm3)	1.15	0.77	1.71	0.51
MGMT promoter: O6-methylguanine–DNA methyl-transferase promoter; GCS: Glasgow coma scale; S: Square of tumour; V: Volume of tumour; HR: Hazard risk; 95%CI: Confidence interval of 95%.				

DISCUSSION

This study aimed to assess the influence of MGMT promoter methylation status on patients with newly diagnosed IDH1 wild-type GBM. In our study, 108 patients with GBM were included, of which 88 patients (80.7%) exhibited MGMT promoter methylation and 21 patients (19.3%) exhibited MGMT promoter unmethylation. Our finding indicates a higher percentage of patients with high methylation of the MGMT gene compared to previous studies. For example, Zawlik et al. showed that 10-44% of 371 patients had MGMT promoter methylation [9], while this rate in Abhinav et al.'s et al. was 11%-59.6% [10]. Previous research indicates that patients with GBM with MGMT promoter methylation demonstrate better response to specific adjunctive therapies, particularly alkylating agents, leading to more prolonged survival [8,11-15].

Our finding aligns with previous research that identified the methylation status of the MGMT promoter as an essential factor in predicting and assessing outcomes for patients with GBM. Analysis of

the methylation status of MGMT showed that people with a methylated MGMT promoter had significantly longer overall survival [15]. Our results were consistent with previous research. Müller et al. (2021) researched patients with GBM who had residual tumour tissue after surgery and found that 31 out of 81 patients (38.27%) had methylated MGMT promoters. The average OS and progression-free survival (PFS) was significantly longer in patients with a methylated MGMT promoter (average OS: 16 months vs 13 months, p= 0.009) [16]. Our study found that MGMT methylation is a favourable indicator for prognosis. Zhang et al. conducted a meta-analysis of 29 studies that examined the impact of MGMT promoter methylation on overall survival (OS). In a univariate analysis of 15 studies, the combined hazard ratios were 0.67 (95% CI: 0.58–0.78), while in a multivariate analysis of 14 studies, the combined hazard ratios were 0.49 (95% CI: 0.38–0.64) [17]. In contrast to other research, our study found that the population we examined had shorter survival rates. Therefore, based on our data, we cannot conclude that increasing I methylation would be able to make up for the reduced survival times resulting from incomplete resection.

Indeed, our finding was in line with several previous studies that recognised the methylation status of the MGMT promoter as a critical predictive and prognostic determinant for outcomes in GBM patients. The examination of MGMT methylation status revealed that individuals with a methylated MGMT promoter exhibited notably extended OS. Our findings were in line with prior studies. Florian Müller Mareike et al. (2021) studied all GBM, IDH-wildtype (WHO grade IV) patients with postoperative residual tumour tissue and found that MGMT promoter was methylated in 31 patients (38.27%). Median OS and PFS were significantly increased in patients with methylated MGMT promoter (mOS: 16 M vs. 13 M, p = 0.009; mPFS: 13 M vs. 5 M, p = 0.003) [18]. Our research revealed that MGMT methylation is a positive prognosis factor. Zhang et al. performed a meta-analysis on 29 studies that reported the influence of MGMT promoter methylation on OS. The combined hazard ratios were 0.67 (95% confidence interval [CI]: 0.58–0.78) in a univariate analysis of 15 studies and 0.49 (95% CI: 0.38–0.64) in a multivariate analysis of 14 studies [17]. However, our study population showed decreased survival times compared to other studies. Thus, our data cannot support that a preferable I methylation could compensate for the loss in survival times from incomplete resection.

Literature pinpointed patient age and clinical condition as notable factors affecting patient prognosis in individuals with GBM [17]. Our study's survival analysis showed that younger patients had higher OS than older patients. Older individuals may experience higher mortality rates due to the presence of co-morbidities and reduced tolerance to surgical procedures and adjuvant therapies [19]. Another potential process linked to the ageing phenomenon is the perturbation of DNA methylation, which manifests as overall hypomethylation across the entire genome, alongside specific methylation at promoter regions [20]. Jovanović et al. identified a significant disparity in overall survival rates between individuals over 50 and their younger counterparts, with median survival ranging from 7 to 19 months [8]. Mateusz Szylberg et al. showed that the younger patients group exhibited a mean OS period of more than two times longer than the older group [21]. A study in Switzerland showed that age emerged as the primary prognostic factor [19]. Our study revealed that patients demonstrating baseline tumour size S<20 cm^2 or V<60 cm^3 and MGMT methylation experienced a significantly longer survival rate than other patient groups. The observed phenomenon may be attributed to the more extensive surgical resection of smaller tumours than tumours of larger volumes. Our study supports previous findings that indicate patients with MGMT methylation and a baseline tumour volume of 32 cm^3 or less had a significantly longer life expectancy [15]. In contrast, one study indicated that the size of tumours in high-grade glioma did not significantly impact prognosis [22]. Other researchers have shown a lack of significant association between preoperative imaging of tumour size and OS despite the notable significance of the extent of resection [23,24].

There were several constraints in this study. We examined the data of a highly similar group based on stringent inclusion criteria, which could lead to a bias in the selection process. The study's results may be less definitive due to the study's design, specifically because of the limited number of patients, particularly in the subgroup analysis. Using a different threshold to distinguish between MGMT promoters methylated and unmethylated GBM could have a significant effect compared to previous research [25].

CONCLUSIONS

Our data underline the impact of the MGMT promoter methylation status in the treatment response of IDH1-wildtype GBM. In addition, age, tumour size and hemisphere were also significant prognostic factors in the overall survival of patients.

Conflicts of interest

The authors declare no conflict of interest.

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