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Clinical implications of *TERT* promoter mutation on *IDH* mutation and *MGMT* promoter methylation in diffuse gliomas

Hyun Sik Kim[a], Mi Jung Kwon[b,f,*], Joon Ho Song[a], Eun Soo Kim[c], Ho Young Kim[d], Kyueng-Whan Min[e]

[a] Department of Neurosurgery, Hallym University Sacred Heart Hospital, Hallym University College of Medicine, Anyang, Republic of Korea
[b] Departments of Pathology, Hallym University Sacred Heart Hospital, Hallym University College of Medicine, Republic of Korea
[c] Department of Radiology, Hallym University Sacred Heart Hospital, Hallym University College of Medicine, Anyang, Republic of Korea
[d] Department of Hematological Oncology, Hallym University Sacred Heart Hospital, Hallym University College of Medicine, Anyang, Republic of Korea
[e] Department of Pathology, Hanyang University Guri Hospital, Hanyang University College of Medicine, Kyoungchun-ro 153, Guri-si, Gyeonggi-do 11923, Republic of Korea
[f] Research Insititute for Complementary & Alternative Medicine, Hallym University, 40 Seokwoo-Dong, Hwaseong, Gyeonggi-do, 445-170, Republic of Korea

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ABSTRACT

IDH mutation and *MGMT* promoter methylation are reliable prognostic and predictive biomarkers in grade II–IV diffuse gliomas. Recurrent mutations in the promoter region of the telomerase reverse transcriptase (*TERTp*) gene have also been found in diffuse gliomas. However, the prognostic and predictive effects of *TERTp* mutation on *IDH* or *MGMT* status are largely unknown.

IDH1/2 and *TERTp* mutations, as well as *MGMT* methylation statuses, were examined via peptide nucleic acid-mediated PCR clamping and *MGMT* methylation-specific PCR in 67 paraffinized tumor samples, respectively.

TERTp mutation was associated with older patients (≥60 years) and frontally located gliomas. Old age, frontal location, and grade IV were found to be predictive factors of *TERTp* mutation. *TERTp* mutation resulted in poor prognosis in overall diffuse gliomas. *TERTp* mutation was not correlated with overall survival (OS) or progression-free survival (PFS) in the diffuse gliomas. However, *TERTp* mutations, in combination with *MGMT* methylation or *IDH* mutation, showed that there were statistical significant survival differences between *MGMT*-unmethylated/*TERTp*-mutated and *MGMT*-unmethylated/*TERTp*-wildtype subgroups in grade II gliomas. There was a statistical significant survival difference of OS between *IDH*-wildtype/*TERTp*-mutated and *IDH*-mutated/*TERTp*-mutated subgroups in grade III gliomas. No significant associations between survival and *MGMT*/*TERTp* or *IDH*/*TERTp* status were found in grade IV gliomas. In conclusion, the combination of *TERTp* with *IDH* or *MGMT* status may be a prognostic indicator depending on grades.

1. Introduction

Diffuse gliomas, the most prevalent primary malignant brain tumors, have been classified by the World Health Organization (WHO) into grades II–IV gliomas [7,12]. Diffuse grade II and III gliomas are generally less aggressive tumors with a median survival of more than 7 years [8]. There is substantial heterogeneity among grade II and III gliomas in terms of pathological features and clinical outcomes [7,12]. Diffuse grade IV glioma, or glioblastoma, represents the most aggressive subtype, and has a poor prognosis of less than 5% 5-year overall survival rate [7]. Significant medical advances in recent years have resulted in the identification of a set of genetic lesions that are characteristic of grades II–IV gliomas; these are well-correlated with histology and clinical outcomes [2]. Recent studies outlined unique genomic profiles for grade II and III gliomas, which show molecular lesions distinct from those typically found in glioblastomas [12]. In this regard, the latest revision on the WHO classification of central nervous system tumors has incorporated molecular classification as part of disease diagnosis. Some examples include mutations in genes encoding isocitrate dehydrogenase (IDH) 1 and 2, as well as co-deletions of 1p and 19q (1p/19q) [12].

Additional biomolecular markers such as methylguanine-DNA-

* Corresponding author at: Department of Pathology, Hallym University Sacred Heart Hospital, Hallym University College of Medicine, Anyang, Gyeonggi-do, 431-070, Republic of Korea.
E-mail address: mulank99@hallym.or.kr (M.J. Kwon).

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methyltransferase (*MGMT*) methylation and telomerase reverse transcriptase promoter (*TERTp*) mutations have been also explored to stratify malignant gliomas into prognostic subgroups [4,6,8,10]. *MGMT* methylation is a well-known favorable prognostic factor for overall survival and is used as a predictive factor for chemotherapy response in glioblastoma patients [1,15]. The telomerase reverse transcriptase gene encodes the catalytic subunit of telomerase, which maintains telomere length [4]. Telomerase activity occurs in more than 90% of cancers [3,9]. Two hotspot mutations in *TERTp*, C228T and C250T, increase telomerase activity, leading to enhanced tumor growth and survival. In addition, upregulation of the *TERT* gene is the major mechanism by which telomerase activation occurs in human cancers, including gliomas [9]. *TERTp* mutations are found in over 70% of primary glioblastomas and oligodendrogliomas; they are less frequent in oligoastrocytomas and WHO grade II/III diffuse astrocytomas [4,9,15–17]. Some genetic alterations can modify *TERT* expression by increasing binding to the *TERT* promoter [3]. However, very few studies have investigated the relationship between *MGMT* methylation and *TERTp* mutation. Since *TERTp* mutations and *MGMT* methylation have been shown to be associated with disease prognosis, we investigated whether these tumor markers can provide additional prognostic information on glioma patients.

2. Materials and methods

Information on 72 diffuse glial tumor patients who underwent surgery at the Hallym University Sacred-Heart Hospital from 2007 to 2017 was obtained from registry files. All patients were chemotherapy-free and targeted drug-naive at the time of surgery. Based on a hematoxylin and eosin (H&E) slide review, only patients with primary brain glial tumor diagnosis with available formalin-fixed paraffin-embedded (FFPE) tumor tissue blocks were included in this study. Finally, 67 patients diagnosed with diffuse glial tumors (grade II–IV) were enrolled in this study.

Clinical information including age, sex, treatment modality, survival, and disease progression were analyzed via clinical records and radiological investigation. To confirm tumor locations, detailed radiological reports, operative reports, and profiles of postoperative magnetic resonance imaging (MRI) were studied. Tumors were classified according to the 2007 WHO classification. This study was approved by the institutional review board at the Hallym University Sacred-Heart Hospital.

2.1. Tumor localization

To determine tumor locations, both MRI profiles and clinical files were retrospectively reviewed by an experienced neuroradiologist (ESK) who was blind to the molecular status of the patient. To simplify the analysis, tumors were primarily assigned into three general locations: frontal, midline, and other [16]. Frontal gliomas only included tumors that were located entirely in the frontal lobe. Midline tumors included those that were entirely located in the corpus callosum, the thalamencephalon, the periventricular locations, and the brainstem [16]. Other gliomas included the insular lobe (including insular, frontotemporal-insular, temporal-insular, and frontal-insular lobes), the temporal lobe (including temporal, frontotemporal, temporoparietal, and temporal-occipital lobes), the parietal lobe (including parietal, frontoparietal, and parietal-occipital lobes), the occipital lobe, and the cerebellum, as previously described [16].

2.2. Detection of IDH1, IDH2, and TERT promoter mutations

Genomic DNA was extracted from 10-μm-thick sections of 10% neutral FFPE tumor tissue blocks using the Maxwell® 16 FFPE Tissue LEV DNA Purification Kit for DNA (Promega, USA), according to manufacturer's instructions.

Genetic variants in *IDH1* (R132) and *IDH2* (R140 and R172) were obtained using the PNAClamp™ IDH1/2 Mutation Detection Kit (PANAGENE, Daejeon, Korea), according to manufacturer's instructions, and as previously described. In brief, all reactions were performed in 20 μl volumes using template DNA, primers, the PNA probe set, and SYBR Green PCR master mix. All necessary reagents were included in the kit. Real-time PCR of PNA-mediated clamping PCR was performed using a CFX 96 (Bio-Rad, USA). The PCR cycling conditions were as follows: 5 min hold at 94 °C, followed by 40 cycles of 94 °C for 30 s, 70 °C for 20 s, and 63 °C for 30 s; last temperature hold was at 72 °C for 30 s *IDH1* and *IDH2* gene mutations were detected using the one-step PNA-mediated real-time PCR clamping technique. In this assay, PNA probes and DNA primers were used together in the clamping reaction. Positive signals were detected via intercalation of SYBRGreen fluorescent dye. The PNA probe sequence, which was complementary to wild-type DNA, was used to suppress amplification of the wild-type target. This in turn enhanced preferential amplification of mutant sequences by competitively inhibiting the binding of DNA primer to wild-type DNA.

PCR efficiency was determined by measuring the threshold cycle (Ct) value. Ct values for the control and mutants were obtained via SYBRGreen amplification plots. Delta Ct (ΔCt) values were calculated as follows: ΔCt1 = [Standard Ct] − [Sample Ct], ΔCt2 = [Sample Ct] − [Non PNA mix Ct]. The standard Ct value was 34 for the *IDH1* PNA mix, and 35 for the *IDH2* PNA mix. The *IDH1* gene was considered to be mutated when ΔCt1 values were greater than 2.0, whereas the *IDH2* gene was considered to be mutated when ΔCt1 values were greater than 2.0 and ΔCt2 values were ≤ 9. When ΔCt1 values were between 0 and 2, a ΔCt2 value was considered to be mutated if the calculated ΔCt2 value was ≤ 4. The non-PNA mix Ct was used to establish efficiency of PCR amplifications; when the non-PNA mix Ct fell within the range of $24 < Ct < 30$ for the *IDH1* gene and $23 < Ct < 30$ for the *IDH2* gene, it was an indication of optimal PCR amplification efficiency.

Mutational analysis of *TERTp* gene variants flanking the C228 and C250 loci (C250 and C228) was performed using the PNAClamp™ TERT Mutation Detection Kit (PANAGENE), according to manufacturer's instructions. The standard Ct values were 33 for the C250 PNA mix and 36 for the C228 PNA mix. The *TERTp* gene was considered to be mutated when ΔCt1 values were greater than 2.0. When ΔCt1 values were between 0 and 2, the gene was considered to be mutated if the calculated ΔCt2 value was ≤ 6. When the non-PNA mix Ct fell within the range of $22 < Ct < 30$ for the *TERTp* gene, it was an indication of optimal PCR amplification efficiency.

2.3. MGMT methylation-specific PCR analysis

MGMT methylation-specific PCR was carried out to determine the methylation status of the *MGMT* promoter, as previously described [13]. In brief, approximately 100–200 ng total DNA was subjected to bisulfite conversion using the EZ DNA methylation-Gold kit (Catalog No. D5005; Zymo Research, Orange, CA, USA). The primer sequences for the *MGMT* gene were as follows: methylated forward: 5′ TTT CGA CGT TCG TAG GTT TTC GC 3′; methylated reverse: 5′ GCA CTC TTC CGA AAA CGA AAC G 3′; unmethylated forward: 5′ TTT GTG TTT TGA TGT TTG TAG GTT TTT GT 3′; and unmethylated reverse: 5′ AAC TCC ACA CTC TTC CAA AAA CAA AAC A 3′. A total of 10–20 ng bisulfite-treated DNA was used for the PCR. Water in place of DNA was used as the control. The PCR cycling parameters for the *MGMT* gene were as follows: 94 °C for 5 min; 35 cycles of 94 °C for 45 s, 59 °C for 45 s, and 72 °C for 1 min; 72 °C for 5 min, and then hold at 4 °C. Analysis was performed by comparing the PCR bands of control methylated DNA and control unmethylated DNA. *MGMT* methylation was considered to be positive when PCR bands were located at 86 bp.

2.4. Statistical analysis

Statistical analyses of categorical variables such as genetic

alterations and clinicopathological features were performed using the chi-square test or the 2-tailed Fisher's exact test. The relationships between continuous variables and genetic alterations were examined by Student's *t* test. Progression-free survival (PFS) was defined as the first day of surgery to tumor progression, death, or end of follow-up. Overall survival (OS) was as first day of surgery until death or end of follow-up. The survival differences among groups were calculated using the Kaplan–Meier method with a log-rank test. We used the Cox proportional hazards model for multivariate PFS and OS analyses. For the subgroup survival analysis, log-rank test with Bonferroni's correction was used. The SPSS statistical software (version 18, Statistical Package for the Social Sciences) was used for all statistical analyses. *P* values < 0.05 were considered to be statistically significant.

3. Results

3.1. Clinicopathologic demographics

A total of 67 patients were recruited, which consisted of 33 men (49.3%) and 34 women (50.7%); their mean age was 56.51 ± 15.81 years (range 18–79 years). The mean duration of follow-up was 60 months. There were 32 patients (47.8%) aged less than 60 years and 35 patients (52.2%) older than 60 years. Lesions were located in the right hemisphere (41.8%), the left hemisphere (40.3%), both hemispheres (14.9%), or at the midline (3.0%). WHO 2016 revised diagnosis of this study group was as follows: 5 diffuse astrocytoma, *IDH*-mutant (7.5%), 4 diffuse astrocytoma, *IDH*-wildtype (6.0%), 5 Anaplastic astrocytoma, *IDH*-mutant (7.5%), 5 anaplastic astrocytoma, *IDH*-wildtype (7.5%), 1 oligodendroglioma, *IDH*-mutant and 1p/19q-codeleted (1.5%), 5 anaplastic oligodendroglioma, *IDH*-mutant & 1p/19q-codeleted (7.5%), 2 glioblastoma, *IDH*-mutant (3.0%), and 40 glioblastoma, *IDH*-wildtype (59.7%). Categorization by WHO 2007 histological grade at diagnosis yielded 9 patients at grade II (13.4%), 16 patients at grade III (23.9%), and 42 patients at grade IV (62.7%).

Biopsy was carried out only on 14 patients (20.9%), and debulking surgery was performed in 53 patients (79.1%); 43 patients (64.2%) were treated with some form of adjuvant radiotherapy and/or chemotherapy, while 24 (35.8%) patients received neither adjuvant radiotherapy nor chemotherapy. There are currently 17 patients alive (25.4%), but 50 patients (74.6%) died (Table 1).

Table 1
Clinicopathological variables of the 67 diffuse glioma patients.

Demographics	No. of patients (%)
Gender	
Male	33 (49.3)
Female	34 (50.7)
Age (y), mean ± SD	56.51 ± 15.806
< 60	32 (47.8)
≥60	35 (52.2)
Tumor location	
Frontal	16 (23.9)
Midline	17 (25.4)
Others	34 (50.7)
WHO 2016 diagnosis	
Diffuse astrocytoma, IDH-mutant	5 (7.5)
Diffuse astrocytoma, IDH-wildtype	4 (6.0)
Anaplastic astrocytoma, IDH-mutant	5 (7.5)
Anaplastic astrocytoma, IDH-wildtype	5 (7.5)
Oligodendroglioma, IDH-mutant & 1p/19q-codeleted	1 (1.5)
Anaplastic oligodendroglioma, IDH-mutant & 1p/19q-codeleted	5 (7.5)
Glioblastoma, IDH-mutant	2 (3.0)
Glioblastoma, IDH-wildtype	40 (59.7)
Surgery	
Biopsy only	14 (20.9)
Debulking surgery	53 (79.1)
Adjuvant treatment	
None	24 (35.8)
Chemotherapy	2 (3.0)
Radiotherapy	10 (14.9)
Chemoradiation	31 (46.3)
Survival outcome	
Alive	17 (25.4)
Deceased	50 (74.6)

SD, standard deviation.

3.2. Frequencies and prognostic values of IDH, TERTp mutations and MGMT methylation

Molecular analyses of PNA-mediated PCR clamping and methylation-specific PCR were successfully performed on all 67 tumor samples. We detected 18 (26.9%) mutations that affected codon 132 of the *IDH1* gene [5 (55.6%) in grade II, 11 (68.8%) in grade III, and 2 (4.8%) in grade IV]. In contrast, no mutation in codon 172 of the *IDH2* gene was found in the 67 gliomas. *TERTp* mutations were identified in 38 (56.7%) samples [3 (33.3%) in grade II, 8 (50%) in grade III, and 27 (64.3%) in grade IV]. The most frequently observed mutational region was C228 in 32 cases (84.2%), followed by C250 in six cases (15.8%). There was only one tumor with a mutation in both C228 and C250. *MGMT* methylation was found in 30 cases (44.8%) [4 (44.4%) in grade II, 10 (62.5%) in grade III, and 16 (38.1%) in grade IV], while 37 (55.2%) samples were found to have unmethylated *MGMT* gene. *MGMT* methylation was frequently observed in glioblastoma, *IDH*-wildtype than other molecular subtypes ($P = 0.029$).

3.3. Correlations between clinicopathological features and IDH, TERTp mutations, and MGMT methylation

Relationships between the clinical and pathological characteristics of glioma patients and their *TERTp*, *IDH*, and *MGMT* statuses were analyzed (Table 2). *TERTp* mutations were more frequently detected in older patients (≥60 years) as compared with younger patients (< 60 years) ($P < 0.001$). *IDH* mutation was associated with grades II–III and *MGMT* methylation ($P < 0.001$ and $P = 0.002$, respectively). *MGMT* methylation was associated with *IDH1* mutation ($P = 0.002$). *TERTp* mutation and *IDH* mutation were more highly associated with frontally located gliomas as compared with *TERTp* or *IDH* wild types ($P = 0.005$ and $P = 0.017$, respectively).

The clinicopathological factors affecting *TERTp* mutation were investigated by multivariate analyses using a logistic regression model (Table 3). The multivariate analyses revealed that older age (≥60 years), frontal location, and grade IV were independent predictive clinicopathological factors for *TERTp* mutation ($P < 0.001$, odds ratio = 14.084, confidence interval (95% CI) = 3.550–55.881; $P = 0.038$, odds ratio = 5.872, 95% CI = 1.101–31.322; and $P = 0.020$, odds ratio = 9.765, 95% CI = 1.438–66.322; respectively).

3.4. Relationship between IDH mutation, TERTp mutation, and MGMT methylation with survival rates

We performed Kaplan–Meier and Cox proportional regression analyses to determine whether *TERTp* mutation, *IDH* mutation, and *MGMT* methylation were correlated with OS or PFS in patients with gliomas. Kaplan–Meier curves showed that *IDH*-wild type tumors had poorer OS and PFS rates (median 13 months and 5 months) as compared with those harboring the *IDH* mutation (median 93 months and 60 months) (log rank test: $P < 0.001$, and $P = 0.001$, respectively) (Fig. 1A–B). Furthermore, patients with *TERTp* mutation also demonstrated significantly reduced OS and PFS (median 15 months and 5 months) as compared with those in *TERTp* wild type patients (median 33 months and 31 months) (log rank test: $P = 0.031$, and $P = 0.008$, respectively) (Fig. 1C–D). OS and PFS were found to be comparable between *MGMT* unmethylated (median 15 months and 7 months) and *MGMT* methylated (median 22 months and 9 months) patients (log rank test:

Table 2
Correlations between clinicopathological characteristics and *TERT* mutation status.

	Total	*TERTp*		*P* value	*IDH*		*P* value	*MGMT*		*P* value
		Mut	Wt		Mut	Wt		M	UM	
	n = 67 (%)	n = 38 (%)	n = 29 (%)		n = 18 (%)	n = 49 (%)		n = 30 (%)	n = 37 (%)	
Sex				0.464			0.280			0.330
Male	33 (49.3)	17 (44.7)	16 (55.2)		11 (61.1)	22 (44.9)		17 (56.7)	16 (43.2)	
Female	34 (50.7)	21 (55.3)	13 (44.8)		7 (38.9)	27 (55.1)		13 (43.3)	21 (56.8)	
Age (years)				< 0.001*			0.097			1.000
< 60	32 (47.8)	10 (26.3)	22 (75.9)		12 (66.7)	20 (40.8)		14 (46.7)	18 (48.6)	
≥60	35 (52.2)	28 (73.7)	7 (24.1)		6 (33.3)	29 (59.2)		16 (53.3)	19 (51.4)	
Tumor location				0.005*			0.017*			0.391
Frontal	16 (23.9)	12 (31.6)	4 (13.8)		8 (44.4)	8 (16.3)		6 (20.0)	10 (27.0)	
Midline	17 (25.4)	4 (10.5)	13 (44.8)		1 (5.6)	16 (32.7)		6 (20.0)	11 (29.7)	
Others	34 (50.7)	22 (57.9)	12 (41.4)		9 (50.0)	25 (51.0)		18 (60.0)	16 (43.3)	
WHO 2016 diagnosis				0.107			< 0.001*			0.029*
DA, IDH-mut	5 (7.5)	2 (5.3)	3 (10.3)		5 (27.8)	0 (0.0)		4 (13.3)	1 (2.7)	
DA, IDH-wt	4 (6.0)	1 (2.6)	3 (10.3)		0 (0.0)	4 (8.2)		0 (0.0)	4 (10.8)	
AA, IDH-mut	5 (7.5)	2 (5.3)	3 (10.3)		5 (27.8)	0 (0.0)		4 (13.3)	1 (2.7)	
AA, IDH-wt	5 (7.5)	1 (2.6)	4 (13.8)		0 (0.0)	5 (10.2)		2 (6.7)	3 (8.1)	
O, IDH-mut & 1p/19q-codel	1 (1.5)	0 (0.0)	1 (3.4)		1 (5.6)	0 (0.0)		0 (0.0)	1 (2.7)	
AO, IDH-mut & 1p/19q-codel	5 (7.5)	5 (13.2)	0 (0.0)		5 (27.8)	0 (0.0)		4 (13.3)	1 (2.7)	
GM, IDH-mut	2 (3.0)	1 (2.6)	1 (3.4)		2 (11.1)	0 (0.0)		2 (6.7)	0 (0.0)	
GM, IDH-wt	40 (59.7)	26 (68.4)	14 (48.3)		0 (0.0)	40 (81.6)		14 (46.7)	26 (70.3)	
Histologic grade				0.105			< 0.001*			0.206
Grade II–III	25 (37.3)	11 (28.9)	14 (48.3)		16 (88.9)	9 (18.4)		14 (46.7)	11 (29.7)	
Grade IV	42 (62.7)	27 (71.1)	15 (51.7)		2 (11.1)	40 (81.6)		16 (53.3)	26 (70.3)	
*IDH*1 gene				0.907			–			0.002*
Mutation	18 (26.9)	10 (26.3)	8 (27.6)		–	–		14 (46.7)	4 (10.8)	
Wild type	49 (73.1)	28 (73.7)	21 (72.4)		–	–		16 (53.3)	33 (89.2)	
MGMT promoter				0.994			0.002*			–
Methylated	30 (44.8)	17 (44.7)	13 (44.8)		14 (77.8)	16 (32.7)		–	–	
Unmethylated	37 (55.2)	21 (55.3)	16 (55.2)		4 (22.2)	33 (67.3)		–	–	
TERT promoter				–			0.907			0.994
Mutation		–	–		10 (55.6)	28 (57.1)		17 (56.7)	21 (56.8)	
Wild type		–	–		8 (44.4)	21 (42.9)		13 (43.3)	16 (43.2)	

TERT, telomerase reverse transcriptase; IDH, isocitrate dehydrogenase; MGMT, O^6-methylguanine-DNA methyltransferase; Mut, mutation; Wt, wild-type; M, Methylation; UM, unmethylation; DA, IDH-mut, Diffuse astrocytoma, IDH-mutant; DA, IDH-wt, Diffuse astrocytoma, IDH-wildtype; AA, IDH-mut, Anaplastic astrocytoma, IDH-mutant; AA, IDH-wt, Anaplastic astrocytoma, IDH-wildtype; O, IDH-mut & 1p/19q-codel, Oligodendroglioma, IDH-mutant & 1p/19q-codeleted; AO, IDH-mut & 1p/19q-codel, Anaplastic oligodendroglioma, IDH-mutant & 1p/19q-codeleted; GM, IDH-mut, Glioblastoma, IDH-mutant; GM, IDH-wt, Glioblastoma, IDH-wildtype.
*Statistically significant, $P < 0.05$.

Table 3
Clinicopathological factors that are associated with *TERT* promoter mutation by multivariate analysis.

	TERTp mutation		*P*
	Odds ratio	95% CI	
Gender (Male vs. female)	1.014	0.292–3.520	0.983
Age (y) (< 60 vs. ≥ 60)	14.084	3.550–55.881	< 0.001*
Tumor location (Non-frontal vs. Frontal)	5.872	1.101–31.322	0.038*
Histologic grade (II–III vs. IV)	9.765	1.438–66.322	0.020*
IDH1 (Wild-type vs. Mutated)	6.414	0.751–54.769	0.089
MGMT promoter methylation (Negative vs. positive)	0.841	0.222–3.188	0.799

TERT, telomerase reverse transcriptase; CI, confidence interval; IDH, isocitrate dehydrogenase; MGMT, O^6-methylguanine-DNA methyltransferase.
*statistically significant, $P < 0.05$.

$P = 0.844$, and $P = 0.718$, respectively) (Fig. 1E–F). We also performed prognostic analysis of *TERTp* mutational status according to tumor locations (frontal vs. non-frontal). *TERTp* mutations were strongly associated with poor OS and PFS in frontal gliomas ($P = 0.032$ and $P = 0.028$, respectively) (Fig. 1G–H). In non-frontal gliomas, *TERTp* mutations were correlated with PFS ($P = 0.018$), but not with OS ($P = 0.098$). However, the multivariate analyses revealed that *TERTp* mutation or frontal located gliomas was not independent prognostic factor for OS and PFS in frontal located gliomas [hazard ratio = 4.526E5, 95% CI, 0.000–2.13E225, $P = 0.960$; hazard ratio = 5.221E5, 95% CI, 0.000–4.95E222, $P = 0.959$, respectively].

Univariate analysis (Cox regression model) showed that four variables, old age (≥60 years), grade IV glioma, *IDH* wild type, and *TERTp* mutation were significantly correlated with OS and PFS (Table 4). In multivariate analyses (Cox regression model), both old age (≥60 years) and grade IV glioma were independent prognostic factors for OS [hazard ratio = 2.210, 95% CI, 1.014–4.817, $P = 0.046$; hazard ratio = 4.127, 95% CI, 1.486–11.460, $P = 0.007$, respectively]. However, only grade IV glioma was an independent prognostic factor for PFS [hazard ratio = 3.313, 95% CI, 1.265–8.678, $P = 0.015$].

3.5. Prognostic impact of TERTp mutation and its combined analysis with IDH mutation and MGMT methylation in grade II, III, and IV gliomas

We further analyzed the prognostic impact of *TERTp* mutation on OS and PFS in each grade gliomas (Fig. 2). *TERTp* mutation was not correlated with OS or PFS of grade II ($P = 0.172$ and $P = 0.218$), grade III ($P = 0.591$ and $P = 0.267$), grade IV gliomas ($P = 0.718$ and $P = 0.514$). We performed subgroup analysis of combined *TERTp* mutational status and *MGMT* status. The *MGMT*-unmethylated/*TERTp*-mutated subgroup tended to have the worst OS and PFS rates in grade II gliomas. There were statistical significant survival differences between *MGMT*-unmethylated/*TERTp*-mutated and *MGMT*-unmethylated/*TERTp*-wildtype subgroups ($P = 0.045$ and $P = 0.030$, respectively). On the other hand, the combination of *TERTp* and *IDH* mutational status

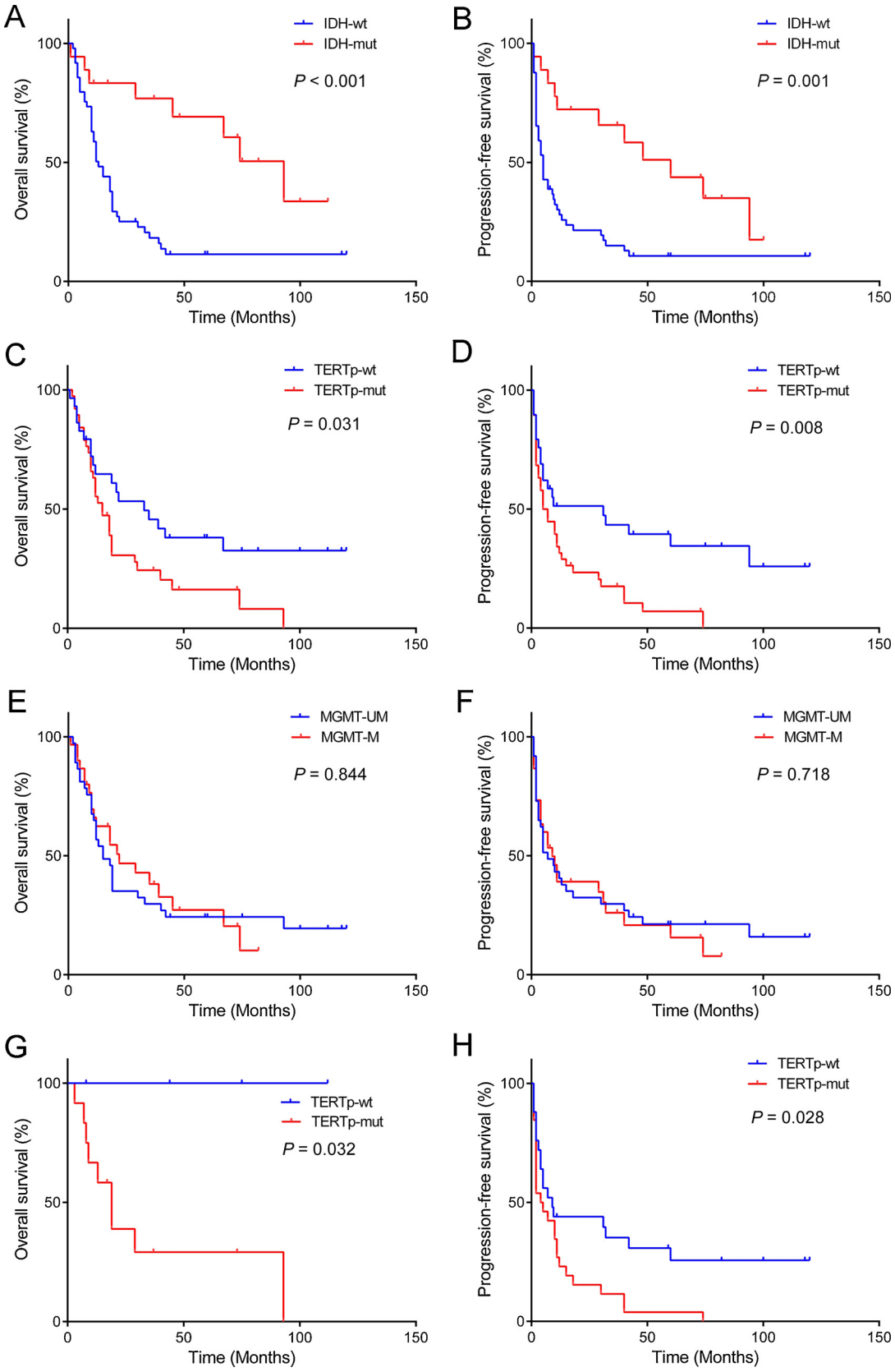

Fig. 1. Relationship between *IDH* mutation, *TERTp* mutation, and *MGMT* promoter methylation and survival. Kaplan–Meier curves were generated to determine the effect of *IDH* mutation (A, B), *TERTp* mutation (C, D) and *MGMT* promoter methylation (E, F) on overall survival (OS) (A, C, E) and progression free survival (PFS) (B, D, F) of patients with diffuse gliomas. OS (G) and PFS (H) based on the *TERTp* mutation status on frontal area gliomas.

Table 4
Clinicopathological and biological factors affecting overall and progression-free survival rates.

	OS				PFS			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
Age at diagnosis (yrs):								
≥ 60 vs. < 60	3.267 (1.757–6.076)	< 0.001*	2.210 (1.014–4.817)	0.046*	3.505 (1.865–6.588)	< 0.001*	1.619 (0.748–3.504)	0.222
Sex:								
Male vs. Female	1.308 (0.743–2.303)	0.352	1.247 (0.655–2.374)	0.502	1.551 (0.898–2.679)	0.115	1.573 (0.852–2.904)	0.148
Tumor location:								
Non-frontal vs. Frontal	0.595 (0.288–1.226)	0.159	1.106 (0.493–2.483)	0.807	0.549 (0.276–1.091)	0.087	0.876 (0.403–1.902)	0.738
Histological grade:								
II, III vs. IV	6.794 (2.934–15.735)	< 0.001*	4.127 (1.486–11.460)	0.007*	5.124 (2.444–10.742)	< 0.001*	3.313 (1.265–8.678)	0.015*
IDH mutation:								
Wild type vs. Mutant	0.266 (0.121–0.581)	0.001*	0.522 (0.168–1.623)	0.261	0.355 (0.180–0.698)	0.003*	0.621 (0.224–1.723)	0.360
MGMT promoter status:								
UM vs. M	0.946 (0.536–1.668)	0.847	1.173 (0.609–2.258)	0.634	1.101 (0.640–1.896)	0.728	1.619 (0.856–3.064)	0.139
TERT gene status:								
Wild type vs. Mutant	1.875 (1.038–3.385)	0.037*	1.055 (0.459–2.422)	0.900	2.121 (1.181–3.808)	0.012*	1.436 (0.670–3.080)	0.352

OS, overall survival; PFS, progression-free survival; HR, hazard ratio; CI, confidence interval; MGMT, O^6-methylguanine-DNA methyltransferase; TERT, telomerase reverse transcriptase; UM, unmethylation; M, methylation.
*Statistically significant, $P < 0.05$.

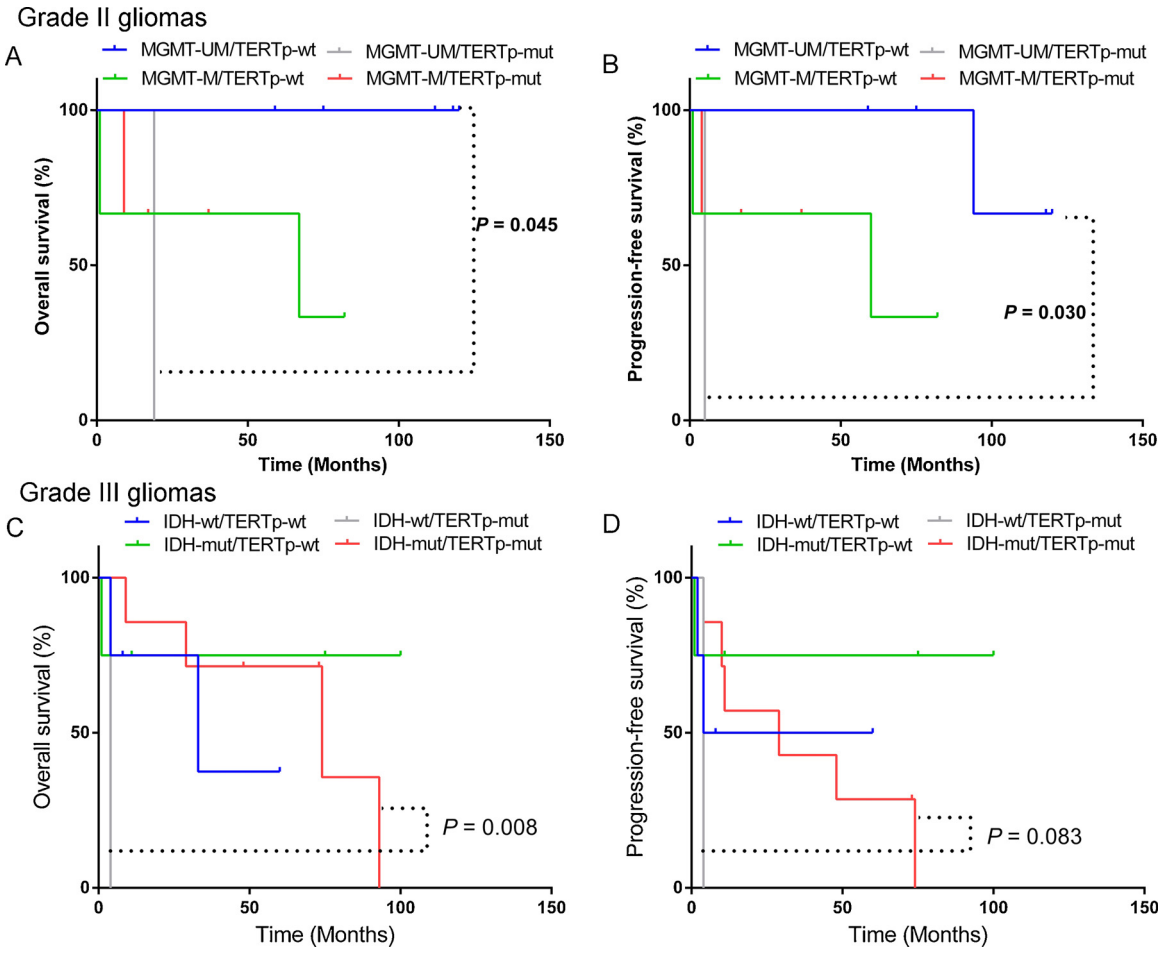

Fig. 2. Prognostic impact of *TERTp* mutation in association with *MGMT* methylation and *IDH* mutation. Overall survival (A) and progression-free survival (B) of the *MGMT*-unmethylated (UM)/*TERTp*-mutated (mut) groups in grade II gliomas. Correlation between *IDH*-wild type (wt)/*TERTp*-mut and overall survival (C) as well as progression-free survival (D) in grade III gliomas.

showed that the *IDH*-wildtype/*TERTp*-mutated group tended to have the worst prognosis in both OS and PFS in grade III gliomas. There was a statistical significant survival difference of OS between *IDH*-wildtype/*TERTp*-mutated and *IDH*-mutated/*TERTp*-mutated subgroups ($P = 0.008$). However, no significant associations between survival (both OS and PFS) and *MGMT*/*TERTp* or *IDH*/*TERTp* combination mutational status were observed in grade IV gliomas (all, $P > 0.05$).

4. Discussion

The present study investigated whether combined analysis of *TERTp* mutation with *IDH* mutation or *MGMT* methylation were associated with the biological behavior of gliomas. Although several previous studies investigated the association of *TERT*p mutation with *IDH* mutation in gliomas only, the present study demonstrated the association between *TERT*p mutation and *MGMT* methylation in low grade gliomas.

The discovery of *TERTp* mutations in a large number of gliomas opened the gate for a more refined molecular classification of gliomas in 2013 [9]. *TERTp* mutations are found in 70–83% of primary glioblastomas [4,9] and less frequently (28–38.5%) found in WHO grade II and III diffuse gliomas [4,16,18,19]. In the present study, we showed that *TERTp* mutation is relatively common in diffuse gliomas, with 44% and 64.3% in grade II–III and grade IV glioma cases, respectively. High *IDH1* mutation (64%) and *MGMT* methylation (56%) were found in grade II–III gliomas; however, low *IDH1* mutation (4.8%) and *MGMT* methylation (38.1%) frequencies were found in grade IV gliomas. We also demonstrated that all gliomas harbored wild type *IDH2* genes. The frequency of *TERTp* mutation in this study appeared to be higher than that found in previous studies [4,16,18,19]. The discrepancy may be explained by the methods used in our study. In previous studies [4,16,18,19], detection of *TERTp* mutation was carried out by conventional PCR, followed by direct sequencing using FFPE samples. This approach has relatively low sensitivity, as mutant alleles must be present in at least 20–50% of the cells to be reproducibly detected [11]. The use of FFPE tissue samples may also result in high false-negative rates [11]. We used a PNA probe-based assay in our study, which is a newly developed *TERTp* mutation detection kit with excellent specificity and sensitivity. By using this method, we identified concomitant C228 and C250 mutations in grade IV glioma, which has been previously reported [15]. The most frequently observed mutated region was C228 (84.2%), followed by C250 (15.8%), among *TERTp* mutated gliomas. Although C228T and C250T mutations were reported to be mutually exclusive in gliomas [8], a recent study detected the presence of both C228T and C250T mutations in a single case of *IDH*-wild type glioblastoma [15]. We observed that *TERTp* mutation was not mutally exclusive to glioblastoma, *IDH*-mutant. *TERTp* mutation was found in 50%% of globlastoma, *IDH*-mutant (1/2) and 65% of glioblastoma, *IDH*-wildtype (26/40), consistent with the reported range (~70%) in primary glioblastomas [4,9,15–17]. Taken together, these results indicated that *TERTp* mutations occur frequently across all types of gliomas [9], which suggested that regulation of telomere elongation by telomerase may play an important role in the pathogenesis of gliomas.

Old age has been reported to be positively correlated with *TERTp* mutations in grade II–III gliomas [4]. Sun et al. [16] described that grades II and III gliomas harboring the *TERTp* mutation are located preferentially in the frontal lobe. In addition, it has also been demonstrated that the frontal location is a risk factor for *TERTp* mutation in grade II–III gliomas [16]. Similarly, we found that old age, frontal location, and grade IV gliomas are risk factors for *TERTp* mutation in diffuse gliomas. Our results indicated that *TERTp* mutations tended to show poor prognosis in frontal gliomas, although multivariate analyses failed to reach the statistical significance. Nevertheless, analysis of the spatial distribution of *TERTp* mutations in low-grade gliomas may enhance our understanding of gliomagenesis.

The prognostic value of *TERTp* mutation in low-grade gliomas is a conflicting issue. *TERTp* mutation had been recognized as a molecular marker in prognostic classification of diffuse gliomas [4]. Similarly, our data showed that *TERTp* mutation is correlated with poor OS and PFS in glioma patients. *TERTp* mutation is associated with poor clinical outcomes in glioblastomas [4]. However, its clinical value and biological characteristics are still uncertain in grade II–III gliomas. Yang et al. [18] reported that *TERTp* mutation is an independent predictive factor for good prognosis in grade II–III gliomas. Therefore, *TERTp* mutations seem to have different prognostic impact in grade II–III and grade IV gliomas. In the present study, *TERTp* mutation was not correlated with OS or PFS in the diffuse gliomas. Interestingly, *TERTp* mutations, in combination with *MGMT* methylation or *IDH* mutation, were prognostic indicators of survival in glioma II and III, respectively: there were statistical significant survival differences between *MGMT*-unmethylated/*TERTp*-mutated and *MGMT*-unmethylated/*TERTp*-wildtype subgroups in grade II gliomas, whereas there was a statistical significant survival difference of OS between *IDH*-wildtype/*TERTp*-mutated and *IDH*-mutated/*TERTp*-mutated subgroups in grade III gliomas. However, no significant associations between survival and *MGMT*/*TERTp* or *IDH*/*TERTp* status were found in grade IV gliomas. The combination of *TERTp* and *IDH* mutational status was a significant prognostic factor in grade III gliomas; this finding of specific association between *IDH*/*TERTp* group and low-grade glioma is consistent with results from a previous study [5]. The *IDH*-wild-type/*TERTp*-mutated group tended to have the worst prognosis in grade III gliomas. *TERTp* mutation may give opposite prognostic indications in *IDH*-mutated and *IDH*-wild-type grade III gliomas [4,18]. Chan et al. [4] reported that *TERTp*-mutated tumors in *IDH* wild-type astrocytomas exhibit unfavorable OS and PFS, whereas *TERTp* mutation is a favorable prognostic in tumors with *IDH* mutation [4]. Eckel-Passow et al. [5] also showed that patients with *IDH*-wild-type/*TERTp*-wild-type have poorer OS when compared with patients with *IDH*-mutated/*TERTp* or *IDH* mutation alone, but show greater OS when compared with patients with *IDH*-wild-type/*TERTp*-mutation [5]. A recently published meta-analysis also suggested that combined *TERTp*-mutated/*IDH*-wild-type mutations can act as a significant biomarker for poor prognosis in grade III gliomas [17]. Therefore, *TERTp* mutations may be an indication of aggressive tumors in the presence of wild-type *IDH*.

Although *MGMT* methylation alone did not show any prognostic significance in gliomas, the combination of *TERTp* mutation and *MGMT* methylation demonstrated prognostic utility in grade II gliomas; this was not observed in grade IV gliomas. The *MGMT*-unmethylated/*TERTp*-mutated subgroup tended to show the worst prognosis in grade II gliomas. A defective *MGMT* may play potentially crucial roles in mutagenesis and carcinogenesis in cancer cells. Furthermore, *MGMT* methylation may be an early event during the development of astrocytic neoplasia [6], which may contribute to the biological behavior of lower grade gliomas. These observations suggested that epigenetic silencing of MGMT by promoter hypermethylation could enhance susceptibility to *TERTp* mutation and may ultimately lead to increased risk of disease recurrence or progression. To date, several studies have shown the correlation between *MGMT* methylation and *TERTp* mutation in diffuse gliomas. A similar correlation has been reported among gastric carcinomas, in which *MGMT* methylation was significantly associated with increased frequency of *KRAS* mutation, lymph node metastasis, advanced tumor stage, and decreased PFS [14]. Therefore, the *TERTp* mutation and *MGMT* methylation combination may be a high risk predictor of recurrence in grade II gliomas. These results suggest that both *TERTp* mutation and *MGMT* methylation could be potential candidates for a new therapeutic target in low-grade gliomas.

This was a single institutional retrospective study, not a trial-based correlative study, and as a result, the small number of patients placed limitations on the study results. Nevertheless, some interesting findings were discovered from the results. The present study showed a different clinical significance of *TERTp* mutation, in combination with *MGMT* methylation or *IDH* mutation depending on grades. The *TERTp* mutation had poor prognostic significance in patients with diffuse gliomas,

which highlighted the potential of *TERTp* as a prognostic molecular marker in gliomas. Results from this study may help guide the selection of treatments for high-risk patients in need of more intensive therapy and raise the possibility of targeted therapy in a subset of gliomas.

Conflicts 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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