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ORIGINAL PAPER

TERT promoter mutation status is necessary and sufficient to diagnose *IDH*-wildtype diffuse astrocytic glioma with molecular features of glioblastoma

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Abstract

The Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy (cIMPACT-NOW) update 3 recommends that histologic grade II and III *IDH*-wildtype diffuse astrocytic gliomas that harbor *EGFR* amplification, the combination of whole chromosome 7 gain and whole chromosome 10 loss (7 + /10 −), or *TERT* promoter (p*TERT*) mutations should be considered as glioblastomas (GBM), World Health Organization grade IV. In this retrospective study, we examined the utility of molecular classification based on p*TERT* status and copy-number alterations (CNAs) in *IDH*-wildtype lower grade gliomas (LGGs, grade II, and III). The impact on survival was evaluated for the p*TERT* mutation and CNAs, including *EGFR* gain/amplification, *PTEN* loss, *CDKN2A* homozygous deletion, and *PDGFRA* gain/amplification. We analyzed 46 patients with *IDH*-wildtype/p*TERT*-mutant (mut) LGGs and 85 with *IDH*-wildtype/p*TERT*-wildtype LGGs. *EGFR* amplification and a combination of *EGFR* gain and *PTEN* loss (*EGFR* + /*PTEN* −) were significantly more frequent in p*TERT*-mut patients ($p < 0.0001$). Cox regression analysis showed that the p*TERT* mutation was a significant predictor of poor prognosis (hazard ratio [HR] 2.79, 95% confidence interval [CI] 1.55–4.89, $p = 0.0008$), but neither *EGFR* amplification nor *EGFR* + /*PTEN* − was an independent prognostic factor in *IDH*-wildtype LGGs. *PDGFRA* gain/amplification was a significant poor prognostic factor in *IDH*-wildtype/p*TERT*-wildtype LGGs (HR 2.44, 95% CI 1.09–5.27, $p = 0.03$, Cox regression analysis). The *IDH*-wildtype LGGs with either p*TERT*-mut or *PDGFRA* amplification were mostly clustered with GBM by DNA methylation analysis. Thus, our study suggests that analysis of p*TERT* mutation status is necessary and sufficient to diagnose *IDH*-wildtype diffuse astrocytic gliomas with molecular features of glioblastoma. The *PDGFRA* status may help further delineate *IDH*-wildtype/p*TERT*-wildtype LGGs. Methylation profiling showed that *IDH*-wildtype LGGs without molecular features of GBM were a heterogeneous group of tumors. Some of them did not fall into existing categories and had significantly better prognoses than those clustered with GBM.

Keywords Lower grade glioma · *IDH*-wildtype · *TERT* promoter mutation · Copy-number alteration

Introduction

The 2016 World Health Organization (WHO) classification of central nervous system tumors represents the updates on diagnostic classes, grades, and criteria [16]. However, the classification and grading of *IDH*-wildtype diffuse astrocytic gliomas remain controversial. The criteria for distinguishing WHO grades II and III have been based on traditional morphologic findings, including mitotic activity and anaplastic nuclear features. Although grades II and III are often collectively called diffuse lower grade gliomas (LGGs), multiple studies have indicated that a substantial subset of *IDH*-wildtype LGGs based on histologic criteria has an aggressive clinical course, with overall patient survival times equal to or only slightly longer than patients

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with *IDH*-wildtype glioblastoma (GBM), WHO grade IV [6, 11, 26]. The Consortium to Inform Molecular and Practical Approaches to CNS Tumor Taxonomy (cIMPACT-NOW) update 3 [5] recommended that histological grade II and III *IDH*-wildtype diffuse astrocytic gliomas that contain either high-level *EGFR* amplification, a combination of whole chromosome 7 gain and whole chromosome 10 loss (7 + /10 −), or *TERT* promoter (p*TERT*) mutations, be designated as "Diffuse astrocytic glioma, *IDH*-wildtype, with molecular features of GBM, WHO grade IV", because these patients show an aggressive clinical course equivalent to GBM WHO grade IV. We and others have also shown that there is no difference in overall survival (OS) between the *IDH*-wildtype, p*TERT* mutant (mut) LGG, and GBM [3]. Thus, molecular delineation of *IDH*-wildtype diffuse astrocytic tumors has become vital in diagnosing adult glioma and treatment decisions.

The present study aimed to independently evaluate the utility of these molecular prognostic markers, p*TERT* mutation, *EGFR* amplification, and 7 + /10 −, and to identify copy-number alterations (CNAs) that could serve as a novel prognostic marker in *IDH*-wildtype LGGs to predict clinical courses corresponding to GBM, regardless of histologic grade, in a large series of our Japanese cohort. Our study suggested that analysis of p*TERT* mutation status is necessary and sufficient to diagnose *IDH*-wildtype diffuse astrocytic gliomas with the molecular features of glioblastoma. The *PDGFRA* status may help further delineate *IDH*-wildtype/p*TERT*-wildtype LGGs. In addition, *IDH*-wildtype LGGs without molecular features of GBM appeared to be a heterogeneous group of tumors, some of which may have distinct clinical and molecular features.

Materials and methods

Patients

In this study, we included a total of 724 patients with gliomas analyzed in our previous study [3]. The inclusion criteria for the analysis of *IDH*-wildtype LGGs were as follows: 18 years of age or older, histological diagnosis of grade II and III diffuse glioma originating in the cranium, absence of *H3F3A* mutation, and clinical data available for survival analysis. Tumors with *BRAF* V600E mutations were included. There were 151 patients with *IDH*-wildtype diffuse glioma WHO grade II or III. For survival analysis, 453 patients with *IDH*-wildtype GBM and 120 with grade II, III *IDH*-mut, and p*TERT*-wildtype astrocytoma were used for comparison. Clinical data collected from each institution were as follows: age at diagnosis, sex, preoperative Karnofsky performance status (KPS), the extent of resection, radiation dose, and chemotherapeutic regimen in the initial treatment. Survival data of patients were updated whenever possible. The study was approved by the Institutional Review Board (IRB) of the National Cancer Center (No. 2013–042) and the corresponding local IRB of the participating centers.

Central pathology review

All cases of LGGs and 260 cases of GBM were subjected to central pathology review by three senior neuropathologists (T.K., M.S., and H.S.). Histological diagnosis was made as a consensus between the three pathologists according to the 2007 WHO classification for central nervous system tumors, similar to the histological diagnosis of the 2016 WHO classification. Another 193 cases of GBM were not subjected to histopathological review; therefore, the local diagnoses were the final diagnoses.

Molecular analysis

Tumor DNA was extracted from frozen tumor tissues for all cases using a DNeasy Blood & Tissue Kit (Qiagen, Tokyo, Japan). The presence of hotspot mutations in *IDH1* (R132) and *IDH2* (R172), in the p*TERT* (−124 and −146), at codons 27 and 34 of *H3F3A*, and codon 600 of *BRAF* was analyzed by Sanger sequencing or pyrosequencing for all cases, as previously reported [2]. To assess copy-number status, we performed multiplex ligation-dependent probe amplification (MLPA) using the SALSA MLPA KIT P105 (version D2) and P088 (version C2), following the manufacturer's protocol (MRC Holland, Amsterdam, The Netherlands) [13]. The P105 kit is designed to detect CNAs typically found in gliomas and includes probes against *PDGFRA*, *EGFR*, *CDKN2A*, *PTEN*, *TP53*, *CDK4*, *MDM2*, and *NFKBIA* genes. The P088 kit was designed to assess mainly 1p/19q codeletion. The CNAs of 1p and 19q were determined by MLPA, microarray-based comparative genomic hybridization, or microsatellite analysis. The methylation status of the *MGMT* promoter was analyzed by pyrosequencing after bisulfite modification of genomic DNA extracted from tumor specimens as described [17], with some modifications in the thermal cycling conditions. Based on an outcome-based study to determine an optimal cut-off to judge *MGMT* promoter methylation in a series of 276 newly diagnosed GBMs, we used a ≥ 16% cut-off for *MGMT* methylation (Ichimura, manuscript in preparation).

Molecular and clinical data analysis in The Cancer Genome Atlas (TCGA) and Memorial Sloan Kettering Cancer Center (MSKCC)

Independent and extensive molecular data and clinical information, including survival data of *IDH*-wildtype LGGs in

TCGA ($n=94$) and MSKCC ($n=73$), were collected from cBioPortal for Cancer Genomics (https://cbioportal.org) [10, 12] and the supplemental data of the previous publication by TCGA [7, 9]. In TCGA data, since the p*TERT* mutation was not examined in every case, only 56 cases with *IDH*-wildtype LGG were analyzed. CNA was determined based on the log2 copy-number value.

Methylation array analysis

The Infinium MethylationEPIC BeadChip Kit was used to obtain genome-wide DNA methylation profiles for tumor samples according to the manufacturer's instructions (Illumina, San Diego, USA). For most samples, 500 ng of DNA was used as the input material. The output data (IDAT files) were checked for general quality, as indicated by the manufacturer. IDAT files were uploaded to the Molecular Neuropathology site to perform DNA methylation-based classification of central nervous system tumors (www.molecularneuropathology.org) [8]. The classifier scores and chromosomal copy-number plots were obtained using the methylation classifier.

For further bioinformatics analysis, all computational analyses were performed using R version 4.0.4. Raw signal intensities were obtained from IDAT files of 64 *IDH*-wildtype LGG samples using the Minfi Bioconductor package version 1.34.0. Unprocessed IDAT files of 2801 samples were downloaded from the NCBI Gene Expression Omnibus (GEO) under accession number GSE109381 and used as reference samples [8]. A correction for the type of material tissue (formalin-fixed paraffin-embedded tissue/frozen) was performed using the removeBatchEffect function (limma package version 3.44.3). The methylated and unmethylated signals were corrected individually, and beta-values were calculated using an offset of 100, as recommended by Illumina. After the probe filtering criteria were applied according to the GitHub repository (https://github.com/mwsill/mnp_training), 428,230 probes were used for the following analysis. To perform unsupervised non-linear dimension reduction, the 1000 most variable probes, according to standard deviation, were selected from 2801 reference samples. The t-distributed stochastic neighbor embedding (t-SNE) [25] plot for 64 *IDH*-wildtype LGGs and 2801 references was made using the Rtsne package (version 0.15), with 2500 iterations and a perplexity value of 30. The predictions of tumor purity for all cases were calculated using the R package RF_Purify with the method 'ABSOLUTE' [14].

Statistical analysis

The characteristics of *IDH*-wildtype, *pTERT*-wildtype LGGs, and *IDH*-wildtype, *pTERT* mut LGGs were compared. The comparison for age distribution was examined using *the Student's t test*. Associations between molecular variables were evaluated using Fisher's exact test. For survival analysis, OS was defined as the time from the date of initial operation to the date of death from any cause. Patients who were still alive at the last follow-up were censored. OS was estimated using the Kaplan–Meier method. Survival curves were compared using the log-rank test. The hazard ratios (HRs) and 95% confidence intervals (CIs) were estimated using the Cox regression model. A p value <0.05 was considered statistically significant. All statistical analyses were performed using JMP version 15 software (SAS Institute, Cary, NC, USA).

Results

Prognostic value of TERT promoter mutation, EGFR amplification, and EGFR + PTEN− in IDH-wildtype LGGs

In 5 of 140 patients with *IDH*-wildtype LGGs (three of 49 patients: p*TERT*-mut, two of 91 patients: p*TERT*-wildtype), additional genomic DNA was not available for copy-number analysis. Thus, a total of 135 patients with *IDH*-wildtype LGGs were analyzed for CNAs in our cohort. In 4 of 89 patients with *IDH*-wildtype/p*TERT*-wildtype LGGs, there were no mutations, CNA, or *MGMT* promoter methylation detected in our examination. Considering the possibility of low tumor cell contents in the surgical specimens used for analysis, we excluded these four patients from the subsequent analysis. Thus, 131 patients with *IDH*-wildtype LGGs were studied. There were 46 *IDH*-wildtype/p*TERT* mut LGGs and 85 *IDH*-wildtype/p*TERT*-wildtype LGGs. Patient clinical information and molecular status are listed in Supplementary Tables S1 and S2.

Table 1 shows the characteristics of *IDH*-wildtype, p*TERT*-wildtype LGGs, and *IDH*-wildtype, p*TERT* mut LGGs. The patients with *IDH*-wildtype and p*TERT* mut LGGs were significantly older ($p=0.0017$). There were no significant differences in gender, WHO grade, KPS, or *MGMT* promoter methylation status. The copy numbers of *PDGFRA*, *EGFR*, *PTEN*, and *CDKN2A* were significantly different between p*TERT*-wildtype and p*TERT* mut. Figure 1 shows the distribution of p*TERT* mutation, *EGFR* amplification, the combination of *EGFR* gain and *PTEN* loss (*EGFR* + /*PTEN* −), and *PDGFRA* gain/amplification. The frequency of *PDGFRA* gain/amplification was significantly higher in p*TERT*-wildtype ($p=0.043$), while the frequencies of *EGFR* amplification and *EGFR* + /*PTEN* − were significantly higher in p*TERT* mut ($p<0.0001$) (Table 2).

To validate the criteria to define the "Diffuse astrocytic glioma, *IDH*-wildtype, with molecular features of GBM, WHO grade IV" recommended in the cIMPACT-NOW

Table 1 Difference of clinical and molecular characteristics between p*TERT* mut and p*TERT* wt in *IDH*-wildtype LGG

IDH wt LGG			p*TERT* mut ($n=46$)	p*TERT* wt ($n=85$)
Age at diagnosis	Median (range)		66 (45–85)	58 (22–81)
Gender	Male		23 (50.0%)	46 (54.1%)
	Female		23 (50.0%)	39 (45.9%)
KPS≧80			29 (63.0%)	62 (72.9%)
WHO grade	II		9 (19.6%)	25 (29.4%)
	III		37 (80.4%)	60 (70.6%)
Surgery	Removal		31 (67.4%)	60 (70.6%)
	Biopsy		15 (32.6%)	25 (29.4%)
Adjuvant therapy	CRT		38 (82.6%)	62 (72.9%)
	Non-CRT		6 (13.0%)	22 (25.9%)
MGMT methylation			16 (34.8%)	23 (27.1%)
CNA	*PDGFRA*	Gain	1 (2.2%)	5 (5.9%)
		Amp	4 (8.7%)	18 (21.2%)
	EGFR	Gain	11 (23.9%)	4 (4.7%)
		Amp	12 (26.1%)	1 (1.2%)
	PTEN	Hemi Del	15 (32.6%)	4 (4.7%)
		Homo Del	2 (4.3%)	0 (0%)
	CDK4	Gain	1 (2.2%)	4 (4.7%)
		Amp	7 (15.2%)	11 (11.9%)
	MDM2	Gain	1 (2.2%)	2 (2.9%)
		Amp	4 (8.7%)	7 (8.2%)
	NFKB1A	Hemi Del	3 (6.5%)	6 (7.1%)
		Homo Del	0 (0%)	1 (1.2%)
	TP53	Hemi Del	8 (17.4%)	23 (27.1%)
		Homo Del	1 (2.2%)	1 (1.2%)
	CDKN2A	Hemi Del	12 (26.1%)	10 (11.8%)
		Homo Del	12 (26.1%)	18 (21.2%)

Amp amplification; *CRT* chemoradiotherapy; *CNA* copy-number alteration; *Hemi Del* hemizygous deletion; *Homo Del* homozygous deletion; *KPS* Karnofsky Performance Status; *LGG* lower grade glioma; *Met* methylation; *pTERT TERT* promoter

update 3, we evaluated the association of *TERT* promoter mutation, *EGFR* amplification, and *EGFR*+/*PTEN*− with the prognosis (Fig. 2a–c). Of note, although the recommendation was the combined whole chromosome 7 gain and whole chromosome 10 loss (+7/−10), it was surrogated by the combination of *EGFR* gain and *PTEN* loss for practicality. The results showed that p*TERT* mutation was the only molecular marker significantly associated with a poor prognosis ($p<0.0001$). Neither *EGFR* amplification nor *EGFR*+/*PTEN*− was significantly associated with OS, although there was a tendency for poor prognosis in patients with *EGFR* amplification or *EGFR*+/*PTEN*− ($p=0.053$ and 0.064, respectively). To further examine the prognostic impact of p*TERT* mutation, *EGFR* amplification, and *EGFR*+/*PTEN*−, we performed multivariate Cox regression analysis using the model, including the possible confounding variables of age at diagnosis, preoperative KPS, WHO grade, surgical procedure, adjuvant chemoradiotherapy, and *MGMT* promoter methylation status. The results showed that the p*TERT* mutation was the sole significant negative predictor of prognosis for the three molecular variables (HR 2.79, 95% CI 1.55–4.89, $p=0.0008$) (Table 3). *MGMT* methylation was significantly associated with a good prognosis (HR 0.39, 95% CI 0.22–0.68, $p=0.0007$). Chemoradiotherapy was significantly associated with poor prognosis (HR 2.27; 95% CI 1.05–5.48; $p=0.038$). Chemoradiotherapy was possibly selectively administered to patients with progressive astrocytoma. Whether surgical removal or biopsy was not associated with prognosis. We analyzed the published data of *IDH*-wildtype LGG in TCGA [6] and MSKCC cohorts [15]. In the TCGA data, chromosome 7+/10- was observed in 1/19 cases (5.3%) of p*TERT*-wildtype and in 26/37 cases (70.3%) of p*TERT* mut. *EGFR* amplification was observed in 3/19 cases (15.8%) of p*TERT*-wildtype and 24/37 cases (64.9%) of p*TERT* mut. In the MSKCC data, chromosome 7+/10− was observed in 2/12 cases (16.7%) of p*TERT*-wildtype and in 32/49 cases (70.3%) of p*TERT* mut. *EGFR* amplification was observed

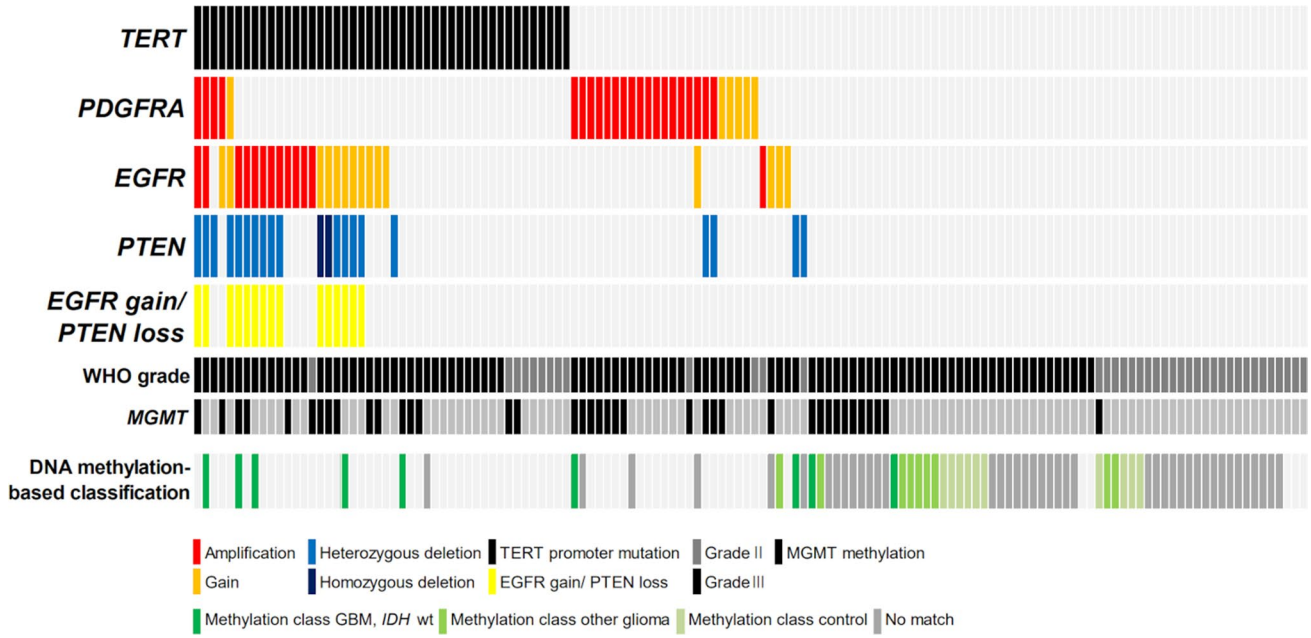


Fig. 1 Distribution of *TERT* promoter mutation and copy-number alterations in *PDGFRA*, *EGFR*, and *PTEN* in *IDH*-wildtype lower grade gliomas. The results of the DNA methylation-based classification by the DKFZ methylation classifier are indicated in the bottom row

Table 2 Frequencies of copy-number alterations between p*TERT* mut and p*TERT* wt in *IDH*-wildtype LGG

IDH wt LGGs		p*TERT* wt ($n=85$)	p*TERT* mut ($n=46$)	p value
PDGFRA	Gain/Amp	23 (27.0%)	5 (10.9%)	0.043
EGFR	Amp	1 (1.2%)	12 (26.1%)	< 0.0001
EGFR + /*PTEN* −	Combined	0 (0%)	15 (32.6%)	< 0.0001

Amp Amplification

in 4/21 cases (19.0%) of p*TERT*-wildtype and 20/52 cases (38.5%) of p*TERT* mut. These data confirmed that chromosome 7 + /10 − ($p < 0.0001$ in TCGA and MSKCC) and amplification of *EGFR* ($p = 0.0013$ in TCGA, $p < 0.0001$ in MSKCC) were mostly among the p*TERT* mut cases.

Association of CNAs with WHO histological grade in IDH-wildtype LGGs

In *IDH*-wildtype LGG, WHO grade III showed a significantly shorter OS than WHO grade II ($p = 0.00012$) (Fig. 2d). We evaluated the association of CNAs with WHO grade in *IDH*-wildtype LGGs (Table 4). These results showed that *PDGFRA* amplification, *EGFR* gain, *PTEN* hemizygous deletion, *CDK4* amplification, and *CDKN2A* homozygous deletion were significantly more common in WHO grade III ($p = 0.014$, 0.011, 0.025, 0.0036, and 0.0046, respectively). There were also significant differences in age at diagnosis, KPS, adjuvant therapy, or *MGMT* promoter methylation status.

Association of CNAs with overall survival in IDH-wildtype pTERT mut LGGs

We evaluated the association between CNAs and OS in *IDH*-wildtype p*TERT* mut LGGs. As shown above, all *EGFR* amplifications except one case and all combined *EGFR* + /*PTEN* − cases were included in the p*TERT* mut-type in *IDH*-wildtype LGGs (Fig. 1). Kaplan–Meier curves showed that neither *EGFR* amplification nor *EGFR* + /*PTEN* − was a prognostic predictor (Fig. 3a, b). Homozygous deletion of *CDKN2A* showed a tendency to predict poor prognosis, although it was not statistically significant ($p = 0.079$) (Fig. 3c). None of the other CNAs were prognostic predictors.

Association of CNAs with overall survival in IDH-wildtype pTERT-wildtype LGGs

Next, we evaluated the association between CNAs and OS in *IDH*-wildtype p*TERT*-wildtype LGGs. As shown in Table 1, copy-number gain or amplification of *PDGFRA* was observed

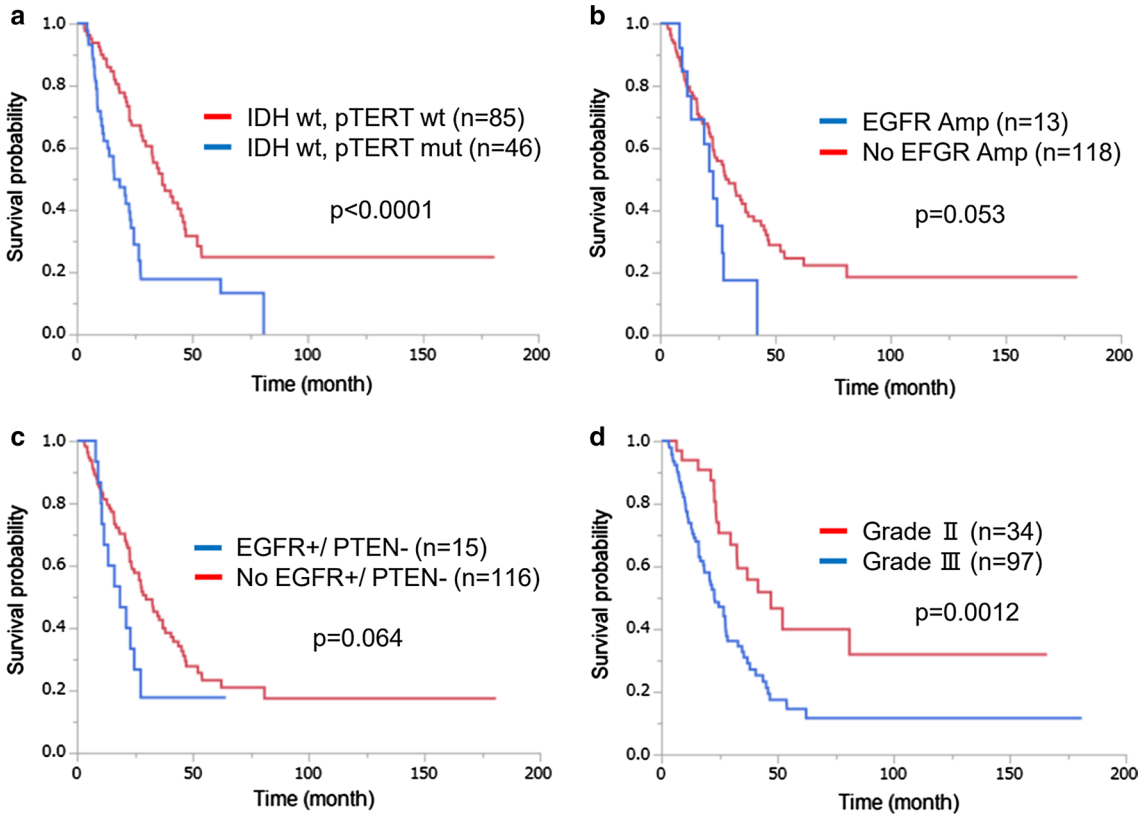


Fig. 2 Kaplan–Meier curves of overall survival (OS) in *IDH*-wildtype lower grade gliomas (LGGs). **a** *IDH*-wildtype p*TERT* mut LGGs (median OS: 16.1 months) showed significantly longer survival than *IDH*-wildtype p*TERT* wt LGGs (median OS: 37.0 months) ($p < 0.0001$). Difference in OS between **b** *IDH*-wildtype *EGFR* amplified (Amp) LGGs (median OS: 22.7 months) and *IDH*-wildtype non-*EGFR* Amp LGGs was not significant (median OS: 28.5 months; $p = 0.053$ months), and **c** *IDH*-wildtype *non-EGFR*+/*PTEN*-LGGs (median OS: 18.5 months) and *IDH*-wildtype non-*EGFR*+/*PTEN*-LGGs was not significant (median OS: 16.0 months; $p = 0.064$). **d** WHO grade III *IDH*-wildtype astrocytoma (median OS: 22.8 months) showed significantly shorter OS than WHO grade II *IDH*-wildtype astrocytoma (median OS: 47.2 months; $p = 0.00012$)

significantly more frequently in p*TERT*-wildtype, *IDH*-wildtype LGGs. In addition, copy-number gain or amplification of *PDGFRA* was a significant predictor of poor prognosis in *IDH*-wildtype p*TERT*-wildtype LGGs ($p = 0.021$) (Fig. 4a). None of the other CNAs were associated with survival. To examine the prognostic effect of copy-number gain or amplification of *PDGFRA*, we performed multivariate Cox regression analysis using the model, including the variables of age at diagnosis, preoperative KPS, WHO grade, surgical procedure, adjuvant chemoradiotherapy, and *MGMT* promoter methylation status. The results showed that *PDGFRA* gain or amplification was an independent predictor of poor prognosis (HR 2.44, 95% CI 1.09–5.27, $p = 0.030$) in addition to age, adjuvant therapy, and *MGMT* methylation (Table 5).

Association between molecular classification and prognosis of LGGs

Based on these results, we divided *IDH*-wildtype LGGs into three groups: *IDH*-wildtype, p*TERT*-wildtype, no *PDGFRA* gain/amplification; *IDH*-wildtype, p*TERT*-wildtype, *PDGFRA* gain/amplification; *IDH*-wildtype, p*TERT* mut, with or without *PDGFRA* gain/amplification. We compared the OS of *IDH*-wildtype p*TERT*-wildtype *PDGFRA* gain/amplification LGGs with *IDH*-wildtype p*TERT* mut LGGs, *IDH*-wildtype p*TERT*-wildtype GBM, or *IDH*-wildtype p*TERT* mut GBM. The results showed that the *IDH*-wildtype, p*TERT*-wildtype, and *PDGFRA* gain/amplification groups showed a short OS equivalent to *IDH*-wildtype p*TERT* mut LGGs, *IDH*-wildtype p*TERT*-wildtype GBM, or *IDH*-wildtype p*TERT* mut GBM (Fig. 4b).

Since the copy-number gain or amplification of *PDGFRA* was found to be a predictor of poor prognosis in *IDH*-wildtype LGGs, we brought together *IDH*-wildtype LGGs with any of the predictors of poor prognoses, such as p*TERT* mut, *PDGFRA* gain/amplification, *EGFR* amplification, or *EGFR*+/*PTEN*− (Group C astrocytoma), although almost all *EGFR* amplification and *EGFR*+/*PTEN*− were included in p*TERT* mut-LGGs. We

Table 3 Cox regression analysis in *IDH* wt LGG

	HR	95% CI	*p* value
Age			
≧65 y.o	2.21	1.34–3.65	0.002
< 65 y.o	Ref	–	–
KPS			
≧80	0.59	0.34–1.07	0.083
≦70	Ref	–	–
WHO grade			
II	0.53	0.27–0.98	0.044
III	Ref	–	–
Surgery			
Removal	0.78	0.47–1.32	0.35
Biopsy	Ref	–	–
Adjuvant therapy			
CRT	2.27	1.05–5.48	0.038
Non-CRT	Ref	–	–
MGMT			
Met	0.39	0.22–0.68	0.0007
Un-met	Ref	–	–
TERT promoter			
Mutation	2.79	1.55–4.89	0.0008
Wildtype	Ref	–	–
EGFR			
Amp	0.93	0.42–1.93	0.86
Non-amp	Ref	–	–
EGFR + /*PTEN* −			
Present	0.63	0.28–1.34	0.24
Absent	Ref	–	–

Amp amplification; *CI* confidence interval; *CRT* chemoradiotherapy; *HR* hazard ratio; *KPS* Karnofsky performance status; *Met* methylation

defined grade II and III *IDH*-mut astrocytoma as Group A astrocytoma, and *IDH*-wildtype LGGs without any poor prognostic predictors as Group B astrocytoma. Comparison of the characteristics between Group B and C astrocytomas showed that the frequencies of elderly patients, WHO grade III, or *MGMT* methylation were significantly higher in Group C astrocytoma ($p < 0.001$, $p = 0.016$ and 0.023, respectively) (Supplementary Table S3). In Group B astrocytoma, patients with WHO grade III showed significantly shorter overall survival than those with WHO grade II ($p = 0.024$) (Supplementary Fig. S1a). In Group C astrocytoma, WHO grade was not significantly associated with OS ($p = 0.087$) (Supplementary Fig. S1b). There was a significant difference in OS between Group A and B astrocytomas ($p < 0.0001$). The OS of Group C astrocytoma was significantly shorter than that of Group A or B astrocytoma ($p < 0.0001$) and equivalent to *IDH*-wildtype GBM (Fig. 4c).

Methylation array analysis of IDH-wildtype LGGs

A total of 54 Group B astrocytomas, for which additional genomic DNA was available, were analyzed for genome-wide DNA methylation analysis. Selected ten Group C astrocytomas, including four tumors with *IDH*-wildtype, p*TERT*-wildtype, *PDGFRA* amplification LGGs, and six tumors with *IDH*-wildtype p*TERT* mut LGGs, were also analyzed (Fig. 1). There was no discrepancy between chromosomal copy-number profiles obtained from the DNA methylation array and the CNA results obtained using MLPA.

Table 6 summarizes the results of the DNA methylation-based classification, and Supplementary Table S4 shows the details (www.molecularneuropathology.org). Among the 54 Group B astrocytomas, 23 cases (42.6%) matched with one of the existing reference groups, including 4 GBM, IDH-wildtype, 3 Glioma, IDH-mutant, 2 anaplastic pilocytic astrocytoma (ANA_PA), 1 anaplastic pleomorphic xanthoastrocytoma (PXA), one ganglioglioma (GG), one CNS high-grade neuroepithelial tumor, one medulloblastoma, and ten control tissues. Among the 31 no-match cases (57.4%), 23 showed a calibrated score above 0.3, and were classified as follows: 11 Glioblastoma, IDH-wildtype, 1 Glioma, IDH-mutant, 1 diffuse midline glioma H3 K27M mutant, 2 dysembryoplastic neuroepithelial tumor, 1 GG, 1 pilocytic astrocytoma, 1 ependymoma, posterior fossa group B, 1 plexus tumor, and 4 control tissue. Eight Group B astrocytomas showed calibrated scores below 0.3 and were not classified as any of the existing categories. Among the Group C astrocytomas, all six *IDH*-wildtype and p*TERT*-mutant LGGs were classified as Glioblastoma, IDH-wildtype, with a calibrated score of above 0.9, except for one case (0.41). One of the four *IDH*-wildtype, p*TERT*-wildtype, and *PDGFRA* amplified LGGs were classified as Glioblastoma, IDH-wildtype, with a calibrated score above 0.9, while the other three were not matched (classified as Glioblastoma, IDH-wildtype, and calibrated scored < 0.9).

The methylation-based t-SNE distribution of *IDH*-wildtype, p*TERT*-wildtype LGGs mainly formed two clusters, with several exceptions (Fig. 5a). One group of tumors formed a cluster within methylation class family Glioblastoma, IDH-wildtype, mainly between subclass midline and subclass RTK I ("GBM" cluster, Fig. 5b). All *IDH*-wildtype, p*TERT* mut, *IDH*-wildtype, p*TERT*-wildtype, and *PDGFRA* amplification LGGs were located within the GBM cluster, except one. Another group of tumors formed a cluster separate from any of the existing reference groups near the methylation class control tissue and several subtypes of LGGs, including LGG_GG (Fig. 5c). We tentatively defined this cluster as an "LGG" cluster. We then compared the OS of GBM cluster cases with LGG cluster cases. Results showed that the LGG cluster cases had significantly longer OS than the GBM cluster cases (Fig. 5d). When the cases classified

Table 4 Difference of clinical and molecular characteristics between WHO grade II and III in *IDH*-wildtype LGG

IDH wt LGG			WHO grade II ($n=34$)	WHO grade III ($n=97$)	p value
Age at diagnosis	Median (range)		55.5 (22–81)	64 (22–85)	0.011
Gender	Male		16 (47.1%)	53 (54.6%)	0.55
	Female		18 (52.9%)	44 (45.4%)	
KPS≧80			31 (91.2%)	60 (61.9%)	0.0011
Surgery	Removal		19 (55.9%)	72 (74.2%)	0.054
	Biopsy		15 (44.1%)	25 (25.8%)	
Adjuvant therapy	CRT		16 (47.1%)	83 (85.6%)	< 0.0001
	Non-CRT		18 (52.9%)	11 (11.3%)	
TERT promoter mutation			9 (26.5%)	37 (38.1%)	0.30
MGMT methylation			5 (14.7%)	34 (35.1%)	0.030
CNA	*PDGFRA*	Gain	1 (2.9%)	5 (5.2%)	1
		Amp	1 (2.9%)	21 (21.6%)	0.014
	EGFR	Gain	0	15 (15.5%)	0.011
		Amp	2 (5.9%)	11 (11.3%)	0.51
	PTEN	Hemi Del	1 (2.9%)	18 (18.6%)	0.025
		Homo Del	0	2 (2.1%)	1
	CDK4	Gain	0	5 (5.2%)	0.57
		Amp	0	18 (18.6%)	0.0036
	MDM2	Gain	0	3 (3.1%)	0.57
		Amp	0	11 (11.3%)	0.065
	NFKB1A	Hemi Del	0	9 (9.3%)	0.11
		Homo Del	1 (2.9%)	0	0.26
	TP53	Hemi Del	4 (11.8%)	27 (27.8%)	0.097
		Homo Del	0	2 (2.1%)	1
	CDKN2A	Hemi Del	3 (8.8%)	19 (19.6%)	0.19
		Homo Del	2 (5.9%)	28 (28.9%)	0.0046

Amp amplification; *CRT* chemoradiotherapy; *CNA* copy-number alteration; *Hemi Del* Hemizygous deletion; *Homo Del* homozygous deletion; *KPS* Karnofsky performance status; *LGG* lower grade glioma; *Met* methylation; *pTERT TERT* promoter

in methylation class control tissue were excluded from the LGG cluster, the remaining LGG cluster cases still showed significantly longer OS than the GBM cluster cases (Fig. 5e).

Discussion

This study showed that almost all cases with *EGFR* amplification or combined *EGFR*+/*PTEN*− in *IDH*-wildtype LGGs also had p*TERT* mutations. Among the molecular characteristics that defined "Diffuse astrocytic glioma, *IDH-wildtype*, with molecular features of GBM (WHO grade IV)," that is, p*TERT* mutation, *EGFR* amplification, and *EGFR*+/*PTEN*−, the only independent prognostic predictor in *IDH*-wildtype LGGs was p*TERT* mutation. Neither *EGFR* amplification nor *EGFR*+/*PTEN*− was significantly associated with OS in *IDH*-wildtype LGGs. In other words, the prognosis of *IDH*-wildtype LGGs with *EGFR* amplification or *EGFR*+/*PTEN*− was dependent on the status of the p*TERT* mutation. In *IDH*-wildtype p*TERT*-wildtype LGGs, gain or amplification of *PDGFRA* was observed significantly more frequently than in p*TERT* mut tumors. The gain or amplification of *PDGFRA* was a significant predictor of poor prognosis in *IDH*-wildtype p*TERT*-wildtype LGGs.

We previously reported that p*TERT* mutation was a significant predictor of poor prognosis in WHO grade II–IV *IDH*-wildtype glioma, independent of the *MGMT* methylation status [3]. In addition, several studies have shown the significance of p*TERT* mutation as a poor prognostic predictor in *IDH*-wildtype glioma [11, 18]. In cIMPACT-NOW update 3, histological grade II and III *IDH*-wildtype diffuse astrocytic gliomas that contain high-level *EGFR* amplification, the combination of whole chromosome 7 gain and whole chromosome 10 loss, or p*TERT* mutations were recommended as WHO grade IV. Our cohort showed that *IDH*-wildtype p*TERT* mut LGGs have a worse prognosis than *IDH*-wildtype p*TERT*-wildtype GBM. This result indicated that p*TERT* status was a more significant prognostic marker than a histologic diagnosis in *IDH*-wildtype astrocytic gliomas. Almost all *EGFR* amplification and

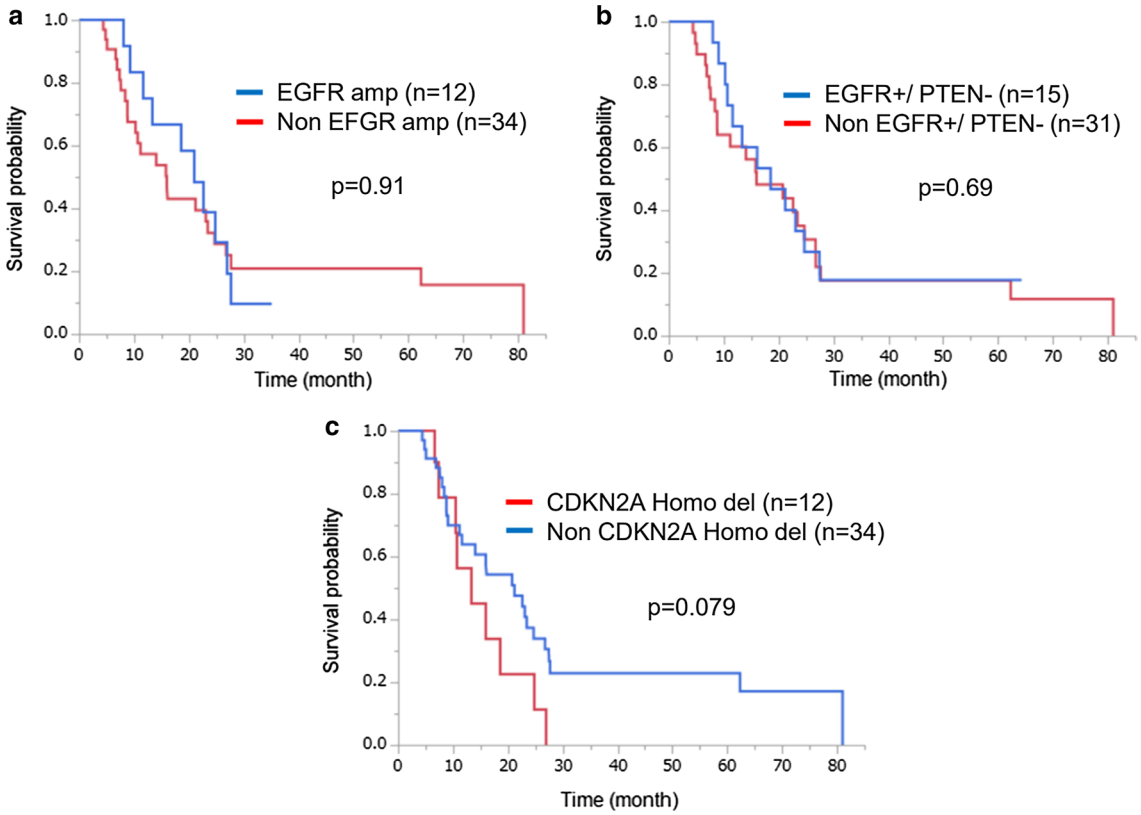


Fig. 3 Kaplan–Meier curves of overall survival (OS) in *IDH*-wildtype p*TERT*-mutant LGGs. Difference in OS in **a** *EGFR* amplification (amp) (median OS: 20.8 months) vs. non-*EGFR* amp (median OS: 16.0 months; $p=0.91$), **b** *EGFR*+/*PTEN* (median OS: 18.5 months) vs. non-*EGFR*+/*PTEN* (median OS: 16.0 months; $p=0.69$), and **c** *CDKN2A* homozygous deletion (HD) (median OS: 13.3 months) vs. non-*CDKN2A* HD (median OS: 21.2 months; $p=0.079$)

EGFR+/*PTEN*− cases in *IDH*-wildtype LGGs were among the p*TERT* mut cases. These results indicate that the most significant prognostic marker in *IDH*-wildtype LGGs was the p*TERT* mutation.

A genome-wide DNA methylation analysis matched five out of the six *IDH*-wildtype p*TERT* mut LGGs (no *EGFR* amplification, no *EGFR*+/*PTEN*−) with the methylation class family Glioblastoma, *IDH*-wildtype (Supplementary Table S4). Taken together, it appears that the presence of p*TERT* mutations alone in *IDH*-wildtype LGG may, in most cases, fulfill the criteria proposed by cIMPACT-NOW update 3 to define "Diffuse astrocytic glioma, *IDH*-wildtype, with molecular features of GBM, WHO grade IV". This has a significant consequence in clinical practice where the resources for a multimodal approach for molecular diagnostics, such as methylation profiling, are limited. Nonetheless, caution is required when interpreting isolated p*TERT* mutations, as this genotype may occasionally be observed in PXA [5, 23]. As has been emphasized in cIMPACT-NOW update 3, these molecular criteria should only be applied to morphologically verified diffusely infiltrative glioma with astrocytic lineage, such as in our cases.

Our study examined the CNAs of *EGFR* and *PTEN* using MLPA in all cases, and the whole of chromosomes 7 and 10 were examined in 64 of 131 cases using DNA methylation analysis. According to a study by Stichel et al. [23], the most common type of chromosome 7 and 10 CNA in *IDH*-wildtype LGGs was whole chromosome 7+/10−, which was 75.8% of any 7/10 CNA. The frequencies of whole 7+/10q− and 7p+/whole 10− were 7.0% and 2.3%, respectively. The presence of *EGFR*+/*PTEN*− indicated the existence of either 7+/10− or 7+/10q− or 7p+/10−. Therefore, *EGFR*+/*PTEN*− represented whole 7+/10− with a probability of over 90%. If it was not *EGFR*+/*PTEN*−, there was no whole 7+/10− either.

All cases with *EGFR*+/*PTEN*− were included in the p*TERT* mut cases. In addition, all *EGFR* amplifications except one case were observed among the *pTERT* mut cases. Copy-number plots obtained from DNA methylation array analysis were consistent with the results of the CNAs obtained from MLPA. There were no whole chromosome 7+/10− cases in Group B astrocytomas. These results were consistent with previous studies regarding LGG [1] and GBM [24]. Aoki et al. reported that the gain of 7p, loss

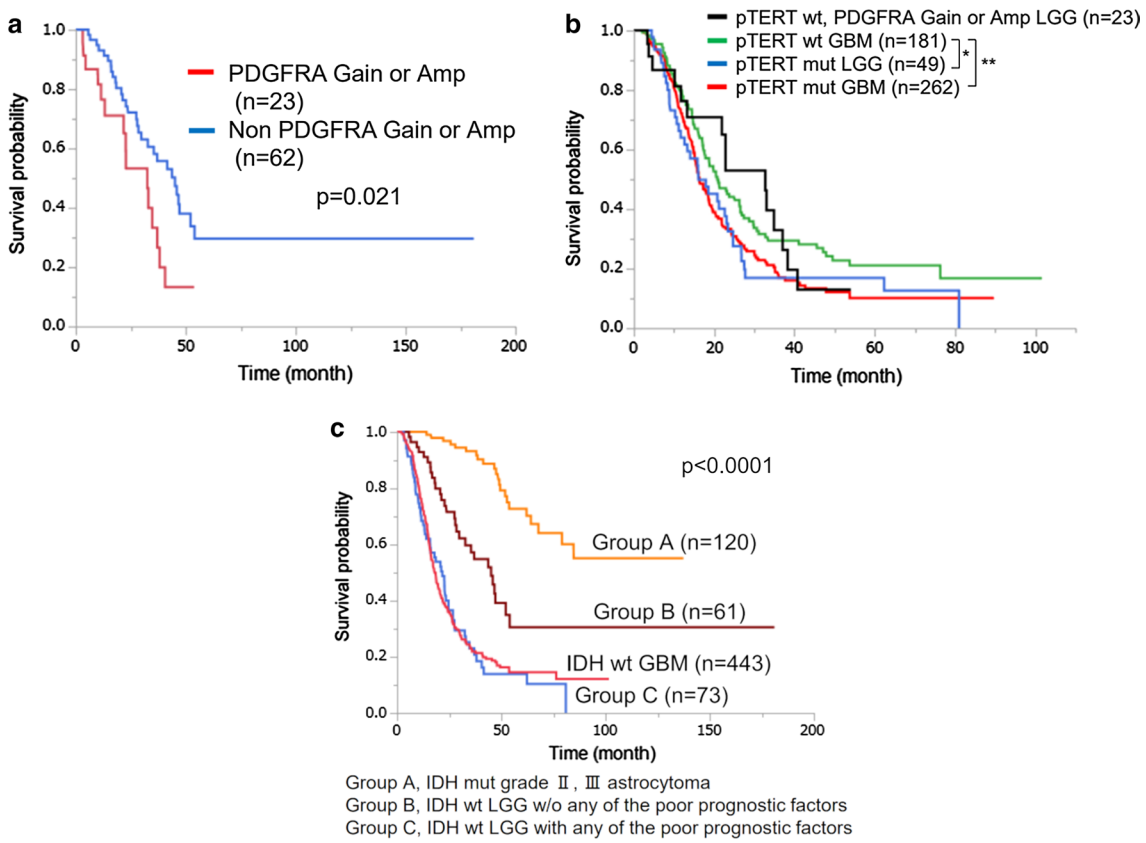


Fig. 4 Kaplan–Meier curves of overall survival (OS). **a** *IDH*-wildtype p*TERT* wt LGGs with *PDGFRA* gain/amplification (median OS: 32.5 months) showed significantly longer OS than those without *PDGFRA* gain or amp (median OS: 45.1 months; $p=0.021$). **b** OS of each molecular group. *IDH*-wildtype, p*TERT*-wildtype, *PDGFRA* gain or Amp LGG had OS equivalent to *IDH*-wildtype, p*TERT*-wildtype GBM (median OS: 21 months), or *IDH*-wildtype, p*TERT*-mutant LGG (median OS: 16.1 months) or *IDH*-wildtype, p*TERT*-mutant GBM (median OS: 16.1 months). $*p=0.044$, $**p=0.0062$ **c** OS of each molecular group. Median OS was not reached for Group A. Group C (median OS: 21.7 months) showed significantly shorter survival than Group B (median OS: 45.1 months; $p<0.0001$). Group A, *IDH*-mutant grade II, III astrocytoma; Group B, *IDH*-wildtype LGG without any of the poor prognostic factors; Group C, *IDH*-wildtype LGG with any of the poor prognostic factors

of 10q, and p*TERT* mutation were strongly mutually associated with *IDH*-wildtype LGGs. Umehara et al. reported that association or tendency toward co-occurrence was observed among the p*TERT* mutation, *EGFR* gain/amplification, and *PTEN* deletion.

These findings were largely confirmed in the TCGA and MSKCC datasets. However, these datasets have limitations: the number of cases in which all necessary genetic data were available, particularly the p*TERT* status, was relatively small, and the patients' follow-up period was not sufficient to allow the assessment of the prognostic impact of the genotype. Validation in a larger number of cases with a complete set of data and longer follow-up periods are warranted.

We showed that the gain or amplification of *PDGFRA* was a significant predictor of poor prognosis in *IDH*-wildtype/p*TERT*-wildtype LGGs. Although *PDGFRA* amplification has been reported to be associated with significantly worse OS in *IDH1* mutant GBM [19], there is no large-scale study evaluating CNAs of *PDGFRA* in *IDH*-wildtype LGGs. The gain or amplification of *PDGFRA* was observed in 21.4% of *IDH*-wildtype LGG and associated with shorter OS (Fig. 4a). Gain or amplification of *PDGFRA* was observed significantly more frequently ($p=0.043$, Table 2) in p*TERT*-wildtype (27.1%) cases than in p*TERT*-mut cases (10.9%) in *IDH*-wildtype LGGs. Umehara et al. reported that the amplification of *PDGFRA* tended to be mutually exclusive to p*TERT* mutation in GBM data [24].

To further elucidate the significance of *PDGFRA* gain/amplification in *IDH*-wildtype/p*TERT*-wildtype LGGs, a genome-wide DNA methylation analysis was performed in four selected cases. One of them was classified as Glioblastoma, IDH-wildtype by the DKFZ methylation classifier, with a calibration score above 0.9, while the other three tumors did not match, although the methylation class of Glioblastoma, IDH-wildtype was suggested (Supplementary Table S4). Methylation-based t-SNE distribution analysis

Table 5 Cox regression analysis in *IDH* wt p*TERT* wt LGG

	HR	95% CI	*p* value
Age			
≧65 y.o	2.2	1.12–4.33	0.023
< 65 y.o	Ref	–	–
KPS			
≧80	0.7	0.30–1.83	0.44
≦70	Ref	–	–
WHO grade			
II	0.71	0.31–1.57	0.40
III	Ref	–	–
Surgery			
Removal	1.11	0.58–2.25	0.76
Biopsy	Ref	–	–
Adjuvant therapy			
CRT	3.6	1.41–11.13	0.0063
Non-CRT	Ref	–	–
MGMT			
Met	0.31	0.12–0.75	0.0085
Un-met	Ref	–	–
PDGFRA			
Gain/Amp	2.44	1.09–5.27	0.030
Non-Gain/Amp	Ref	–	–

Amp amplification; *CI* confidence interval; *CRT* chemoradiotherapy; *HR* hazard ratio; *KPS* Karnofsky performance status; *Met* methylation

clustered all four *IDH*-wildtype, p*TERT*-wildtype, *PDGFRA* gain/amplification LGGs within the GBM cluster between GBM_MID and GBM_RTKIII (Fig. 5b). Thus, *PDGFRA* gain/amplification is likely to serve as an additional marker to molecularly define GBM in *IDH*-wildtype gliomas, although its biology requires further exploration.

In addition, we showed that almost all CNAs analyzed in this study were more frequently observed in WHO grade III than WHO grade II in *IDH*-wildtype LGG. There were 26/28 cases (92.9%) of WHO grade III in *IDH*-wildtype LGG with *PDGFRA* gain or amplification. These results indicated that copy-number gain or amplification of *PDGFRA* in *IDH*-wildtype LGG was associated with a higher WHO grade. The prognosis of WHO grade II and III *IDH*-wildtype LGG was significantly different in our Kaplan–Meier and multivariate Cox regression analyses. However, in *IDH*-wildtype/p*TERT*-wildtype LGG, WHO grade was not a significant prognostic predictor in multivariate Cox regression analysis. Whether the apparent poorer prognosis of grade III patients than grade II in *IDH*-wildtype/*pTERT*-wildtype LGGs can be attributed to molecular alterations such as CNA, including *PDGFRA* gain/amplification, remains to be seen.

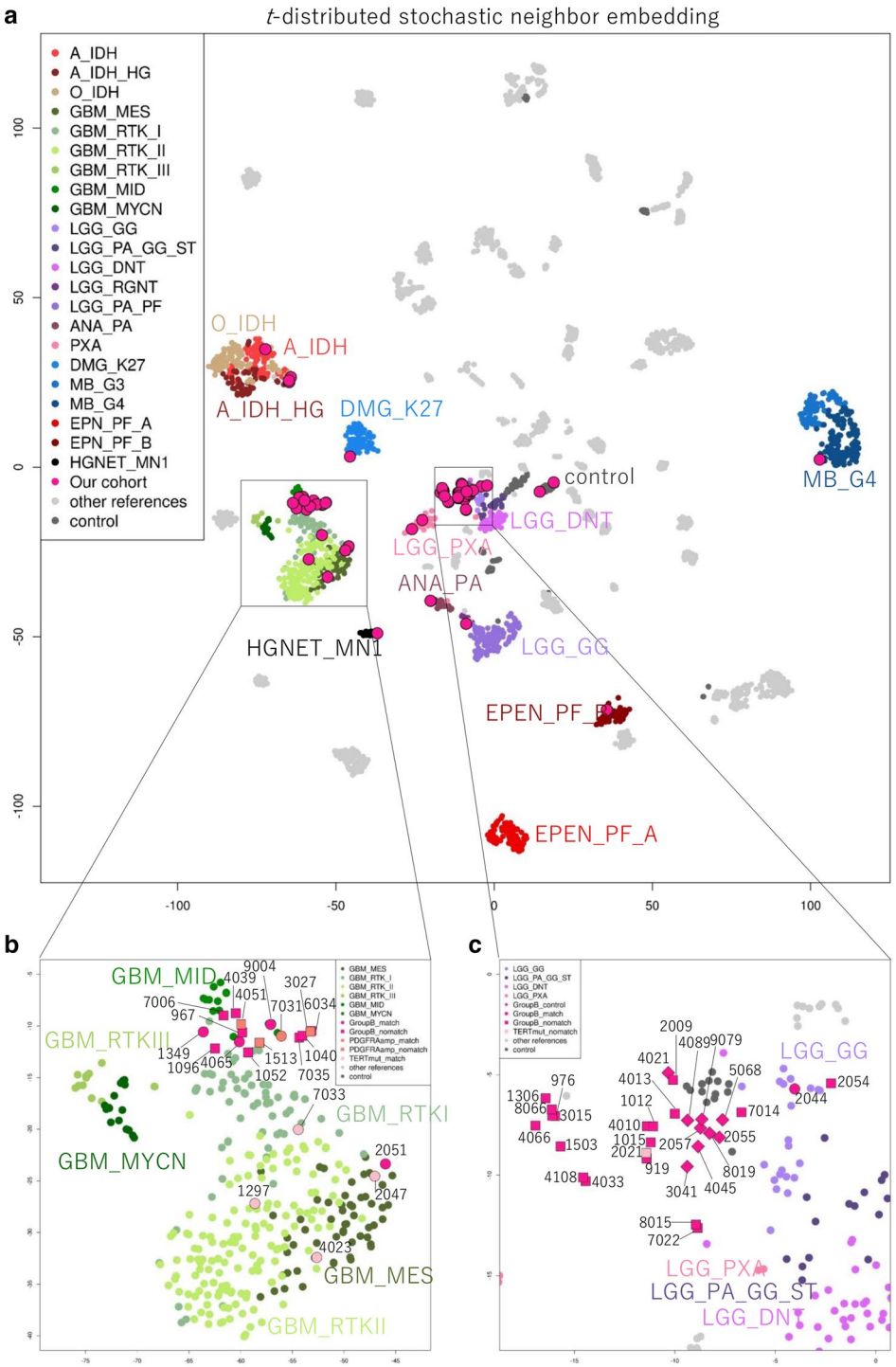
It has been reported that *CDKN2A/B* homozygous deletion is a poor prognostic factor in *IDH*-mut WHO grade III astrocytoma [4, 22]. However, there is no large cohort evaluating the prognostic value of *CDKN2A/B* in *IDH*-wildtype astrocytoma. In our study, although *CDKN2A* homozygous deletion was not a statistically significant prognostic predictor in *IDH*-wildtype LGGs, there was a tendency to predict poor prognosis in *IDH*-wildtype/p*TERT* mut LGGs ($p = 0.079$). Again, an independent validation with a larger cohort is warranted.

It is noteworthy that *IDH*-wildtype LGG without any of the above predictors for poor prognosis (Group B, Fig. 4c) showed significantly longer OS than *IDH*-wildtype GBM or *IDH*-wildtype LGG with one or more of the poor prognostic predictor (Group C, Fig. 4c) while showing significantly shorter OS than *IDH*-mut LGG (Group A, Fig. 4c). Furthermore, patients with WHO grade II astrocytoma had significantly longer survival than those with grade III astrocytoma

Table 6 DNA methylation-based classification of *IDH* wt LGGs

	Methylation class	*n*
Group B astrocytoma ($n = 54$)	No match	31
	Methylation class control tissue	10
	Methylation class family Glioblastoma, IDH-wildtype	4
	Methylation class family Glioma, IDH-mutant	3
	Methylation class anaplastic pilocytic astrocytoma	2
	Methylation class CNS high-grade neuroepithelial tumor with MN1 alteration	1
	Methylation class (anaplastic) pleomorphic xanthoastrocytoma	1
	Methylation class low-grade glioma, ganglioglioma	1
	Methylation class family Medulloblastoma group 3 and 4	1
IDH wt, p*TERT* wt, *PDGFRA* Amp astrocytoma ($n = 4$)	No match	3
	Methylation class family Glioblastoma, IDH-wildtype	1
IDH wt, p*TERT* mut astrocytoma ($n = 6$)	Methylation class family Glioblastoma, IDH-wildtype	5
	No match	1

Amp amplification; *LGG* lower grade glioma; *pTERT TERT* promoter


a
t-distributed stochastic neighbor embedding
A_IDH
A_IDH_HG
O_IDH
GBM_MES
GBM_RTK_I
GBM_RTK_II
GBM_RTK_III
GBM_MID
GBM_MYCN
LGG_GG
LGG_PA_GG_ST
LGG_DNT
LGG_RGNT
LGG_PA_PF
ANA_PA
PXA
DMG_K27
MB_G3
MB_G4
EPN_PF_A
EPN_PF_B
HGNET_MN1
Our cohort
other references
control
O_IDH
A_IDH
A_IDH_HG
DMG_K27
control
LGG_DNT
LGG_PXA
ANA_PA
HGNET_MN1
LGG_GG
EPEN_PF_B
EPEN_PF_A
MB_G4
b
GBM_MID
GBM_RTKIII
GBM_MYCN
GBM_RTKI
GBM_MES
GBM_RTKII
c
LGG_GG
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LGG_PA_GG_ST
LGG_DNT

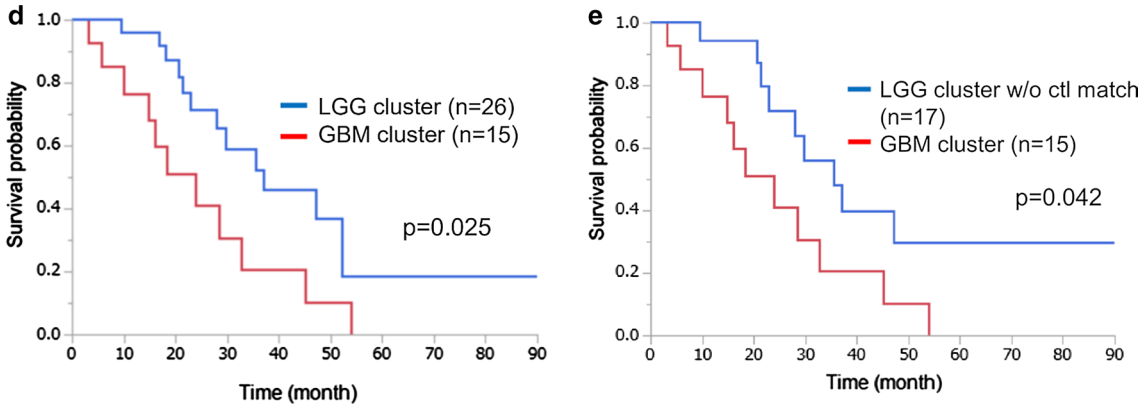

Fig. 5 DNA methylation-based unsupervised clustering of *IDH*-wildtype astrocytomas. **a** Our cohort ($n=64$) and the reference cohort ($n=2801$) from 91 methylation classes [GSE109381] were plotted using t-distributed stochastic neighbor embedding (t-SNE) dimensionality reduction. Individual samples of reference cohorts relevant to our cohorts ($n=22$) were color-coded according to the respective matched class colors. The other 60 reference methylation classes that were not associated with our cohorts and 9 methylation class control tissues were compiled and collectively indicated as dark gray and light gray plots, respectively, to highlight the relevant classes (the class names are indicated). The clusters highlighted in **b** and **c** are indicated by squares. **b** Of the 64 samples from our cohort, 20 were clustered within the glioblastoma cluster, including four *PDGFRA* amplified cases and four p*TERT*-mutant cases. **c** Twenty-nine tumors clustered close but not overlapping with the LGG and control tissue clusters. All but one such cases were categorized as Group B *IDH*-wildtype astrocytoma which were devoid of any of the markers, including *PDGFRA* amplification and p*TERT* mutation, which defined molecular GBM. **d** Kaplan–Meier curves of overall survival (OS) in LGG cluster (median OS: 37.0 months) vs. GBM cluster (median OS: 23.8 months; $p=0.025$). **e** Kaplan–Meier curves of overall survival (OS) in LGG cluster except the control match cases (median OS: 35.5 months) vs. GBM cluster (median OS: 23.8 months; $p=0.042$). The class abbreviations were as follows: A_IDH; methylation class IDH glioma, subclass astrocytoma: A_IDH_HG; methylation class IDH glioma, subclass high-grade astrocytoma: O_IDH; methylation class IDH glioma, subclass 1p/19q codeleted oligodendroglioma: GBM_MES; methylation class glioblastoma, IDH-wildtype, subclass mesenchymal: GBM_RTK_I; methylation class glioblastoma, IDH-wildtype, subclass RTK I: GBM_RTK_II; methylation class glioblastoma, IDH-wildtype, subclass RTK II: GBM_RTK_III; methylation class glioblastoma, IDH-wildtype, subclass RTK III: GBM_MID; methylation class glioblastoma, IDH-wildtype, subclass midline: GBM_MYCN; methylation class glioblastoma, IDH-wildtype, subclass MYCN: LGG_GG; methylation class low-grade glioma, ganglioglioma: LGG_PA_GG_ST; methylation class low-grade glioma, subclass hemispheric pilocytic astrocytoma and ganglioglioma: LGG_DNT; methylation class low-grade glioma, dysembryoplastic neuroepithelial tumor: LGG_RGNT; methylation class low-grade glioma, rosette forming glioneuronal tumor: LGG_PA_PF; methylation class low-grade glioma, subclass posterior fossa pilocytic astrocytoma: ANA_PA; methylation class anaplastic pilocytic astrocytoma: PXA; methylation class (anaplastic) pleomorphic xanthoastrocytoma: DMG_K27; methylation class diffuse midline glioma H3 K27M mutant: MB_G3; methylation class medulloblastoma, subclass group 3: MB_G4; methylation class medulloblastoma, subclass group 4: EPN_PF_A; methylation class ependymoma, posterior fossa group A: EPN_PF_B; methylation class ependymoma, posterior fossa group B: HGNET_MN1; methylation class CNS high-grade neuroepithelial tumor with MN1 alteration: PDGFRAamp; *PDGFRA* amplified: TERTmut; p*TERT* mutant

in Group B (Supplementary Fig. S1a). In Group C, no significant difference in overall survival between grades II and III was observed (Supplementary Fig. S1b). However, WHO grade III was significantly enriched in Group C, most likely reflecting that this group of tumors was biologically equivalent to GBM.

We then analyzed 54 Group B astrocytomas for genome-wide DNA methylation. When the DKFZ DNA methylation-based classification was applied, 23 Group B cases matched with some of the existing reference groups, including 4 methylation class family Glioblastoma, IDH-wildtype, and 10 methylation class control tissue. Among them, one PXA and one GG had BRAF V600E mutations. Thirty-one cases of Group B astrocytoma did not reach the cut-off of ≥ 0.9, and were interpreted as no match. More than half of the unmatched Group B astrocytomas formed a separate cluster nearby but did not overlap with other existing LGG reference groups (Supplementary Table S4 and Fig. 5c). None of them had BRAF mutation. These cases (defined as "LGG") showed significantly longer OS than those that were clustered with GBM (Fig. 5d, e). Of note, no patients belonged to the age group that was typically observed among pediatric-type diffuse low-grade gliomas. These findings suggest that *bona fide IDH*-wildtype diffuse astrocytoma may exist as a separate entity from Group C *IDH*-wildtype astrocytomas, which are molecularly equivalent to GBM. The presence of "true" *IDH*-wildtype low-grade astrocytomas that show significantly longer OS than GBM has also been suggested elsewhere [21]. This group of tumors deserves further investigation to refine the classification of diffuse astrocytic tumors.

Group B *IDH*-wildtype astrocytomas were presented as a heterogeneous group of tumors by methylation profiling. Apart from the molecularly defined GBM and the newly defined LGG, as described above, isolated cases were classified into other existing entities such as ANA_PA (Supplementary Table S4), as has been previously reported [20]. However, three tumors that were classified as Glioma, IDH-mutant by the methylation classifier, were devoid of *IDH* mutations, as determined by Sanger sequencing or pyrosequencing. The tumor classified as Medulloblastoma group 3 and 4 was a supratentorial tumor developed in a 74-year-old patient who was diagnosed with anaplastic astrocytoma WHO grade III based on central pathology review. Ten tumors were classified as methylation class control tissue. Although under-sampling is an important issue in molecular analysis, tumor cell content, estimated using the methylation array data in these tumors, ranged from 30 to 60%, which was comparable to those in other tumors. Some of these tumors showed elevated *MGMT* methylation, suggesting that they contained a certain proportion of tumor cells. Furthermore, the prognosis of these cases was equivalent to that of the other cases of the LGG cluster defined above (Fig. 5d). Thus, *IDH*-wildtype astrocytomas without molecular features of GBM may not necessarily fall into existing categories and therefore need better classification. Our data are also in agreement with another study in which a considerable number of *IDH*-wildtype grade II and III astrocytomas were interpreted as no prediction in DNA methylation-based classification [23].

This study inevitably has inherent limitations as a retrospective analysis susceptible to selection biases, such as introducing the initial inclusion criteria for molecular data availability. Another limitation is the different molecular techniques utilized in our cohorts, including TCGA and MSKCC, where a more comprehensive analysis was performed. On the other hand, MLPA, which we used to evaluate CNAs, is a reliable and cost-effective method utilized in routine diagnosis. These results were consistent with the CNA profiles obtained from the methylation array analysis. It may serve as a powerful tool to efficiently detect typical CNA to delineate the poor prognosis group in adult gliomas.

In conclusion, we report here that p*TERT* mutation is the most useful marker to predict molecularly defined GBM with the poorest prognosis in *IDH*-wildtype LGGs. It is the most critical marker to identify the newly proposed entity of "Diffuse astrocytic glioma, *IDH*-wildtype, with molecular features of GBM, WHO grade IV" among the *IDH*-wildtype diffuse astrocytomas. It is noteworthy that there were *IDH*-wildtype diffuse astrocytomas with a significantly better prognosis than GBM, suggesting that *IDH*-wildtype diffuse astrocytomas are a highly heterogeneous group of tumors that need to be further molecularly delineated by additional methods including methylation profiling.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00401-021-02337-9.

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