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RESEARCH ARTICLE

Brain
Pathology

Molecular landscape of *IDH*-wild type, p*TERT*-wild type adult glioblastomas

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Abstract

Telomerase reverse transcriptase (*TERT*) promoter (p*TERT*) mutation has often been described as a late event in gliomagenesis and it has been suggested as a prognostic biomarker in gliomas other than 1p19q codeleted tumors. However, the characteristics of isocitrate dehydrogenase (*IDH*) wild type (wt) (*IDH*wt), p*TERT*wt glioblastomas are not well known. We recruited 72 adult *IDH*wt, p*TERT*wt glioblastomas and performed methylation profiling, targeted sequencing, and fluorescence in situ hybridization (FISH) for *TERT* structural rearrangement and ALT (alternative lengthening of telomeres). There was no significant difference in overall survival (OS) between our cohort and a the Cancer Genome Atlas (TCGA) cohort of *IDH*wt, p*TERT* mutant (mut) glioblastomas, suggesting that p*TERT* mutation on its own is not a prognostic factor among *IDH*wt glioblastomas. Epigenetically, the tumors clustered into classic-like (11%), mesenchymal-like (32%), and LGm6-glioblastoma (GBM) (57%), the latter far exceeding the corresponding proportion seen in the TCGA cohort of *IDH*wt, p*TERT*mut glioblastomas. LGm6-GBM-clustered tumors were enriched for platelet derived growth factor receptor alpha (*PDGFRA*) amplification or mutation ($p = 0.008$), and contained far fewer epidermal growth factor receptor (*EGFR*) amplification ($p < 0.01$), 10p loss ($p = 0.001$) and 10q loss ($p < 0.001$) compared with cases not clustered to this group. LGm6-GBM cases predominantly showed ALT ($p = 0.038$). In the whole cohort, only 35% cases showed *EGFR* amplification and no case showed combined chromosome $+7/-10$. Since the cases were already p*TERT*wt, so the three molecular properties of *EGFR* amplification, $+7/-10$, and p*TERT* mutation may not cover all *IDH*wt

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glioblastomas. Instead, *EGFR* and *PDGFRA* amplifications covered 67% and together with their mutations covered 71% of cases of this cohort. Homozygous deletion of cyclin dependent kinase inhibitor 2A *(CDKN2A)/B* was associated with a worse OS ($p = 0.031$) and was an independent prognosticator in multivariate analysis ($p = 0.032$). In conclusion, adult *IDH*wt, p*TERT*wt glioblastomas show epigenetic clustering different from *IDH*wt, p*TERT*mut glioblastomas, and *IDHwt* glioblastomas which are p*TERTwt* may however not show *EGFR* amplification or +7/−10 in a significant proportion of cases. *CDKN2A/B* deletion is a poor prognostic biomarker in this group.

KEYWORDS
chromosome +7/−10, *EGFR*, epigenetic profiling, glioblastoma, *IDH*-wildtype, *PDGFRA*, *TERT* promoter

1 | INTRODUCTION

Tumor cells may display telomere dysfunction and shortened telomeres that lead to chromosome instability. *TERT* (telomerase reverse transcriptase) activation regenerates telomeres sufficiently to maintain them above the critical threshold and stabilize the tumor genome [1]. *TERT* promoter (p*TERT*) mutation leads to telomerase activation, and increased *TERT* expression [2]. But p*TERT* mutation only accounts for about 30% of instances of *TERT* activation across different cancers, the other methods being structural variants and chromosomal rearrangements to enhancer elements or oncogene activation or other mechanisms [1]. And p*TERT* mutations are often found in cells of low rates of renewal like brain cells and are rare in malignancies with very high rates of proliferation [1].

About 70%–80% of *IDH*wt (wild type for isocitrate dehydrogenase) glioblastomas are p*TERT* mutant (mut) [2–6]. p*TERT* mutation is often regarded as the last stage of gliomagenesis when the tumor presents clinically by survival-promoting mutations [4, 7]. p*TERT* mutation has been found to be inversely correlated with *IDH* in glioblastoma and therefore the incidence of p*TERT* promoter mutation is high in *IDH*wt glioblastoma and low in Grade 4 astrocytoma, *IDH*-mutant [2, 8–10]. This has also been our and others' experience where in, Grade 4 astrocytoma, *IDH*-mutant incidence of *TERT* promoter mutation is very low [11–13]. In Grade 4 astrocytoma, *IDH*-mutant, telomere maintenance is usually achieved by ALT (alternative lengthening of telomeres) that is typically associated with mutations of the gene *ATRX* (alpha thalassemia/mental retardation syndrome X-linked) [11, 14, 15]. Ceccarelli et al. showed that *ATRX* mutation, rather than p*TERT* mutation, is associated telomere length [16]. p*TERT* mutations are also described to be associated with a worse survival in some studies [17, 18] but not with others [5, 19] and ALT was associated with a better survival in glioblastomas [20].

Typically, p*TERT*mut glioblastomas are enriched with epidermal growth factor receptor (*EGFR*) amplification, chromosome 7 gain and chromosome 10 loss (+7/−10) [16] and p*TERT* mutation is usually mutually exclusive with *ATRX* (alpha thalassemia/mental retardation syndrome X-linked) mutation [1, 10]. Enigmatically, among 1p19q non-codeleted astrocytomas, p*TERT* mutation appears to have a favorable prognostic impact [21]. In *IDH*wt lower-grade gliomas, p*TERT* mutation is not predictive of a poorer outcome in one study [22] but in other studies, it is a poor prognostic marker [23–25].

For glioblastomas which are p*TERT*wt, there is relatively little information and the mechanism of telomere maintenance remains unknown. Here, the term "p*TERT*wt" is designated just for glioblastomas that are wildtype for the promoter of the gene *TERT*. To our knowledge, there were only two publications dedicated to *IDH*wt, p*TERT*wt glioblastomas. Diplas et al. identified *SMARCAL1* inactivating mutation, in addition to *ATRX* mutation, as a genetic mechanism of ALT [3]. *SMARCAL1* is also a member of the SW1/SNF family of chromatin remodelers like *ATRX* [26] and mutations of *SMARCAL1* lead to ALT telomere maintenance [3]. The authors also identified another mechanism of telomerase activation in glioblastomas via chromosomal rearrangements of *TERT*. The location of breakpoint was variable but the translocations led to increased *TERT* expression. Next-generation sequencing was performed in this study in some of the *IDH*wt, p*TERT*wt glioblastomas in this publication but methylation profiling was not performed [3]. Williams et al. studied a small series 16 cases and found them to be from younger patients than p*TERT*mut glioblastoma patients and they showed frequent P13K pathway mutations [6].

In this study, we examined a cohort of *IDH*wt, p*TERT*wt adult glioblastomas with methylation profiling, DNA-targeted sequencing, fluorescence in situ hybridization (FISH) for ALT (alternative lengthening of telomeres), and FISH for *TERT* structural rearrangement. We aim to provide a better understanding of the molecular landscape of *IDH*wt, p*TERT*wt glioblastomas and to identify genomic or genetic tests which can help risk stratification.

2 | MATERIALS AND METHODS

2.1 | Patients

We recruited unselectively adult *IDH*wt glioblastomas (aged 18 or above) from the archives of the neurosurgical teams at the Prince of Wales Hospital, Chinese University of Hong Kong from April 2013 to March 2020, and Hua Shan Hospital, Fudan University, Shanghai, from January 2015 to December 2017. The reasons for the difference in years between the two institutions was the difference in annual caseloads for the two neurosurgical teams and we aimed to collect a cohort that was within the limits of our research resource capacity. Histology of all recruited cases was reviewed (Hong Chen and Ho-Keung Ng) to confirm histological features of glioblastoma as per WHO 2021 Classification, namely mitoses, cellular atypia, necrosis, and/or microvascular proliferation [27]. The approach to gliomas in both hospitals was maximal safe resection in the first instance. Ethics approvals were obtained from the Joint Chinese University of Hong Kong—New Territories East Cluster Clinical Research Ethics Committee, and the Ethics Committees of Huashan Hospital, Fudan University, Shanghai. Data on patient demographics and treatments were retrieved from institutional paper and electronic records. Survival data were obtained from follow-up clinic visits and direct contact with patients or close relatives via phone.

2.2 | Sanger sequencing of *IDH1* and *IDH2*

The *IDH* status of the 72 glioblastomas in this study was first identified in our routine diagnostic practice. At our hospitals, *IDH* status of determined by Sanger sequencing for the codon 132 of *IDH1* and codons 140 and 172 of *IDH2*. As described below, these cases were further evaluated with target sequencing in which the panel was designed to examine all coding sequences of *IDH1* and *IDH2* genes.

2.3 | *TERT* promoter mutation analysis

TERT promoter mutations were evaluated by Sanger sequencing according to our previous publication [28]. In brief, crude cell lysate was obtained from formalin-fixed paraffin-embedded (FFPE) sections. Nucleic acid in the cell lysate was amplified using forward primer, reverse primer, and KAPA HiFi HotStart ReadyMix (Sigma). The forward primer was 5′-GTCCTGCCCCTTCA CCTT-3′ and the reverse primer was 5′-CAGCGCTGC CTGAAACTC-3′. PCR products were then purified and sequenced with BigDye Terminator Cycle Sequencing kit (Life Technologies).

2.4 | Illumina infinium methylation EPIC BeadChip array

FFPE sections were sent to Sinotech Genomics Co., Ltd, Shanghai, where the DNA was extracted, bisulfite modified, and subjected to DNA methylation profiling by EPIC Illumina Infinium Human (850 k) array.

2.5 | DNA methylation pre-processing

We performed noob (normal-exponential convolution using out-of-band probes) background correction [29] and dye bias correction using the minfi package [30]. The DNA methylation score for each locus was presented as a beta (β) value ($\beta = (M/[M + U])$), where M and U indicated the mean methylated and unmethylated signal intensities for each locus, respectively. β-values ranged from zero to one, with a score of zero indicating no DNA methylation and a score of one indicating complete DNA methylation. A detection p-value also accompanied each data point and compared the signal intensity difference between the analytical probes and a set of negative control probes on the array. Any data point with a corresponding p-value >0.05 was deemed not to be statistically significantly different from the background and was thus marked as "NA".

2.6 | Classification of glioma samples based on TCGA DNA methylation subtypes

Samples were classified into the previously published glioma *IDH*wt molecular subtypes (classic-like, mesenchymal-like, and LGm6-glioblastoma [GBM]) using the CpG methylation signatures and method previously described [16]. We used a predictive model to classify the samples into one of the three *IDH*wt molecular subtypes, using the R packages and the randomForest. Cases were assigned to the methylation groups according to the highest score.

2.7 | Identification of copy number variations with EPIC 850 k array

Assessment of copy number variations (CNVs) was performed according to our previous publication [11]. The "conumee" R package in Bioconductor (http://www.bioconductor.org/packages/release/bioc/html/conumee.html) was employed to evaluate CNVs. A log2 ratio $\pm$ 0.35 was used as the cutoff of amplification/loss and a log2 ratio $-$ 0.415 was used as the cutoff for homozygous loss [31]. GISTIC v2.0 analysis was conducted to identify significantly recurrent copy number amplification/gain and deletions at arm-level and focal-

level, defined as affected regions spanning less than 50% of a chromosome arm.

2.8 | Targeted sequencing

DNA was obtained from FFPE sections using GeneRead DNA FFPE kit (Qiagen). The quality and quantity of extracted DNA were evaluated by QIAseq DNA Quanti-MIZE Assay (Qiagen). Samples that passed quality control were subjected to library preparation with a custom QIAseq Targeted DNA Panel, which examined coding exons or hotspots of 91 glioma-relevant genes (Table S1). The DNA libraries were then further qualified before the libraries were sequenced with MiSeq v3 (Illumina).

Paired-end reads were aligned to the hg19 (GRCh37) build of the human reference genome with BWA-MEM algorithm on GeneGlobe platform (Qiagen). Variants were called using smCounter2 and annotated using wANNO-VAR. Variants with the following criteria were excluded: not passing quality filters, with variant allele fractions of ≤10%, with variant allele counts of ≤5, or with minor allele frequencies of >1% in the overall human population or East Asians or documented in public databases (1000 Genomes, ExAc, gnomAD exome, and genome databases). Mutation events were checked with the Catalog of Somatic Mutations in Cancer (COSMIC) as per other previous studies on brain tumors [32–35].

2.9 | *TERT* structural rearrangement by FISH

TERT rearrangement was evaluated by FISH. FISH break-apart probes were designed according to previous publication [3]. In brief, 4-μm thick FFPE sections were deparaffinized in xylene, treated with 1 M sodium thiocyanate, digested in pepsin solution, rinsed in milli-Q water and dehydrated. The labeled probes were denatured and hybridized to the section overnight. Sections were then washed, stained with Vectashield mounting medium, and visualized under a Zeiss Axioplan fluorescence microscope. Samples were considered positive when break-apart signal was noticed in >5% of evaluated nuclei [3].

2.10 | Telomere-specific FISH analysis

ALT phenotype was examined with the Telomere PNA FISH kit (K532511, Dako) on 4-μm thickness FFPE sections. Tumor areas on the slides were identified and marked for evaluation. ALT-positive cases were identified when ≥5% of tumor cells exhibited large, very bright intranuclear foci of telomere FISH signals [36, 37]. Endothelial cell nuclei were used for normal internal control purposes.

2.11 | TCGA clinical data

Clinical data of a total of 326 glioblastoma *IDH*wt, p*TERT*mut samples with available survival data were downloaded from The Cancer Genome Atlas (TCGA) Data Portal using TCGAbiolinks package (v2.18) function and imported into R (https://www.r-project.org) for further analysis [38]. All cases were aged above 18.

2.12 | Statistical analysis

Statistical analysis was performed on IBM SPSS software. Overall survival (OS) was defined as the period of time between operation and death or the last follow-up. Chi-square or Fisher's test was applied to determine correlation between molecular alterations and clinical parameters and between different molecular alterations. Survival curves were evaluated by the Kaplan–Meier (KM) method, and log–rank test was done to compare survival distribution between groups. Multivariate analysis was performed by Cox proportional hazards model. A $p < 0.05$ (two-sided) value was considered statistically significant.

3 | RESULTS

3.1 | Characteristics of the study cohort

During the period described, 285 cases of *IDH*wt adult glioblastomas were retrieved from the two neurosurgical teams for review and sequencing. By Sanger sequencing, 72 cases of *IDH*wt, p*TERT*wt glioblastomas were identified and retrieved for this study. Methylation profiling was performed in all 72 cases. FISH for ALT and *TERT* structural rearrangement were performed in 71 cases due to lack of materials. DNA targeted sequencing was unsuccessful in 5 cases, due to small quantity of DNA available.

A summary of the molecular findings in this cohort, including CNVs and different means of telomere maintenance is shown in Figure 1. Intensity data (IDAT) of the 72 cases is uploaded at https://www.surgery.cuhk.edu.hk/btc/hsbc/.

3.2 | LGm6-GBM subtype of the TCGA classification is the main group within *IDH*wt, p*TERT*wt glioblastomas, and *TERT* promoter mutation was not a prognosticator when compared with TCGA

The clinical profile of the cohort is presented in Table 1. The mean and median of this cohort were 51.9 and 57 years old, respectively. Male to female ratio was 1.25:1. Most tumors were located in the hemisphere.
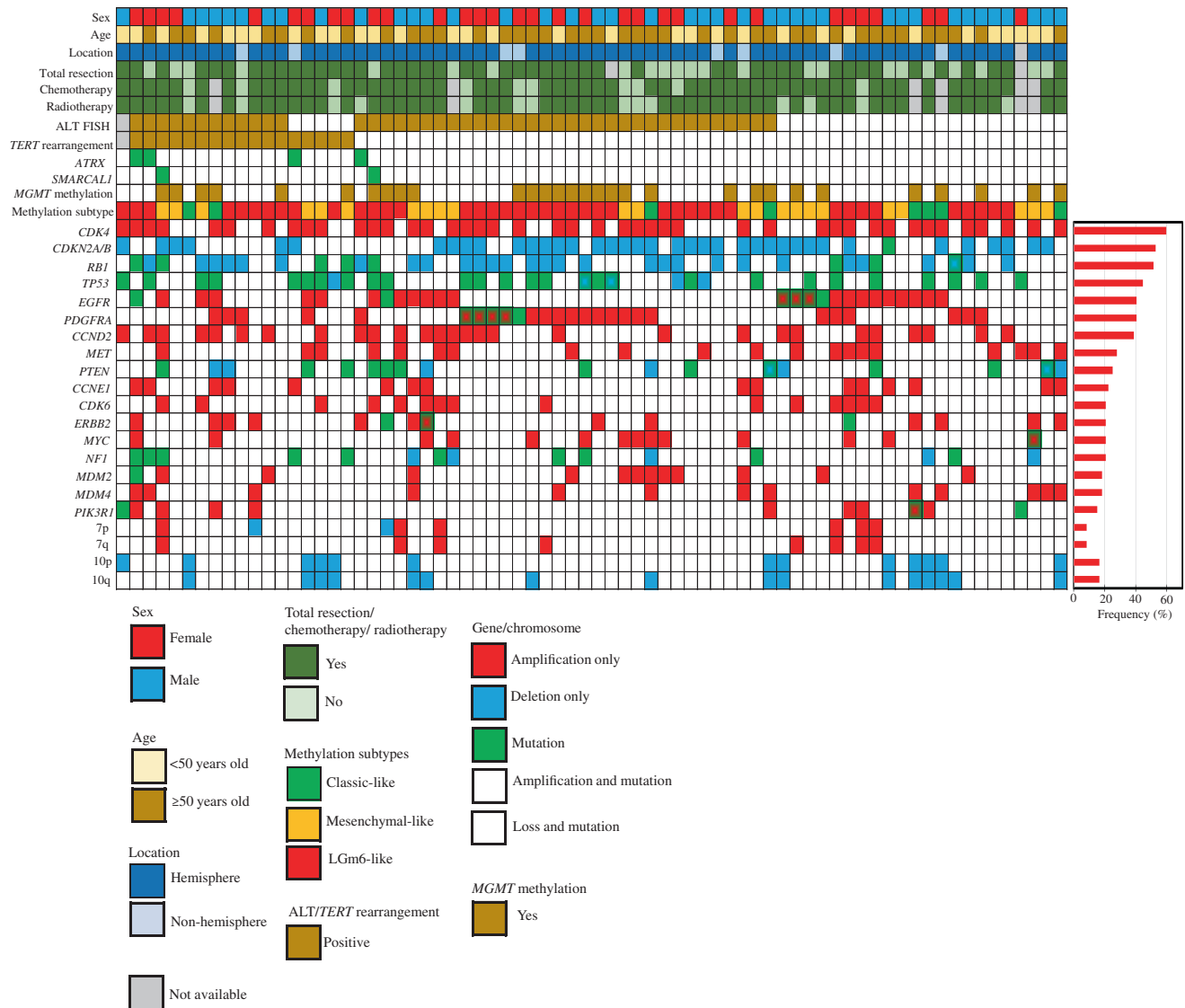
FIGURE 1 Oncoprint of the clinical features and molecular alterations of *IDH*wt, p*TERT*wt glioblastomas. *IDH*wt, isocitrate dehydrogenase wild type; p*TERT*, telomerase reverse transcriptase promoter;

About 65% of the patients underwent gross total resection as evaluated by post-operation MR imaging. Chemotherapy and radiotherapy were given to 78% and 72% of the patients. OS and progression-free survival (PFS) data were available in 98.6% and 87.5%, respectively. The mean and median OS of the cohort were 18.4 and 15 months, respectively. Univariate analysis revealed that gross total resection, chemotherapy, and radiotherapy were the prognosticators for OS and PFS (Table 2).

We compared the OS of this cohort with the cohort of the adult *IDH*wt, p*TERT*mut glioblastomas (*n* = 326) retrieved from TCGA and found no significant difference in OS between the two cohorts (Figure 2A). This suggests that p*TERT* mutation on its own is not a prognostic factor in *IDH*wt glioblastomas. TCGA only contained five cases of p*TERT*wt

glioblastomas and they were all *IDH*mut. Interestingly, the mean age of the TCGA *IDH*wt, p*TERT*mut glioblastomas is 61.8 years old and is significantly higher than the mean age of this present *IDH*wt, p*TERT*wt cohort of 51.94 years old (*p* = 0.001).

Epigenetically, the cohort can be clustered according to methylation subtypes for glioblastomas [16] into classic-like (*n* = 8, 11%), mesenchymal-like (*n* = 23, 32%) and LGm6-GBM (*n* = 41, 57%) according to TCGA nomenclature. The distribution of the clustering score of each individual sample can be found in Figure S1. There was however no survival difference between the different epigenetic subtypes (Figures 2B and C). The TCGA cohort of *IDH*wt, p*TERT*mut glioblastomas were however epigenetically clustered as classic-like (122/326, 37.4%), mesenchymal-like (165/326, 50.6%), and LGm6-GBM (39/326, 12%) [16]. There was clearly an over-representation of the

TABLE 1 Clinical characteristics of 72 patients

| | | Methylation subtypes | | | |
| | | Non-LGm6-GBM | | | |
Features	All cases (*n* = 72)	Classic-like (*n* = 8)	Mensenchymal-like (*n* = 23)	LGm6-GBM (*n* = 41)	*p*-Value
Age					
<50 years old	32	1	10	21	0.130
≥50 years old	40	7	13	20	
Sex					
Male	40	6	14	20	0.325
Female	32	2	9	21	
Location					
Hemisphere	63	7	21	35	0.479
Non-hemispheric	8	1	1	6	
Not available	1	0	1	0	
Operation					
Gross total resection	47	5	12	30	0.249
Non-total resection	23	3	10	10	
Not available	2	0	1	1	
Chemotherapy					
Yes	56	4	18	34	0.733
No	10	1	2	7	
Not available	6	3	3	0	
Radiotherapy					
Yes	52	3	16	33	0.564
No	14	2	4	8	
Not available	6	3	3	0	
Overall survival (months) (mean/median)	18.4/15.0	19.3/22.7	13.9/10.2	20.8/16.1	0.137

LGm6-GBM cluster in our cohort of *IDH*wt, p*TERT*wt glioblastomas (57% vs. 12%).

Twenty-nine cases of LGm6-GBM-clustered cases (73%, 29/40) in this cohort showed ALT by FISH (Figures 3A, B). Within the LGm6-GBM cluster, *PDGFRA* amplification or mutation could be seen in 54% (22/41) of cases, far exceeding the proportion found in the TCGA *IDH*wt, p*TERT*mut glioblastoma cohort (27%), and the non-LGm6-GBM cases in this cohort (Table 2, 23%, *p* = 0.008). Conversely, *EGFR* amplification was seen only in 6 cases (14.6%) in the LGm6-GBM, contrasting, respectively, with 63% in the online TCGA data on *IDH*wt, p*TERT*mut glioblastomas and 61% in the non-LGm6-GBM cases of this cohort (Table 2 and Table 3). Similarly, 10q loss was seen only in 7% (3 cases) in the LGm6-GBM subtype in contrast to nearly 90% in the same TCGA cohort and 42% in the non-LGm6-GBM cases in this cohort (Table 2). LGm6-GBM cases and non-LGm6-GBM cases in this cohort were also different in their prevalence with *MDM4* and *RB1* alterations (Table 2).

3.3 | The mechanism of telomere maintenance of a proportion of *IDH*wt, p*TERT*wt glioblastomas remains unaccounted for

For the mechanism of telomere maintenance, we performed FISH to detect *TERT* structural rearrangement and ALT in all samples with the exception of one case lacking sufficient tissue (Figures 3A–D).

Overall, 44 cases (62%) showed ALT, 4 cases showed *ATRX* mutations, 2 cases (3%) showed *SMARCAL1* mutation, and 17 cases (24%) showed *TERT* structural rearrangement, and these cases overlapped (Figure S2). The LGm6-GBM subtype was enriched with cases showing ALT (*p* = 0.038), and *TERT* rearrangement and *ATRX/SMARCAL1* mutations were not associated with methylation subtypes. Nevertheless, with the exceptional of one case, all *ATRX/SMARCAL1* mutations were detected in LGm6-GBM subtype. Similarly, all *ATRX/SMARCAL1* mutant, except one case, were positive for ALT. *ATRX* mutation and *SMARCAL1* mutation were mutually exclusive. And *TERT* structural rearrangement

FIGURE 2 Survival analyses. (A) The survival outcome of *IDH*wt, p*TERT*wt glioblastomas in this study cohort and *IDH*wt, p*TERT*mut glioblastomas in TCGA cohort showed no difference. (B) OS and (C) PFS were not different among methylation subtypes in this study cohort. (D) *IDH*wt, p*TERT*wt glioblastomas of this study cohort harboring cyclin dependent kinase inhibitor 2A (*CDKN2A*)/*B* loss had a shorter OS. (E) *CDKN2A*/*B* loss showed a trend toward a shorter PFS. *IDH*wt, isocitrate dehydrogenase wild type; OS, overall survival; PFS, progression-free survival; p*TERT*, telomerase reverse transcriptase promoter.
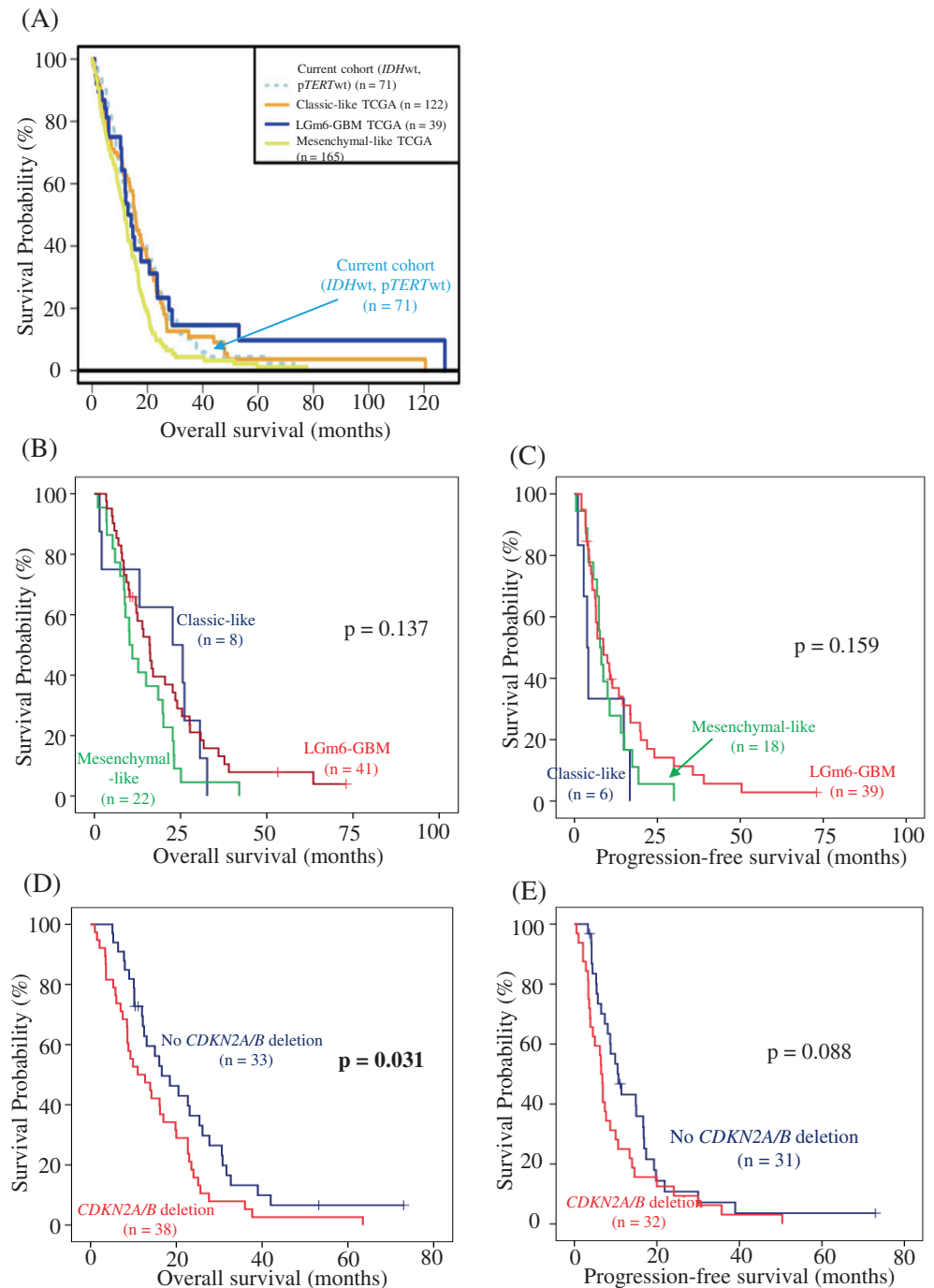
was correlated with *ATRX*/*SMARCAL1* mutations ($p = 0.011$). One-quarter of *TERT*-rearranged tumors carried *ATRX*/*SMARCAL1* mutation, whereas only 4% of non-*TERT*-rearranged tumors carried *ATRX*/*SMARCAL1* mutation. Most of the *TERT*-rearranged positive tumors were also positive for ALT (12/17, 71%). *TERT* rearrangement was not a prognosticator (Table S4). We did not make separate calculations for *ATRX*, *SMARCAL1* mutations or *TERT* amplification as the numbers of cases for these groups were too small. *PDGFRA* amplification was enriched in cases showing ALT (20/44,

45%) compared with the non-ALT group (7/27, 26%). *EGFR* amplification was more frequent in ALT-negative cases (52%) than positive cases (25%, $p = 0.021$).

For the whole cohort, 23 cases (32%) the mechanism of telomere maintenance was not accounted for by either ALT, *TERT* structural rearrangement or *ATRX* or *SMARCAL1* mutations. This group of "mechanism of telomere maintenance not yet found" showed no association with any methylation subtypes and could be found in all of them. Interestingly, they showed an enrichment of *EGFR* amplification (52%, $p = 0.033$, Table S5).
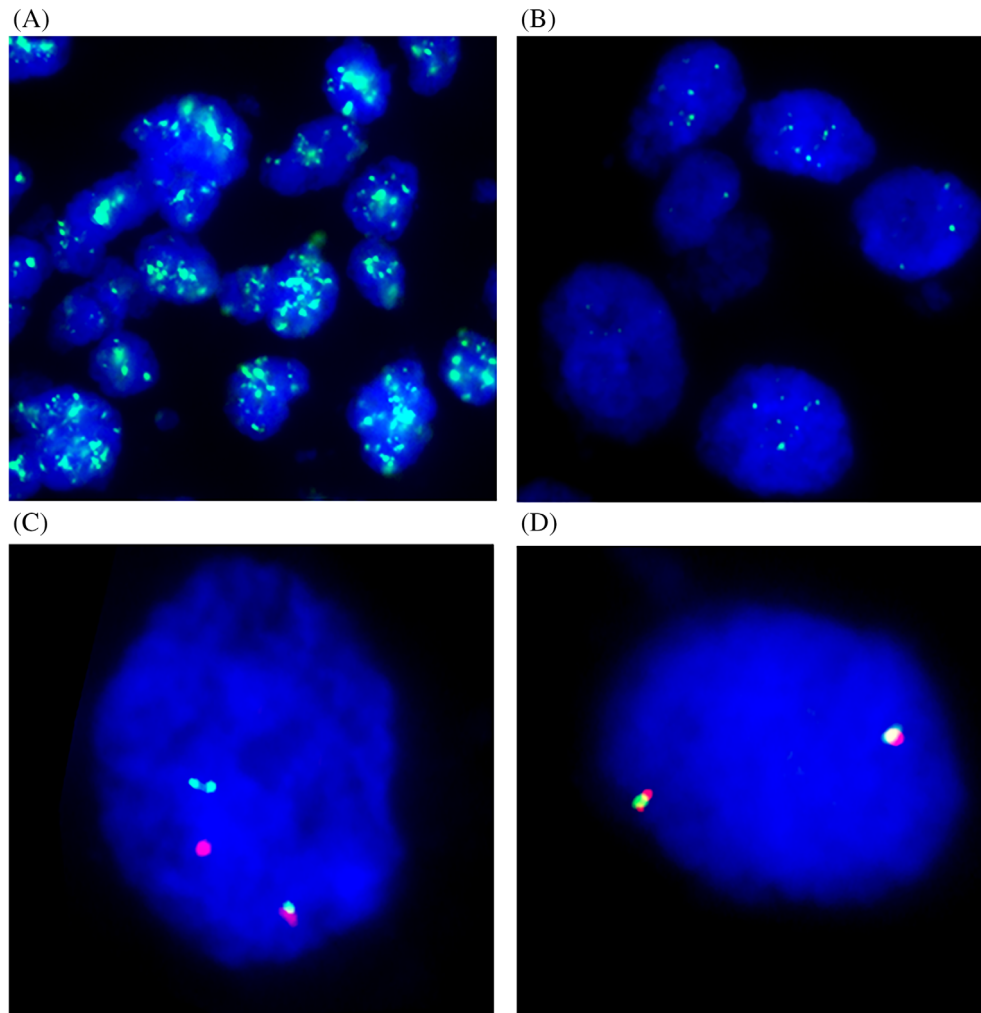
3.4 | CNVs analysis and targeted sequencing

Significant chromosomal alterations as well as CNV for the genes as obtained from genome-wide methylation profiling of 72 cases are shown in Figure 1, Figure S3A and Table S6. Gains of chromosome 20p ($p = 0.025$) and 20q ($p = 0.007$) were associated with non-LGm6-GBM methylation subtypes (Table 2; Table S6). Also, non-LGm6-GBM tumors were enriched for losses of 14q ($p = 0.015$) and 17p ($p = 0.015$). Losses at chromosome 10p (35%, $p < 0001$) and 10q (42%, $p < 0.001$) were significantly associated with non-LGm6-GBM methylation subtypes (Table 2).

At focal-level (Figure S3B), we found nine significantly recurrent amplification regions on chromosomes 4q12, 4p13, 4q21.3, 6p21.32, 7p11.2, 9p22.3, 12q13.13, 12q14.1, and 12q15. Genes located in these regions include *CDK4*, *EGFR*, *MDM2*, and *PDGFRA*. We also identified regions of recurrent loss at chromosomes 1p36.33, 1q21.2, 3p22.2, 5q35.3, 8q23.1, 9p24.3, 10q26.3, 12q13.13, 13q22.1, 15q11.2, and 19q13.42. Overall, *CDK4* amplification was the most common gene for CNVs and was detected in nearly 60% of the cohort

(Table S3). Other CNVs at gene level included amplification of *CCND2*, *EGFR*, and *PDGFRA* and homozygous losses of *CDKN2A/B* and *RB1* and they were detected in 35%–53% of the cohort. *TERT* amplification was identified in only three cases (Table 2). *PDGFRA* amplification was associated with the LGm6-GBM subtype ($p = 0.014$) whereas *EGFR* amplification was prevalent in the non-LGm6-GBM subtype (Table S3).

DNA-targeted sequencing for 91 genes (Table S1) was successful in 67 (93.1%) cases. All mutations are listed in Table S7 and the more frequent mutations are shown in Figure 1. Diplas et al. found that 20% (5 cases) of their *IDH*wt, p*TERT*wt glioblastomas contained *BRAF*V600E mutation, a biomarker of potential therapeutic significance but we were only able to identify 2 such cases (3%) in this cohort. We did not detect a prognostic implication of single-gene mutations in the whole cohort and also among different methylation subtypes (Table S8). *ATRX* mutation was detected in 4/67 (6%) of the cases. All *ATRX* mutations were found in tumors of the LGm6-GBM subtype. *ATRX* mutation and chromosome $+7$ or -10 were also found to be mutually exclusive.

TABLE 2 Selected molecular alterations and methylation subtypes of 72 *IDH*wt, p*TERT*wt glioblastomas

Molecular changes	Methylation subtypes		
	Non-LGm6-GBM[a] (frequency and number of cases)	LGm6-GBM (frequency and number of cases)	*p*-Value (non-LGm6-GBM vs. LGm6-GBM tumors)
EGFR amplification			
Yes	61% (19)	15% (6)	**<0.001**
No	39% (12)	85% (35)	
PDGFRA amplification			
Yes	23% (7)	51% (21)	**0.014**
No	77% (24)	49% (20)	
MDM4 amplification			
Yes	29% (9)	10% (4)	**0.035**
No	71% (22)	90% (37)	
RB1 loss			
Yes	26% (8)	54% (22)	**0.018**
No	74% (23)	46% (19)	
EGFR amplification or mutations			
Yes	65% (20)	20% (8)	**<0.001**
No	35% (11)	80% (33)	
PDGFRA amplification or mutations			
Yes	23% (7)	54% (22)	**0.008**
No	77% (24)	46% (19)	
TP53 loss or mutations			
Yes	29% (9)	56% (23)	**0.022**
No	71% (22)	44% (18)	
MMR mutations			
Yes	4% (1)	18% (7)	0.088
No	96% (26)	82% (33)	
TERT rearrangement			
Yes	26% (8)	26% (9)	0.746
No	74% (23)	74% (26)	
TERT amplification			
Yes	6% (2)	2% (1)	0.399
No	94% (29)	98% (40)	
Chromosome 7p gain/amplification			
Yes	6% (2)	10% (4)	0.615
No	94% (29)	90% (37)	
Chromosome 7q gain/amplification			
Yes	10% (3)	12% (5)	0.736
No	90% (28)	88% (36)	
Whole chromosome 7 gain/amplification			
Yes	6% (2)	10% (4)	0.615
No	94% (29)	90% (37)	
Chromosome 10p loss			
Yes	35% (11)	5% (2)	**0.001**
No	65% (20)	95% (39)	
Chromosome 10q loss			
Yes	42% (13)	7% (3)	**<0.001**
No	58% (18)	93% (38)	

(Continues)

TABLE 2 (Continued)

Molecular changes	Methylation subtypes		
	Non-LGm6-GBM[a] (frequency and number of cases)	LGm6-GBM (frequency and number of cases)	p-Value (non-LGm6-GBM vs. LGm6-GBM tumors)
Whole chromosome 10 loss			
Yes	35% (11)	2% (1)	**<0.001**
No	65% (20)	98% (40)	
Chromosome 20p loss			
Yes	23% (7)	5% (2)	**0.025**
No	77% (24)	95% (39)	
Chromosome 20p loss			
Yes	23% (7)	2% (1)	**0.007**
No	77% (24)	98% (40)	

Abbreviations: *EGFR*, epidermal growth factor receptor; *IDH*wt, isocitrate dehydrogenase wild type; MMR, mismatch repair; MDM4, MDM4 regulator of p53; PDGFRA, platelet derived growth factor receptor alpha; p*TERT*, telomerase reverse transcriptase promoter; RB1, RB transcriptional corepressor 1; TP53, tumor protein p53.

[a]Non-LGm6-GBM tumors included classic-like and mensenchymal-like tumors.

Overall, we detected 4.15 ± 6.187 mutations per sample. With the exception of one tumor, MMR (mismatch repair genes-*MSH2, MSH6, MLH1,* and *PMS2*) mutations were found in the LGm6-GBM tumors (17.5% vs. 3.7%). We performed sanger sequencing to confirm MMR mutations in 6 cases with enough materials to confirm the findings of NGS (Figures S4A-C). Review of the clinical history of these cases showed no other cancer and these tumors were all treatment naïve. The mean age of patients in this cohort (43.0 years old as mentioned above) was high for the possibility of a germline disease. As expected, cases positive for MMR mutations showed a higher mutation load than those that were negative ($p = 0.017$; 9.00 ± 11.832 mutations/sample vs 3.49 ± 4.794 mutations/sample). *ATRX* mutated cases showed a higher mutation load than those without ($p < 0.001$; 22.25 ± 13.022 mutations/sample vs. 3.00 ± 3.172 mutations/sample). A possible explanation is the presence of a link between *ATRX* deficiency and impaired non-homologous chromosomal end joining, rendering cells sensitive to DNA-damaging agents [39]. *TERT*-rearrangement positive patients also carried higher number of mutations compared to the negative patients ($p = 0.009$; 7.63 ± 10.658 vs. 3.02 ± 3.396).

3.5 | Most *IDH*wt, p*TERT*wt glioblastomas do not have *EGFR* amplification or −7/−10

Overall, gain/amplification of chromosome 7 was only found in 6 tumors and loss of chromosome 10 was detected in another 12 tumors. None of the tumors in this cohort harbored combined whole chromosome +7/−10. For the entire cohort, only 25 cases (35%) showed *EGFR* amplification and since the cases of this cohort were already p*TERT*wt, this suggests that three molecular features of *TERT* promoter mutation, *EGFR* amplification or

combined whole chromosome +7/−10, may not molecularly cover all cases of *IDH*wt glioblastomas [40, 41]. Interestingly, *EGFR* and *PDGFRA* amplification together covered 67% of the whole cohort and *EGFR* and *PDGFRA* amplification/mutation together covered 71% of cases successfully sequenced and the coverage would be broader.

For correlation with clinical outcomes, *MGMT* methylation was detected in 30/72 (41.7%) and was not associated with OS ($p = 0.421$) and PFS ($p = 0.589$) (Figures S5A,B). *CDKN2A/B* homozygous deletion was associated with a worsened OS ($p = 0.031$, Figure 2E) and a trend for worse survival with PFS ($p = 0.088$, Figure 2F). Multivariate analysis including age, sex, location, resection, chemotherapy, radiotherapy, *MGMT* methylation, *EGFR*, *PDGFRA*, and *CDKN2A/B* revealed that *CDKN2A/B* deletion was an independent prognosticator (Table 3). They were the only genes the mutations or amplifications or losses of which were associated with OS (Tables S3 and S8).

4 | DISCUSSION

While p*TERT* mutations account for about 70%–80% of glioblastomas and are generally regarded as a late or terminal event of gliomagenesis [4, 7], whether it has a prognostic significance within glioblastomas is still uncertain. We compared our cohort with the survival of the TCGA adult glioblastoma cohort which were *IDH*wt, p*TERT*-mut. No survival difference could be found between the cohorts. Similar finding of a lack of prognostic significance for p*TERT* mutation among *IDH*wt glioblastomas was also reported by Ceccarelli et al. [16]. Diplas et al. also showed no difference in survival between *IDH*wt, p*TERT*mut, and *IDH*wt, p*TERT*wt glioblastomas, although with smaller cohorts [3]. *IDH*wt, p*TERT*wt glioblastomas also seem to occur in younger patients

TABLE 3 Multivariate analysis of clinical and molecular features of *IDH*wt, p*TERT*wt glioblastomas

Features	OS		PFS	
	HR (95% CI)	*p*-Values	HR (95% CI)	*p*-Values
Age	1.011 (0.990-1.032)	0.303	0.999 (0.977-1.021)	0.931
Sex				
Male	1	0.697	1.281 (0.698-2.353)	0.424
Female	1.127 (0.617-2.059)			
Location				
Hemisphere	1	0.114	0.974 (0.383-2.477)	0.956
Non-hemisphere	0.447 (0.165-1.214)			
Operation				
Gross total resection	1	**0.047**	1	**0.003**
Non-total resection	1.824 (1.007-3.302)		2.598 (1.369-4.931)	
Chemotherapy				
Yes	1	**0.003**	1	**0.003**
No	6.644 (1.901-23.225)		6.711 (1.917-23.494)	
Radiotherapy				
Yes	1	0.688	1	0.147
No	1.220 (0.462-3.223)		2.095 (0.771-5.692)	
MGMT				
Unmethylated	1	0.096	1	0.745
Methylated	0.609 (0.340-1.092)		0.908 (0.509-1.622)	
EGFR alterations				
No	1	0.456	1	0.627
Yes	1.269 (0.679-2.372)		1.177 (0.610-2.270)	
PDGFRA alterations				
No	1	0.866	1	0.884
Yes	0.947 (0.506-1.775)		0.952 (0.489-1.851)	
CDKN2A/B homozygous deletion				
No	1	**0.023**	1	**0.020**
Yes	2.068 (1.107-3.864)		1.996 (1.115-3.572)	

Abbreviations: HR, hazard ratio; OS, overall survival; PFS, progression-free survival.

than *IDH*wt, p*TERT*mut patients in our series, and similar finding was observed in other studies [5, 6].

Importantly, in this cohort, we found that only 25 cases (35%) showed *EGFR* amplification and no case showed combined whole chromosome +7/−10. As these cases were already p*TERT*wt, so the WHO 2021-listed molecular diagnostic features of *IDH*wt glioblastomas, namely either *TERT* promoter mutation, *EGFR* amplification or combined whole chromosome +7/−10 cannot cover all cases of *IDH*wt glioblastomas in this context. It should be noted that even in Stichel and von Deimling study found the sensitivity of the three markers was only 77.8% for coverage of *IDH*wt glioblastomas [42]. Interestingly, in this cohort, *EGFR* and *PDGFRA* amplifications covered 67% of cases and *EGFR*/*PDGFRA* amplification or mutation together covered 71% of cases successfully sequenced, suggesting that adding *PDGFRA* alterations in the umbrella of molecular features of

*IDH*wt glioblastomas may be more inclusive. Interestingly, a recent paper by Fujimoto et al. showed that *PDGFRA* gain/amplification was an independent predictor of poor prognosis in *IDH*wt, p*TERT*wt lower-grade gliomas [43], highlighting the potential diagnostic value of *PDGFRA* alteration in *IDH*wt lower-grade gliomas and our results are consistent with their findings. And *MGMT*, a major favorable prognosticator in glioblastoma as shown by many studies [44, 45], provided no survival benefit in this cohort. This concurs with the results of another study [19].

Other than mutations at the promoter of *TERT*, structural rearrangement upstream of *TERT* locus has been associated with telomerase activity [3, 46]. Diplas et al. found that other than *ATRX* mutation, *SMARCAL1* mutation is another mechanism for ALT in gliomas [3]. For mechanism of telomere maintenance, overall in this cohort, 44 cases (61%) showed ALT, 4 cases showed

ATRX mutations, 2 cases showed *SMARCAL1* mutations and 17 cases (24%) showed *TERT* structural rearrangement, and LGm6-GBM cases predominantly showed ALT (73%). *ATRX* and *SMARCAL1* were mutually exclusive similar to findings of Diplas et al. In 23 cases (32%) in our cohort, the mechanism of telomere maintenance was not accounted for and *ATRX* and *SMARCAL1* mutations did not account for 88% of cases exhibiting ALT. We concede that the methods for telomere maintenance in cancer are diverse and we have not covered all possible mechanisms for telomerase activation in this study [1]. Similarly, in a smaller series, Williams et al. [6] only found *ATRX* mutations in 6/16 (37.5%) cases of p*TERT*wt glioblastomas. Interestingly, the group of "mechanism not yet found" cases in our cohort showed an enrichment of *EGFR* amplification (52%), this being biomarker of high-grade gliomas [47, 48].

For the whole cohort, homozygous deletion of *CDKN2A/B* was associated with a worse OS ($p = 0.031$) and it was an independent prognosticator in multivariate analyses. *CDKN2A/B* deletion was found in all methylation groups and in both ALT and non-ALT tumors. This is interesting in that *CDKN2A/B* deletion is also an independent poor prognostic marker for *IDH* mutant lower-grade astrocytomas [31, 49] and this is the only gene where mutations or CNVs showed a survival significance in this cohort.

Other than providing detailed molecular landscape for adult *IDH*wt, p*TERT*wt glioblastomas, the findings of this study also have implications for diagnostic practice where it may be impracticable or too expensive to perform methylation studies. Our findings show that the current molecular features to define molecular glioblastomas were seen only in a subset of *IDH*wt glioblastomas which are already p*TERT*wt, and that *EGFR* and *PDGFRA* amplification or mutation studies may cover most cases of *IDH*wt glioblastomas when the tumor is already *TERT*wt. Among *IDH*wt glioblastomas that are p*TERT*wt, our findings show that *CDKN2A/B* homozygous deletion, a test that is easily performed by FISH and is already routinely used in diagnostic laboratories for *IDH* mutant astrocytomas, is an independent poor prognosticator in this group of adult gliomas.

AUTHOR CONTRIBUTIONS
Study conception and design: EML and HKN performed study concept and design; EML, ZFS, KKL, NYC, HC, JYC, MFP, DTC and YM performed experiment work and data acquisition; EML, TMM, JSK, HN and HKN performed data analysis and interpretation; KKL and HKN prepared the manuscript. All authors read and approved the final paper.

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CONFLICT OF INTEREST
All authors declare no conflict no interest.

ETHICS STATEMENT
This study was approved by the Joint Chinese University of Hong Kong - New Territories East Cluster Clinical Research Ethics Committee, and the Ethics Committees of Huashan Hospital, Fudan University, Shanghai.

DATA AVAILABILITY STATEMENT
The IDAT files generated during the current study are available at https://www.surgery.cuhk.edu.hk/btc/hsbc/.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.