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Prognostic Stratification of GBMs Using Combinatorial Assessment of IDH1 Mutation, MGMT Promoter Methylation, and TERT Mutation Status: Experience from a Tertiary Care Center in India[1]

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Abstract

This study aims to establish the best and simplified panel of molecular markers for prognostic stratification of glioblastomas (GBMs). One hundred fourteen cases of GBMs were studied for IDH1, TP53, and TERT mutation by Sanger sequencing; EGFR and PDGFRA amplification by fluorescence *in situ* hybridization; NF1expression by quantitative real time polymerase chain reaction (qRT-PCR); and MGMT promoter methylation by methylation-specific PCR. IDH1 mutant cases had significantly longer progression-free survival (PFS) and overall survival (OS) as compared to IDH1 wild-type cases. Combinatorial assessment of MGMT and TERT emerged as independent prognostic markers, especially in the IDH1 wild-type GBMs. Thus, within the IDH1 wild-type group, cases with only MGMT methylation (group 1) had the best outcome (median PFS: 83.3 weeks; OS: not reached), whereas GBMs with only TERT mutation (group 3) had the worst outcome (PFS: 19.7 weeks; OS: 32.8 weeks). Cases with both or none of these alterations (group 2) had intermediate prognosis (PFS: 47.6 weeks; OS: 89.2 weeks). Majority of the IDH1 mutant GBMs belonged to group 1 (75%), whereas only 18.7% and 6.2% showed group 2 and 3 signatures, respectively. Interestingly, none of the other genetic alterations were significantly associated with survival in IDH1 mutant or wild-type GBMs.
Based on above findings, we recommend assessment of three markers, *viz*., IDH1, MGMT, and TERT, for GBM prognostication in routine practice. We show for the first time that IDH1 wild-type GBMs which constitute majority of the GBMs can be effectively stratified into three distinct prognostic subgroups based on MGMT and TERT status, irrespective of other genetic alterations.

Translational Oncology (2016) 9, 371–376

Introduction

Glioblastoma (GBM), the most common malignant primary brain tumor in adults, has a diverse prognosis despite aggressive therapeutic intervention [1,2]. There has been a great increase in the understanding of molecular alterations, both genetic and epigenetic, in GBMs. However, the number of clinically relevant molecular markers for GBM prognostication remains limited. Also, the best panel of molecular markers to be used in routine practice remains debatable. Among the molecular alterations, isocitrate dehydrogenase 1 (IDH1) mutation has been shown to be a prognostic marker

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associated with longer overall (OS) as well as progression-free survival (PFS) [3–5]. The methylation of the O-6-methylguanine-DNA methyltransferase (MGMT) promoter is another well-established prognostic and predictive marker [6]. Recently, mutations in the promoter region of telomerase reverse transcriptase (TERT), the gene encoding catalytic subunit of telomerase, have been described in gliomas especially in GBMs and correlated with poor clinical outcome [7–10]. Few recent studies have suggested various prognostic subgroups. Thus, Molenaar et al. [11] reported a two-gene predictor for GBM survival and, based on the combination of IDH1 and MGMT status, stratified GBMs into three prognostically distinct genotypes. Another study by Kellia et al. [12] demonstrated three prognostic molecular subgroups of GBMs based on TERT and IDH1 status. Recently, Eckel-Passow et al. [13] combined TERT mutation with IDH1 mutation and 1p/19q co-deletion status and described five distinct prognostic subgroups of gliomas. GBMs having only IDH1 mutation had the best prognosis, whereas those with TERT mutation only had the worst PFS/OS [13]. However, the drawback of all these studies is that they do not simultaneously incorporate other important genetic alterations in GBMs such as TP53 mutations and RTK and NF1 alterations for survival analysis, which may be important confounding factors in determining clinical outcomes.

Hence, the present study was undertaken to assess the key genetic alterations in GBMs (*viz.*, amplification of EGFR and PDGFRA and mutation of IDH-1, TP53, and NF-1 genes), along with MGMT promoter methylation and TERT mutation status, to determine their prognostic significance in combination and thus identify the best panel of markers for prognostication of GBMs.

Material and Methods

All cases diagnosed as GBMs between 2006 and 2012 were identified from a detailed review of records of the Neuropathology Laboratory of the Department of Pathology, All India Institute of Medical Sciences, New Delhi, India. Cases of adult GBM wherein adequate tumor tissue was available in formalin-fixed, paraffin-embedded (FFPE) blocks along with snap-frozen tumor tissue stored at −80°C were taken up for the study. All experiments using patient samples were approved by the ethics committee of All India Institute of Medical Sciences, New Delhi. Hematoxylin and eosin–stained slides of these cases were reviewed, and a concordant agreement was established for the confirmation of the diagnosis between three experienced histopathologists based on the WHO classification (2007). Based on these criteria, 114 cases of GBMs were selected for the present study.

Patient Data

Patient records were reviewed to obtain demographic data, including age, sex, tumor location, treatment received, and follow-up. The cases were divided into young adult (age ≤ 40 years) and older adult groups (age > 40 years). All the patients included for survival analysis (n = 73) underwent total to near-total surgical resection and received radiotherapy along with concurrent chemotherapy and had a Karnofsky Performance Score (KPS) of ≥70. Postoperative radiation therapy was started within 4 to 6 weeks of surgery. A dose of 50 Gy in 25 fractions was prescribed for CTV1 (enhancing tumor + edema+2.5-cm margin all around as seen in the preoperative T2-weighted magnetic resonance imaging scan) followed by a boost of 10 Gy in 5 fractions over 1 week. Concurrent temozolomide was given at a dose of 75 mg/m^2 daily in empty stomach following antiemetic and ranitidine 1 hour before radiation. The maintenance temozolomide was started after a gap of 1 month in patients without any recurrence. The first cycle was given at 150 mg/m^2 and, depending on the tolerance, increased to 200 mg/m^2 in the next cycle for a minimum of 6 cycles every 4 weeks. The incidence date was defined as the date of surgery. The recurrences were defined as the cases, which showed evidence of progression by magnetic resonance imaging that required a second resection or adjuvant treatment. Patient outcome was characterized in terms of PFS and OS.

IDH1, TP53, and TERT Gene Mutation Analysis by Sanger Sequencing

For sequencing analysis, DNA was isolated from frozen tumor tissue using standard protocol. For TP53 mutational analysis, coding regions from exons 2 to 11 were evaluated using the direct sequencing protocol as described in the International Agency for Research on Cancer p53 database and described earlier [14]. Mutations in exon 4 of IDH1 were determined by direct sequencing in all the cases as described in the previous study [15].

For TERT mutation, the forward primer TERT-F (5′-GTCCT GCCCCTTCACCTT-3′) and reverse primer TERT-R (5′-CAGCG CTGCCTGAAACTC-3′) were used to amplify a 163-bp fragment spanning the two mutational hotspots [chr5, 1 295 228 (C228T) and 1 295 250 (C250T)] in TERT promoter region. Polymerase chain reaction (PCR) was performed in a total of 25 μl using 0.3 mM of each dNTP, 2.5 mM MgCl2, 0.3 mM of each primer, 0.2 U of DNA polymerase, and 10 ng of DNA in 25 μl of reaction mixture. PCR product was further purified and cleaned using ExoSAP as per manufacturer's protocol. Subsequently, 2 μl of the purified amplification product was submitted to bidirectional sequencing using the BigDye Terminator Cycle Sequencing kit v1.1 (Life Technologies). The products were resolved in Genetic Analyzer 3130xl and analyzed by Sequencing Analysis software.

Analysis of NF-1 Expression using Real-Time PCR

As sequencing of NF1 gene is difficult owing to the large size and multiple mutation hotspots, downregulation of NF-1 mRNA expression (expression ≤ 0.5 folds of control) was considered as a signature marker of gene mutation. RNA was extracted from frozen tissue specimens by using commercially available mirVanamiRNA Isolation Kit (M/S. Ambion, USA) following manufacturer's protocol. One microgram of the total RNA from all samples was reverse-transcribed to cDNA using SuperScript VILO cDNA Synthesis Kits (Life Technologies). To determine the expression profile of NF1, real-time PCR was performed using primers designed via Primer3 software (v.0.4.0) [5′-GCATTTCTACCAGTAACCTTGATGATAC-3′ (F) and 5′-TCTGAACAAACAGTTAATTCCTGTAACC-3′ (R)]. Quantification was performed on Light Cycler 480 (Roche Diagnostic, Basel, Switzerland) using SYBR-Green chemistry. Expression levels of mRNA were calculated using comparative cycle threshold (Ct) method. Ct values of the target mRNAs were normalized in relation to reference genes (TATA Box Binding Protein). The fold change was calculated using the equation $2^{-\Delta\Delta Ct}$.

Assessment of EGFR and PDGFRA Amplification Using Fluorescence In Situ Hybridization (FISH)

FISH analysis was carried out on FFPE sections as previously described [16]. Briefly, a dual-color FISH assay was performed on paraffin-embedded sections with a locus-specific probe for EGFR

(spectrum orange) paired with a corresponding reference centromeric probe for chromosome 7 (spectrum green) (M/S Vysis, Inc.). For analysis of PDGFRA amplification, Tricolor Rearrangement Probe was used (Spectrum Aqua for PDGFRA, Spectrum Green and Orange for control) (M/S Vysis, Inc.). Signals were enumerated in 200 nonoverlapping nuclei. Amplification was considered when >10% of tumor cells showed either test:control signal ratio >2 or innumerable tight clusters of signals of the locus probe.

Assessment of MGMT Promoter Methylation Status Using Methylation-Specific PCR

DNA methylation pattern of the MGMT gene promoter was determined by methylation-specific PCR. This procedure involved chemical modification of unmethylated cytosine to uracil, followed by a nested two-stage PCR. The detailed procedure has been described in the previous study [17].

Statistical Analysis

Statistical tests were carried out using SPSS version 17 software (SPSS Inc., Chicago, IL) or Excel (Microsoft). Chi-square test and Fisher's exact test were applied to examine the association between the qualitative variables. To test the relationship with continuous variables, the Mann-Whitney U test was used. PFS and OS were calculated from the incidence date until the date of recurrence or death (PFS) or the date of death due to tumor (OS). Patients who were recurrence-free at the last follow-up were considered as a censored event in the analysis. To assess clinical and/or molecular alteration associated with PFS, survival curves were calculated according to the Kaplan-Meier method, and the differences between curves were assessed using the log-rank test. Variables with a significant P value were used to build a multivariate Cox model. In all analysis, two-sided P values less than .05 were considered significant.

Results

Patient Characteristics

The study included 114 cases of adult supratentorial GBMs. The mean age at diagnosis was 48 years (range: 21-77 years), and the male to female ratio was 2.3:1. Majority (67.5%; 77/114) of the patients were older adults (>40 years), whereas the rest (32.5%; 37/114) were young adults (≤40 years). Follow-up was available in 73 cases, and median PFS and OS were 56.1 weeks and 83.3 weeks, respectively. The young adult patients (n = 24) had a significantly better survival as compared to older adults (n = 49) [median PFS: 69.7 weeks vs 45.4 weeks; median OS: not reached (NR) vs 83.3 weeks] (Figure 1, *A* and *B*).

MGMT Promoter Methylation, TERT Mutation Status, and Correlation with Age and Prognosis

MGMT status was assessed in the GBM cases with follow up (n = 73). Methylation of MGMT promoter was identified in 48.6% (36/73) of cases. This epigenetic alteration did not show any association with age. The frequency of MGMT promoter methylation was significantly higher in IDH1 mutated cases (81.3%; 13/16) as compared to IDH1 wild-type (40.4%; 23/57) (P value = .005). As a corollary, 36% (13/36) of the MGMT promoter methylated cases showed IDH1 mutation. The cases with MGMT promoter methylation also had significantly longer survival as compared to unmethylated cases (median PFS: 82.7 weeks vs 23.8 weeks; median OS: NR vs 58.3 weeks) (Figure 1, *C* and *D*).

TERT promoter mutation was identified in 51% (37/73) of cases which included 72% C228T and 28% C250T mutations. This genetic alteration was significantly more common in older adults as compared to young adults (61.2% vs 25%; P value = .006). On correlation with molecular alteration, TERT mutation was found to be extremely rare in IDH1 mutated group (12.5%; 2/16) (P value = .001), whereas it was more commonly associated with EGFR amplification (74%; 17/23) and NF1 alteration (66.7%;12/18). The cases with TERT mutation had significantly shorter survival as compared to those with TERT wild-type (median PFS: 32.8 vs 82.5 weeks; median OS: 58.3 weeks vs NR) (Figure 1, *E* and *F*).

Frequency of Genetic Alterations and Correlation with Age

The frequency of genetic alterations, age distribution, and prognostic outcome of each genetic alteration separately is depicted in Table 1. EGFR amplification was most frequent (40%), whereas TP53, IDH1, and NF1 mutations were noted in approximately 20% of cases each. Furthermore, IDH1 mutation, TP53 mutation, and PDGFRA amplification were more prevalent in young adults, whereas EGFR amplification and NF1 alteration were commoner in older adults. No significant association was identified between different genetic alterations with site and gender.

Prognostic Stratification Based on Molecular Genetic Alterations

For prognostic stratification, we first subdivided GBM cases into IDH1 mutant and IDH1 wild-type. IDH1 mutated cases had significantly longer survival as compared to IDH1 wild-type cases (median PFS: 91.3 vs 40.7 weeks; median OS: NR vs 61.7 weeks) (Figure 1, *G* and *H*). Next, the IDH1 wild-type cases were further prognostically subclassified based on EGFR, PDGFRA, TP53, and NF1 status. Interestingly, there was no significant difference among these genetic subgroups in terms of survival (Table 2; Figure 1, *I* and *J*). In the IDH1 mutant group, none of the cases showed EGFR/PDGFRA amplification or NF1 downregulation. A total of 42.1% (8/19) of cases showed TP53 mutation, but there was no significant correlation with prognosis.

Combinatorial Assessment of IDH1, MGMT, and TERT for Prognostication

Next, we assessed the combinatorial prognostic implication of TERT and MGMT in IDH1 mutant and wild-type cases. Interestingly, the IDH1 wild-type group could be effectively divided into three distinct prognostic subgroups based on MGMT and TERT status, with cases showing only MGMT promoter methylation (group 1; n = 12) having the best outcome (median PFS and OS: 83.3 weeks and NR) and cases with only TERT mutation (group 3; n = 24) having the worst prognosis (median PFS and OS: 19.7 and 32.8 weeks). The prognosis of the cases with both these alterations (double positive) or none of these alterations (double negative) was intermediate between the above-mentioned groups. Hence, these two groups were clubbed together as group 2 (median PFS and OS: 47.5 and 89.2 weeks) (Figure 1, *K* and *L*). On multivariate analysis also, these prognostic subgroups showed similar association with survival irrespective of other genetic alterations and age (Table 3).

In the IDH1 mutant group, TERT mutation was rare and only a single case belonged to group 3; hence, it was excluded for analysis. However, cases with only MGMT methylation (group 1; n = 12) demonstrated a trend toward better survival than group 2 (n = 3) [hazard ratio (HR): 1.47 and 1.22, respectively, for PFS and OS]. However, the difference was not statistically significant possibly because of the small number of cases in group 2.

Thus, the most important panel for prognostication in GBMs appears to be a combination of IDH1, MGMT, and TERT. Majority

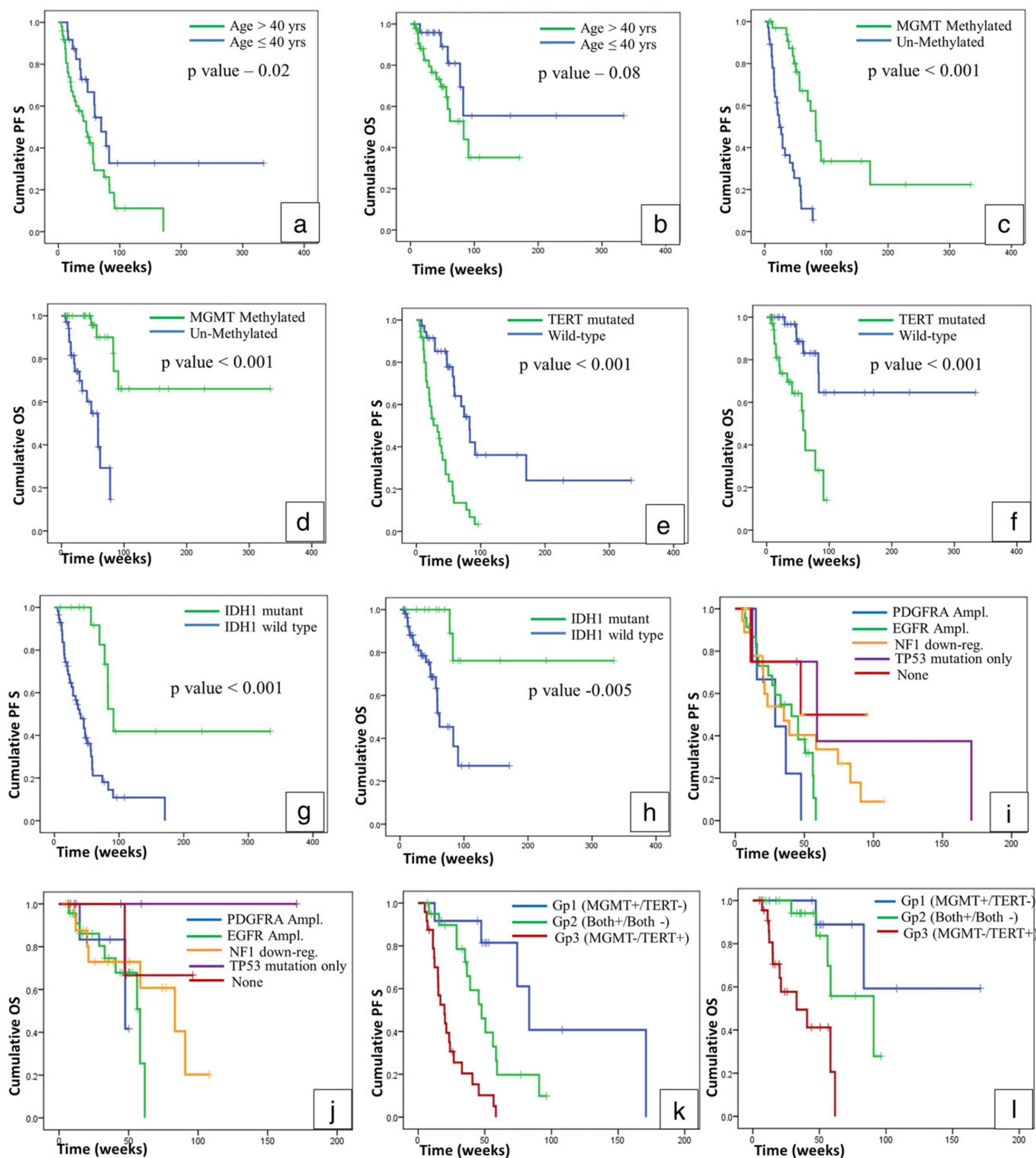

Figure 1. PFS and OS represented by Kaplan-Meier plots for young versus older adult age group (A, B); MGMT promoter methylated versus unmethyalted cases (C, D); TERT mutated versus wild-type cases (E, F); IDH1 mutated versus wild-type cases (G, H); EGFR, PDGFRA, TP53, and NF1 alterations in IDH1 wild-type cases (I, J); and three prognostic subgroups of GBMs based on MGMT and TERT status in IDH1 wild-type group (K, L).

of the GBMs (83%) are IDH1 wild, and therefore, their further stratification based on MGMT and TERT is indeed very important.

Discussion

GBMs are extremely heterogeneous tumors with multiple complex genetic abnormalities. Among various prognostic biomarkers, IDH1-mutations appeared most important and defined a distinct subgroup of GBMs with favorable outcome [18,19]. Similarly, the present study also highlighted the prognostic significance of IDH1. However, other molecular genetic alterations, namely, TP53 mutation, NF1 mutation, EGFR amplification, and PDGFRA amplification, did not demonstrate any significant association with survival in the IDH1 wild-type group. MGMT promoter methylation status has been one of the well-known positive prognostic as well as an independent predictive marker for

Table 1. Frequency and Demographic Profile of Different Genetic Alterations

Molecular Alteration	Frequency	Age (Years) Mean (Range)	M:F
IDH1 mutation	16.7% (19/114)	37 (22-60)	8.5:1
TP53 mutation	16.7% (19/114)	41 (21-66)	8.5:1
PDGFRA amplification	8.8% (10/114)	35 (23-55)	4:1
NF1 downregulation	21% (24/114)	52 (28-74)	2.1:1
EGFR amplification	39.5% (45/114)	54 (38-77)	2.2:1

therapeutic response to TMZ in high-grade astrocytic tumors [6,20]. Its relevance for prognostication of GBMs was also confirmed in the present series. TERT is another frequently mutated gene in GBMs with prognostic implication. In the present study, the frequency of TERT promoter mutation was slightly lower as compared to the reported Western literature. However, similar to previous studies, this genetic alteration was found to be significantly more frequent in older adults as compared to young adults [8]. Hence, the lower frequency of TERT promoter mutation in the present series may be attributable to the lower mean age of our cohort. Our data also showed that TERT mutation was associated with poorer outcome in GBMs, consistent with previous data on gliomas [12].

Few studies in recent years have prognostically stratified GBMs based on combination of various biomarkers. Interestingly, all these studies included IDH1 mutation in the marker panel for prognostication. Based on the IDH1 and MGMT status, Molenaar et al. [11] stratified GBMs (n-98) into three prognostically different subgroups. Patients with IDH1 mutation and MGMT methylation had the longest survival, followed by patients with IDH1 mutation or MGMT methylation alone, whereas patients without both these alterations had the shortest survival. Similar prognostic stratification using MGMT and IDH1 was also described by Hartmann et al. [21] in cases of high-grade astrocytoma (grade III and IV; n = 338). Interestingly, the authors did not observe any significant difference in prognosis between IDH1 wild-type GBMs and anaplastic astrocytomas. Killela et al. [12] analyzed IDH1/2 and TERT status in 415 cases of gliomas of different grades and showed that, based on these genetic alterations, grade III and IV tumors can be effectively classified into four prognostic subgroups irrespective of tumor histomorphology and WHO grade. The cases with both TERT and IDH mutation (morphologically oligodendroglioma) had the best prognosis, whereas cases with only TERT mutation had the worst prognosis. Interestingly, within the GBM group, concomitant IDH and TERT mutation was extremely rare. Hence, GBM cases were subdivided into three prognostic subgroups. Cases with only IDH mutation had the best prognosis (OS = 42.3 months), followed by cases with IDH/TERT wild-type (16.6 months) and only TERT mutation (11.3 months). Recently, Eckel-Passow et al. [13] subdivided 1087 cases of gliomas (615 grade II or III and 472 grade IV) based on IDH, TERT, and 1p/19q co-deletion. Among the GBMs, majority (74%) showed only TERT mutation, 17% were triple negative, 7% had only IDH mutation, and only 2% showed combined TERT and IDH mutation. Similar to the previous studies, cases with only IDH1 mutation had the best and cases with only TERT mutation had the worst survival.

All the above-mentioned studies including ours highlight the importance of IDH in prognostic stratification of GBMs. Unfortunately, IDH1 mutation is rare in primary GBMs and seen mainly in secondary GBMs which constitute only 10% to 15% of all GBMs [22].On the other hand, there is considerable heterogeneity among the IDH1 wild-type cases in terms of clinical outcome, indicating the necessity of further stratification. For the first time, the present study demonstrated that IDH1 wild-type cases which constitute majority of GBMs (approximately 83% in present series) can effectively be subclassified into three prognostic subgroups based on MGMT and TERT status. Interestingly, IDH1 wild-type cases with only MGMT methylation (group 1) had survival similar to the IDH1 mutated cases, whereas IDH1 wild-type cases with TERT mutation demonstrated worst survival. IDH1 mutated cases need not be further stratified based on MGMT and TERT because majority of these cases are MGMT methylated and rarely have TERT mutation.

Therefore, based on the results of the present study, we recommend routine assessment of a panel of only three markers, namely, IDH1 mutation, MGMT promoter methylation, and TERT mutation status for GBM prognostication. All cases of GBMs should be analyzed for IDH1mutation first by Sanger sequencing or immunohistochemistry with mutant-specific antibody followed by assessment of TERT and MGMT status especially in IDH wild-type cases. All these assessments require a small quantity of tumoral DNA and can be done on DNA extracted from FFPE tissue. The International Society of Neuropathology-Haarlem Consensus Guidelines state that "It is also entirely possible that genetic tests not discussed at this meeting (eg, *TERT* mutation) will be incorporated into diagnostic definitions at the time of the eventual WHO classification revisions" [23]. Our study thus supports the incorporation of these tests into the final revised WHO classification.

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Table 2. Correlation of EGFR, TP53, NF1,and PDGFRA Status with PFS and OS in IDH1 Wild-Type GBMs (n = 57)

Genetic Alterations	Median PFS (Wks)	*P* Value	Median OS (Wks)	*P* Value
Only TP53 mut (n = 4)	59.1	TP53 vs EGFR = .06 TP53 vs PDGRR = .11	Not reached	TP53 vs EGFR = .06 TP53 vs PDGRR = .22
EGFR amp (n = 23)	40.7	TP53 vs NF1 = .31 TP53 vs none = .88	58.3	TP53 vs NF1 = .14 TP53 vs none = .41
PDGFR amp (n = 6)	28.8	EGFR vs PDGFRA = .23 EGFR vs NF1 = .38	47.5	EGFR vs PDGFRA = .97 EGFR vs NF1 = .22
NF1 (n = 18)	35.1	EGFR vs none = .24 NF1 vs PDGFR = .35	83.2	EGFR vs none = .42 NF1 vs PDGFR = .90
None (n = 6)	47.3	NF1vs none = .30 PDGFRA vs none = .19	Not reached	NF1 vs none = .63 PDGFRA vs none = .61

Table 3. Prognostic Subgroup of IDH1 Wild-Type GBMs Based on MGMT and TERT Status

Prognostic Subgroups	PFS				OS			
	Univariate Analysis		Multivariate Analysis*		Univariate Analysis		Multivariate Analysis*	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Gp1– MGMT+/TERT– (*n* = 12)	Reference	–	Reference	–	Reference	–	Reference	–
Gp2– both+/both– (*n* =21)	2.97 (0.97-9.09)	.05	2.67 (0.81-8.75)	.10	3.45 (0.79-14.95)	.09	2.42 (0.42-13.97)	.32
Gp3– MGMT–/TERT+ (*n* = 24)	11.95 (3.81-37.51)	<.001	10.44 (3.21-33.09)	<.001	23.83 (5.43-104.47)	<.001	11.12 (1.99-61.99)	.006

* Adjusted for age and other genetic alterations.

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