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Human TERT promoter mutation enables survival advantage from MGMT promoter methylation in IDH1 wild-type primary glioblastoma treated by standard chemoradiotherapy

HuyTram N. Nguyen, Amy Lie, Tie Li, Reshmi Chowdhury, Fei Liu, Byram Ozer, Bowen Wei, Richard M. Green, Benjamin M. Ellingson, He-jing Wang, Robert Elashoff, Linda M. Liau, William H. Yong, Phioanh L. Nghiemphu, Timothy Cloughesy, Albert Lai

Department of Neurology, David Geffen School of Medicine at UCLA, University of California, Los Angeles, California (H.T.N., A.L., T.L., R.C., F.L., B.O., P.L.N., T.C., A.L.); Department of Pathology, David Geffen School of Medicine at UCLA, University of California, Los Angeles, California (B.W., W.H.Y.); Kaiser Permanente Southern California, Los Angeles, California (R.M.G.); Department of Radiological Sciences, David Geffen School of Medicine at UCLA, University of California, Los Angeles, California (B.M.E.); Department of Biomathematics, David Geffen School of Medicine at UCLA, University of California, Los Angeles, California (H.W., R.E.); Department of Neurosurgery, David Geffen School of Medicine at UCLA, University of California, Los Angeles, California (L.M.L.)

Corresponding Author: Albert Lai, MD, PhD, UCLA Neurology, 710 Westwood Plaza, RNRC 1–230, Los Angeles, CA 90095 (albertlai@mednet.ucla.edu).

Abstract

Background. Promoter mutation in the human telomerase reverse transcriptase gene (*hTERT*) occurs in ~75% of primary glioblastoma (GBM). Although the mutation appears to upregulate telomerase expression and contributes to the maintenance of telomere length, its clinical significance remains unclear.

Methods. We performed *hTERT* promoter genotyping on 303 isocitrate dehydrogenase 1 wild-type GBM tumors treated with standard chemoradiotherapy. We also stratified 190 GBM patients from the database of The Cancer Genome Atlas (TCGA) by *hTERT* gene expression. We analyzed overall and progression-free survival by Kaplan–Meier and Cox regression.

Results. We detected *hTERT* promoter mutation in 75% of the patients. When included as the only biomarker, *hTERT* mutation was not prognostic in our patient cohort by Cox regression analysis. However, when *hTERT* and O^6-DNA methylguanine-methyltransferase (*MGMT*) were included together, we observed an interaction between these 2 factors. To further investigate this interaction, we performed pairwise comparison of the 4 patient subcohorts grouped by *hTERT-MGMT* status (MUT-M, WT-M, MUT-U, and WT-U). *MGMT* methylated patients showed improved survival only in the presence of *hTERT* promoter mutation: MUT-M versus MUT-U (overall survival of 28.3 vs 15.9 mos, log-rank $P < .0001$ and progression-free survival of 15.4 vs 7.86 mo, log-rank $P < .0001$). These results were confirmed by Cox analyses. Analogously, the cohort from TCGA demonstrated survival benefit of *MGMT* promoter methylation only in patients with high *hTERT* expression. In addition, *hTERT* mutation was negatively prognostic in our *MGMT* unmethylated patients, while the analogous association with high expression was not observed in the cohort from TCGA.

Conclusion. The prognostic influence of *MGMT* promoter methylation depends on *hTERT* promoter mutation. This interaction warrants further mechanistic investigation.

Key words

hTERT promoter mutation | *MGMT* promoter methylation | overall survival | primary glioblastoma | progression free survival

Glioblastoma (GBM) is the most common and lethal form of primary brain cancer, whose prognosis remains poor despite ongoing advances in treatment. Recent reports indicate that 70%–80% of GBM genomes harbor either C228T or C250T mutations in the promoter region of the human telomerase reverse transcriptase gene (*hTERT*).[1–9] These mutations upregulate the *hTERT* gene product in GBM[5,10] and other cancers.[2,3,11,12] In doing so, *hTERT* mutation may represent the predominant mechanism underlying the maintenance of telomere length necessary to achieve replicative immortality in GBM cells. Despite accumulating studies demonstrating such a functional role of *hTERT* promoter mutation, consensus on their prognostic value in GBM has not been established.[1–10,13,14] Several studies showed worse overall survival (OS) and progression-free survival (PFS) in the *hTERT* mutant group,[2–9,13] while others[1,10,14] showed no difference in survival between *hTERT* mutant and *hTERT* wild-type (wt) patients. These disparate findings may be explained by small cohort size and lack of genetic, pathological, and treatment homogeneity in these cohorts.

Multiple retrospective studies have investigated the association of *hTERT* promoter mutation with several known biomarkers in GBM and found that the significance of *hTERT* promoter mutation depends on the genetic background of the tumor. For example, *hTERT* promoter mutation is associated with better outcome in gliomas with mutation in the isocitrate dehydrogenase 1 gene (*IDH1*),[3,6] and GBM with epidermal growth factor receptor (*EGFR*) amplification.[4] In addition, *hTERT* promoter mutational status appears to influence the clinical significance of *EGFR* amplification: *EGFR* amplification was reported to associate with better outcome in *hTERT* mutant patients, but with poorer outcome in *hTERT*-wt patients.[4,8] These results support the notion that *hTERT* promoter mutation is likely to be an important genetic event. However, its clinical significance may manifest differently depending on genetic context.

In the past decade, promoter methylation in the O^6-methylguanine-DNA-methyltransferase gene (*MGMT*) has provided an avenue to stratify GBM outcome. Methylation of *MGMT* promoter disrupts the tumor's DNA repair mechanism by silencing *MGMT* expression,[15,16] which subsequently sensitizes GBM to temozolomide, an alkylating agent, and portends a survival benefit for patients receiving standard chemoradiotherapy.[15,17,18] Nencha and colleagues[8] studied both *hTERT* promoter mutation and *MGMT* promoter methylation in GBM patients but reported no association in incidence of the 2 markers; however, this study included only 239 (37%) of the 651 patients following standard chemoradiotherapy, and 50 patients of the 651 were *IDH1* mutant. Given the importance of *MGMT* promoter methylation in predicting GBM patient outcome, multiple other studies have also reported no significant association in incidence between *MGMT* promoter methylation and *hTERT* promoter mutation[1,4,8,9]; however, no study has addressed the clinical significance of *hTERT* promoter mutation in the context of *MGMT* promoter methylation.

As the vast majority of primary GBM are *IDH1*-wt, we investigated *hTERT* promoter mutation as a potential prognostic marker in a large homogeneous primary GBM cohort, limited to only *IDH1*-wt patients treated with standard chemoradiotherapy. By analyzing data from 303 *IDH1*-wt treatment naïve GBM patients treated at the University of California Los Angeles (UCLA) and Kaiser Permanente Los Angeles (KPLA), we observed an interaction between *hTERT* promoter mutation and *MGMT* promoter methylation. The survival benefit of *MGMT* promoter methylation was seen only in *hTERT* mutant GBM, and *hTERT* promoter mutation was a negative prognostic indicator in *MGMT* unmethylated patients. Similarly, analysis of 190 patients from the database of The Cancer Genome Atlas (TCGA) showed that the benefit of *MGMT* promoter methylation was seen only in high *hTERT* expressing GBM.

Materials and Methods

Patient Data Collection

UCLA/Kaiser cohort.

We retrospectively identified 303 *IDH1*-wt primary glioblastoma patients from UCLA and KPLA for *hTERT* promoter sequencing and survival analyses. Two hundred and twenty-eight patients came from a previously reported cohort.[16] All patients were diagnosed from March 2001 to October 2013, had DNA isolated from treatment naïve formalin-fixed, paraffin-embedded tumor samples, and received first-line treatment with concurrent radiation and temozolomide. Two hundred and seventy-seven patients (91%) received sufficient dose of radiotherapy (≥5400cGy) and 11 patients (4%) received whole brain radiation or planned to receive less than sufficient radiation dose. Among the remaining 15 patients, 2 developed adverse events secondary to radiation therapy, hence received less than sufficient dose, while the rest showed unclear reasons for early termination due to lack of clinical follow-up around the radiotherapy period. Pathological diagnosis was reviewed at UCLA ($n = 258$) or collected from outside-institution pathological reports ($n = 45$).

The Cancer Genome Atlas cohort.

Gene expression of *hTERT* and OS data were collected from TCGA for 190 de novo, *IDH1*-wt, primary GBM patients, all of whose diagnoses were after 2005; they all received temozolomide after surgical resection and had *MGMT* promoter methylation reported by Brennan et al.[19] The majority of patients ($n = 171$) received radiotherapy, while the remaining patients ($n = 19$) had unclear documented radiation treatment. As only a small subset of *IDH-WT* primary GBM patients from the database of TCGA were genotyped for *hTERT* promoter sequence ($n = 30$ in Ceccarelli et al[20]), we used *hTERT* low versus high gene expression as an approximate surrogate for wild-type versus mutant promoter, respectively, based on previous studies showing relative increased *hTERT* expression in *hTERT* mutant GBM.[1,5,6,9,10,13]

Detection of Biomarker Status

Genomic DNA from formalin-fixed, paraffin-embedded sample blocks was isolated using the Recoverall Total

Nucleic Acid Isolation Kit (Ambion) and the DNeasy Blood & Tissue Kit (Qiagen), respectively. PCR amplification of *hTERT* promoter was performed using forward primer, 5′–AGCACCTCGCGGTAGTGG–3′ and reverse primer, 5′–GGCCGATTCGACCTCTCT–3′. The product was confirmed by agarose gel electrophoresis and cleaned up using the MinElute PCR Purification Kit (Qiagen). Purified PCR products were sequenced using the BigDye Terminator v1.1 and analyzed on a 3730 sequencer, both from Applied Biosystems. All PCR products were sequenced using forward primer as described above. Reverse primer was used to confirm only samples that failed sequencing with forward primer.

IDH1 genotype and *MGMT* promoter methylation status were previously reported for 225 patients from the previous study,[16] while the remainder were tested using Sanger sequence and methylation-specific PCR as previously described[16,21] in our laboratory or from routine clinical testing by a lab (LabCorp) certified by the Clinical Laboratory Improvement Amendments as described in Vlassenbroeck et al.[22] Among 303 UCLA/Kaiser patients, all carried *IDH1*-wt. Eighty-four patients were subsequently sequenced for *IDH2* genotype and confirmed to have *IDH2*-wt. Based on the low incidence of *IDH2* mutation in *IDH1*-wt GBM, which was found to be ~0.6% from our institutional experience, *IDH2* genotyping was discontinued for the remaining 219 cases. The collection of the brain tumor samples was approved by the UCLA institutional review board, and informed consent was obtained from all patients.

Statistical and Survival Analyses

The primary objective was to assess OS for both cohorts and PFS for only the UCLA/Kaiser cohort. OS was determined from the date of tumor diagnosis by surgery to the date of death/censor. Patients who were lost to follow-up with unobtainable dates of death or had the last follow-up before the freeze date on September 21, 2015 were censored on their last known clinical visit or imaging study. Patients with the last follow-up after the freeze date were censored on September 21, 2015. We followed the same protocol described by Lalezari et al[16] to calculate PFS, using the freeze date of September 21, 2015. The majority of PFS ($n = 220$) were available from a previous study[16]; only patients who were stable at the time of the previous study were reviewed again for PFS. An additional 49 patients who had MRI scans available were reviewed as previously described.[16] In cases where scans were not available ($n = 30$), the original neuro-oncologist progression date was used. Four patients from the UCLA/Kaiser cohort were excluded from PFS analysis due to lack of radiological follow-up.

Patient characteristics were summarized using descriptive statistics and compared between *hTERT* mutant versus wild-type subjects using the Wilcoxon rank sum test for continuous variates and the chi-square test for categorical variables. Statistical analyses were carried out using SAS v9.4. Since this was an exploratory study, alpha level was not adjusted for multiple comparisons, and significance level was assigned at $P < .05$.

Survival curves were generated using Kaplan–Meier analysis in the R package. The Cox proportional hazard regression model was used to study the prognostic significances of *hTERT* promoter mutation, both with and without *MGMT* promoter methylation, on OS and PFS, while other important clinical factors were adjusted (such as age, gender, KPS, extent of resection status, and whether the patient received bevacizumab treatment). Upfront bevacizumab factor was included in the Cox regression model evaluating PFS to control for early benefit from bevacizumab observed in PFS.[23]

Multiple Cox regression models were developed. Besides the clinical factors, *hTERT* promoter mutation and *MGMT* promoter methylation were included in the model individually first, then jointly, including *hTERT* promoter mutation by an *MGMT* promoter methylation interaction term. Following the interaction in the Cox regression analysis, we performed single degree of freedom analysis for the 4 patient groups defined by *hTERT* promoter mutation and *MGMT* promoter methylation status while controlling for other clinical factors and patient groups. In the last model, we performed pairwise comparisons while controlling for other clinical factors, to evaluate the effect of *hTERT* promoter mutation or *MGMT* promoter methylation in the context of each other, such as *MGMT* methylated versus unmethylated hazard ratio (HR) for given *hTERT* mutational status and *hTERT* mutant versus wild-type HR for given *MGMT* methylation status.

To validate the stability of the interaction between the 2 biomarkers and the dependence of each marker's survival benefit on the other, we used bootstrap analysis to obtain unbiased estimates of HR and its 95% confidence interval.[24–26] We performed random resampling with replacement from the original dataset to obtain a new dataset with equal size and carried out Cox regression analysis using the new data. We repeated the above steps 1000 times and calculated the mean HR and 95% CI for each parameter using the analysis results of the 1000 bootstrap datasets. Bootstrap analysis was carried out using SAS v9.4.

Several studies reported a strong association between *hTERT* promoter mutation and high *hTERT* gene expression in GBM[1,5,6,9,10,13]; hence, we analyzed OS of the primary GBM cohort from TCGA with available *hTERT* gene expression data. The same methods were applied to investigate the significance of *hTERT* gene expression level on predicting *IDH1*-wt GBM patient outcome from the dataset of TCGA.

Results

Human TERT Promoter Mutation Alone Is Not Associated with IDH1-wt Primary GBM Patient Survival

In order to resolve conflicting reports regarding the significance of *hTERT* promoter mutation in primary GBM, we sought to investigate *hTERT* promoter mutation as a potential biomarker in a large homogeneous GBM patient cohort. We genotyped 303 *IDH1*-wt GBM samples and detected a total of 228/303 *hTERT* mutations (75%), distributed as 177 patients (78%) with C228T mutation and 51 patients (22%) with C250T mutation. Patients carrying the C228T mutation had improved median PFS, but not OS, compared with patients with C250T mutation (Fig. 1A–B). Based on this

observation, we combined both mutations into a single *hTERT* mutant group for further survival analysis. Patient demographics and clinical summary are presented in Table 1.

By Kaplan–Meier analysis, patients with *hTERT* promoter mutation demonstrated similar median OS of 18.5 months (n = 228) versus 17.8 months (n = 75) in *hTERT*-wt patients (log-rank P = .3845), and similar median PFS of 9.63 months (n = 224) versus 8.45 months (n = 75) in *hTERT*-wt patients (log-rank P = .5346; Fig. 1C–D). Confirming the univariate analysis, the Cox regression model also showed that *hTERT* mutation alone was not an independent predictor of outcome in *IDH1*-wt primary GBM patients; OS and PFS hazard ratios were 1.08 (P = .6549) and 1.03 (P = .8399), respectively (Table 2). Our result indicates that *hTERT* promoter mutation alone was not prognostic of patient survival in *IDH1*-wt GBM.

Human TERT Promoter Mutation Shows Statistical Interaction with MGMT Promoter Methylation

Promoter mutation of *hTERT* alone was not prognostic of GBM outcome in our cohort, but accumulating evidence supports the notion that the prognostic value of the *hTERT* promoter mutation depends on the overall genetic background of the patient's tumor.[3,4,6,8] Therefore, we sought to investigate the prognostic value of *hTERT* promoter mutation in combination with *MGMT* promoter methylation. As expected, we observed survival advantage of *MGMT* promoter methylation in the entire cohort by Kaplan–Meier and Cox analyses (Fig. 2A–B and Table 2). A Cox regression model including both factors was performed and showed an interaction between *hTERT* promoter mutation and *MGMT* promoter methylation: OS and PFS hazard ratios were 0.37 (P = .0032) and 0.41 (P = .0125; Table 3). This result was validated by bootstrap analysis (Table 3).

To further understand the interaction between *hTERT* promoter mutation and *MGMT* promoter methylation, we performed single degree of freedom analysis of the interaction and applied a P value threshold of .0167 in order to control for type 1 (multiple comparisons) error. Three single degrees of freedom were defined as patients with *hTERT*.MUT-*MGMT*.M, *hTERT*.WT-*MGMT*.M, or *hTERT*.MUT-*MGMT*.U, who were then compared with the reference group designated as patients with *hTERT*.WT-*MGMT*.U. When the same clinical factors were controlled, we observed that patients with *hTERT*.MUT-*MGMT*.M showed the best outcome with the lowest HRs of OS (0.53, P = .0047) and PFS (0.57, P = .0121), while patients with *hTERT*.

Table 1 Demographic characteristics of 303 *IDH1*-wt primary GBM patients in UCLA/Kaiser cohort

UCLA/Kaiser Patient Characteristics (n = 303)		*hTERT* MUT (n = 228, 100%)	*hTERT* WT (n = 75, 100%)	P value
Age	Mean ± SD	58.6 ± 9.8	54.3 ± 14.3	* P = .0433
	Median, IQR	58.9, 52.6–65.9	56.7, 45.6–65.5	
Gender	Male	130 (57.0%)	47 (62.7%)	‡ P = .3892
	Female	98 (43.0%)	28 (37.3%)	
KPS status	100	39 (17.1%)	8 (10.8%)	* P = .0117
	90	115 (50.5%)	33 (44.6%)	
	80	52 (22.8%)	15 (20.2%)	
	70	14 (6.1%)	9 (12.2%)	
	≤60	8 (3.5%)	9 (12.2%)	
	[NA]		[1]	
Extent of resection	Gross total resection	108 (48.0%)	47 (63.5%)	‡ P = .0205
	Subtotal resection/ Biopsy	117 (52.0%)	27 (36.5%)	
	[NA]	[3]	[1]	
MGMT	M	93 (40.8%)	25 (33.3%)	‡ P = .2507
	U	135 (59.2%)	50 (66.7%)	
Upfront bevacizumab	Yes	55 (24.2%)	13 (17.3%)	‡ P = .2151
	No	172 (75.8%)	62 (82.7%)	
	[NA]	[1]		
Bevacizumab at recurrence	Yes	130 (57.0%)	29 (38.7%)	‡ P = .0058
	No	98 (43.0%)	46 (61.3%)	
Received bevacizumab (any time)	Yes	151 (66.2%)	38 (50.7%)	‡ P = .0158
	No	77 (33.8%)	37 (49.3%)	

Abbreviations: IQR, interquartile range; MUT: mutant; M: methylated; U: unmethylated; NA: not available
*Wilcoxon rank sum test ‡ chi-square test.

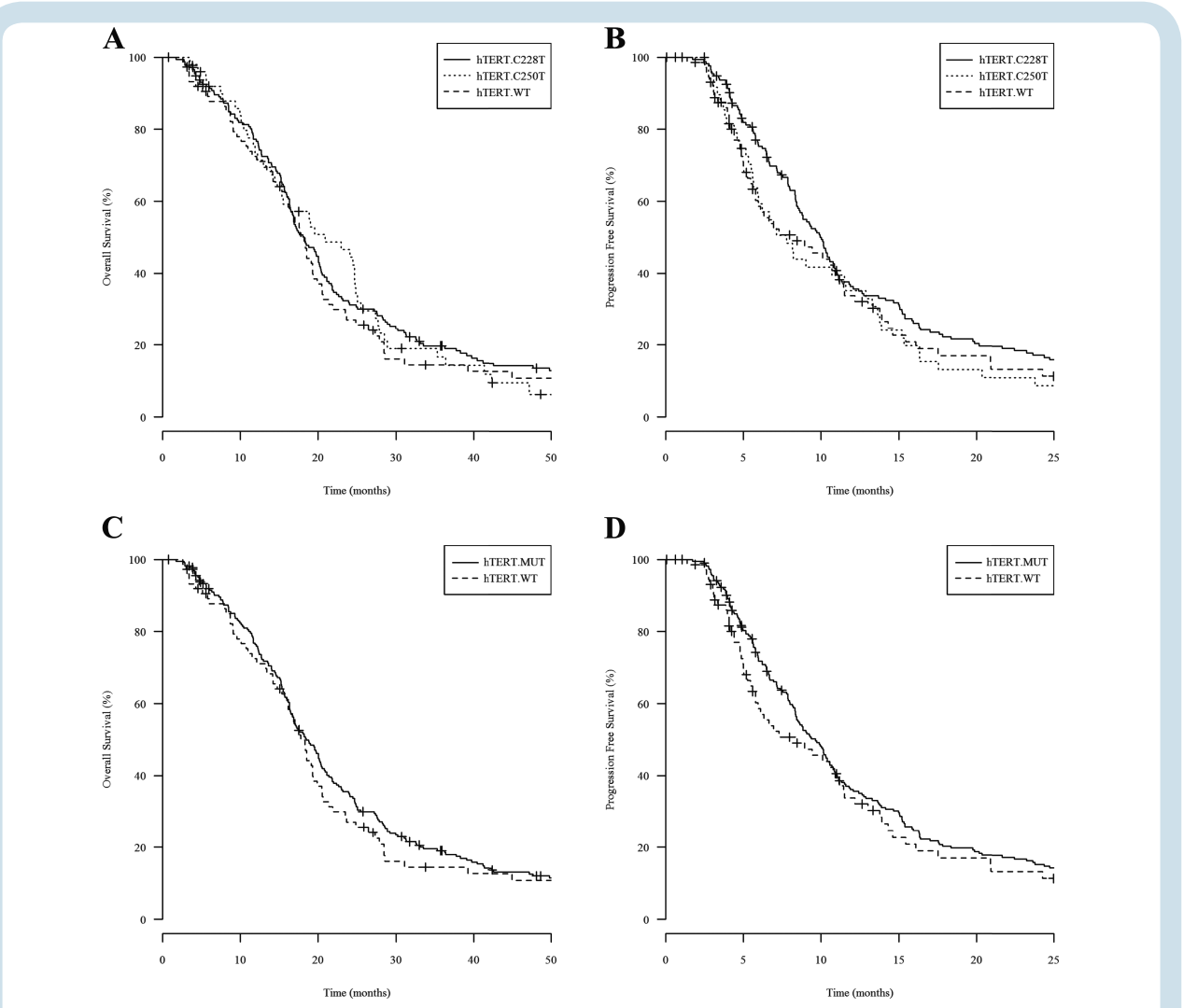

Fig. 1 Kaplan–Meier analysis of 303 UCLA/Kaiser patients evaluating the survival benefit of *hTERT* promoter mutation. (A) and (B) show OS and PFS, respectively, for patients with *hTERT* C228T variant (*hTERT* C228T, median OS=18.2 mo and PFS=10.0 mo), patients with *hTERT* C250T variant (*hTERT* C250T, median OS=20.9 mo and PFS=7.79 mo), and patients with *hTERT* wild-type (*hTERT* WT, median OS and PFS = 17.8 mo and 8.45 mo). Log-rank *P* values comparing median OS and PFS between *hTERT* C228T versus C250T were 0.7910 and 0.0306, respectively. (C) and (D) show OS, in months, and PFS, in months, respectively, for patients carrying *hTERT* promoter mutation (*hTERT*.MUT) and patients with *hTERT* wild-type (*hTERT*.WT).

MUT-*MGMT*.U showed the worst outcome with the highest HRs of OS (1.68, $P = .0098$; Table 3). Our results indicate that patients with both genetic variations carried the best prognosis, which was also reflected in Kaplan–Meier analysis comparing survival of 4 patient subcohorts representing the different combinations of *hTERT-MGMT* (MUT-M, WT-M, MUT-U, and WT-U) (Fig. 2C–D). Interestingly, these results also indicate that *hTERT* promoter mutation is negatively prognostic in the unmethylated *MGMT* population.

Human TERT Promoter Mutation Appears Necessary for the Prognostic Value of MGMT Promoter Methylation

To further understand these results, we performed pairwise comparisons of the 4 cohorts grouped by the various *hTERT* (MUT/WT) and *MGMT* (M/U) combinations. By comparing MUT-M versus MUT-U alongside WT-M versus WT-U, we found that the survival benefit associated with *MGMT* promoter methylation remained only in patients carrying the *hTERT* promoter mutation (Fig. 2C–D). Among 228 *hTERT* mutant patients, *MGMT* methylated patients had median OS of 28.3 months ($n = 93$) versus 15.9 months ($n = 135$) in *MGMT* unmethylated patients (log-rank $P < .0001$), and median PFS of 15.4 months ($n = 89$) versus 7.86 months ($n = 135$) in *MGMT* unmethylated patients (log-rank $P < .0001$). Among 75 *hTERT*-wt patients, *MGMT* methylated patients showed median OS of 19.1 months ($n = 25$) versus 17.8 months ($n = 50$) in *MGMT* unmethylated patients (log-rank $P = .4290$), and median PFS of 10.8 months ($n = 25$) versus 6.94 months ($n = 50$) in *MGMT* unmethylated patients (log-rank $P = .6875$). Confirming these univariate findings, Cox regression analysis also showed that in

Table 2 Cox regression analysis evaluating prognostic value of *hTERT* promoter mutation and *MGMT* promoter methylation individually in UCLA/Kaiser cohort

Factors–UCLA/Kaiser Cohort		Estimated HR (95% CI) *P* value		Estimated HR (95% CI) *P* value	
		OS	PFS	OS	PFS
Age		1.01 (1.00, 1.03), *P* = .0296	1.01 (0.99, 1.02), *P* = .2679	1.02 (1.01, 1.04), *P* = .0003	1.02 (1.01, 1.03), *P* = .0189
Gender	Male vs Female	1.19 (0.92, 1.53), *P* = .1883	1.08 (0.82, 1.38), *P* = .6214	1.17 (0.91, 1.50), *P* = .2313	1.02 (0.79, 1.32), *P* = .8984
KPS	100–80	0.26 (0.15, 0.45), *P* < .0001	0.38 (0.21, 0.68), *P* = .0013	0.31 (0.18, 0.53), *P* < .0001	0.45 (0.25, 0.80), *P* = .0065
	70	0.47 (0.24, 0.95), *P* = .0340	0.60 (0.29, 1.21), *P* = .1527	0.51 (0.26, 1.02), *P* = .0553	0.69 (0.34, 1.41), *P* = .3078
	≤60	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
EOR	GTR vs others	0.61 (0.47, 0.78), *P* = .0001	0.66 (0.51, 0.85), *P* = .0016	0.66 (0.52, 0.86), *P* = .0017	0.72 (0.55, 0.93), *P* = .0134
Bevacizumab treatment	Y vs N	0.81 (0.61, 1.07), *P* = .1353		0.89 (0.68, 1.18), *P* = .4136	
Upfront bevacizumab	Y vs N		0.78 (0.58, 1.05), *P* = .1013		0.81 (0.60, 1.08), *P* = .1530
MGMT	M vs U			0.39 (0.30, 0.52), *P* < .0001	0.43 (0.33, 0.57), *P* < .0001
hTERT	MUT vs WT	1.08 (0.78, 1.48), *P* = .6549	1.03 (0.75, 1.44), *P* = .8399		

EOR: extent of resection; GTR: gross total resection; Y: Yes; N: No; M: methylated; U: unmethylated; MUT: mutant.

hTERT mutant patients, *MGMT* promoter methylation was prognostic of OS and PFS: OS and PFS hazard ratios were 0.32 ($P < .0001$) and 0.36 ($P < .0001$), whereas in *hTERT*-wt patients, *MGMT* promoter methylation was not prognostic: OS and PFS hazard ratios were 0.85 ($P = .5855$) and 0.88 ($P = .6931$; Table 4). These results were validated by bootstrap analysis (Table 4). Our results show that *IDH1*-wt GBM patients treated with first-line radiation and temozolomide benefited from *MGMT* promoter methylation only in the context of *hTERT* promoter mutation.

Interestingly, several additional observations can be made if we first separated patients by *MGMT* status (ie, comparing WT-U vs MUT-U alongside WT-M vs MUT-M). We observed by Kaplan–Meier analysis that *hTERT* mutant patients showed improved survival in *MGMT* methylated patients (log-rank $P = .0231$ for OS and $P = .0318$ for PFS), and trended toward worse survival in *MGMT* unmethylated patients (log-rank $P = .0973$ for OS and $P = .1395$ for PFS). However, Cox regression analysis showed that *hTERT* promoter mutation represented an independent predictor for both OS and PFS only in *MGMT* unmethylated patients; OS and PFS hazard ratios for *hTERT* mutant patients were 1.68 ($P = .0098$) and 1.57 ($P = .0287$), respectively (Table 4). Thus, as seen in the single degree of freedom analysis and pairwise analysis of WT-U versus MUT-U alongside WT-M versus MUT-M, *hTERT* mutation was prognostic of poorer outcome in the unmethylated *MGMT* population. These results were validated by bootstrap analysis (Table 4).

From the patient cohort at UCLA and Kaiser, we observed that *hTERT* mutation can be both beneficial (in enabling benefit of *MGMT* methylation) and harmful (in the context of *MGMT* unmethylated patients).

Human TERT High Gene Expression Appears Necessary for the Improved Prognosis of MGMT Promoter Methylation Based on Analysis of TCGA GBM Database

The GBM database of TCGA lacks *hTERT* promoter mutation information. As *hTERT* promoter mutation showed a strong association with high gene expression in GBM,[1,5,6,9,10,13] we analyzed the database from TCGA of primary GBM patients who had both *hTERT* gene expression and *MGMT* promoter methylation data available.[19] Starting with the entire GBM cohort ($n = 577$), we retained 190 patients who were diagnosed after 2005, and received temozolomide after surgical resection. Most patients ($n = 171$, 90%) received radiotherapy, while the remaining patients ($n = 19$, 10%) had unclear documented radiation treatment. Gene expression of *hTERT* was dichotomized using the expression level above the 25th percentile as indication for high *hTERT* gene expression, while patients with equal or lower than 25th percentile expression were stratified into the low *hTERT* gene expressing group. The 25th percentile cutoff point was selected based on the observation that 75% of primary GBM carried *hTERT* promoter mutation in our discovery UCLA/Kaiser cohort. This cutoff point yielded 48 patients with low *hTERT* gene expression and 142 patients with high *hTERT* gene expression. Detailed patient characteristics are presented in Supplementary Table 1.

Table 3 Cox regression analysis and bootstrap validation evaluating the interaction between *MGMT* promoter methylation and *hTERT* promoter mutation in predicting patient outcome, and the single degree of freedom analysis of UCLA/Kaiser cohort

Factors–UCLA/Kaiser Cohort		OS HR (95% CI), *P* value	Bootstrap HR (95% CI)	PFS Survival HR (95% CI), *P* value	Bootstrap PFS HR (95% CI)
Age		1.02 (1.01, 1.03), *P* = .0056	1.02 (1.01, 1.04)	1.01 (0.99, 1.02), *P* = .1877	1.01 (0.99, 1.02)
Gender	Male vs female	1.20 (0.93, 1.54), *P* = .1601	1.22 (0.91, 1.61)	1.03 (0.80, 1.34), *P* = .8132	1.04 (0.80, 1.36)
KPS	100–80	0.24 (0.13, 0.43), *P* < .0001	0.24 (0.08, 0.50)	0.36 (0.20, 0.66), *P* = .0010	0.37 (0.18, 0.69)
	70	0.41 (0.20, 0.83), *P* =.0127	0.44 (0.11, 0.99)	0.56 (0.27, 1.17), *P* = .1239	0.60 (0.24, 1.18)
	≤60	1.00 (reference)	1.00 (reference)	1.00 (reference)	1.00 (reference)
EOR	GTR vs others	0.67 (0.52, 0.87), *P* = .0026	0.67 (0.51, 0.87)	0.72 (0.55, 0.94), *P* = .0144	0.72 (0.54, 0.93)
Bevacizumab treatment	Y vs N	0.80 (0.60, 1.07), *P* = .1332	0.79 (0.54, 1.09)		
Upfront bevacizumab	Y vs N			0.78 (0.58, 1.05), *P* = .0979	0.77 (0.58, 0.98)
MGMT	M vs U	0.85 (0.48, 1.52), *P* = .5855	0.89 (0.44, 1.65)	0.88 (0.47, 1.65), *P* = .6931	0.96 (0.41, 2.06)
hTERT	MUT vs WT	1.68 (1.13, 2.50), *P* = .0098	1.73 (1.13, 2.53)	1.57 (1.05, 2.36), *P* = .0287	1.59 (0.99, 2.37)
hTERT by *MGMT* interaction		0.37 (0.19, 0.72), *P* = .0032	0.39 (0.16, 0.76)	0.41 (0.21, 0.83), *P* = .0125	0.43 (0.16, 0.87)
OR					
hTERT-MGMT (single degree of freedom analysis)	MUT-M	0.53 (0.34, 0.82), *P* = .0047		0.57 (0.37, 0.89), *P* = .0121	
	WT-M	0.85 (0.48, 1.52), *P* = .5855		0.88 (0.47 1.65), *P* = .6931	
	MUT-U	1.68 (1.13, 2.50), *P* = .0098		1.57 (1.05, 2.36), *P* = .0287	
	WT-U	1.00 (reference)		1.00 (reference)	

EOR: extent of resection; GTR: gross total resection; Y: Yes; N: No; M: methylated; U: unmethylated; MUT: mutant.

Analogous to our cohort stratified by genotype, *hTERT* gene expression level in isolation did not predict patient survival. Patients with high *hTERT* expression showed a median OS of 14.9 months ($n = 142$) versus 12.7 months ($n = 48$) in patients with low expression (log-rank $P = .1587$) (Supplementary Fig. 1A). Kaplan–Meier analysis also demonstrated the survival benefit of *MGMT* promoter methylation only in patients with high *hTERT* gene expression. In 142 patients with high *hTERT* expression, patients with *MGMT* methylation had median OS of 17.8 months ($n = 68$) versus 13.9 months ($n = 74$) from patients with unmethylated *MGMT* (log-rank $P = .0026$), whereas in 48 patients with low *hTERT* expression, *MGMT* methylated patients had median OS of 12.7 months ($n = 21$) versus 12.2 months ($n = 27$) from patients with unmethylated *MGMT* (log-rank $P = .9967$; Supplementary Fig. 1B). In addition, the Cox regression model demonstrated that *MGMT* promoter methylation was prognostic for patient survival only in the context of high *hTERT* expression (OS HR = 0.61, $P = .0303$) and not in patients with low *hTERT* expression (OS HR = 0.84, $P = .6407$) (Supplementary Table 2).

We obtained similar results using the median expression as the cutoff point for *hTERT* dichotomization (Supplementary Table 1 and 2; Supplementary Fig. 2A–B). The cohort from TCGA supports the observations in our discovery cohort that the survival benefit from *MGMT* promoter methylation is present only in the context of increased *hTERT* expression, which has been shown to associate with *hTERT* promoter mutation.[1,5,6,9,10,13] However, low *hTERT* gene expression was not found to be associated with poorer survival in *MGMT* unmethylated patients as observed in our discovery cohort.

Discussion

Despite the high frequency of *hTERT* promoter mutation in primary GBM, its clinical significance remains unclear. By retrospectively genotyping the *hTERT* promoter region for 303 *IDH1*-wt primary GBM patients treated with radiation and temozolomide, we detected *hTERT* promoter mutation in 75% of our patients, confirming the findings from other reported cohorts.[2–10] Our results showed that *hTERT* promoter mutation as the sole molecular marker did not predict OS or PFS in the UCLA/Kaiser patient

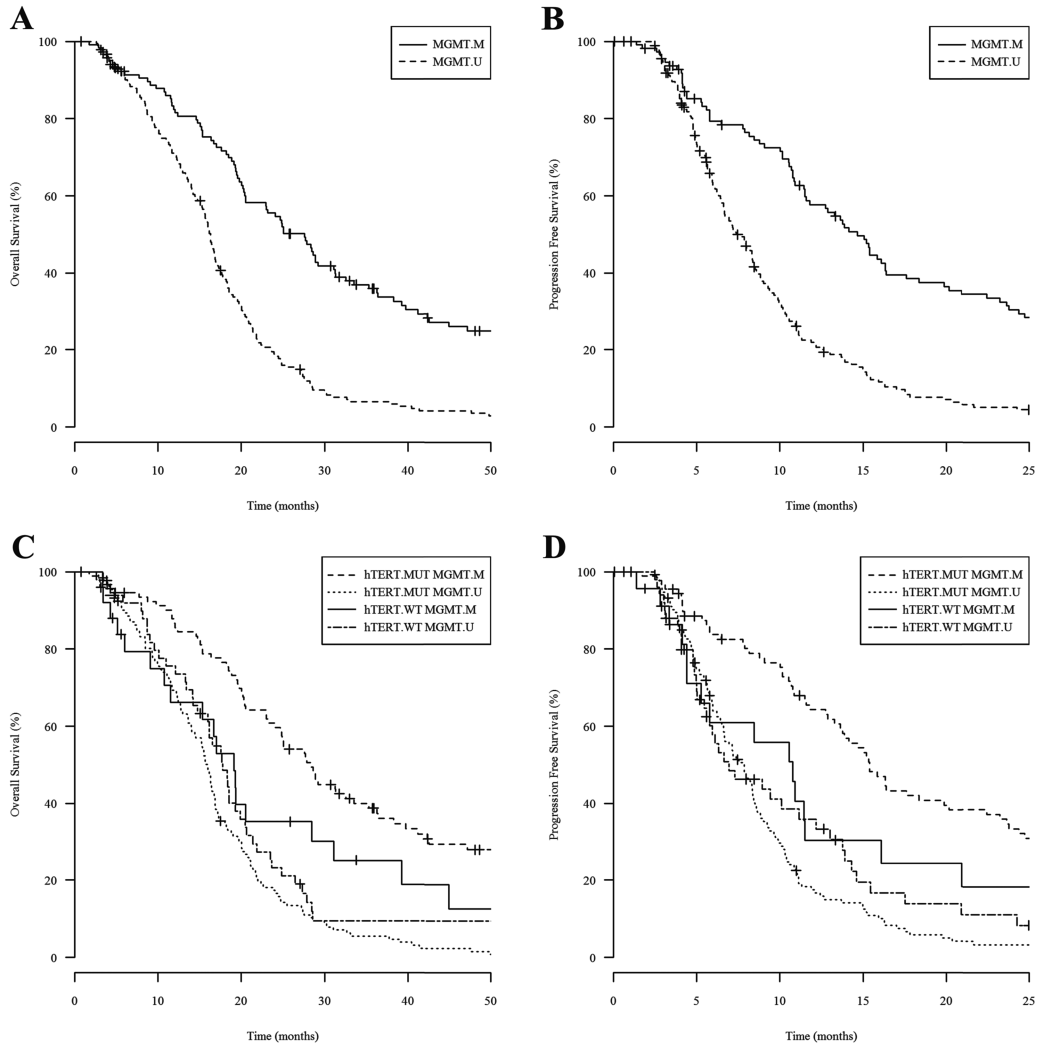

Fig. 2 Kaplan–Meier analysis of 303 UCLA/Kaiser patients while substratifying by *MGMT* promoter methylation alone or in combination with *hTERT* promoter mutation. (A) and (B) show OS and PFS, respectively, for patients with *MGMT* promoter methylation (*MGMT*.M) and patients without *MGMT* promoter methylation (*MGMT*.U). *MGMT* methylated patients showed median OS of 27.6 months ($n = 118$) and PFS of 14.7 months ($n = 114$), while *MGMT* unmethylated patients showed median OS of 16.3 months ($n = 185$) and PFS of 7.36 months ($n = 185$). Log-rank *P* values comparing OS and PFS between *MGMT* methylated versus unmethylated patients both reach significant values ($P < .0001$). (C) and (D) show OS (mo) and PFS (mo), respectively, for four subgroups of patients stratified by both factors, including patients with *hTERT* mutation and *MGMT* methylated (*hTERT*.MUT *MGMT*.M), patients with *hTERT* mutation and *MGMT* unmethylated (*hTERT*.MUT *MGMT*.U), patients with *hTERT* wild-type and *MGMT* methylated (*hTERT*.WT *MGMT*.M), and patients with *hTERT* wild-type and *MGMT* unmethylated (*hTERT*.WT *MGMT*.U).

cohort, supporting the findings from a smaller study of 53 patients, in which, like our study, only *IDH1*-wt GBM patients treated with standard chemoradiotherapy were included.[1] Unexpectedly, however, when *MGMT* promoter methylation and *hTERT* promoter mutation were included together, we discovered a statistical interaction that led us to the observations that *hTERT* promoter mutation enabled the *MGMT* methylation benefit and was a negative prognostic marker in *MGMT* unmethylated patients.

This dual prognostic nature of *hTERT* promoter mutation may explain conflicting results reported in the literature on whether *hTERT* promoter mutation is a negative prognostic marker. Our results clearly indicate that interpretation of studies correlating *hTERT* promoter mutation with survival must consider both the percentage of patients receiving temozolomide and the percentage of patients with methylated/unmethylated *MGMT* promoters. Since patients carrying methylated *MGMT* promoter showed minimal survival benefit over *MGMT* unmethylated patients in the absence of first-line temozolomide treatment,[15] *MGMT* methylated patients who were not exposed to temozolomide after tumor resection might behave similarly to *MGMT* unmethylated patients, allowing the *hTERT* promoter mutation to predominate as a negative prognostic factor. In addition, even when all patients received temozolomide upfront, if more *MGMT* unmethylated patients were included in the

Table 4 Prestratified by *MGMT* methylation and *hTERT* mutation log-rank test, Cox pairwise comparison and bootstrap validation while controlling for other clinical factors (age, gender, KPS, EOR, and bevacizumab treatment) in UCLA/Kaiser cohort

hTERT - *MGMT* Status	Overall Survival			Progression-free Survival		
	Median Survival Log-rank *P* value	HR (95% CI) *P* value	Bootstrap HR (95% CI)	Median Survival Log-rank *P* value	HR (95% CI) *P* value	Bootstrap HR (95% CI)
MUT-M vs MUT-U	28.3 mo vs 15.9 mo *P* < .0001	0.32 (0.23, 0.43) *P* < .0001	0.31 (0.21, 0.42)	15.4 mo vs 7.86 mo *P* < .0001	0.36 (0.27, 0.49) *P* < .0001	0.36 (0.24, 0.49)
WT-M vs WT-U	19.1 mo vs 17.8 mo *P* = .4290	0.85 (0.48, 1.52) *P* = .5855	0.90 (0.44, 1.65)	10.8 mo vs 6.94 mo *P* = .6875	0.88 (0.47, 1.65) *P* = .6931	0.96 (0.41, 2.06)
MUT-U vs WT-U	15.9 mo vs 17.8 mo *P* = .0973	1.68 (1.13, 2.50) *P* = .0098	1.73 (1.13, 2.53)	7.86 mo vs 6.94 mo *P* = .1395	1.57 (1.05, 2.36) *P* = .0287	1.59 (0.99, 2.37)
MUT-M vs WT-M	28.3 mo vs 19.1 mo *P* = .0231	0.63 (0.37, 1.06) *P* = .0810	0.64 (0.32, 1.14)	15.4 mo vs 10.8 mo *P* = .0318	0.65 (0.37, 1.14) *P* = .1324	0.65 (0.29, 1.17)

MUT: *hTERT* mutant; WT: *hTERT* wildtype; M: *MGMT* methylated; U: *MGMT* unmethylated.

cohort, the survival outcome would be skewed, again leading to the conclusion that *hTERT* promoter mutations are associated with poor survivals in these studies.[2–4,6,8,9,13]

We also analyzed an independent cohort of 190 *IDH1*-wt primary GBM patients, who had both *hTERT* gene expression and *MGMT* promoter methylation data available from the database of TCGA,[19] and investigated the interaction of the 2 biomarkers. Despite the strong association between the promoter mutation and increased gene expression in GBM,[1,5,6,9,10,13] some *hTERT*-wt tumors show a relatively high level of *hTERT* mRNA expression under different mechanisms (ie, hypermethylation of *hTERT* promoter).[27] In addition, only a small subset (n = 30) of *IDH1*-wt primary GBM from the database of TCGA were genotyped for *hTERT* promoter sequence, and all samples were reported to carry *hTERT* promoter mutation.[20] Therefore, *hTERT* gene expression from the cohort of TCGA may not represent an adequate surrogate for *hTERT* promoter mutation and limits the ability of these results to validate findings in our cohort. However, this analysis has utility as a complementary approach to support the results obtained by *hTERT* genotyping in our discovery cohort. TCGA cohort demonstrated similar results, showing that high *hTERT* gene expression in isolation did not predict patient OS but enabled *MGMT* promoter methylation survival benefit, which was not observed in patients with low *hTERT* gene expression. Low *hTERT* gene expression, however, was not found associated with poorer survival in *MGMT* unmethylated patients as observed in our discovery cohort. This lack of confirmation might be due to the expression threshold for dichotomization or the lack of homogeneity in salvage treatment across patients in the dataset from TCGA.

The interaction between *hTERT* mutation and *MGMT* methylation may have biological implications. Promoter mutation of *hTERT* is associated with higher *hTERT* gene and telomerase activity,[1,5,6,9,10,13] whose main function is telomere maintenance.[28,29] *MGMT* encodes for a DNA repair enzyme that repairs alkylation of the O^6 position on guanine.[30] Promoter methylation silences *MGMT* gene expression and predicts improved outcome in primary GBM patients treated with radiation and temozolomide.[15–17,31] Thus, our observation that the survival advantage of *MGMT* promoter methylation only occurs in *hTERT* mutant patients suggests that temozolomide sensitivity may depend on telomerase activity in addition to reduced *MGMT* repair activity. We hypothesize that high *hTERT* gene expression via promoter mutation could enhance the tumor sensitivity to temozolomide treatment. This is supported by a study showing that telomerase inhibition using catalytically inactive and dominant-negative forms of *hTERT* increased resistance of melanoma cells to temozolomide.[32] This hypothesis warrants further experimental investigation, with the possible clinical implication that pharmacological inhibitors of telomerase activity may be unsuitable for concurrent use with temozolomide.

The worse survival associated with *hTERT* promoter mutation in *MGMT* unmethylated patients may also have biological consequences by suggesting that *hTERT* promoter mutation could intrinsically promote glioblastomas to behave more aggressively. Further studies looking at association of *hTERT* promoter mutation with other radiological and pathological phenotypes in GBM, such

as proliferative rate and Ki67 index, might show promising findings. Moreover, this observation suggests the use of telomerase inhibitors that are currently under clinical investigation, such as imetelstat,[33,34] in treating *IDH1*-wt primary GBM tumors, which do not carry *MGMT* promoter methylation.

While we believe that our study, by virtue of the large patient cohort, homogeneous treatment, complete *MGMT* promoter methylation status, and analysis of the dataset of TCGA, provides useful insight regarding the use of *hTERT* promoter mutation as a prognostic marker, we acknowledge that this is a retrospective study and could include selection biases or treatment variation that may affect the results. While we attempted to minimize the differences in treatment by selecting only patients receiving radiation and temozolomide after tumor resection, patients might be exposed to a variety of other salvage chemotherapies throughout their treatment, which might contribute unknown benefit or disadvantage to patient survival. Our findings need to be validated in an independent cohort in which *hTERT* genotype is assessed.

In summary, using a large cohort of homogeneously treated *IDH1*-wt GBM patients, we provide evidence that the *hTERT* promoter mutation has different prognostic implication depending on the *MGMT* promoter methylation status of the patient. The co-occurrence of *hTERT* promoter mutation and *MGMT* promoter methylation classified a subgroup of *IDH1*-wt GBM with the best prognosis and may mechanistically contribute to the beneficial effects of *MGMT* promoter methylation. On the other hand, *hTERT* mutation may potentially portend a more aggressive GBM when *MGMT* is unmethylated. Further investigation is required to understand these interactions and ultimately to determine whether *hTERT* inhibition will have a clinical role.

Supplementary Material

Supplementary material is available online at *Neuro-Oncology* (http://neuro-oncology.oxfordjournals.org/)

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