Preclinical and clinical implications of *TERT* promoter mutation in glioblastoma multiforme

DA EUN JEONG[1], SEON RANG WOO[2,3], HYUN NAM[3], DO-HYUN NAM[1,3], JAE-HO LEE[4] and KYEUNG MIN JOO[1,2]

[1]Department of Health Sciences and Technology, Samsung Advanced Institute for Health Sciences and Technology, Sungkyunkwan University; Departments of [2]Anatomy and Cell Biology and [3]Neurosurgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 06531; [4]Department of Anatomy, Keimyung University School of Medicine, Daegu 42601, Republic of Korea

Received September 8, 2016; Accepted August 8, 2017

DOI: 10.3892/ol.2017.7196

Abstract. The promoter region of the telomerase reverse transcriptase gene (*TERT*) is mutated in a subpopulation of patients with glioblastoma multiforme (GBM). In the present study, preclinical and clinical implications of the mutation were analyzed in 25 GBMs to evaluate its utility as a therapeutic target. Associations between the *TERT* promoter mutation and a number of preclinical/clinical characteristics were analyzed. Notably, the *TERT* promoter mutation was identified in 92.3% of GBMs where dissociated cells revealed *in vitro* sphere formation capacity; while the *TERT* promoter mutation was identified in 33.3% of GBMs without *in vitro* sphere formation capacity (P=0.004). In addition, this significantly increased mutation rate was observed in GBMs with *in vivo* tumorigenic potential (80% vs. 0%; P=0.004). Furthermore, patients with GBM exhibiting the *TERT* promoter mutation demonstrated significantly decreased overall survival rate compared with patients lacking this mutation (81.7 vs. 152.6 weeks; P=0.026). The results of the present study indicated that the *TERT* promoter mutation is associated with the self-renewal capacity of GBM cells and clinical aggressiveness of GBMs, which may be translated to a targeting therapy against TERT to inhibit the self-renewal of GBM cells.

Correspondence to: Professor Kyeung Min Joo, Department of Anatomy and Cell Biology, Samsung Medical Center, Sungkyunkwan University School of Medicine, 50 Ilwon, Gangnam, Seoul 06531, Republic of Korea
E-mail: kmjoo@skku.edu

Professor Jae-Ho Lee, Department of Anatomy, Keimyung University School of Medicine, 1095 Dalgubeaol Boulevard, Daegu 42601, Republic of Korea
E-mail: anato82@dsmc.or.kr

Key words: glioblastoma multiforme, telomerase reverse transcriptase, telomerase reverse transcriptase promoter mutation, sphere formation, self-renewal capacity

Introduction

Glioblastoma multiforme (GBM; World Health Organization grade IV glioma) is the most common type of primary brain tumor worldwide (1). In spite of radical surgery combined with concomitant chemoradiation therapy based on temozolomide, the median survival rate of patients with GBM remains ~1 year (2). Furthermore, clinical trials have demonstrated only limited benefits of targeted regimens, indicating that the identification of the genetic and molecular characteristics of GBM is required to develop more effective treatment strategies (2-4).

Genetic studies including a large number of patients with GBM have revealed various genetic alterations in GBM, including isocitrate dehydrogenase gene mutations and mutations in the promoter region of telomerase reverse transcriptase gene (*TERT*) (4,5). Of these, *TERT* promoter mutations have been identified in a subpopulation of GBM and were revealed to be significantly associated with poor clinical prognosis (2,5,6). In addition, these mutations were associated with increased expression of *TERT* (7,8). These results indicated the potential for personalized therapy against *TERT* in GBM on the basis of mutation status.

In vitro and *in vivo* preclinical models derived from surgical samples of GBMs have revealed the molecular and functional features of the parental tumors, and may represent the GBM population experimentally (9). Therefore, patient-derived preclinical models exhibiting GBMs with or without *TERT* promoter mutations may enable experimental examination of personalized TERT-targeted treatments. In the present study, a patient-derived GBM preclinical model library, including GBMs with and without *TERT* promoter mutations, was established, and preclinical and clinical implications were determined.

Materials and methods

GBM patients, primary cell culture and stereotactic transplantation. Surgical specimens and clinical records were obtained from 25 patients with primary GBM from May 2004 to June 2006 at the Samsung Medical Center (Seoul, Korea). All tissue samples were collected with written informed consent

under a protocol approved by the Institutional Review Board of the Samsung Medical Center (Seoul, Korea). GBMs were pathologically diagnosed by specialized neuropathologists, on the basis of the World Health Organization criteria (10). For genomic analysis, parts of the specimens were snap-frozen and preserved in liquid nitrogen until use. Genomic DNA and mRNA were extracted using the DNeasy Kit (Qiagen GmbH, Hilden, Germany) and the RNeasy kit (Qiagen GmbH, Hilden, Germany).

Sections of the surgical samples were enzymatically dissociated into single cells as previously described (11). Dissociated GBM cells were cultured in neurobasal media (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 1X N2 and 2X B27 supplements (Invitrogen; Thermo Fisher Scientific, Inc.), human recombinant basic fibroblast growth factor and epidermal growth factor (25 ng/ml each; R&D Systems, Inc., Minneapolis, MN, USA) (12). Because clonogenic growth as neurospheres is an *in vitro* indicator of the self-renewal of GBM cells, sphere formation (diameter, ≥50 μm), within 4 weeks, was used to identify the *in vitro* sphere formation capacity of dissociated GBM cells. For neurosphere formation, cells were seeded at a range of 1-200 cells per well. Following 4 weeks, the number of wells without spheres were counted and analyzed.

Dissociated GBM cells were stereotactically (2 mm left and 1 mm anterior to the bregma, 2 mm deep from the dura) transplanted into the brains of immuno-deficient NOG mice (between 2.5×10^4 and 1.0×10^5 cells/10 μl Hank's Balanced Salt solution for each mice, n=4-9 for each sample) (13). For the intracranial injection, 250 6-8 week-old female immune-deficient NOG mice (4-9 mice per group) were obtained from Orient Bio, Inc. (Seongnam, Korea). Mice were housed under controlled temperature (22±2˚C) and a 12 h light/dark cycle in laminar flow cabinets with filtered air, and handled using aseptic procedures and food and water were provided ad libitum. The average weight of the mice prior to the procedure was 20 g. Mice were sacrificed and their brains were processed for pathological diagnosis, following the procedures previous reported (9). Animal experiments were approved by the Institutional Review Boards of the Samsung Medical Center and conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

TERT promoter mutation analysis. Genomic DNA was isolated from tumor samples a using QIAamp DNA mini kit (Qiagen GmbH). The *TERT* promoter region was amplified from isolated genomic DNA using the polymerase chain reaction (PCR), as described previously (14). PCR was performed using AmpliTaq Gold DNA polymerase (Applied Biosystems; Thermo Fisher Scientific, Inc.). PCR products were electrophoresed on 1.5% agarose gel and stained with ethidium bromide to validate the sizes of the bands. Subsequently, direct DNA sequencing for the *TERT* promoter region was performed using an ABI 3730 DNA sequencer by Bionics Co., Ltd. (Seoul, Korea). The sequences for *TERT* were as follows: Sense, 5'-CACCCGTCCTGCCCCTTCACCTT-3' and antisense, 5'-GGCTTCCCACGTGCGCAGCAGGA-3'.

Relative telomere length determination. Telomere length was analyzed using quantitative PCR (qPCR) (7990HT Fast Real-time, Applied Biosystems; Thermo Fisher Scientific, Inc.). For telomere length determination, DNA were extracted from cells. To quantitatively determine telomere length relative to nuclear DNA, specific primers for telomere (T) and nuclear DNA-encoded β-globin (S) were selected according to a previous study (14). The β-globin gene was used as the reference gene. qPCR was performed using a LightCycler 480 II system (Roche Diagnostics, Basel, Switzerland) with SYBR PCR master mix (Toyobo Life Science, Osaka, Japan). For telomere PCR, there were 18 cycles at 95˚C for 15 sec and at 54˚C for 2 min. Relative telomere length was determined by calculating T/S values using the formula $T/S=2^{-\Delta Cq}$, where ΔCq=average $Cq_{telomere}$-average $Cq_{\beta\text{-globin}}$. Each measurement was repeated in triplicate and 5 serially diluted control samples were included in each experiment. The primer sequences used in this experiment were follows: β-globin sense, 5'-TGTGCTGGC CCATCACTTTG-3; β-globin antisense, 5'-ACCAGCCAC CACTTTCTGATAGG-3'; telomere sense, 5'-CGGTTTGTT TGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3'; and telomere antisense, 5'-GGCTTGCCTTACCCTTACCCTTAC CCTTACCCTTACCCT-3'.

GBM subtype prediction. According to The Cancer Genome Atlas data of the mRNA expression of 840 genes in 173 patients with GBM, the subtypes (proneural, neural, classical and mesenchymal) of the 25 GBMs used in the present study were determined (15). The Cancer Genome Atlas data were downloaded from tcga-data.nci.nih.gov/docs/publications/gbm_exp. The Nearest Template Prediction algorithm was used to predict the class of a sample with statistical significance (false discovery rate, <0.2) using a predefined set of markers specific to multiple classes (GenePattern Modules; version 2; Broad Institute) (15). For the dataset in the present study, the 788 overlapping genes, out of the 840 genes, were used to predict the subtype.

Genetic alteration analysis. Microarray-based comparative genomic hybridization (aCGH) was performed using the Agilent SurePrint G3 Human CGH 4x180K arrays (Agilent Technologies, Inc., Santa Clara, CA, USA). aCGH feature extraction files were processed and normalized fold changed of matched normal samples using Agilent Genomic WorkBench 7.0.4.0 (Agilent Technologies, Inc.). The DNAcopy R package (version 1.48.0; performed in Bioconductor) was used to estimate DNA copy numbers (16). From the copy numbers at the segment level, the copy number for each gene was measured using the mean value of the copy numbers of all exonic segments of the gene (9).

Somatic mutations were detected using the Agilent SureSelect kit (Agilent Technologies, Inc.) to capture exonic DNA fragments. The Illumina HiSeq 2000 was used to generate 2×10^1 bp paired-end reads (Illumina, Inc., San Diego, CA, USA). The sequenced reads in FASTQ files were mapped to the human reference genome assembly (hg19), using the Burrows-Wheeler Aligner (version 0.6.2) (17). The initial alignment BAM files were subjected to regular preprocessing prior to mutation calling: Sorting reads by genomic coordinates, removing duplicated reads, locally realigning reads around potential small indels and recalibrating base quality scores using SAMtools (18), Picard (version 1.73; Broad

Institute), and Genome Analysis ToolKit (GATK version 2.5.2; Broad Institute). For mutation calling, MuTect (GATK version 1.1.4; Broad Institute) and SomaticIndelDetector (GATK version 2.2; Broad Institute) were used to make high-confidence predictions regarding somatic mutations from the tumor and paired blood. Copy number data were obtained using the ngCGH python package (version 0.4.4) to generate aCGH-like data from whole exome sequencing (WES) data. The matched blood WES data were used as a reference to calculate fold-changes in copy numbers in tumors. In cases without matched blood WES, created 'pseudo-normal' profile blood WES data were generated using the same sequencing platform and analysis pipeline as were used for the tumor data. Downstream analysis (segmentation and calculation of copy number) were conducted as described for aCGH data.

Using the Illumina TruSeq RNA Sample Prep kit (Illumina, Inc.), RNA-seq libraries were prepared for all cases. The trimmed reads in FASTQ files were aligned with hg19 using GSNAP (version 2012-12-20) with two output formats: GSNAP native format (exon-skipping analysis) and SAM format (point mutation analysis) (19). The resulting GSNAP native format files were analyzed to isolate the 'split' reads spanning non-canonical splicing junctions, with a minimal anchor of five nucleotides on each exon. In cases demonstrating plural reading splits between two exons, the event was termed a skipped exon event between the two exons. The SAM format files were sorted using the same preprocessing procedures as those applied for the WES data, with the exception of local realignments were restricted to exonic regions to prevent the mislabeling of normal splicing events as misaligned indels. Potential point mutations were identified using UnifiedGenotyper (GATK version 1.2.0; Broad Institute).

Statistical analysis. The SPSS statistical package, version 19.0, was used for statistical analyses (IBM Corp., Armonk, NY, USA). χ^2, Fischer's exact tests, Mann Whitney U tests and Spearman's correlation analysis were used to analyze the associations between variables. Survival curves, estimated using the Kaplan-Meier method (univariate analysis), were compared using the log-rank test. Overall survival was defined as the time between diagnosis and mortality (as a result of any cause). Progression-free survival was defined as the time between diagnosis and disease recurrence. P<0.05 was considered to indicate a statistically significant difference. Data are presented as the mean ± standard deviation.

Results

TERT promoter mutation status of GBMs is associated with sphere formation capacity. First, *TERT* promoter mutations were investigated in the 13 GBMs with *in vitro* sphere formation capacity to establish patient-derived GBM preclinical model libraries, including GBMs with and without *TERT* promoter mutations. In parallel, *in vivo* tumor formation, gene mutation status and global gene expression were analyzed. Notably, *TERT* promoter mutations were identified in 92.3% (12/13) GBMs (Table IA). All TERT mutations were revealed to be C228T, but 1 GBM exhibited C228T and C250T mutations. It was revealed that 1 GBM without *TERT* promoter mutation exhibited α-thalassemia/mental retardation syndrome X-linked (*ATRX*) amplification, although it was previously demonstrated that *ATRX* amplification and *TERT* promoter mutation were mutually exclusive (6). On the basis of this, it was hypothesized that the genetic alteration in *ATRX* is equivalent to *TERT* promoter mutation (6).

The *TERT* promoter mutation rate (92.3%) in GBMs with *in vitro* sphere formation capacity was not expected because *TERT* promoter mutations were observed in between 28-84% of GBMs (20). Accordingly, the present study hypothesized that *TERT* promoter mutation is associated with the *in vitro* sphere formation capacity of GBMs. To test the hypothesis, *TERT* promoter mutations in GBMs without *in vitro* sphere formation capacity were subsequently analyzed.

TERT promoter mutations are decreased in GMBs without sphere formation capacity. *TERT* promoter mutations were identified in 33.3% (4/12) GBMs without *in vitro* sphere formation capacity (Table IB). All mutations were C228T (Table IB). This frequency was significantly decreased compared with that in GBMs with *in vitro* sphere formation capacity (P=0.004). Other preclinical characteristics and genetic changes were not associated with *TERT* promoter mutation in GBMs without *in vitro* sphere formation capacity (data not shown).

TERT promoter mutation is associated with age and sex. Out of 25 GBMs (Table II), *TERT* promoter mutation was demonstrated in 64.0% (16/25). *TERT* promoter mutation was significantly associated with increased age (P=0.050) and sex; being more prevalent in male patients (P<0.001). Notably, GBMs with *in vivo* tumorigenic potential demonstrated a significantly increased *TERT* promoter mutation rate (P=0.004) compared with those without. Although the values were not statistically significant, *TERT* promoter mutations were at an increased frequency in GBMs with epidermal growth factor receptor (*EGFR*) gene mutation (P=0.117), *EGFR* amplification (P=0.102), cyclin dependent kinase inhibitor 2A (*CDKN2A*) deletion (P=0.116) and phosphatase and tensin homolog deletion (P=0.102).

Telomere length is not associated with TERT promoter mutation status. Relative telomere length (0.92±0.49) was analyzed in the GBMs. However, the length did not differ according to *TERT* promoter mutation status (0.91±0.42 vs. 0.93±0.77; P=0.598; Fig. 1). When the GBMs were divided into two groups according to *in vitro* sphere formation capacity, *TERT* promoter mutation revealed limited association with the relative telomere length (Fig. 1).

To explore the association between telomere length and clinicopathological parameters, GBMs were divided into two groups according to the median value (0.92) of relative telomere length. In the analysis, telomere length was not associated with any clinicopathological characteristics or molecular changes (data not shown).

Positive TERT promoter status is associated with poor survival. The clinical prognosis of *TERT* promoter mutation-positive GBMs (n=16) was compared with that of *TERT* promoter mutation-negative GBMs (n=9). The median overall survival in GBMs exhibiting *TERT* promoter mutation was 81.7 [95% confidence interval (CI),

Table I. Association between *TERT* promoter mutation and other gene mutation status in GBMs.

A, Sphere formation-positive GBMs

	Sample												
Gene mutation status	1	2	3	4	5	6	7	8	9	10	11	12	13
TERT mutation	+	+	+	+	+	+	+	+		+	+	+	+
C228T	+	+	+	+	+	+	+	+		+	+	+	+
C250T						+							
Sphere formation	+	+	+	+	+	+	+	+	+	+	+	+	+
ATRX amplification						ND			+	ND			
ATRX mutation	+	+			+		+	+	+	ND			+
EGFR mutation		+		+				+		ND	+	+	+
EGFR amplification	+	+		+	+	ND			+	ND	+	+	+
IDH1 mutation										ND			
TP53 mutation	+				+		+	+		ND			
PTEN deletion	+		+	+	+	ND	+		+	ND	+	+	+
PTEN mutation					+		+			ND			
CDKN2A deletion	+	ND	+	+	+	ND	+	+	+	ND	+	+	+

B, Sphere formation-negative GBMs

	Sample											
Gene mutation status	1	2	3	4	5	6	7	8	9	10	11	12
TERT mutation		+								+	+	+
C228T		+								+	+	+
C250T												
Sphere formation												
ATRX amplification												
ATRX mutation	ND	ND	ND	ND	ND	ND			ND	ND		+
EGFR mutation	ND	ND	ND	ND	ND	ND			ND	ND	+	
EGFR amplification	+	+					+				+	+
IDH1 mutation	ND	ND	ND	ND	ND	ND			ND	ND		
TP53 mutation	ND	ND	ND	ND	ND	ND			ND	ND		+
PTEN deletion						+	+				+	+
PTEN mutation	ND	ND	ND	ND	ND	ND		+				+
CDKN2A deletion	+	+				+	+		+		+	+

TERT, telomerase reverse transcriptase gene; GBM, glioblastoma multiforme; *ATRX*, α-thalassemia/mental retardation syndrome X-linked; *EGFR*, epidermal growth factor receptor; *IDH1*, isocitrate dehydrogenase 1; *TP53*, protein 53; *PTEN*, phosphatase and tensin homolog; *CDKN2A*, cyclin dependent kinase inhibitor 2A; *ND*, Not determined.

61.71-101.85] weeks, which was significantly decreased compared with that in GBMs without *TERT* promoter mutation (median, 152.6 weeks; 95% CI, 84.05-221.16; P=0.026; Fig. 2A). According to the median value of telomere length (0.92), GBMs were stratified into longer and shorter groups to analyze the prognostic value of telomere length. Overall survival in patients with GBM with a longer telomere length (median, 75.70 weeks; 95% CI, 40.65-110.75) was reduced compared with those with shorter telomere length (median, 125.04 weeks; 95% CI, 84.02-166.05; P=0.041; Fig. 2B). In contrast, progression-free survival did not differ according to *TERT* promoter mutation (median, 51.19 vs. 43.32; 95% CI, 27.56-74.82 vs. 29.09-57.54; P=0.463; Fig. 2C) and telomere length (median, 47.43 vs. 43.88; 30.47-64.40 vs. 26.88-60.88; P=0.560; Fig. 2D]. When survival analysis was performed separately according to other variables [*in vitro* sphere formation capacity, *in vivo* tumor formation, age (<60 vs. ≥60), sex and subtype], there was no significant prognostic difference in overall survival or progression-free survival (data not shown).

Table II. Clinicopathological and experimental characteristics of GBMs with or without TERT promoter mutation.

Variable	TERT promoter mutation, n (%)		P-value
	+	-	
Age, years	56.5±8.3	47.2±14.6	0.050
Sex			<0.001
Male	12 (100)	0 (0)	
Female	4 (30.8)	9 (69.2)	
Tumor size, cm	4.70±1.63	4.79±0.75	0.943
ATRX mutation			1.00
+	7 (87.5)	1 (12.5)	
-	6 (75.0)	2 (25.0)	
ATRX amplification			0.391
+	0 (0)	1 (100)	
-	14 (63.6)	8 (36.4)	
EGFR mutation			0.117
+	11 (91.7)	1 (8.3)	
-	4 (57.1)	3 (42.9)	
EGFR amplification			0.102
+	10 (76.9)	3 (23.1)	
-	4 (40.0)	6 (60.0)	
CDKN2A deletion			0.116
+	12 (70.6)	5 (29.4)	
-	1 (20.0)	4 (80.0)	
PTEN mutation			1.00
+	3 (75.0)	1 (25.0)	
-	10 (83.3)	2 (16.7)	
PTEN deletion			0.102
+	10 (76.9)	3 (23.1)	
-	4 (40.0)	6 (60.0)	
Subtype			0.176
Classical	6 (100)	0 (0)	
Mesenchymal	2 (40.0)	3 (60.0)	
Proneural	3 (60.0)	2 (40.0)	
Not determined	5 (55.6)	4 (44.4)	
In vivo tumor formation			0.004
+	12 (80.0)	3 (20.0)	
-	0 (0)	5 (100)	

Data are presented as the mean ± standard deviation. *TERT*, telomerase reverse transcriptase gene; GBM, glioblastoma multiforme; *ATRX*, α-thalassemia/mental retardation syndrome X-linked; *EGFR*, epidermal growth factor receptor; *PTEN*, phosphatase and tensin homolog; *CDKN2A*, cyclin dependent kinase inhibitor 2A.

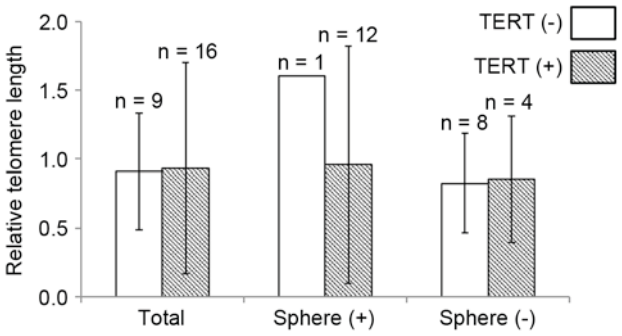

Figure 1. Relative telomere length analysis according to *TERT* promoter mutation in glioblastoma multiforme cases with and without *in vitro* sphere formation capacity. *TERT*, telomerase reverse transcriptase gene.

Discussion

The present study revealed that *TERT* promoter mutations in GBMs are significantly associated with the *in vitro* sphere formation capacity and *in vivo* tumorigenic potential of dissociated GBM cells. *In vitro* and *in vivo* preclinical models using GBM cells primarily cultured from surgical samples have provided an improved understanding of the biology of the disease (9). Several preclinical characteristics of primary cultured GBM cells, including *in vitro* sphere formation capacity and *in vivo* tumorigenic potential, were revealed to be associated with clinical aggressiveness in corresponding patients (9). The associations may be utilized to determine molecular and/or functional mechanisms of clinical aggressiveness of GBMs.

In the present study, *in vitro* and *in vivo* preclinical GBM models exhibiting *TERT* promoter mutation-positive and -negative GBMs were established. As preclinical models may summarize the clinicopathological features of patient with GBMs (9,21-23), the preclinical models may be utilized to predict the treatment effects of *TERT*-targeting therapies for *TERT* promoter mutation-positive GBMs, compared with those for *TERT* promoter mutation-negative GBMs. In the present study, the majority of GBMs with *in vitro* sphere formation capacity exhibited *TERT* promoter mutations (92.3%). By contrast, the *TERT* promoter mutation rate in GBMs without *in vitro* sphere formation capacity (33.3%) was significantly decreased. This significant difference was observed between GBMs with and without *in vivo* tumorigenic potential.

The *in vitro* sphere-forming assay has been widely used in stem cell biology as an experimental method for determining the self-renewal and differentiation potential of stem cells (9,11). Therefore, a significant association between *TERT* promoter mutation and the *in vitro* sphere-forming capacity of GBM cells, identified in the present study, suggests that mutations in the *TERT* promoter region are associated with the biology of GBM cells to enhance self-renewal capacity. Self-renewal capacity is a key feature of GBM cancer stem cells that exert recurrence following anti-cancer treatments (24,25). Therefore, *TERT* promoter mutations resulting in overexpression of *TERT* may be associated with treatment resistance of GBMs. In addition, the possible association between *TERT* promoter mutations and treatment resistance of GBMs is supported by the survival analysis results in the present study, which revealed that GBMs with *TERT* promoter mutation have significantly decreased overall survival.

Previous studies have demonstrated the negative clinical impacts of *TERT* promoter mutation or longer telomere length in a number of types of cancer, including GBMs (15,20,21,25,26). *TERT* promoter mutations may generate novel binding motifs for E26 transformation-specific/T-cell factor transcription

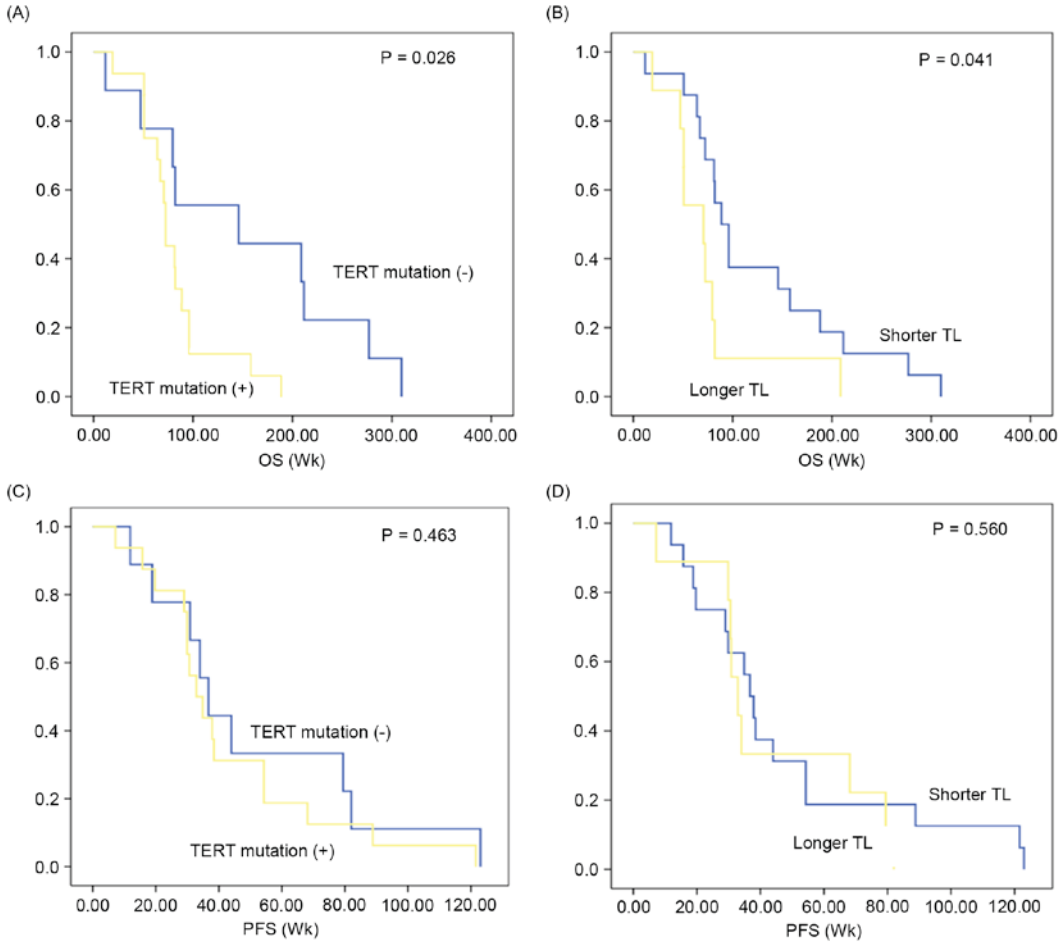

Figure 2. Prognostic impact of *TERT* promoter mutation and telomere length in GBMs. OS of patients with GBM, depending on (A) *TERT* promoter mutation status and (B) TL. PFS of patients with GBM, depending on (C) *TERT* promoter mutation status and (D) TL. *TERT*, telomerase reverse transcriptase gene; GBM, glioblastoma multiforme; TL, telomere length; PFS, progression-free survival; Wk, week.

factors, and cause two- to four-fold increases in transcriptional activity and telomere length (14,26,27). These data suggested that overexpression of *TERT* by its promoter mutation may increase the self-renewal capacity of GBM cancer stem cells and induce poor clinical outcomes. The results of the present study did not reveal a significant association between *TERT* promoter mutation and relative telomere length in the GBM samples. Previous studies demonstrated that the association between telomere length and *TERT* expression is complex and may be regulated by a number of other factors, including the activities of signaling pathways and alterations of genes (4,28,29).

Previous studies have demonstrated that *TERT* promoter is the most common type of mutation in GBMs, suggesting that it may be an early event in GBM carcinogenesis (2,4,5,30-32). In the present study, *TERT* promoter mutations were identified in 64% of GBMs, and were associated with the age and sex of patients with GBM. These results are consistent with those of previous studies (4,5). The association between *TERT* promoter mutations and other genetic alterations has been observed in previous studies (4,5). These studies suggested that *TERT* promoter mutations revealed a significant inverse association with isocitrate dehydrogenase 1 mutation and *P53* mutation, but a positive association with *EGFR* amplification and *CDKN2* deletion. Though the results of the present study were not statistically significant due to the limited sample size of primary GBMs, *TERT* promoter mutations tend to be associated with *EGFR* amplification and *CDKN2A* deletion. This result supports the reliability of our results.

The present study revealed the association between *TERT* promoter mutation and preclinical characteristics of GBM, including *in vitro* sphere-forming capacity and *in vivo* tumorigenic potential. As *TERT* promoter mutation is a prognostic marker of GBM, the identification of preclinical characteristics of *TERT* promoter mutations may reveal the functions of *TERT* and telomere length in the self-renewal of GBM cells, and treatment resistance of GBM. Furthermore, the results may provide a foundation for the development of innovative telomerase-based therapeutic strategies for treatment-resistant GBMs.

Acknowledgements

The present study was supported by the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education (grant no. NRF-2014R1A6A3A04058057). Additional support was supported by the National Research Foundation of Korea (NRF) Grant funded by the Korean Government (MSIP) (grant no. NRF-2016R1A5A2945889).

References

1. Mosrati MA, Malmström A, Lysiak M, Krysztofiak A, Hallbeck M, Milos P, Hallbeck AL, Bratthäll C, Strandéus M, Stenmark-Askmalm M and Söderkvist P: TERT promoter mutations and polymorphisms as prognostic factors in primary glioblastoma. Oncotarget 6: 16663-16673, 2015.
2. Reitman ZJ, Pirozzi CJ and Yan H: Promoting a new brain tumor mutation: TERT promoter mutations in CNS tumors. Acta Neuropathol 126: 789-792, 2013.
3. Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, Miller CR, Ding L, Golub T, Mesirov JP, *et al*: Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. Cancer Cell 17: 98-110, 2010.
4. Labussière M, Boisselier B, Mokhtari K, Di Stefano AL, Rahimian A, Rossetto M, Ciccarino P, Saulnier O, Paterra R, Marie Y, *et al*: Combined analysis of TERT, EGFR, and IDH status defines distinct prognostic glioblastoma classes. Neurology 83: 1200-1206, 2014.
5. Nonoguchi N, Ohta T, Oh JE, Kim YH, Kleihues P and Ohgaki H: TERT promoter mutations in primary and secondary glioblastomas. Acta Neuropathol 126: 931-937, 2013.
6. Killela PJ, Reitman ZJ, Jiao Y, Bettegowda C, Agrawal N, Diaz LA Jr, Friedman AH, Friedman H, Gallia GL, Giovanella BC, *et al*: TERT promoter mutations occur frequently in gliomas and a subset of tumors derived from cells with low rates of self-renewal. Proc Natl Acad Sci USA 110: 6021-6026, 2013.
7. George J, Banik NL and Ray SK: Knockdown of hTERT and concurrent treatment with interferon-gamma inhibited proliferation and invasion of human glioblastoma cell lines. Int J Biochem Cell Biol 42: 1164-1173, 2010.
8. Beck S, Jin X, Sohn YW, Kim JK, Kim SH, Yin J, Pian X, Kim SC, Nam DH, Choi YJ and Kim H: Telomerase activity-independent function of TERT allows glioma cells to attain cancer stem cell characteristics by inducing EGFR expression. Mol Cells 31: 9-15, 2011.
9. Joo KM, Kim J, Jin J, Kim M, Seol HJ, Muradov J, Yang H, Choi YL, Park WY, Kong DS, *et al*: Patient-specific orthotopic glioblastoma xenograft models recapitulate the histopathology and biology of human glioblastomas in situ. Cell Rep 3: 260-273, 2013.
10. Louis DN, Ohgaki H, Wiestler OD, Cavenee WK, Burger PC, Jouvet A, Scheithauer BW and Kleihues P: The 2007 WHO classification of tumours of the central nervous system. Acta Neuropathol 114: 97-109, 2007.
11. Joo KM, Kim SY, Jin X, Song SY, Kong DS, Lee JI, Jeon JW, Kim MH, Kang BG, Jung Y, *et al*: Clinical and biological implications of CD133-positive and CD133-negative cells in glioblastomas. Lab Invest 88: 808-815, 2008.
12. Pollard SM, Yoshikawa K, Clarke ID, Danovi D, Stricker S, Russell R, Bayani J, Head R, Lee M, Bernstein M, *et al*: Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens. Cell Stem Cell 4: 568-580, 2009.
13. Ito M, Hiramatsu H, Kobayashi K, Suzue K, Kawahata M, Hioki K, Ueyama Y, Koyanagi Y, Sugamura K, Tsuji K, *et al*: NOD/SCID/gamma(c)(null) mouse: An excellent recipient mouse model for engraftment of human cells. Blood 100: 3175-3182, 2002.
14. Liu T, Wang N, Cao J, Sofiadis A, Dinets A, Zedenius J, Larsson C and Xu D: The age- and shorter telomere-dependent TERT promoter mutation in follicular thyroid cell-derived carcinomas. Oncogene 33: 4978-4984, 2014.
15. Oh YT, Cho HJ, Kim J, Lee JH, Rho K, Seo YJ, Choi YS, Jung HJ, Song HS, Kong DS, *et al*: Translational validation of personalized treatment strategy based on genetic characteristics of glioblastoma. PLoS One 9: e103327, 2014.
16. Olshen AB, Venkatraman ES, Lucito R and Wigler M: Circular binary segmentation for the analysis of array-based DNA copy number data. Biostatistics 5: 557-572, 2004.
17. Li H and Durbin R: Fast and accurate short read alignment with Burrow-Wheeler transform. Bioinformatics 25: 1754-1760, 2009.
18. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G and Durbin R; 1000 Genome Project Data Processing Subgroup: The sequence Alignment/Map format and SAMtools. Bioinformatics 25: 2078-2079, 2009.
19. Wu TD and Nacu S: Fast and SNP-tolerant detection of complex variants and splicing in short reads. Bioinformatics 26: 873-881, 2010.
20. Vinagre J, Pinto V, Celestino R, Reis M, Pópulo H, Boaventura P, Melo M, Catarino T, Lima J, Lopes JM, *et al*: Telomerase promoter mutations in cancer: An emerging molecular biomarker? Virchows Arch 465: 119-133, 2014.
21. Fichtner I, Rolff J, Soong R, Hoffmann J, Hammer S, Sommer A, Becker M and Merk J: Establishment of patient-derived non-small cell lung cancer xenografts as models for the identification of predictive biomarkers. Clin Cancer Res 14: 6456-6468, 2008.
22. John T, Kohler D, Pintilie M, Yanagawa N, Pham NA, Li M, Panchal D, Hui F, Meng F, Shepherd FA and Tsao MS: The ability to form primary tumor xenografts is predictive of increased risk of disease recurrence in early-stage non-small cell lung cancer. Clin Cancer Res 17: 134-141, 2011.
23. Lee HW, Lee JI, Lee SJ, Cho HJ, Song HJ, Jeong DE, Seo YJ, Shin S, Joung JG, Kwon YJ, *et al*: Patient-derived xenografts from non-small cell lung cancer brain metastases are valuable translational platforms for the development of personalized targeted therapy. Clin Cancer Res 21: 1172-1182, 2015.
24. Hale JS, Otvos B, Sinyuk M, Alvarado AG, Hitomi M, Stoltz K, Wu Q, Flavahan W, Levison B, Johansen ML, *et al*: Cancer stem cell-specific scavenger receptor CD36 drives glioblastoma progression. Stem Cells 32: 1746-1758, 2014.
25. Borah A, Raveendran S, Rochani A, Maekawa T and Kumar DS: Targeting self-renewal pathways in cancer stem cells: Clinical implications for cancer therapy. Oncogenesis 4: e177, 2015.
26. Horn S, Figl A, Rachakonda PS, Fischer C, Sucker A, Gast A, Kadel S, Moll I, Nagore E, Hemminki K, *et al*: TERT promoter mutations in familial and sporadic melanoma. Science 339: 959-961, 2013.
27. Huang FW, Hodis E, Xu MJ, Kryukov GV, Chin L and Garraway LA: Highly recurrent TERT promoter mutations in human melanoma. Science 339: 957-959, 2013.
28. Chiba K, Johnson JZ, Vogan JM, Wagner T, Boyle JM and Hockemeyer D: Cancer-associated TERT promoter mutations abrogate telomerase silencing. Elife 4, 2015.
29. Ko E, Seo HW, Jung ES, Kim BH and Jung G: The TERT promoter SNP rs2853669 decreases E2F1 transcription factor binding and increases mortality and recurrence risks in liver cancer. Oncotarget 7: 684-699, 2016.
30. Huse JT: TERT promoter mutation designates biologically aggressive primary glioblastoma. Neuro Oncol 17: 5-6, 2015.
31. Simon M, Hosen I, Gousias K, Rachakonda S, Heidenreich B, Gessi M, Schramm J, Hemminki K, Waha A and Kumar R: TERT promoter mutations: A novel independent prognostic factor in primary glioblastomas. Neuro Oncol 17: 45-52, 2015.
32. Spiegl-Kreinecker S, Lötsch D, Ghanim B, Pirker C, Mohr T, Laaber M, Weis S, Olschowski A, Webersinke G, Pichler J and Berger W: Prognostic quality of activating TERT promoter mutations in glioblastoma: Interaction with the rs2853669 polymorphism and patient age at diagnosis. Neuro Oncol 17: 1231-1240, 2015.