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Expression of Telomeres in Astrocytoma WHO Grade 2 to 4: TERRA Level Correlates with Telomere Length, Telomerase Activity, and Advanced Clinical Grade[1,2]

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Abstract

Cancer cells bypass replicative senescence, the major barrier to tumor progression, by using telomerase or alternative lengthening of telomeres (ALT) as telomere maintenance mechanisms (TMMs). Correlation between ALT and patient survival was demonstrated for high-grade astrocytomas. Transcription from subtelomeres produces telomeric repeat-containing RNA (TERRA), a natural inhibitor of telomerase activity (TA). This led us to evaluate correlations of TERRA and TMM with tumor grade and outcome in astrocytoma patients. SYBR Green real-time reverse transcription–polymerase chain reaction assays for quantitation of total and chromosome 2p and 18p specific TERRA levels were developed. Tumor samples from 46 patients with astrocytoma grade 2 to 4, tissue controls, and cell lines were assessed. TMMs were evaluated by measuring TA and by detecting long telomeres due to ALT. In glioblastoma multiforme (GBM) grade 4, total TERRA levels were similar to cell lines but 14-, 31-, and 313-fold lower compared with grade 3, grade 2, and nonmalignant tissue, respectively. Total TERRA levels differed from chromosomal levels. Low 2p TERRA levels correlated with dense promoter methylation of subtelomeric CpG islands, indicating that TERRA expression in gliomas may be chromosome specific and epigenetically regulated. Total TERRA levels correlated with diagnosis, with low or absent TA and the presence of ALT, and were tentatively associated with favorable patient prognosis in our cohort ($P = .06$). TA and short telomeres identified a subset of GBM with a median survival of only 14.8 months. TERRA and TA may be prognostic in astrocytic tumors.

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Introduction

Telomeres are the ends of linear chromosomes that serve as a protective cap to avoid permanent proliferation arrest, termed *replicative senescence*. In vertebrates, telomeres consist of tandem repeats of TTAGGG hexanucleotide sequences and some repeats are lost in each cell division [1]. The activation of a telomere maintenance mechanism (TMM) results in immortalization and has been identified as a crucial hallmark of cancer cells [2,3].

Tumors use two TMMs, one based on telomerase activity (TA) and the second, which does not use telomerase, named alternative lengthening of telomeres (ALT) [4]. TA is the predominant mechanism used by roughly 85% of human cancer [5], and its role in cancer has been the focus of many reviews [6,7]. ALT is less commonly used, but it has shown high prevalence in sarcomas and astrocytomas [8,9]. A characteristic finding of ALT in affected tumor cells is that the mean telomere length (TL) analyzed by terminal restriction fragment (TRF) Southern blots is substantially longer—approximately twice as long as in normal cells—indicating that the telomeres had undergone

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extensive lengthening [10]. TA-positive cells regulate TLs around a mean of 5 to 10 kb with normal distribution, whereas in ALT cells, the telomeres are very large—up to 20 kb with considerable heterogeneity in length.

The first report demonstrating telomerase as a prognostic marker in human malignancies was published on childhood neuroblastoma [11]. The question of whether telomerase and telomere dysfunction are useful prognostic markers is still debated [12,13]. Glioblastoma multiforme (GBM, grade 4 astrocytoma) has a poor prognosis, with a median survival less than 2 years despite multimodality therapy [14].

TA was reported in 78% (60/77 cases) of GBM but was less commonly observed in astrocytoma grade 3 tumors, with 28% (5/18 cases) evidencing activity, and absent in astrocytoma grade 2 tumors (0/10 cases) [15]. Similar correlations between TA and histologic grade were published by other authors as well [16]. Although additional studies reported that 30% to 50% of GBM express TA [17,18]. However, TA studied in a larger GBM cohort did not correlate with survival of patients [19]. Previous studies reported the presence of ALT in a high percentage of astrocytoma grades 2 and 3 and a positive association between ALT and survival in GBM patients [8,19,20]. ALT detected by TL has some limitations because this pattern is not identified in all cells using ALT, and long telomeres are seen in cells using TA when telomerase genes *hTERT* and *hTR* are expressed at supraphysiological levels. Comparing different methods detecting ALT, the long heterogeneous TL pattern remains the best-established marker for ALT in human cells, including tumors [9].

Recently, a class of noncoding telomeric repeat-containing RNA (TERRA or TelRNA) transcripts has been reported as key components of telomeres [21,22]. TERRA transcription by RNA polymerase II originates in the subtelomeres of chromosome ends, and its expression depends on the epigenetic state of CpG island promoters [23]. Considering all chromosomal ends, more than 20 TERRA transcripts are predicted by *in silico* analyses and are defined as telomeric transcriptome [24]. Reduced TERRA expression and epigenetic regulation with an increase in DNA methylation at the proximal subtelomere were found associated with TA in a study of normal human fibroblasts and cancer cell lines suggesting that TERRA may inhibit telomerase [25]. Preliminary data of TERRA expression demonstrated downregulation in advanced stages of human tumors of larynx, colon, and lymph node compared with normal tissues [22]. The results are consistent with the notion that TERRA may inhibit telomerase because these tumors are using frequently TA as TMM.

In this study, we analyzed TERRA expression and subtelomeric methylation in a panel of astrocytoma grades 2 to 4 tumors and cell lines and compared the results with TMM status and clinical variables. We hypothesized that TERRA expression would correlate with prolonged survival in patients with astrocytomas. Total TERRA expression was determined after reverse transcription by a SYBR Green real-time polymerase chain reaction (PCR) method published for measuring relative telomeric repeat DNA content [26,27] and compared with TERRA levels from subtelomeric regions of chromosomes 2p and 18p. Methylation levels at the subtelomere of chromosome 2p were determined by bisulfite allelic sequencing and correlated with 2p TERRA expression. TERRA transcript levels were compared with nonmalignant brain tissue and decreased by tumor grade and correlated with ALT and TA, as studied by TL and the telomeric repeat amplification protocol (TRAP) assay, respectively. TA was not detected in astrocytoma grades 2 and 3 but in 50% of GBM. However, in grades 2 and 3 tumors, the mean TL measured by TRF analyses and PCR was increased more than 17 kb, indicating a strong prevalence for ALT and better outcome in these tumors as reported [19]. Indeed, elevated TERRA levels, long telomeres, and absence of TA correlated with low-grade and survival. In conclusion, we found that TERRA expression and TA correlated with outcome of this patient series, suggesting that the correlation of TERRA and TA as prognostic markers in astrocytomas should be further investigated.

Materials and Methods

Patient Material and Cell Lines

Tissue samples were resected surgically from 46 patients with astrocytoma at the Department of Neurosurgery of the General Hospital Vienna, Austria, between 2000 and 2007. The local research ethics committee approved the study. Follow-up information was collected until December 2008 and was available for 42 of 46 patients. Patients' age at diagnosis and at last follow-up and survival were calculated from the date of their first operation at which World Health Organization grades 2 to 4 astrocytoma was confirmed histologically. Tissue samples from brain regions (hippocampus, cortex, and temporal mark) were included from two patients experiencing drug-refractory epilepsy as nontumor (NT) controls. The samples were snap frozen in liquid nitrogen after surgical removal and stored at –80°C or in liquid nitrogen until use. A synopsis of tumor tissue material and its relation to patient data is given in Table 1.

Cell lines originating from astrocytoma CRL-1718 (CCF-STTG1), CRL-2020 (DBTRG/05MG), CRL-1690 (T98G), HTB17 (U-373 MG), and HTB138 (Hs683) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were grown under standard conditions. LN-140 cell line (kindly provided by Dr Tribolet, Lausanne, Switzerland) and MR-1 and MGC glioblastoma cells (a gift from Dr T. Kurata, Tokyo, Japan) were grown in minimal essential medium–Eagle with 10% fetal calf serum. KG-MH, KM-YH, YT-BO, and YU-PM were established from patient material and were grown in RPMI-1640 with 10% fetal calf serum as described [28]. HTB-186 (Daoy) and HTB-85 (SAOS-2) were derived from ATCC and originated from desmoplastic cerebellar medulloblastoma and primary human osteosarcoma, respectively.

RNA, DNA, and Protein Extraction

RNA and DNA extraction from tissue samples was carried out using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the

Table 1. Patient Data.

	Diagnosis*		
	GBM	AA	DA
WHO grade	4	3	2
No. patients	28	6	12
Male	10	3	8
Female	18	3	4
Median (min-max) age (yr)	64 (8-80)	42 (31-72)	42 (2-68)
Clinical follow-up available	25[†]	6	11
Median follow-up time (mo)	17	33	61
Favorable outcome	8	4	11
Deceased	17	2	0
Median survival (mo)	17.4	49.9	>50[‡]

*GBM indicates glioblastoma multiforme; AA, anaplastic astrocytoma; DA, diffuse astrocytoma.
[†]All diagnosed with primary GBM.
[‡]Not reached after 50 months.

manufacturer's instructions. RNA and DNA contents were determined by photometric measurement. The purity of the extracted RNA and DNA was assessed by calculating the ratio of extinctions at 260 nm/280 nm [29]. Values between 1.7 and 2.2 were tolerated. In addition, the quality of the RNA was assessed by denaturing agarose gel analysis with EtBr. The intensity of the bands representing the 28S and 18S rRNA were determined with FluorImager595 (Molecular Dynamics, Sunnyvale, CA). The ratio of intensities of the 28S/18S rRNA signal was calculated. Values between 1.5 and 2.5 were acceptable. Protein extracts were isolated from 10^5 to 10^6 growing cells from cell lines, were washed with phosphate-buffered saline, and briefly mixed using a vortex mixer with 200 µl of CHAPS buffer [30]. Alternatively, 50 to 100 mg of thawed tissue samples was immediately mechanically homogenized on ice with three-time pulses for 5 seconds using Ultra-TURRAX-T8 dispersing tool S8N-5G (IKA, Staufen, Germany) in 200 µl of CHAPS buffer including 20 U of ribonuclease inhibitor RNasin (Promega, Madison, WI). Buffer extracts were incubated on ice for 30 minutes and centrifuged at 12,000*g* for 20 minutes at 4°C. One hundred sixty microliters of supernatants was transferred to fresh tubes, snap frozen on dry ice, and stored at −80°C until further use. The remaining pellets or intact cells were applied for high-quality DNA isolation with Maxwell 16 DNA Purification Kit (Promega). Protein concentrations were determined according to Coomassie Protein Assay Reagent Product protocol (Pierce, Rockford, IL).

TL Analysis

Relative TL was determined by real-time PCR as described [26,27]. Reactions contained 35 ng of DNA isolated with TRIzol reagent and SYBR Green Power MasterMix (Applied Biosystems, Foster City, CA). PCR was performed on ABI PRISM 7000 Sequence Detection System (Applied Biosystems) under cycling conditions as described previously [27]. Reactions were set up in triplicates with each 100 nM telomere-specific (T) primers Tel1b 5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGGT-3′ and Tel2b 5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′ as described [27] or in duplicates with each 100 nM single-copy *36B4* gene (S) primers 36B4u 5′-CAGCAAGTGGGAAGGTGTAATCC-3′ and 36B4d 5′-CCCATTCTATCATCAACGGGTACAA-3′ [26]. C_t values with a SD greater than 0.5 were repeated. Relative quantities (RQ) of T/S values were calculated in relation to median levels measured in NT tissue samples according to Pfaffl's method [31]. Efficiencies used for calculating Tel and *36B4* PCR were determined by standard curves of template DNA dilution series with 0.83 and 0.94, respectively.

Absolute TL measurement was done by TRF analysis with Telo*TAGGG*-Telomere-Length-Assay (Roche, Basel, Switzerland) according to the manufacturer's instructions. In brief, 1 µg of high-quality DNA was digested with *Rsa*I and *Hin*fI restriction enzymes and separated by 1% agarose gel electrophoresis. Restriction efficiency was validated by DNA staining and visualization with FluorImager595 (Molecular Dynamics) before blot analysis on positively charged nylon membranes. Digoxigenin (DIG)-labeled oligo probes were used for hybridization to detect telomere sequences by luminescence system with ChemiSmart 5100 and ChemiCapt software (BioRad, Hercules, CA). Background corrections on TIFF result files were performed with ImageJ software (National Institutes of Health, Bethesda, MD). Mean TRFs were determined in kilobase pairs according to the manufacturer's instructions (Roche) based on signal intensities from ImageQuant5.0 software (Molecular Dynamics).

TERRA Expression Quantification

Transcript levels of total, 2p, and 18p TERRA were measured after complementary DNA (cDNA) synthesis with RevertAid First Strand Kit (Fermentas, Sankt Leon-Rot, Germany) by real-time PCR on ABI PRISM 7500 Fast Sequence Detection System with FAST SYBR Green MasterMix (Applied Biosystems). One microgram of RNA was converted with random hexanucleotide primers into cDNA. Aliquots corresponding to 40 ng of RNA were analyzed in triplicates by real-time PCR with 200 nM primers each. Total TERRA levels were amplified with telomere-specific (T) primers under conditions as described for relative TL measurement. Primers for specific amplification of chromosome 2p and 18p TERRA were designed between telomere ends and known CpG islands [25]. Primers for 2p TERRA are as follows: chr2_F 5′-TAAGCCGAAGCCTAACTCGTGTC-3′ and chr2_R 5′-GTAAAGGCGAAGCAGCATTCTCC-3′; those for 18p-TERRA are as follows: chr18_F 5′-CCTAACCCTCACCCTTCTAAC-3′ and chr18_R 5′-TACCTCGCTTTGGGACAAC-3′. Amplicons were verified by sequencing. Relative quantities (RQ) were determined in relation to levels of *36B4* and to the median expression found in NT tissue samples according to Pfaffl's method [31]. Efficiencies of PCRs were determined and used for quantitative RQ calculation.

Bisulfite Allelic Sequencing

One microgram of DNA was subjected to bisulfite conversion by Epitect reagent (Qiagen, Hilden, Germany). PCR was performed on iCycler (BioRad) with touchdown PCR protocol as described [25]. Aliquots corresponding to 20 ng of bisulfite-converted DNA were analyzed for methylation of CpG islands of chromosome 2p subtelomere region. Twenty-microliter reactions were set up with primer HC_MP2fwd 3′-GTATTGTAGGTGTATAGTTGTATAAG-5′ and a modified primer HC_MP2rev 3′-TAATACGACTCACTATAGGGGATC-GATCTAAAATTCCACCTATCTCTATAC-5′. HC_MP2rev was extended at the 5′-end by T7 primer sequence to allow direct sequencing. PCR fragments of 156 bp in size were purified by 3% agarose (BIOzym, Hessisch Oldendorf, Germany) gel electrophoresis. The extent of methylation was determined by direct sequencing of PCR fragments (Qiagen). In brief, all sequencing reactions were set up with 8 ng of DNA template and 10 pmol of T7 primer per reaction and cycled with BigDye3.1 Terminator (Applied Biosystems) on GeneAmp PCR System 9700 (Applied Biosystems) and purified using DyeEx (Qiagen). Data collection was performed on 3730xl DNA Analyzer (Applied Biosystems) equipped with 50-cm capillary arrays and POP7 polymer. Raw data channels from ab1 result files were processed using customized software (Qiagen) for compensation of different dye migration, baseline correction, peak detection, and base calling. For each base, the area was calculated from the corresponding peak to measure the base's quantity. Bases that correspond to variable positions of CpG sites were identified by comparison with a reference sequence. Ratios of methylated *versus* unmethylated CpG sites were reported with accuracy in the range of ±5% when signal-to-noise ratio was 20 or higher.

Telomerase Activity Analysis

TA was quantified as the total product generated (TPG) units per 0.6 µg of protein extract analyzed by TRAP assay as described [32,33]. In brief, 0.6 µg of protein extract in CHAPS lysis buffer was used for each assay in a 20-µl volume setup. Each reaction contained buffer, dNTPs, 0.8 U of titanium Taq DNA polymerase (Clontech, Mountain View, CA) and primers for telomerase products (telomerase substrate [TS] and anchored return CX [ACX] primers) and for PCR control (telomerase substrate–nontelomerase [TSNT]

internal control oligonucleotide and nontelomerase [NT] internal control primer). Reactions were run in duplicates on iCycler (BioRad) under the following conditions: 30 minutes at 30°C, 1 minute at 95°C, 32× (30 seconds at 94°C, 30 seconds at 59°C, 60 seconds at 72°C). Amplicons were separated on 12% polyacrylamide gels, stained with VistraGreen (Amersham Biosciences, NJ) and visualized by FluorImager595 (Molecular Dynamics). Staining intensities of the telomeric DNA ladder were corrected by signals obtained from controls with protein lysates heated to 85°C for 10 minutes to inactivate TA. Furthermore, intensities were normalized with values obtained from PCR controls. TSR8 oligonucleotide contains sequences for TS primer and eight telomeric repeat products and was used in separate reactions as standard for TA quantitation in TPG units. One TPG unit corresponds to 0.001 amoles (~600 molecules) of TSR8 extended by at least four telomeric repeats within 30 minutes at 30°C. Transcript levels for 18S rRNA were determined with TaqMan chemistry (Applied Biosystems) on 10-fold dilutions of protein extracts as described [34] to avoid false-negative results. Samples with levels of 18S rRNA less than 10% of the mean of all results were scored not determinable for TA.

Statistical Analysis

All statistical tests were two sided, and $P \leq .05$ was considered significant. Statistical analysis was performed with GraphPad Prism software package (Version 5; GraphPad Software, Inc, San Diego, CA). Correlations among RQ and activity values were assessed after log transformation with the Pearson correlation calculation. Mann-Whitney *U* test was used for RQ group comparisons. Mean TL in kilobase pairs was calculated from relative PCR values by linear regression analyses from a subset of samples analyzed for TRF. Kaplan-Meier analysis and the log-rank test were used to illustrate differences between overall survivals to parameters assessed by experiments.

Results

TERRA Expression and Comparison with Histopathology Grading

Tissue samples from patients with diagnosis for astrocytoma WHO grade 2 to 4 and NT tissues (Table 1) were analyzed for TERRA expression. A panel of 14 tumor cell lines, including 12 that originated from astrocytoma, was also included.

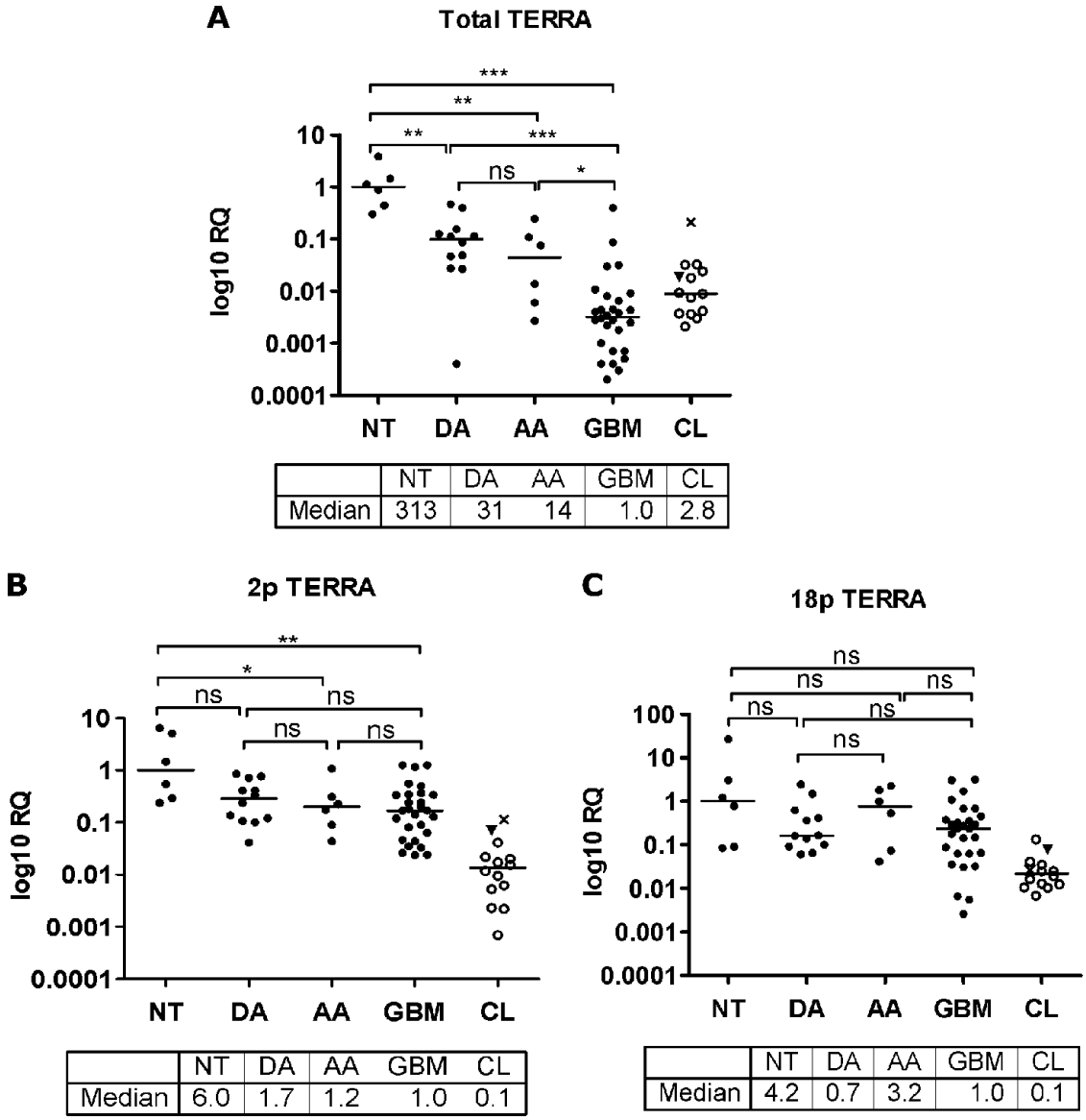
The PCR assay for TL measurement used cDNA instead of genomic DNA for total TERRA expression analysis. In addition to this assay, amplifying all expressed telomere repeats, we also established subtelomeric region PCR assays to detect chromosome-specific TERRA from chromosomal ends 2p and 18p. RNA was isolated from snap-frozen tissue and used for further analyses when it was of adequate quality as described under Materials and Methods. RQs for TERRA expression were determined in comparison to the expression of ribosomal gene *36B4* and to the median TERRA expression values in NT tissues (Figure 1). Similar results were obtained when *β-actin* was used instead of *36B4* as a housekeeping control gene. RQ values for 2p and 18p TERRA normalized on *36B4* correlated strongly with values normalized on *β-actin* after log transformation (Pearson r = 0.92, $P < .0001$; not shown). Total TERRA expression gradually decreased in astrocytoma as the grade increased (Figure 1*A*). In detail, GBM demonstrates 14-, 31-, and 313-fold decrease compared with anaplastic astrocytoma (AA), diffuse astrocytoma (DA), and NT tissue, respectively. Although not significant, the expression of TERRA from chromosome end 2p trended the relation with tumor grade (Figure 1*B*). 2p TERRA expression in GBM decreased six-fold compared with NT tissue. In contrast, 18p TERRA expression demonstrates no relation with tumor grade. Log-transformed RQ values of total TERRA correlated *in vivo* with both transformed values of 2p and 18p TERRA, Pearson r = 0.53 and 0.34, respectively (not shown). The interesting correlation of 2p TERRA in contrast to the weak correlation of 18p TERRA may indicate a prevalence of 2p over 18p TERRA. Furthermore, expression levels of chromosome-specific TERRA transcripts were measured generally more than 100-fold less compared with levels of total TERRA. For example, NT cases demonstrate mean C_t values of 24.2, 30.7, and 33.1 for total, 2p, and 18p TERRA, respectively. Calculating the relative amount of TERRA by the exponent of the C_t value differences with base 2 results in an estimated 90- and 480-fold lower expression of 2p and 18p TERRA compared with total TERRA levels. Summing up, this may indicate the existence of different regulation mechanisms for chromosome-specific TERRA expression in astrocytoma.

Total TERRA expression in cell lines derived from astrocytoma tumors demonstrates RQ values similar to that measured in tumor tissues. However, in these cell lines, median RQ values of TERRA from 2p and 18p were lower when compared with their expression *in vivo*. From all cell lines analyzed, the osteosarcoma cell line SAOS-2 demonstrated the highest expression of total and 2p but not 18p TERRA. CpG islands of the promoters for 2p and 18p TERRA of SAOS-2 cells were determined recently *in vitro* as having low and high levels of methylation, respectively [25]. Thus, the observed chromosome-specific TERRA expression may reflect the methylation status of TERRA promoters also *in vivo*.

Subtelomere Methylation and TERRA Transcription of Chromosome 2p

The detailed epigenetic state of a CpG island within the subtelomeric region positioned approximately 1400 bp from the chromosomal 2p end [25] was determined by bisulfite allelic sequencing in a subset of grade 2/3 (n = 5) and grade 4 (n = 7) astrocytoma tumor samples (Figure 2). Sequence strategy, primer, and CpG positions are outlined (Figure 2*A*). Ratios of methylated *versus* unmethylated were obtained for 12 of 13 CpG positions analyzed, grouped for low and high 2p TERRA expression and visualized using a heat map. Average percentage methylation of the CpG island on chromosome 2p revealed dense cytosine methylation with little interindividual variation across all analyzed tumor tissues of 87% ± 5% (mean methylation ± SD) with occasional less methylated CpG positions occurring (not shown). Two cell lines served as controls. T98G cells contain low 2p TERRA level of RQ = 0.0218 and exhibit at CpG positions 1 to 9 very dense average methylation of 96% ± 3%. In contrast to these cells, SAOS-2 cells contain five-fold more 2p TERRA levels of RQ = 0.1134 and, at CpG positions 7 to 13, demonstrate a low average methylation of 17% ± 12% similar to a recent report [25].

Tumor tissues with low and high 2p TERRA levels selected for epigenetic analyzes demonstrated 3.6-fold difference between median transcript levels (Figure 2*B*). Mean methylation at the 12 CpG positions of 2p subtelomeric region analyzed for groups with low and high 2p TERRA expression differ with 89% and 83%, respectively (P = .0127). No significant difference in the epigenetic state between analyzed tumors of low and high grade was evident (P = .6506). Dense methylation of the analyzed CpG island close to the potential 2p TERRA promoter correlated with low TERRA expression.



	NT	DA	AA	GBM	CL
Median	313	31	14	1.0	2.8

	NT	DA	AA	GBM	CL
Median	6.0	1.7	1.2	1.0	0.1

	NT	DA	AA	GBM	CL
Median	4.2	0.7	3.2	1.0	0.1

Figure 1. TERRA expression in DA, AA, GBM, and cell lines (CL) compared to NT. The median RQ value for each individual group is indicated with horizontal line and is set as 1 for NT. Open circles indicate glioma cell lines; black triangle, Daoy cells; black cross, SAOS-2 cells. (A) Total TERRA shows down-regulation following tumor grades and cell lines. (B) 2p TERRA shows down-regulation for AA and GBM, and cell lines. (C) 18p TERRA shows no correlation with tumor grade. Results of statistics are indicated as extremely significant (***$P < .001$), very significant (**$P = .001$ to .01), significant (*$P = .01$ to .05), and not significant (ns, $P > .05$).

Comparison of TERRA Expression with Telomerase Activity and TL

TA and TL were correlated with TERRA transcript levels (Figure 3). TA was detectable by TRAP assays in tumor tissue samples in 14 GBM and ranged between 2 and 34 TPG units (Figure 3*A*). TA was not detectable in tumor tissue samples diagnosed for AA and DA. Of 14 analyzed tumor cell lines, 11 demonstrated TA with values up to 126 TPG units. The cell lines with no detectable TA were identified as DBTRG/05MG, YT-BO, and SAOS-2—the last one has been recognized to use ALT in place of TA [25].

TL was determined by quantitative real-time PCR assay as RQ values of telomere (T)-to-single-copy gene (S) ratios of the analyzed samples in relation to the median ratios as measured in the samples of NT tissues (Figure 3*B*). TL was increased 3.0-, 3.3-, and 2.8-fold in low-grade DA and AA tumors and cell lines compared with GBM. In contrast, TL in NT tissues was 0.15-fold the TL in GBM. TRF analyses resulted in a mean TL in kilobase pairs for a set of 12 tumor samples (Figure W1*A*). Log-transformed TL values of both methods correlated with $R^2 = 0.4271$ and $P = .0212$, similar to published data [27]. Linear regression analyses resulted a fitting line of [log]TRF = 0.6595 ± 0.2416*x*([log]RQ) + 0.3850 ± 0.2176 and allowed to calculate TL from RQ values as mean TRF lengths in kilobase pairs ± SD (Figure W2). Nontumor tissues exhibit short mean TRF lengths of 3.1 ± 2.5 kbp, and tumor tissues of astrocytoma grades 2, 3, and 4 exhibit 21.6 ± 14.9, 17.4 ± 4.5, and 8.8 ± 4.0 kbp, respectively, demonstrating that mean TL was increased in low-grade DA and AA tumors compared with GBM and NT tissue. Mean TRF lengths of the cell lines demonstrated with 18.4 ± 10.7 kbp comparable values. Remarkable, cell lines without detectable TA, like DBTRG/05MG, YT-BO, and SAOS-2, exhibit the longest telomeres of all cell lines

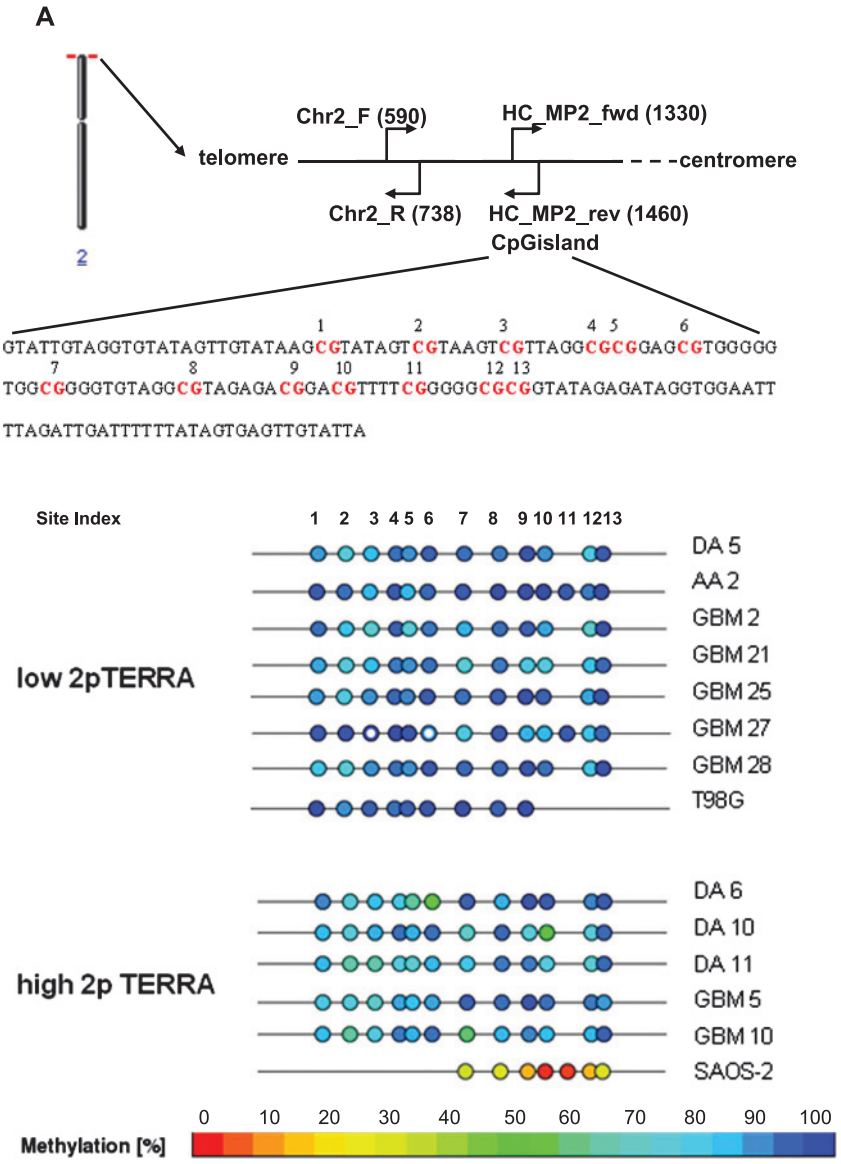


Figure 2. Subtelomere methylation and expression of chromosome 2p. (A) Upper panel illustrates the localization and sequence of chromosome 2p subtelomeric region studied. Locations of primers chr2_F and chr2_R used for quantification of 2p TERRA levels by real-time reverse transcription–polymerase chain reaction are indicated. Sequence represents the amplicon from 2p used for bisulfite allelic sequencing of 13 CpG positions labeled in red and flanked from both sides by indicated primers. Numbers in brackets indicate primer positions relative to the telomere. Lower panel shows the mean methylation of individual CpG positions in a panel of astrocytoma tissue samples and cell lines grouped for high and low expression of 2p TERRA. Colors of circles indicate the percentage of methylation as defined by heat map. (B) Upper panel shows significant difference of median RQ values (horizontal line) in the samples grouped for low and high expressions of 2p TERRA (Mann-Whitney test, $P = .0025$). Lower panels illustrate the difference in mean methylation (horizontal line) over all tested 13 CpG positions compared to 2p TERRA expression (left) and to tumor type (right) analyzed by unpaired *t* test with Welch correction.

analyzed. TRF analyses of YT-BO cells demonstrate the same TL profile as is seen in SAOS-2 (Figure W1*B*). These long telomeres suggest that the YT-BO astrocytoma cell line may use ALT as TMM [9].

The tumors were grouped according their TMM status (TA positive or negative, ALT based on high or low TL) and compared with TERRA expression (Figure 3*C*). Moreover, 17% (8/46) of tumors use TA as TMM, 37% (17/46) use ALT, 13% (6/46) use both TA and ALT, and for 33% (15/46), no TMM could be identified. Tumors with TA demonstrate 1.3-, 6-, and 12-fold decrease in total TERRA expression compared to tumors using both TA and ALT, no TMM, and ALT, respectively. Thus, total TERRA expression depends on the TMM used in astrocytomas and is highest if TA is absent. Furthermore, TA levels and RQ values of total TERRA demonstrate a moderate negative correlation after log transformation (Pearson $r = -0.54$) in the GBM cases with detectable TA (Figure 3*D*).

TMM, TERRA Expression, and Patient Survival

For most of the patients, overall survival data were available (Table 1). Median overall survival time correlated with tumor grade. Astrocytoma grades 4, 3, and 2 demonstrated median survival times of 17.4, 49.9, and longer than 50 months, respectively.

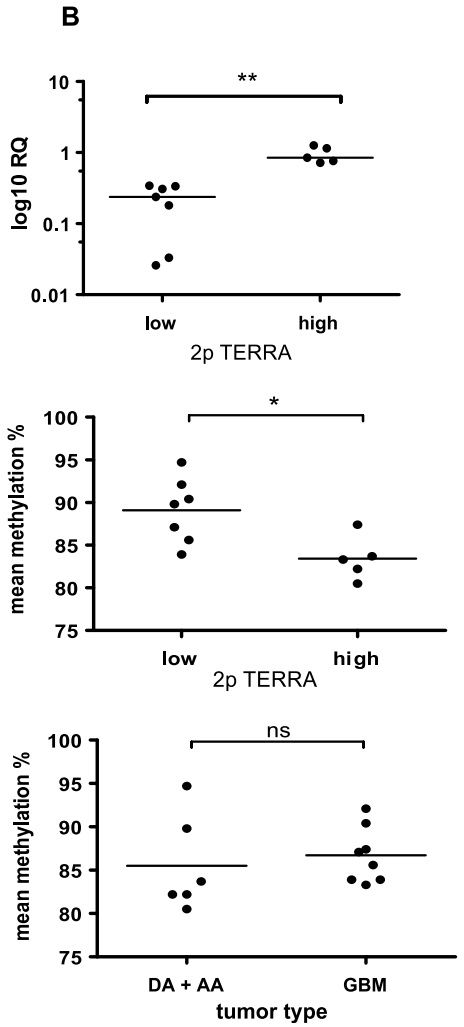


Figure 2. (*continued*).

Kaplan-Meier analyses were performed including individuals with all tumor grades of the study and with GBM only. No differences between groups defined by TA, TL, and TERRA expression were observed (Figure W3). However, TA, TL, and total TERRA showed a nonsignificant association with patient outcome when all tumor grades were included. Mantel-Cox test of these analyses revealed $P < .1$. In detail, absence of TA, long TL, and low TERRA expression were associated with better prognosis ($P = .0969$, $P = .0548$, and $P = .0563$, respectively). Survival analyses were performed between four TMM groups defined in Figure 3*C*, and a significant trend related to defined combinations between TA and TL was observed with $P = .0194$ (Figure 4*A*). Detection of TA and short telomeres predicted worse prognosis with a median survival time of 14.8 months. In contrast, the absence of telomerase and long telomeres, indicating ALT, predicted patients with long (>66 months) overall survival time. Both groups differ significantly in prediction of survival ($P = .0041$). Similar analyses on four groups defined by total TERRA expression levels and TA status resulted in the observation of a significant trend between groups with $P = .0148$ (Figure 4*B*). Detection of TA and low TERRA levels predicted worse prognosis with a median survival time of 15.6 months. In contrast, detection of TA and high TERRA levels predicted best outcome. The survival difference between the group of individuals with tumors showing TA and low TERRA levels and the group showing TA and high TERRA levels analyzed by Mantel-Cox test did not reach significance ($P = .09$). However, the survival difference between the group of individuals with grade 2 to 4 tumors showing TA and low TERRA and all other individuals was observed ($P = .0144$). We repeated all analyses with the GBM data set (Figure 4, *C* and *D*). Analyzing four groups defined by TL levels and TA did not demonstrate any significant correlations with P value of 0.0934 (Figure 4*C*). In contrast, analyzing four groups of GBM defined by TERRA levels and TA resulted in a significant trend for survival between these groups, with $P = .0222$ (Figure 4*D*). Our findings indicate that TERRA expression together with TA in astrocytoma may be prognostic for survival.

Discussion

In this study, we show that TERRA expression reflects telomerase-dependent and -independent TMM operating in astrocytoma and is associated with a favorable outcome. Patients with GBM still have a dismal prognosis despite aggressive treatment strategies [14], and new markers that show clinical significance are urgently needed [35].

Total TERRA expression inversely correlates with tumor grade as measured by telomeric repeat-specific PCR assay originally developed for determination of relative TL [26,27]. In comparison to TERRA levels from chromosome ends 2p and 18p measured by gene-specific PCR assays, significant more than 100-fold less amounts were detected. This is supported by the finding that the bulk of telomeric repeats in TERRA transcripts has an average length of 200 bases [36] and that beside chromosomes 2p and 18p further chromosome ends are transcribed [23,24]. The TERRA transcript levels from the two analyzed chromosomes 2p and 18p differ in correlation with tumor grade and thus indicate that chromosome ends are transcribed and regulated individually in astrocytoma. Our finding of a diagnostic value of TERRA levels in astrocytoma WHO grade 2 to 4 correspond with preliminary data in advanced stages of human tumors of larynx, colon, and lymph node [22].

Further, we show that, in astrocytoma, TERRA expression from chromosome 2p correlates with cytosine methylation of CpG islands in the promoter, suggesting epigenetic difference as a mechanism involved in regulation in tumors. These *in vivo* results were supported by our finding of pronounced epigenetic differences at TERRA promoter from chromosome 2p between two analyzed cell lines, T98G and SAOS-2, expressing low and high TERRA levels, respectively, and which are known to use TA and ALT, respectively. Similar epigenetic differences were observed *in vitro* between telomerase-positive and ALT human cell lines [25,37] and highlight that the epigenetic state of the telomere may influence TMM as shown in mice [38].

We evaluated the TMM by analyzing TA and TL as a marker for ALT in clinical material and cell lines as reported [19]. Fifty percent of GBM demonstrated TA-positive tumors and TA was undetectable in all tested astrocytoma grades 2 and 3, as has been reported by others [15–18]. In contrast, longer telomeres, a feature of tumor cells using ALT, were found in some of the other GBM samples studied and consistently in grade 2 and 3 tumors of our series. These latter results are in line with reports that detected ALT in 88% (7/8) of astrocytoma grades 2 and 3 and in 24% (26/109) or 15% (86/573) of GBM [8,20]. A recent report supports the finding of almost exclusive ALT-positive tumors in astrocytoma grades 1 to 3 with 93% [39]. We mainly studied cell lines derived from high-grade tumors, and most demonstrated TA. In contrast, two studied cell lines revealed no detectable TA, but long

telomeres were recognized and thus may represent candidates for ALT cell models [9]. Supporting our *in vitro* findings, a cell line was established recently from GBM, which demonstrated the ALT phenotype and furthermore showed characteristics of glioma stem cells [40]. Thus, *in vitro* both TA and ALT were shown to maintain the telomeres of GBM and were associated with treatment-resistant glioma stem cells. These cell lines are currently analyzed for TMM and tumor stem cell properties at our laboratory. Tumors grouped according their TL and TA status correlated with TERRA expression. TERRA expression was high in tumors with long telomeres and absent TA and low in tumors with short telomeres and detectable TA, suggesting that TERRA is associated with TA and ALT as TMM in astrocytoma. This finding together with the resulting inverse linear correlation between TA units and TERRA expression suggests an inhibitory function of TERRA on TA in astrocytoma. Indeed, TERRA was recognized *in vitro* as a natural ligand and direct inhibitor of telomerase [41]. Direct inhibition of telomerase enzymatic activity has shown efficiency against glioma cell lines and xenografts [42–44], and first telomerase-based cancer therapy approaches are tested in clinical studies [45]. Thus, TERRA could be a further candidate for clinical prognosis, like *O*-methylguanine methyltransferase promoter methylation for alkylating agent in chemotherapy [35].

TMM status did correlate with overall survival of all tumors studied. TA was identified exclusively in a subset of GBM and together with short telomeres resulted in the worst median survival time of 14.8 months. In contrast, total TERRA levels correlated with TMM, in detail with low or absent TA and presence of long telomeres indicating ALT and were associated with a favorable patient

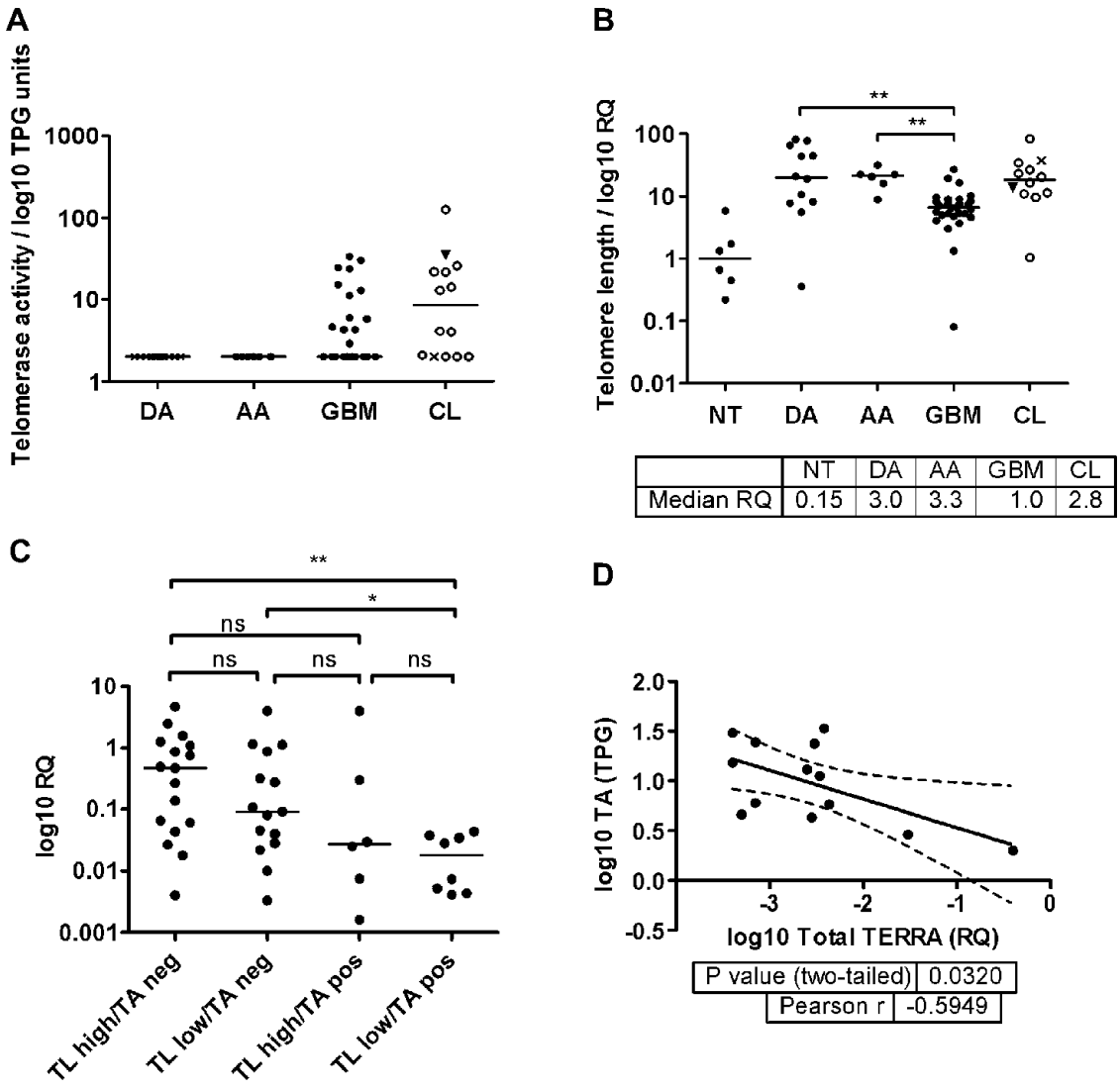


Figure 3. TA and TL compared to TERRA expression in astrocytoma. (A) TA was detectable in GBM and most of cell lines studied. Horizontal line represents the median and in groups DA and AA indicate the detection limit. (B) The median RQ value for relative TL of each individual group is indicated with a horizontal line and is set as 1 for NT. (C) TERRA expression and TMM. Tumors from patients were grouped: TA detected (TA pos), TA undetected (TA neg), long telomeres (TL high) indicating ALT, and short telomeres (TL low) indicating absence of ALT. Median TL of all tumors studied defined groups for long and short telomeres. The median RQ value for total TERRA expression of each individual group is indicated with a horizontal line. (D) Linear regression analyses of TA TPG units and total TERRA RQ values show a negative relation. Slashed lines indicate 95% confidence interval; black line, linear regression line best fitting the data.

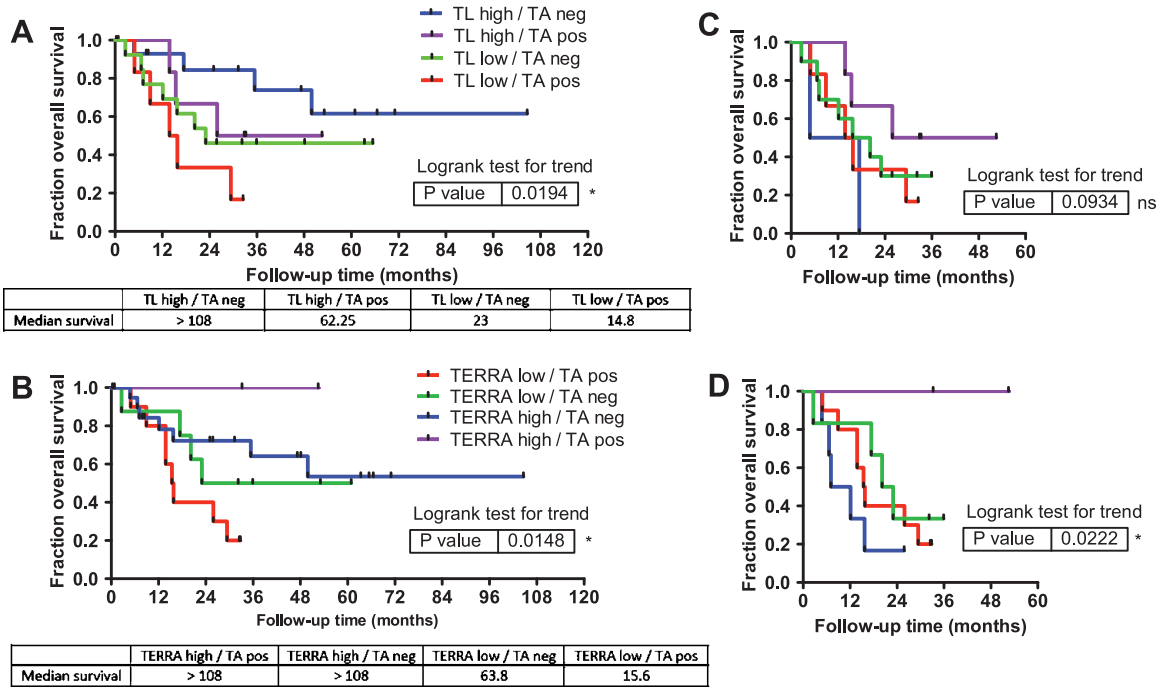


Figure 4. Kaplan-Meier analysis for overall survival defined as time from diagnosis until astrocytoma-related death. Median survival time for each group in months is listed below graphs. Undefined means not reached within follow-up time. *P* values indicate significance of survival differences as a trend between the groups analyzed by log-rank test. (A) Patients were pooled into four groups depending on TMM status: telomerase detectable (TA pos) or not detectable (TA neg) and long (TL high) or short (TL low) telomere length indicating ALT. Significant correlation for a trend between TMM and patient outcome was observed ($P = .0194$). (B) Patients were pooled into four groups: telomerase detectable (TA pos) or not detectable (TA neg) and high and low total TERRA expression. A significant correlation for a trend between groups and patient outcome was observed ($P = .0146$). Groups of TMM (C) and of TA/TERRA (D) were applied for GBM survival analyses, without ($P = .0934$) and with ($P = .0222$) the finding of significant correlation for a trend between groups and patient outcome, respectively.

prognosis. High TA and expression were reported to be significant predictors of worse prognosis in astrocytoma of higher grades [17]. In contrast, long telomeres and ALT have been associated with better outcome in grade 4 tumors [19,20] and are detected more common in astrocytoma of lower grades [8]. Survival of patients with GBM grouped for TA, TL, and TERRA expression did not result in a correlation with outcome. However, analyzing combined groups defined by TERRA and TA did result in a trend for survival between these groups, for both patients with astrocytoma including all grades and with GBM. In detail, detection of TA and low TERRA levels predicted worse prognosis with a median survival time of 15.6 months. In contrast, detection of TA and high TERRA levels predicted best outcome. Our data suggest that TERRA expression together with TA in astrocytoma might be of prognostic significance. Future studies including higher numbers of GBM may allow the identification of an association between survival and TERRA expression within subtypes of GBM, similar to those published for GBM subtypes using ALT as TMM with clear survival benefit [19,20], and for GBM subtypes identified based on gene signatures with differences in methylation phenotype and therapy response [46].

In summary, our data indicate that total TERRA levels are downregulated in astrocytoma involving epigenetic silencing by promoter methylation and reflect TMM status with prognostic potential. Furthermore, we show that TERRA originating from different chromosome ends is independently regulated. It remains to be investigated how individual TERRA transcripts may influence the TMM of all or single chromosomes. Our results of TERRA expression and TA showed correlation with outcome in this patient series, suggesting that further investigation into the correlation of TERRA and TA as prognostic markers in gliomas is worthwhile. Further, our findings suggest the importance of analyzing TERRA and TA especially in clinical trials with telomerase as a treatment target investigating responsiveness and resistance.

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References

[1] Samassekou O, Gadji M, Drouin R, and Yan J (2010). Sizing the ends: normal length of human telomeres. *Ann Anat* **192**, 284–291.
[2] Colgin LM and Reddel RR (1999). Telomere maintenance mechanisms and cellular immortalization. *Curr Opin Genet Dev* **9**, 97–103.
[3] Hanahan D and Weinberg RA (2000). The hallmarks of cancer. *Cell* **100**, 57–70.
[4] Neumann AA and Reddel RR (2002). Telomere maintenance and cancer—look, no telomerase. *Nat Rev Cancer* **2**, 879–884.
[5] Shay JW and Bacchetti S (1997). A survey of telomerase activity in human cancer. *Eur J Cancer* **33**, 787–791.
[6] Blackburn EH, Greider CW, and Szostak JW (2006). Telomeres and telomerase: the path from maize, *Tetrahymena* and yeast to human cancer and aging. *Nat Med* **12**, 1133–1138.

[7] Koziel JE, Fox MJ, Steding CE, Sprouse AA, and Herbert BS (2011). Medical genetics and epigenetics of telomerase. *J Cell Mol Med* **15**, 457–467.

[8] Henson JD, Hannay JA, McCarthy SW, Royds JA, Yeager TR, Robinson RA, Wharton SB, Jellinek DA, Arbuckle SM, Yoo J, et al. (2005). A robust assay for alternative lengthening of telomeres in tumors shows the significance of alternative lengthening of telomeres in sarcomas and astrocytomas. *Clin Cancer Res* **11**, 217–225.

[9] Henson JD and Reddel RR (2010). Assaying and investigating alternative lengthening of telomeres activity in human cells and cancers. *FEBS Lett* **584**, 3800–3811.

[10] Bryan TM, Englezou A, Gupta J, Bacchetti S, and Reddel RR (1995). Telomere elongation in immortal human cells without detectable telomerase activity. *EMBO J* **14**, 4240–4248.

[11] Hiyama E, Hiyama K, Yokoyama T, Matsuura Y, Piatyszek MA, and Shay JW (1995). Correlating telomerase activity levels with human neuroblastoma outcomes. *Nat Med* **1**, 249–255.

[12] Heaphy CM and Meeker AK (2011). The potential utility of telomere-related markers for cancer diagnosis. *J Cell Mol Med* **15**, 1227–1238.

[13] Mabruk MJ and O'Flatharta C (2005). Telomerase: is it the future diagnostic and prognostic tool in human cancer? *Expert Rev Mol Diagn* **5**, 907–916.

[14] Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, Belanger K, Brandes AA, Marosi C, Bogdahn U, et al. (2005). Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* **352**, 987–996.

[15] Falchetti ML, Larocca LM, and Pallini R (2002). Telomerase in brain tumors. *Childs Nerv Syst* **18**, 112–117.

[16] Langford LA, Piatyszek MA, Xu R, Schold SC Jr, and Shay JW (1995). Telomerase activity in human brain tumours. *Lancet* **346**, 1267–1268.

[17] Boldrini L, Pistolesi S, Gisfredi S, Ursino S, Ali G, Pieracci N, Basolo F, Parenti G, and Fontanini G (2006). Telomerase activity and hTERT mRNA expression in glial tumors. *Int J Oncol* **28**, 1555–1560.

[18] Chen YJ, Hakin-Smith V, Teo M, Xinarianos GE, Jellinek DA, Carroll T, McDowell D, MacFarlane MR, Boet R, Baguley BC, et al. (2006). Association of mutant TP53 with alternative lengthening of telomeres and favorable prognosis in glioma. *Cancer Res* **66**, 6473–6476.

[19] Hakin-Smith V, Jellinek DA, Levy D, Carroll T, Teo M, Timperley WR, McKay MJ, Reddel RR, and Royds JA (2003). Alternative lengthening of telomeres and survival in patients with glioblastoma multiforme. *Lancet* **361**, 836–838.

[20] McDonald KL, McDonnell J, Muntoni A, Henson JD, Hegi ME, von Deimling A, Wheeler HR, Cook RJ, Biggs MT, Little NS, et al. (2010). Presence of alternative lengthening of telomeres mechanism in patients with glioblastoma identifies a less aggressive tumor type with longer survival. *J Neuropathol Exp Neurol* **69**, 729–736.

[21] Azzalin CM, Reichenbach P, Khoriauli L, Giulotto E, and Lingner J (2007). Telomeric repeat containing RNA and RNA surveillance factors at mammalian chromosome ends. *Science* **318**, 798–801.

[22] Schoeftner S and Blasco MA (2008). Developmentally regulated transcription of mammalian telomeres by DNA-dependent RNA polymerase II. *Nat Cell Biol* **10**, 228–236.

[23] Nergadze SG, Farnung BO, Wischnewski H, Khoriauli L, Vitelli V, Chawla R, Giulotto E, and Azzalin CM (2009). CpG-island promoters drive transcription of human telomeres. *RNA* **15**, 2186–2194.

[24] Chawla R and Azzalin CM (2008). The telomeric transcriptome and SMG proteins at the crossroads. *Cytogenet Genome Res* **122**, 194–201.

[25] Ng LJ, Cropley JE, Pickett HA, Reddel RR, and Suter CM (2009). Telomerase activity is associated with an increase in DNA methylation at the proximal subtelomere and a reduction in telomeric transcription. *Nucleic Acids Res* **37**, 1152–1159.

[26] Cawthon RM (2002). Telomere measurement by quantitative PCR. *Nucleic Acids Res* **30**, e47.

[27] O'Callaghan N, Dhillon V, Thomas P, and Fenech M (2008). A quantitative real-time PCR method for absolute telomere length. *Biotechniques* **44**, 807–809.

[28] Holzmann K, Ambrosch I, Elbling L, Micksche M, and Berger W (2001). A small upstream open reading frame causes inhibition of human major vault protein expression from a ubiquitous mRNA splice variant. *FEBS Lett* **494**, 99–104.

[29] Baelde HJ, Cleton-Jansen AM, van Beerendonk H, Namba M, Bovee JV, and Hogendoorn PC (2001). High quality RNA isolation from tumours with low cellularity and high extracellular matrix component for cDNA microarrays: application to chondrosarcoma. *J Clin Pathol* **54**, 778–782.

[30] Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, and Shay JW (1994). Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011–2015.

[31] Pfaffl MW (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**, e45.

[32] Kim NW and Wu F (1997). Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). *Nucleic Acids Res* **25**, 2595–2597.

[33] Sagmeister S, Eisenbauer M, Pirker C, Mohr T, Holzmann K, Zwickl H, Bichler C, Kandioler D, Wrba F, Mikulits W, et al. (2008). New cellular tools reveal complex epithelial-mesenchymal interactions in hepatocarcinogenesis. *Br J Cancer* **99**, 151–159.

[34] Clark GM, Osborne CK, Levitt D, Wu F, and Kim NW (1997). Telomerase activity and survival of patients with node-positive breast cancer. *J Natl Cancer Inst* **89**, 1874–1881.

[35] Tabatabai G, Stupp R, van den Bent MJ, Hegi ME, Tonn JC, Wick W, and Weller M (2010). Molecular diagnostics of gliomas: the clinical perspective. *Acta Neuropathol* **120**, 585–592.

[36] Porro A, Feuerhahn S, Reichenbach P, and Lingner J (2010). Molecular dissection of telomeric repeat-containing RNA biogenesis unveils the presence of distinct and multiple regulatory pathways. *Mol Cell Biol* **30**, 4808–4817.

[37] Vera E, Canela A, Fraga MF, Esteller M, and Blasco MA (2008). Epigenetic regulation of telomeres in human cancer. *Oncogene* **27**, 6817–6833.

[38] Gonzalo S, Jaco I, Fraga MF, Chen T, Li E, Esteller M, and Blasco MA (2006). DNA methyltransferases control telomere length and telomere recombination in mammalian cells. *Nat Cell Biol* **8**, 416–424.

[39] Slatter T, Gifford-Garner J, Wiles A, Tan X, Chen YJ, MacFarlane M, Sullivan M, Royds J, and Hung N (2010). Pilocytic astrocytomas have telomere-associated promyelocytic leukemia bodies without alternatively lengthened telomeres. *Am J Pathol* **177**, 2694–2700.

[40] Silvestre DC, Pineda JR, Hoffschir F, Studler JM, Mouthon MA, Pflumio F, Junier MP, Chneiweiss H, and Boussin FD (2011). Alternative lengthening of telomeres in human glioma stem cells. *Stem Cells* **29**, 440–451.

[41] Redon S, Reichenbach P, and Lingner J (2010). The non-coding RNA TERRA is a natural ligand and direct inhibitor of human telomerase. *Nucleic Acids Res* **38**, 5797–5806.

[42] Hashizume R, Ozawa T, Gryaznov SM, Bollen AW, Lamborn KR, Frey WH II, and Deen DF (2008). New therapeutic approach for brain tumors: intranasal delivery of telomerase inhibitor GRN163. *Neuro Oncol* **10**, 112–120.

[43] Marian CO, Cho SK, McEllin BM, Maher EA, Hatanpaa KJ, Madden CJ, Mickey BE, Wright WE, Shay JW, and Bachoo RM (2010). The telomerase antagonist, imetelstat, efficiently targets glioblastoma tumor-initiating cells leading to decreased proliferation and tumor growth. *Clin Cancer Res* **16**, 154–163.

[44] Ozawa T, Gryaznov SM, Hu LJ, Pongracz K, Santos RA, Bollen AW, Lamborn KR, and Deen DF (2004). Antitumor effects of specific telomerase inhibitor GRN163 in human glioblastoma xenografts. *Neuro Oncol* **6**, 218–226.

[45] Roth A, Harley CB, and Baerlocher GM (2010). Imetelstat (GRN163L)-telomerase-based cancer therapy. *Recent Results Cancer Res* **184**, 221–234.

[46] Verhaak RG, Hoadley KA, Purdom E, Wang V, Qi Y, Wilkerson MD, Miller CR, Ding L, Golub T, Mesirov JP, et al. (2010). Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1. *Cancer Cell* **17**, 98–110.

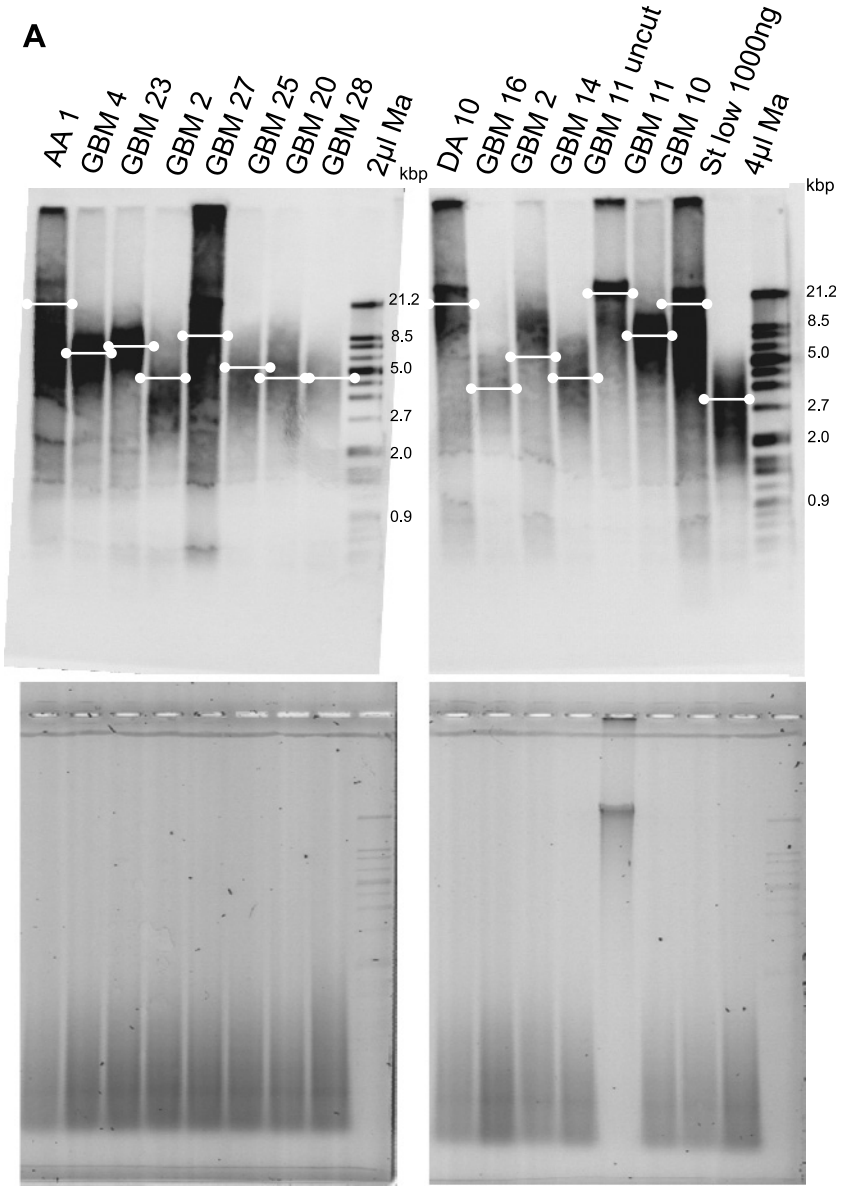
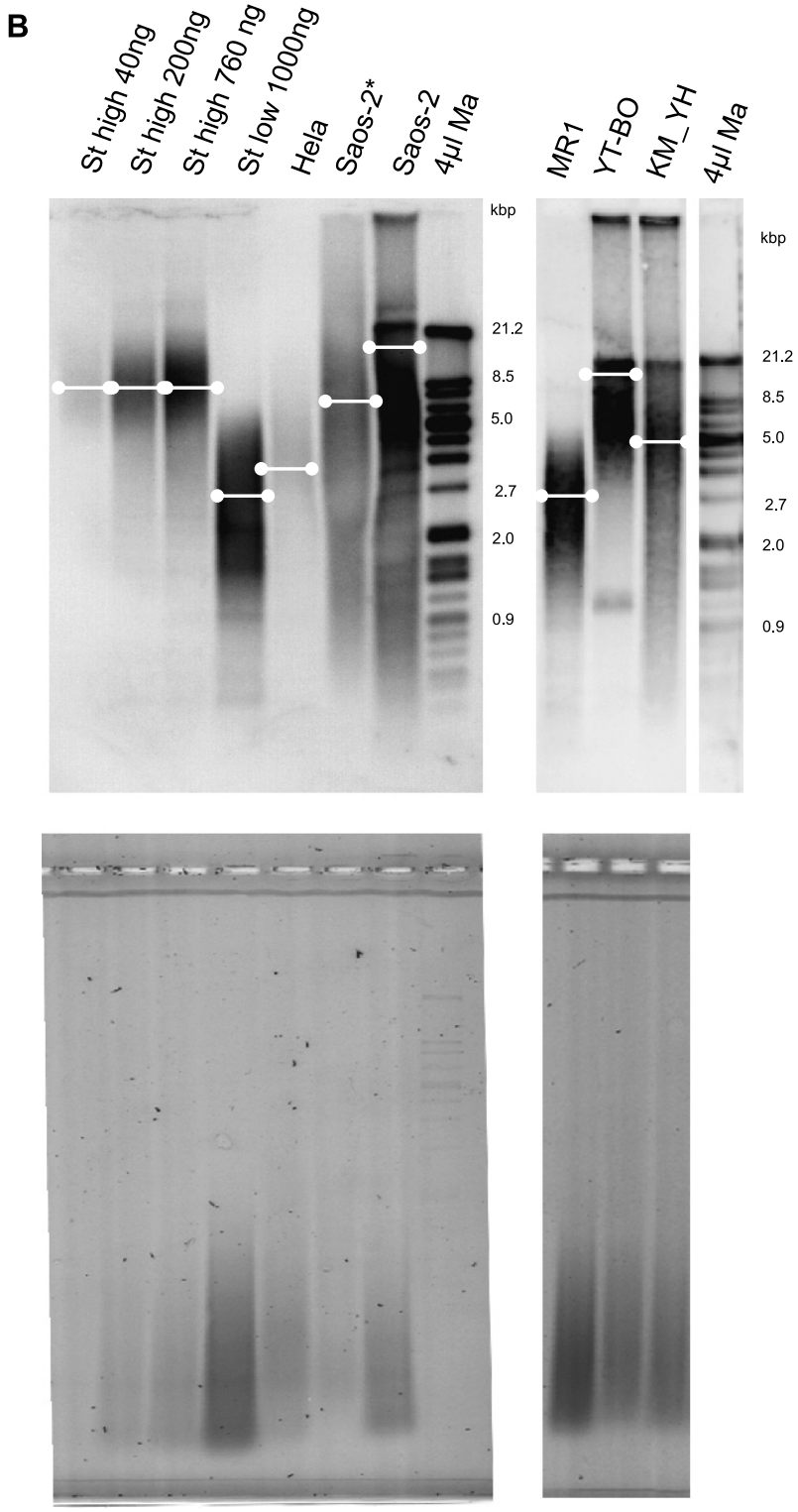


Figure W1. Mean TL analyses by TRF Southern blots. (A) TRF blots of a set of tumor tissue samples. (B) TRF blots of cell lines. Lanes with molecular weight size markers (Ma) and standards from Telo-TAGGG-Telomere-Length-Assay with long (St high) and short (St low) telomeres applied with different amounts are indicated. DNA stained with EtBr was visualized in agarose gels before blot analysis and shown below each TRF blot. GBM11 was applied without restriction digest (GBM11 uncut) to validate the system. Results obtained with applied different amounts of telomere standard (St high; 40, 200, and 760 ng) indicate the broad reproducibility of the method. For further validation, DNA extracted from SAOS-2 by TRIzol reagent was applied in lane SAOS-2* and resulted in a different telomere pattern when compared to DNA extracted by high-quality DNA isolation system (lane SAOS-2). Thus, only high-quality DNA isolation was applied for TRF analyses. Mean TRFs in kilobase pairs were determined, and they are indicated as white bars.



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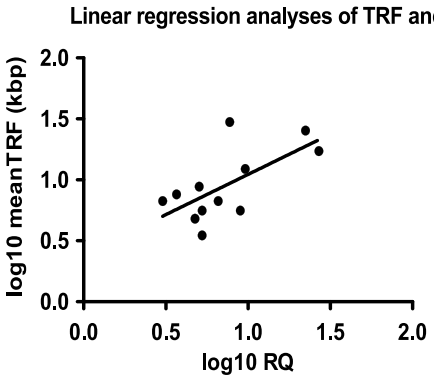

$$y = 0.6595 \pm 0.2146 * x + 0.3850 \pm 0.2176$$

$$R^2 = 0.427; P = 0.0212$$

Conversion of RQ into mean TRF

TL (kbp)	NT	DA	AA	GBM	CL
mean TRF	3.1	21.6	17.4	8.8	18.4
SD	2.5	14.9	4.5	4.0	10.7

Figure W2. Linear regression analyses of TRF and quantitative PCR results. Relative T/S-RQ values determined by quantitative PCR and mean TRF lengths in kilobase pairs determined by Southern blots correlated after log transformation. The resulting equation for the linear regression line best fitting the data, the correlation coefficient, and the *P* value are shown. The table sums up the mean TRF values in kilobase pairs and SD for individual groups after conversion of the relative T/S-RQ values with the equation.

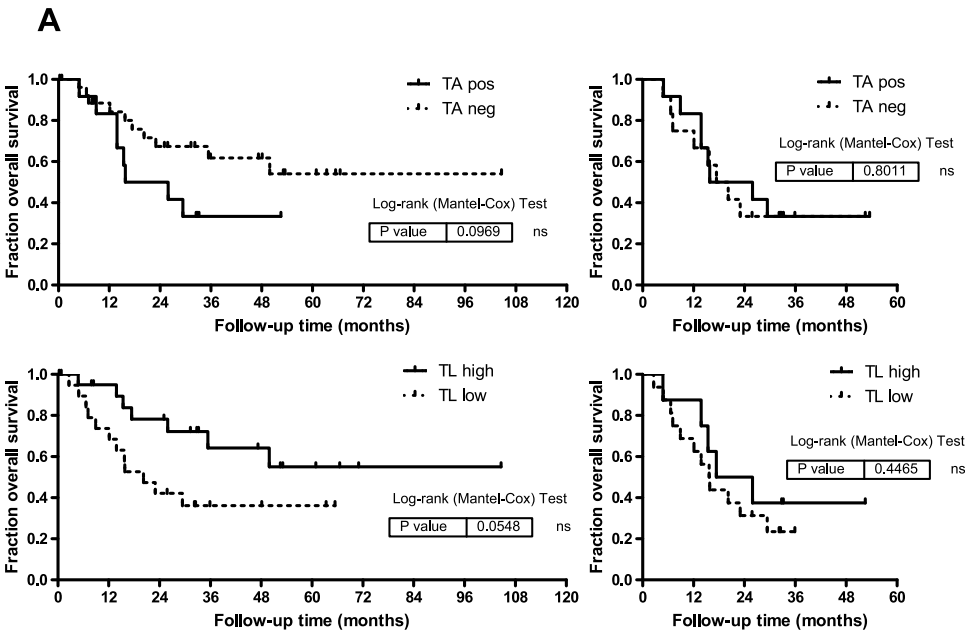

Figure W3. Kaplan-Meier analysis for overall survival defined as time from diagnosis until astrocytoma-related death. Individuals were pooled into two groups and analyzed for association with survival using patients with astrocytoma of all grades studied (left) or GBM (right). *P* values indicate significance of survival differences between the groups. (A) For TA, patients were grouped in positive or negative, when TA was detectable or absence, respectively. For TL, patients were pooled in groups of long and short telomeres, in TL high and TL low, respectively. (B) For total, 2p, and 18p TERRA, patients were pooled in groups of high and low expression.

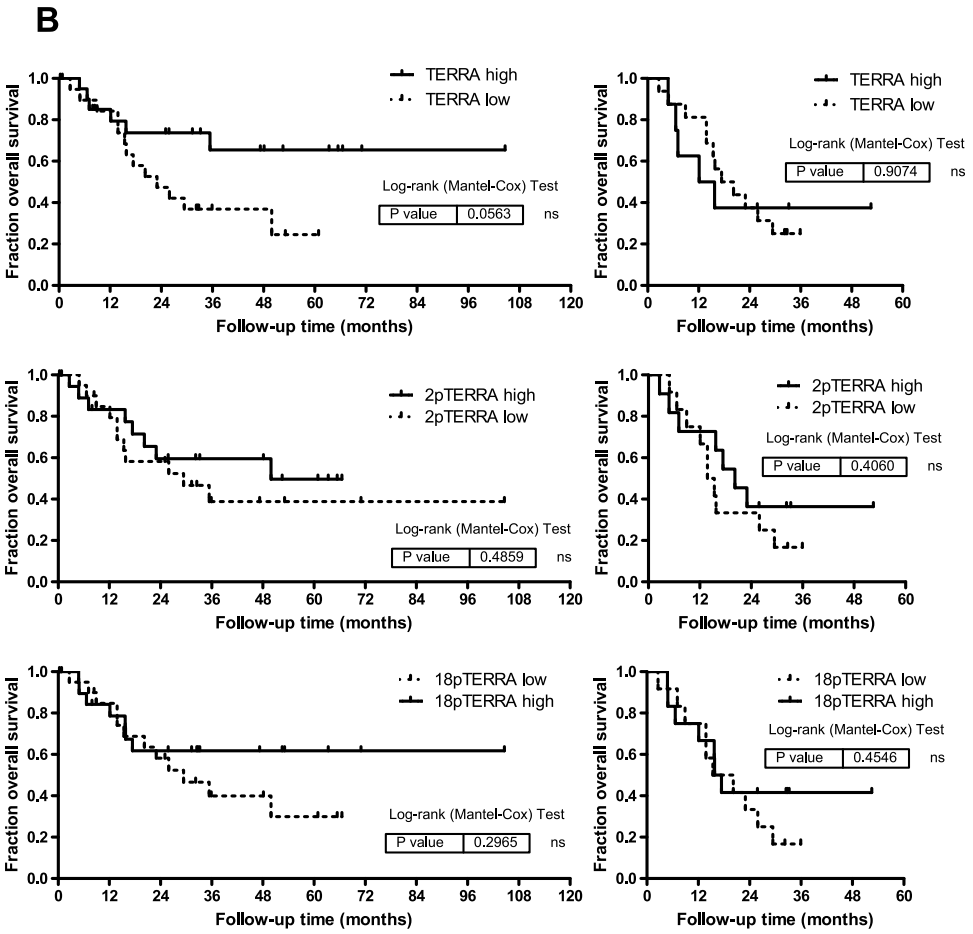

Figure W3. (*continued*).