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Expression of Telomeric Repeat Binding Factor-1 in Astroglial Brain Tumors

OBJECTIVE: In human somatic cells, telomeres shorten with successive cell divisions, resulting in progressive genomic instability, altered gene expression, and cell death. Recently, telomere-specific deoxyribonucleic acid-binding proteins, such as telomeric repeat binding factor-1 (TRF1), have been proposed as candidates for the role of molecules regulating telomerase activity, and they have been suggested to play key roles in the maintenance of telomere function. The present study was designed to assess TRF1 expression in human astroglial brain tumors and to speculate on the clinical implications of its expression.

METHODS: Twenty flash-frozen surgical specimens obtained from adult patients who underwent craniotomy for microsurgical tumor resection, histologically verified as World Health Organization Grade II to IV astrocytomas, were used. Expression of TRF1 in astrocytomas of different grades was studied by means of both immunohistochemical and Western blotting analysis. The correlation between the extent of TRF1 expression and histological grading, performance status, and length of survival of patients underwent statistical analyses.

RESULTS: TRF1 was expressed in all tumor samples. The level of its expression was variable, decreasing from low-grade through high-grade astrocytomas ($P = 0.0032$). TRF1 expression correlated with the patient's length of survival ($P < 0.001$) and performance status ($P < 0.001$) and proved to be an independent indicator of length of survival.

CONCLUSION: Our findings suggest that the loss of TRF1 expression capability, as a result of down-regulation of TRF1 expression in malignant gliomas cells, may play a role in the malignant progression of astroglial brain tumors.

KEY WORDS: Astroglial tumor, Brain tumor, Glioma, Telomere, Telomeric repeat binding factor

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Astroglial tumors represent the largest tumor entity in the central nervous system. Despite notable advances achieved during recent decades in both surgical and chemotherapeutic/radiotherapeutic approaches, an improved survival has not been clearly documented. The World Health Organization (WHO) guidelines for diffusely infiltrating astrocytomas distinguish three grades of malignancy (Grades II–IV) on the basis of histopathological features that predict patients' survival (26, 27). The transition from a low-grade astrocytoma (LGA) to a glioblastoma is associated with a stepwise accumulation of genetic mutations. Although age, preoperative Karnofsky Performance Scale (KPS) status, extent of resection, and histopathological malignancy grade constitute widely recognized prognostic factors, additional indicators are needed to more accurately determine the patient's prognosis and to identify novel therapeutic approaches that can optimize the patient's outcome. Moreover, it is impossible to predict the clinical outcome for the individual patient on the basis of histological grade alone, because astrocytomas of the same tumor grade may behave differently. In the staging of carcinogenesis, two phenomena have been proposed. One is malignant transformation, which is the result of a multistep process in which cells acquire mutations of genes that activate proto-oncogenes or negate the action of tumor suppressor genes. The other phenomenon in carcinogenesis is immortalization

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(20). It has recently been speculated that changes in telomere domain can result in genetic disorders and genomic variability (33). Telomeres consist of long tandem arrays of TTAGGG repeats bound by proteins, placed at the end of linear chromosomes, which are involved in several essential biological functions (4, 12). Telomeres protect chromosomes from recombination, end-to-end fusion, and recognition as damaged deoxyribonucleic acid (DNA), providing a means for complete replication of chromosomes. Furthermore, telomeres contribute to the functional organization of chromosomes within the nucleus, participate in the regulation of gene expression, and serve as a molecular clock that controls the replicative capacity of human cells and their entry into senescence (4, 12). In human somatic cells, telomeres shorten with successive cell divisions, resulting in progressive genomic instability, altered gene expression, and cell death (42) via p53-dependent or -independent mechanisms (10, 51). This shortening in normal human cells could monitor their replicative history (12, 21, 46). In cancer cells, telomere length is dependent on the balance between the loss of telomeric repeats during DNA replication and the elongation of telomeric repeats mediated by telomerase (54). In human tumors, telomere length is generally reduced. In human gliomas, hypervariability in length was observed (29, 33, 37). These findings lead to the speculation that changes in telomere domain contribute to the fundamental molecular and structural alterations that create genomic instability in tumors (33). Telomerase is a ribonucleoprotein that synthesizes telomeric repeats onto chromosome ends by use of an endogenous ribonucleic acid as a template and provides the molecular basis for unlimited proliferative potential (7, 8, 36). Two major subunits of the human telomerase core complex have been identified, namely, hTERC and hTERT. The first identified subunit, hTERC, serves as a template for telomere elongation by telomerase, whereas the second subunit, hTERT, contains a reverse transcriptase domain that catalyzes this reaction (10). Recently, telomere-specific DNA-binding proteins, such as TRF1, have been put forward as additional candidates for the role of molecules modifying telomerase activity, and they have been suggested to play key roles in the maintenance of telomere function (1, 36, 48). TRF1 is a mammalian telomeric protein that binds to the duplex array of TTAGGG repeats at chromosome ends. TRF1 has a number of biochemical similarities to Rap1p, a distantly related DNA-binding protein that functions at telomeres in yeast (5, 9). Like Rap1p, TRF1 requires two Myb motifs for DNA binding. Human TRF1 was found to form a homodimer through interactions involving the N-terminal half of the protein. Like the Rap1p protein, TRF1 may have an architectural role at telomeres. DNA bending by those telomeric proteins could induce a higher-order structure at telomeres that is required for their function. In particular, T-loop formation induced by TRF1 may act as a negative regulator of telomere lengthening operated by the telomerase (19). Thus, overexpression of TRF1 inhibits telomere elongation in telomerase-positive cells (45), resulting in gradual and progressive telomere shortening to the "mortality stages," the proliferative barriers that lead to a nondividing state and cell death (9, 45, 47). TRF1 accepts adenosine diphosphate ribosylation catalyzed by the tankyrase PARP. The adenosine diphosphate ribosylation of TRF1 diminishes its ability to bind to telomeric DNA, allowing telomerase to elongate telomeres and extending cellular life span (13, 40, 44). The mutation or deletion of TRF1 can result in telomere elongation and extend cell survival (47). Overexpression of a dominant negative TRF1, which removes endogenous TRF1 from telomeres, results in telomere lengthening in telomerase-positive cells (24, 47). Therefore, the expression of TRF1 is considered a physiological homeostatic mechanism that controls the proliferative potential of normal cells by inhibiting the activity of telomerase (45).

The main goal of the present study was to assess the extent of TRF1 expression in human astroglial brain tumors of different histological grading. An attempt to correlate the level of TRF1 expression with the patient's clinical features was also made.

PATIENTS AND METHODS

Patient Population

This study included tumor samples, histologically verified as WHO Grade II to IV astrocytomas, obtained from adult patients who had undergone craniotomy for microsurgical brain tumor resection at the Neurosurgical Clinic of the University of Messina School of Medicine (*Table 1*). All tumors were located in the supratentorial compartment.

Only patients who had undergone large, gross total resection of their neoplasms (>95% of the tumor volume) were eligible for the study. The patients affected by anaplastic astrocytoma (AA) and glioblastoma multiforme (GBM) were treated, after surgical removal of the tumor, by fractionated whole-brain irradiation (60 Gy). Patients with LGAs did not undergo chemotherapy or radiotherapy after tumor resection. Samples obtained from single or multiple stereotactic biopsies were not included in the present study. We carefully excluded tumors containing components that were suspect for oligodendroglioma. No case of recurrent tumors and no patient who underwent adjuvant therapy (radiotherapy and/or chemotherapy) before surgery were used in the present study.

Tissue Samples

Twenty surgical specimens were evaluated. All tumor tissue samples were obtained rapidly from resection specimens within 15 minutes after surgical tissue removal. Three to seven anatomically separate areas of tumor tissue were sampled from each resection specimen, according to the volume of resected tissue available. Specimens were taken from viable areas of tumor, avoiding areas of gross necrosis. Tissue samples for the histological, immunohistochemical, and Western blot analysis were taken from the same general region as the tumor during surgery. Tissue was placed in cryovials and immediately flash-frozen in liquid nitrogen in the operating room and stored at −70°C. Both the adjacent tissue samples

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TABLE 1. Summary of demographic and clinical data of 20 patients with different grades of astrocytoma[a]

Patient no.	Age (yr)/sex	WHO grade	Localization	Duration of symptoms (wk)	KPS score	Survival (wk)	TRF1 (WB)
1	39/M	II	L T	12	100	208[b]	690.7
2	42/M	II	R F	12	100	208[b]	673.5
3	31/F	II	L FT	1	100	208[b]	652.6
4	62/F	III	L FT	20	100	123	564
5	76/M	III	L O	12	80	62	522.6
6	70/M	III	R TO	16	60	48	375
7	29/M	III	L T	16	100	178	672.6
8	72/F	III	R T	8	100	158	722
9	72/M	III	R FT	24	100	158	537
10	74/M	III	R PO	12	80	53	543
11	68/F	III	L T	4	60	45	386
12	74/F	III	L PO	4	100	130	715
13	62/M	IV	L FPT	8	80	65	536
14	72/M	IV	R FPT	20	80	41	130
15	47/M	IV	L FT	12	100	27	280
16	79/F	IV	R T	8	90	37	433
17	61/M	IV	L O	4	80	60	522
18	73/M	IV	R FTP	4	80	32	128.5
19	50/M	IV	L TP	2	100	30	298
20	81/F	IV	L TP	12	90	88	452

[a] WHO, World Health Organization; L, left; R, right; F, frontal; P, parietal; T, temporal; O, occipital; KPS, Karnofsky Performance Scale; TRF1, telomeric repeat binding factor-1; WB, western blotting.
[b] Still surviving at the end of follow-up (208 wk).

matched to the frozen tissue and additional tissue submitted *in toto* from the resection specimens were used for histological typing and grading. Normal brain tissue was used as control.

TRF1 Immunohistochemical Assessment

TRF1 assessment was performed on corresponding mirror-image sections with a goat polyclonal antibody, anti-TRF1 (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). As a revealing system, secondary antibody biotin-streptavidin-peroxidase (AEC kit; Universal Quick Kit; Vector Laboratories, Burlingame, CA) was used. Briefly, surgical specimens were mounted in embedding medium for frozen tissue specimens and sectioned to 5-μm thickness with a 2800 Frigocut Cryostat (Reichert-Jung, Heidelberg, Germany). Sections were mounted on glass slides and fixed in ice-cold acetone for 5 minutes. Each section was then rinsed three times in phosphate-buffered saline, incubated for 5 minutes in 3% hydrogen peroxide (H_2O_2) to inhibit endogen peroxidase, and then incubated overnight at 4°C with the primary antibody against TRF1. After being rinsed three times in phosphate-buffered saline, sections were incubated (15 min at room temperature) with a secondary antibody (Vectastain Universal Quick Kit; Vector), rinsed three times in phosphate-buffered saline, then labeled (15 min at room temperature) with streptavidin-peroxidase preformed complex (Universal Quick Kit; Vector). Positive signal was stained brown-red with 3-amino-9-ethylcarbazole (AEC; Vector), counterstained with hematoxylin, and mounted on aqueous mounting medium. As negative control, the primary antibody was substituted with nonimmune serum. Samples were then observed with a Zeiss

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photomicroscope (Carl Zeiss Co., Oberkochen, Germany). The percentage of cells exhibiting positive staining for TRF1 was determined for each case. The area that seemed to have the highest density of labeled cells was selected for counting. Only clearly reactive nuclei were counted as positive. The number of positive nuclei in a total of 100 cells was counted in five high-power fields (×200). The mean of the obtained value was determined for each case. Sections were independently evaluated by two of the authors, who were blinded to the patient's identity. We intentionally decided not to use the intensity of staining as a grading criterion, because an "intensity score" is highly subjective and prone to variations among runs. A preliminary analysis of these independent evaluations exhibited substantial interobserver correlation.

Protein Extraction, Electrophoresis, and Immunoblotting

Frozen tumor tissues (~50 mg) were harvested by homogenization with a Potter homogenizer in 15 volumes of ice-cold triple detergent lysis buffer (20 mmol/L *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, 9.2 mmol/L ethyleneglycol- *O*,*O*'-bis-[2-amino-ethyl]- *N*,*N*,*N*',*N*'-tetra-acedic acid, 1 mmol/L ethylenediamine tetra-acetic acid, 150 mmol/L NaCl, 0.1% sodium dodecyl sulfate, 1% Igepal 40, 0.5% deoxycholic acid, pH 7.5; protease inhibitors: 0.5 mmol/L phenylmethylsulfonyl fluoride, apronin, pepstatin, and leupeptin [10 μg/ml each]).

The concentration of total proteins in the samples was determined by a Lowry method assay with albumin standards. Approximately 50 μg of total proteins was resolved in 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and proteins were transferred laterally to nitrocellulose membranes in a transfer buffer containing 0.192 mol/L glycine and 0.025 mol/L Tris (pH 8.3) with 10% methanol at a constant voltage of 100 V for 1 hour at 4°C. Blots were blocked for 1 hour at room temperature in 5% nonfat milk in Tris-buffered saline and 0.05% Tween-20.

Immunoblots were probed with goat polyclonal antibody anti-TRF1 (Santa Cruz Biotechnology, Inc.), which recognizes TRF1 at 76 kD. After incubation with primary antibody (1:200) at room temperature for 2 hours, blots were incubated with a rabbit anti-mouse immunoglobulin G secondary antibody (1: 1000; Dako, Carpinteria, CA) conjugated to peroxidase at room temperature for 1 hour. Enhanced chemiluminescence reagents were used to visualize immunolabeling on Kodak Biomax ML chemiluminescent film (Amersham Biosciences, Little Chalfont, Buckinghamshire, England). We used three different exposures for each blot.

Quantification of TRF1 Level

Semiquantitative evaluation of protein levels detected by immunoblotting was performed by computer-assisted densitometric scanning (AlphaImager 4.2 Digital Imaging System, Milan, Italy). Different times of exposure were used for each blot (15–25 s), and longer exposures were performed in an attempt to detect very low levels of TRF1 in normal brain tissue. Data were acquired as integrated densitometric values and expressed as percentages of the densitometric levels obtained on scans from normal brain tissue used as control visualized on the same blot in arbitrary densitometric units (ADU). We used two different negative controls for each blot.

Statistical Data Analysis

Statistical data analysis was performed by use of one-way analysis of variance with the Tukey post hoc correction for multiple comparisons to compare the level of expression of TRF1, as quantified by Western blot analysis, in LGAs, AAs, and GBMs. The Spearman nonparametric correlation test was used to assess the correlation between TRF1 expression and the other included variables (WHO grading, survival, KPS score, age). The χ^2 log-rank test was used to compare the survival curves of patients with different expression of TRF1, and results were displayed by use of a Kaplan-Meier curve. Finally, to assess whether TRF1 was an independent indicator of survival, a multivariate analysis was performed using the logistic regression model. Survival was used as the independent variable and TRF1 expression, age, and KPS score as potential independent variables. Those variables were transformed into binary variables to be used in the logistic regression model.

Computer software programs (INSTAT [version 3.0] and PRISM [version 4.0], GRAPHPAD, San Diego, CA; and MedCalc [version 7.2.1.0], Mariakerke, Belgium) were used to perform the data analysis. A probability value of $P < 0.05$ was considered statistically significant. All values were expressed as mean ± standard deviation.

RESULTS

Twenty tumor samples were included in the study. The tumor samples were resected from 13 men and 7 women, whose ages ranged from 31 to 81 years; the mean age of these patients at surgery was 61.3 ± 16.1 years. KPS scores ranged from 60 to 100% (mean, 89 ± 13.4%). According to the revised WHO classification (22, 23), tumors were diagnosed as LGA (3 patients), AA (9 patients), and GBM (8 patients).

Three patients with LGAs had an average age of 37.3 ± 5.6 years; KPS score was 100% in all cases, and all patients' length of survival was longer than 208 weeks. Nine patients with AAs had an average age of 66.3 ± 14.6 years; the KPS score was 86.7 ± 17.3%, and the patients' length of survival was 106.1 ± 53.9 weeks. Eight patients with GBMs had an average age of 65.6 ± 12.7 years; the KPS score was 87.5 ± 8.9%, and the patients' length of survival was 47.5 ± 21.4 weeks. Total length of follow-up was 208 weeks after surgery.

Immunohistochemical TRF1 Expression

TRF1 immunoreactivity was expressed at variable intensity and distribution by tumor cells in 8 (40%) of 20 cases. In most cells, the signal was generally weak and was observed in both the nucleus and the cytoplasm. The percentage of tumor cells

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exhibiting positive staining for TRF1 was 3.2 ± 1.4%. TRF1 expression varied from 6 to 10% in LGAs, from 0 to 8% in AAs, and from 0 to 1% in GBMs (*Fig. 1*). TRF1 expression was absent in normal brain tissue samples. Because of the low percentage of immunostaining cells in TRF1-positive samples, immunohistochemical results for TRF1 were not considered for statistical analysis.

Western Blot Analysis for TRF1

A 76-kD TRF1 band was evidenced in all analyzed tumor samples and was absent in normal brain tissue (*Fig. 2A*). Densitometric analysis of bands revealed that such expression varied among tumor samples, with a mean value of 500.6 ± 187.7 ADU. Mean TRF1 expression levels in LGAs were 672.4 ± 19.1 ADU; in AAs, those levels were 559.7 ± 127.3 ADU; and in GBMs, the TRF1 levels were 369.8 ± 200.7 ADU (*Fig. 2B*), with a significant statistical difference ($P = 0.0032$).

Correlation of TRF1 Expression with Grading, KPS Score, and Length of Survival

The correlation between TRF1 expression levels, as measured by use of Western blot analysis, and histological grading of tumor samples was assessed by use of the Spearman's nonparametric correlation test. A statistically significant inverse correlation was found ($r^2 = 0.47$; $P < 0.001$) (*Fig. 3*). The same statistical test was used to measure the correlation between TRF1 expression levels and KPS score and age. A statistically significant correlation was recorded between TRF1 levels and KPS score, with an $r^2 = 0.21$ and $P = 0.039$ (*Fig. 4*). No statistically significant correlation was found between TRF1 expression and age.

The Kaplan-Meier analysis showed that patients with TRF1 expression of less than 500 ADU (mean value) had shorter

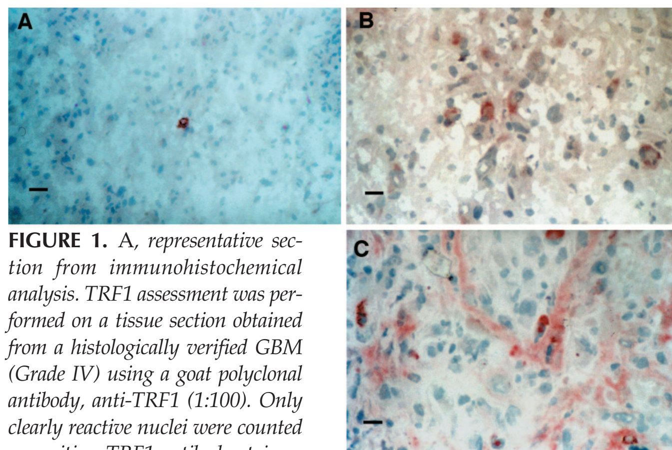

FIGURE 1. A, *representative section from immunohistochemical analysis. TRF1 assessment was performed on a tissue section obtained from a histologically verified GBM (Grade IV) using a goat polyclonal antibody, anti-TRF1 (1:100). Only clearly reactive nuclei were counted as positive. TRF1 antibody stains a single cancer cell* (red-brown), *whereas the other cells are negative (original magnification, ×63; scale bar = 160 μm).* B, *representative section from immunohistochemical analysis performed on a tissue section obtained from a histologically verified AA. TRF1 antibody stains a few cancer cells at low intensity* (red-brown). *The vacuolar aspects are a result of freezing artifact (original magnification, ×250;* scale bar *= 40 μm). The number of positive nuclei in a total of 100 cells was counted in five high-power fields. TRF1 expression was scored as 8% of positive cells.* C, *representative section from immunohistochemical analysis performed on a tissue section obtained from a histologically verified low-grade astrocytoma. TRF1 antibody stains both nuclei and cytoplasm of a few cancer cells* (red-brown) *and stains microvasculature at lower intensity (original magnification, ×250;* scale bar *= 40 μm). The number of positive nuclei in a total of 100 cells was counted in five high-power fields. TRF1 expression was scored as 10% of positive cells.*

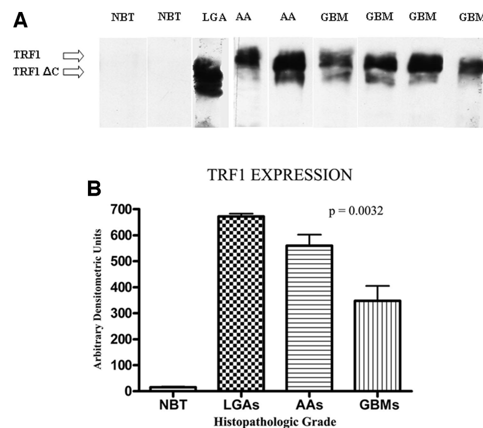

FIGURE 2. A, *representative Western blot. Immunoblots were probed with goat polyclonal antibody anti-TRF1 at 76 kD. After incubation with primary antibody (1:200) at room temperature for 2 hours, blots were incubated with a rabbit anti-mouse immunoglobulin G secondary antibody (1:1000) conjugated to peroxidase at room temperature for 1 hour. Variable levels of TRF1 were detected in tumor specimens, with decreasing expression levels from low-grade through high-grade astrocytomas. Time of exposure was 15 minutes. Apparent double-banding in some of the tumor samples was because of the intrinsic characteristic of the polyclonal antibody detecting full-length and C-terminally deleted TRF1 (TRF1 ΔC).* NBT, *normal brain tissue;* LGA, *low-grade astrocytoma.* B, *semiquantitative densitometric analysis of the expression of TRF1 in different astroglial brain tumors. Bar graph showing the different TRF1 expression levels (x axis) according to the histopathological diagnosis (y axis) in three low-grade astrocytomas (672.4 ± 19.1), nine AAs (559.7 ± 127.3), and eight GBMs (369.8 ± 200.7).* Error bars *indicate standard deviation. A one-way analysis of variance with the Tukey post hoc correction for multiple comparisons was used to compare the levels of expression of TRF1, as quantified by Western blot analysis, in LGAs, AAs, and GBMs (*$P = 0.0032$*).*

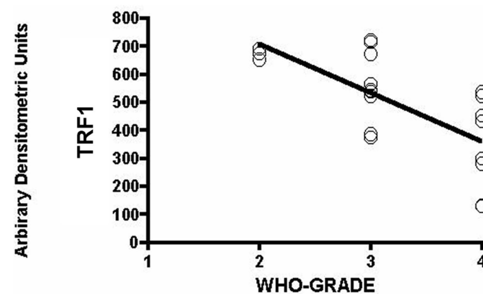

FIGURE 3. *Graph showing correlation between TRF1 expression levels, based on semiquantitative densitometric analysis, and the WHO tumor grade.* $P < 0.001$*; Spearman* $r^2 = 0.47$*.*

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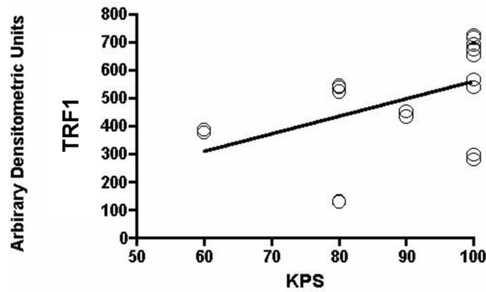

FIGURE 4. *Graph showing correlation between TRF1 expression levels, based on semiquantitative densitometric analysis, and the KPS score.* $P < 0.0039$; *Spearman* $r^2 = 0.21$.

survival times than those with values of greater than 500 ADU (log-rank test $P < 0.001$) (*Fig. 5*). To assess whether TRF1 expression was an independent prognostic factor, a linear logistic regression analysis was performed. To perform the analysis, length of survival was used as the dependent variable and compared with three different independent variables: age, KPS score, and TRF1 expression, age and KPS score being well-known independent prognostic factors in human gliomas. TRF1 expression (using the binary value of <500 or >500 ADU) turned out to be an independent prognostic factor for length of survival ($P = 0.032$; odds ratio, 14.0).

DISCUSSION

The aim of our study was to assess the expression of TRF1 in diffusely infiltrating astrocytomas of different histological grades. Using Western blot analysis, we found that TRF1 is expressed at variable levels in astroglial brain tumors, whereas its expression was not detectable in normal brain tissue. Moreover, its expression seems to decrease from low-grade through high-grade astrocytomas. As in almost all tumors, malignant brain tumors are reported to be associated with higher telomerase activity than benign tumors, such as neuromas and meningiomas (41), or normal brain (50). Telomerase expression has also been associated with high proliferative index, grade of tumor, age, vascular endothelial proliferation (28), and poor outcome (22, 32), and it increases with malignancy from low-grade to high-grade astrocytomas (29). In light of the recently identified role for telomerase in the process of tumorigenesis, and in particular in the progression of glioma malignancy, moving toward the study of molecular mechanisms controlling telomerase activity was the next logical research step and constituted the rationale of the present investigation. Furthermore, it has been reported that telomerase activity does not always correlate with the telomere length found in cancer cells (35, 54). This means that the length of the telomere may be regulated by factors other than telomerase activity. In particular, in human brain tumors, it was recently speculated that a number of gliomas are telomerase-negative, suggesting that, in addition to the telomerase-dependent mechanism, a telomerase-independent mechanism for telomere length maintenance may be present in human gliomas (35). These findings confirm that the regulation of telomerase access to telomeres in human cells is not yet fully understood. In the past few years, an increasing number of telomere-associated proteins and their interacting partners have been identified (15, 17, 39). Collectively, these telomeric proteins may function to protect telomere integrity and functionality, to connect the DNA damage repair network with the controls of cellular senescence, to monitor telomere homeostasis, and to modify the access of telomerase to telomeres. Recent studies indicate that telomere-associated proteins can regulate telomerase accessibility in either positive or negative ways (16). The first identified telomeric protein, TRF1, specifically binds to duplex telomeric DNA and is involved in T-loop formation (19), thus acting as a negative regulator of telomere length. Overexpression of TRF1 inhibits telomere elongation in telomerase-positive cells (45). TRF1 is involved in a negative feedback mechanism that stabilizes telomere length (9, 11, 18, 43, 45, 47). Antisense experiments with human telomerase ribonucleic acid indicate that telomerase inhibition may lead to telomere shortening and cell death in human cell lines (35). However, telomerase activity is absent in a number of human tumors, such as in some astroglial brain tumors (35). This means that a telomerase-independent mechanism for the regulation of telomere lengthening possibly exists in these telomerase-negative tumors (35). Recent in vitro studies indicate that not only hTERT but also TRF1 is an important regulator of telomerase activity in pancreatic cancer cells (52). Overexpression of TRF1 in the tetracycline-responsive human fibrosarcoma cell line HTCC75 resulted in a gradual decline in telomere length (38), and it has been reported that the forced tethering of a large number of TRF1 molecules to a single telomere induces a significant shortening of telomere length (1). Yokota et al. (54) suggest that TRF1 is up-regulated in hepatocellular carcinoma with elongated telomeres. These findings confirm the hypothesis that in human cancer cells, an overexpression of TRF1 may result in a progressive telomere shortening. An important question is how TRF1, which binds along the length of the telomere, modulates telomerase, an enzyme that acts at telomere termini. One mechanism that could be considered in this context is that accessibility of a DNA end to telomerase is diminished by the presence of TRF1

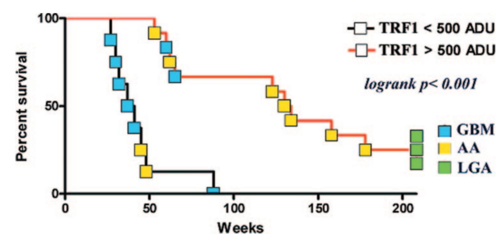

FIGURE 5. *Kaplan-Meier survival curves for groups of diffusely infiltrating astrocytomas classified according to their TRF1 expression. Follow-up was ended at 208 weeks after surgery. Survival was significantly longer in patients affected by astrocytic tumors with TRF1 expression levels >500 ADU than in those with a lower expression log-rank* $P < 0.001$.

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on or near the telomere terminus. An alternative proposal has recently emerged from the finding that telomeres fold back, forming a large duplex lariat called the T loop (19). In the T loop, the 3' single-stranded telomeric overhang of TTAGGG repeats is tucked into the duplex part of the telomeric repeat tract. The T loop is proposed to sequester telomeres from activities that might act on chromosome ends, including telomerase. In vitro studies suggest that telomerase requires an accessible 3' overhang (30, 31, 49), a structure predicted to be absent from T loops. Therefore, T loops could control the action of telomerase at individual chromosome ends. On the basis of biochemical studies, the formation of T loops was proposed to involve both TRF1 and TRF2. TRF1 induced bending, looping, and pairing of duplex telomeric DNA (5, 6, 18, 19), facilitating the folding back of the telomere.

As far as we know, no other studies regarding TRF1 expression in brain tumors are available in the current literature. To address this issue, we recently analyzed TRF1 expression in a pilot panel of human brain tumors, including benign neoplasms such as meningiomas and malignant histotypes such as AA (3, 14). Typically, TRF1 was expressed to various extents in meningiomas and was far less detectable in AA (WHO Grade III) (3). This previous observation suggested that TRF1 expression was inversely correlated to proliferation and malignancy. In the present investigation, we analyzed TRF1 expression in 20 human brain glioma specimens, including LGAs (WHO Grade II), AAs (WHO Grade III), and GBMs (WHO Grade IV). TRF1 was detected in all tumor samples, whereas it was not detected in normal brain tissue. Moreover, its expression seemed to be inversely correlated with histological grading. Of note, it is somewhat paradoxical that TRF1 levels are undetectable in normal brain tissue but that lower levels correlate with higher tumor grade. However, our observation of an absence of a constitutive expression of TRF1 in the nonneoplastic brain tissue is consistent with similar observations in gastrointestinal, lung, and adrenal tissues (2, 23, 36).

Furthermore, a tissue-specific behavior can be suggested because of the contrasting observations reported in the current literature regarding the role of TRFs. Actually, a down-regulation of TRF1 gene expression may be important to maintain telomere length in gastric cancer (53), whereas up-regulation of specific telomere-binding proteins, such as TRF2, may contribute to telomere maintenance in malignant lymphoma (25).

Our observations are consistent with those of Matsutani et al. (34) on gastric cancer. In that study, TRF expression levels were higher in cancer cells compared with nonneoplastic mucosa. Furthermore, the authors assessed that there are cancer cells with long telomeres, high telomerase activity, and higher TRF expression, and there are cancer cells with short telomeres, low telomerase activity, and lower TRF1 expression levels. Ohyashiki et al. (38) reported that TRF1 expression was significantly elevated in patients with acute lymphoblastic leukemia compared with those in acute myeloid leukemia and that TRF1 expression tended to be higher in patients without telomere shortening and in those with hTERT expression. Together, these observations suggest that TRF1 may act to monitor telomere length under the condition of up-regulated telomerase activity in some neoplastic cells.

According to the model of Smogorzewska et al. (45), telomeres can exist in two states: an "open" state, which allows telomerase to elongate the telomere, and a "closed" state, in which the enzyme cannot access or extend the telomere terminus. The switching between these two states is proposed to be governed by the telomere binding proteins TRF1 and TRF2 in human cells, which act as negative regulators through promoting the closed state. It can be suggested that in some nonneoplastic cells, short telomeres would not bind sufficient amounts of TRF1 or TRF2. In cancer cells, as telomeres are elongated by telomerase, they will bind a greater number of the negative regulators, increasing the chance of a switch to the closed state. This is therefore consistent with our observations that glioma cells express higher levels of TRF1 compared with normal brain tissue and with our previous observations that TRF1 is expressed at a high level in meningiomas as well (3, 14).

After switching to the closed state, namely the T-loop state, telomerase would no longer be able to elongate the telomere terminus. The telomere will gradually lose sequences with each cell division, leading to a smaller number of bound negative regulators and an improved chance of switching back to the open state. As a result, each individual telomere will approach a steady-state length determined by the activity of telomerase, the expression level of TRF1 and TRF2, and other regulatory factors (e.g., tankyrase). Eventually, this sequence loss would result in diminished binding of TRF1 and TRF2 to the telomere, which would consequently form T loops at a lower rate (or at a reduced frequency). The resulting (temporary) persistence of an unfolded telomere, a substrate for telomerase, would then again lead to telomere elongation (45).

TRF proteins are therefore necessary to limit telomere elongation. In the early stages of carcinogenesis of human gliomas, this mechanism of control can be induced by the telomere elongation by telomerase; in later stages, this control mechanism can be lost.

To completely ascertain the truth of this hypothesis, the mutational status of cells, the correlation with genomic instability, and telomere length as well as telomerase activity should be analyzed in these cells. The lack of these data represents a strong limit of the study. Nevertheless, this article represents a preliminary study to assess whether TRF1 may play a role in the multifactorial oncogenesis of human gliomas. Further studies are warranted to draw definitive conclusions.

CONCLUSION

TRF1 was expressed in astroglial brain tumors of different grades, whereas it was not expressed in normal brain tissue. Such expression decreased from low-grade through high-grade astrocytomas. This finding may suggest that the loss of TRF1 expression capability, being the result of down-

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regulation of TRF1 expression in malignant gliomas cells, may play a role in the cell immortalization of astroglial brain tumors. Confirmation of a role for TRF1 in the carcinogenesis of diffusely infiltrating astrocytomas would require a large-scale study using molecular techniques, showing that this parameter does indeed influence the tumor sequence. The next step for the near future may be to determine how to incorporate such biological information into the treatment of diffusely infiltrating astrocytomas.

REFERENCES

1. Ancelin K, Brunori M, Bauwens S, Koering CE, Brun C, Ricoul M, Pommier JP, Sabatier L, Gilson EA: Targeting assay to study the cis functions of human telomeric proteins: Evidence for an inhibition of telomerase by TRF1 and for activation of telomere degradation by TRF2. **Mol Cell Biol** 22:3474–3487, 2002.
2. Aragona M, Buda Ca, Panetta S, Morelli M, Giudice A, Campagna Fl, Pontoriero A, Cascinu S, La Torre F: Immunohistochemical telomeric-repeat binding factor-1 expression in gastrointestinal tumors. **Oncol Rep** 7:987–990, 2000.
3. Aragona M, De Divitiis O, La Torre D, Panetta S, D'avella D, Pontoriero A, Morelli M, La Torre I, Tomasello F: Immunohistochemical TRF1 expression in human primary intracranial tumors. **Anticancer Res** 21:2135–2139, 2001.
4. Aragona M, Maisano R, Panetta S, Giudice A, La Torre F: Telomere length maintenance involvement in aging and cancerogenesis. **Int J Oncol** 17:981–989, 2000.
5. Bianchi A, Smith S, Chong L, Elias P, de Lange T: TRF1 is a dimer and bends telomeric DNA. **EMBO J** 16:1785–1794, 1997.
6. Bianchi A, Stansel RM, Fairall L, Griffith JD, Rhodes D, de Lange T: TRF1 binds a bipartite telomeric site with extreme spatial flexibility. **EMBO J** 18:5735–5744, 1999.
7. Blackburn EH: Structure and function of telomeres. **Nature** 350:569–573, 1991.
8. Blasco MA, Funk W, Villeponteau B, Greider CW: Functional characterization and developmental regulation of mouse telomerase RNA. **Science** 269:1267–1272, 1995.
9. Broccoli D, Smogorzewska A, Chong L, de Lange T: Human telomeres contain two distinct Myb-related proteins, TRF1 and TRF2. **Nat Genet** 17:231–235, 1997.
10. Chin L, Artandi SE, Shen Q, Tonn A, Lee SL, Gottlieb GJ, Greider CW, DePinho RA: p53 deficiency rescues the adverse effects of telomere loss and cooperates with telomere dysfunction to accelerate carcinogenesis. **Cell** 97:527–538, 1999.
11. Chong L, van Steensel B, Broccoli D, Erdjument-Bromage H, Hanish J, Tempst P, de Lange T: A human telomeric protein. **Science** 270:1663–1667, 1995.
12. Cong Y-S, Wright WE, Shay JW: Human telomerase and its regulation. **Microbiol Mol Biol Rev** 66:407–425, 2002.
13. d'Adda di Fagagna F, Hande PM, Tong WM, Lansdorp PM, Wang ZQ, Jackson SP: Functions of poly(ADP-ribose) polymerase in controlling telomere length and chromosomal stability. **Nat Gen** 23:76–80, 1999.
14. de Divitiis O, La Torre D: Inverse correlation of TRF1 expression and cell proliferation in human primary intracranial tumors. **J Neurosurg Sci** 45:1–6, 2001.
15. de Lange T: Protection of mammalian telomeres. **Oncogene** 21:532–540, 2002.
16. Evans SK, Lundblad V: Positive and negative regulation of telomerase access to the telomere. **J Cell Sci** 113:3357–3364, 2000.
17. Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, Adams RR, Chang E, Allsopp RC, Yu J, Le S, West MD, Harley CB, Andrews WH, Greider CW, Villeponteau B: The RNA component of human telomerase. **Science** 269:1236–1241, 1995.
18. Griffith J, Bianchi A, de Lange T: TRF1 promotes parallel pairing of telomeric tracts in vitro. **J Mol Biol** 278:79–88, 1998.
19. Griffith JD, Comeau L, Rosenfield S, Stansel RM, Bianchi A, Moss H, de Lange T: Mammalian telomeres end in a large duplex loop. **Cell** 97:503–514, 1999.
20. Harada K, Kurisu K, Tahara H, Tahara E, Ide T, Tahara E: Telomerase activity in primary and secondary glioblastoma multiforme as a novel molecular tumor marker. **J Neurosurg** 93:618–625, 2000.
21. Harley CB, Vaziri H, Counter C, Allsopp RC: The telomere hypothesis of cellular aging. **Exp Gerontol** 27:375–382, 1992.
22. Hiraga S, Ohnishi T, Izumoto S, Miyahara E, Kanemura Y, Matsumura H, Arita N: Telomerase activity and alterations in telomere length in human brain tumors. **Cancer Res** 58:2117–2125, 1998.
23. Kanauchi H, Nobuyuki W, Ginzinger DG, Yu M, Wong MG, Clark OH, Duh QY: Diagnostic and prognostic value of Fas and telomeric-repeat binding factor-1 genes in adrenal tumors. **J Clin Endocrinol Metab** 88:3690–3693, 2003.
24. Karlseder J, Smogorzewska A, de Lange T: Senescence induced by altered telomere state, not telomere loss. **Science** 295:2446–2449, 2002.
25. Klapper W, Krams M, Qian W, Janssen D, Parwaresch R: Telomerase activity in B-cell non-Hodgkin lymphomas is regulated by hTERT transcription and correlated with telomere-binding protein expression but uncoupled from proliferation. **Br J Cancer** 89:713–719, 2003.
26. Kleihues P, Burger PC, Scheithauer BW: *Histological Typing of Tumor of the Central Nervous System*. Berlin, Springer-Verlag, 1993, ed 2.
27. Kleihues P, Louis DN, Scheithauer BW, Rorke LB, Reifenberger G, Burger PC, Cavenee WK: The WHO classification of tumors of the nervous system. **J Neuropathol Exp Neurol** 61:215–225, 2002.
28. Kleinschmidt-DeMasters BK, Hashizumi TL, Sze CI, Lillehei KO, Shroyer AL, Shroyer KR: Telomerase expression shows differences across multiple regions of oligodendroglioma versus high grade astrocytomas but shows correlation with Mib-1 labelling. **J Clin Pathol** 51:284–293, 1998.
29. Le S, Zhu JJ, Anthony DC, Greider CW, Black PMcL: Telomerase activity in human gliomas. **Neurosurgery** 42:1120–1124, 1998.
30. Lee MS, Blackburn EH: Sequence-specific DNA primer effects on telomerase polymerization activity. **Mol Cell Biol** 13:6586–6599, 1993.
31. Lingner J, Cech TR: Purification of telomerase from *Euplotes aediculatus*: Requirement of a primer 3' overhang. **Proc Natl Acad Sci U S A** 93:10712–10717, 1996.
32. Maitra A, Yashima K, Rathi A, Timmons CF, Rogers BB, Shay JW, Gazdar AF: The RNA component of telomerase as a marker of biologic potential and clinical outcome in childhood neuroblastic tumours. **Cancer** 85:741–749, 1999.
33. Manuelidis L: Genomic stability and instability in different neuroepithelial tumors: A role for chromosome structure? **J Neurooncol** 18:225–239, 1994.
34. Matsutani N, Yokozaki H, Tahara E, Tahara H, Kuniyasu H, Haruma K, Chayama K, Yasui W, Tahara E: Expression of telomeric repeat binding factor 1 and 2 and TRF1-interacting nuclear protein 2 in human gastric carcinomas. **Int J Oncol** 19:507–512, 2000.
35. Morii K, Tanaka R, Onda K, Tsumanuma I, Yoshimura J: Expression of telomerase RNA, telomerase activity, and telomere length in human gliomas. **Biochem Biophys Res Commun** 239:830–834, 1997.
36. Nakanishi K, Kawai T, Kumaki F, Hiroi S, Mukai M, Ikeda E, Koering CE, Gilson E: Expression of mRNAs for telomeric repeat binding factor (TRF)-1 and TRF2 in atypical adenomatous hyperplasia and adenocarcinoma of the lung. **Clin Cancer Res** 9:1105–1111, 2003.
37. Nürnberg P, Thiel G, Weber F, Epplen JT: Changes of telomere lengths in human intracranial tumors. **Hum Genet** 91:190–192, 1993.
38. Ohyashiki JH, Hayashi S, Yahata N, Iwama H, Ando K, Tauchi T, Ohyashiki K: Impaired telomere regulation mechanism by TRF1 (telomere-binding protein), but not TRF2 expression, in acute leukemia cells. **Int J Oncol** 18:593–598, 2001.
39. Pandita TK: ATM function and telomere stability. **Oncogene** 21:611–618, 2002.
40. Pennisi E: A possible new partner for telomerase. **Science** 282:1395–1397, 1999.
41. Sano T, Asai A, Mishima K, Fujimaki T, Kirino T: Telomerase activity in 144 brain tumours. **Br J Cancer** 77:1633–1637, 1998.
42. Shay JW, Pereira-Smith O, Wright WE: A role for both RB and p53 in the regulation of human senescence. **Exp Cell Res** 196:33–39, 1991.

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43. Shore D: Telomeres: Unsticky ends. **Science** 281:818–819, 1998.
44. Smith S, Giriat I, Schmitt A, de Lange T: Tankyrase, a poly(ADP-ribose) polymerase at human telomeres. **Science** 282:1484–1487, 1998.
45. Smogorzewska A, van Steensel B, Bianchi A, Oelmann S, Schaefer MR, Schnapp G, de Lange T: Control of human telomere length by TRF1 and TRF2. **Mol Cell Biol** 20:1659–1668, 2000.
46. Takakura M, Kyo S, Sowa Y, Wang Z, Yatabe N, Maida Y, Tanaka M, Inoue M: Telomerase activation by histone deacetylase inhibitor in normal cells. **Nucleic Acids Res** 29:3006–3011, 2001.
47. van Steensel B, de Lange T: Control of telomere length by the human telomeric protein TRF1. **Nature** 385:740–743, 1997.
48. van Steensel B, Smogorzewska A, de Lange T: TRF2 protects human telomeres from end-to-end fusions. **Cell** 92:401–413, 1998.
49. Wang H, Blackburn EH: De novo telomere addition by Tetrahymena telomerase in vitro. **EMBO J** 16:866–879, 1997.
50. Weil RJ, Wu YY, Vortmeyer AO, Moon YW, Delgado RM, Fuller BG, Lonser RR, Remaley AT, Zhuang Z: Telomerase activity in microdissected human gliomas. **Mod Pathol** 12:41–46, 1999.
51. Wright WE, Shay JW: Time, telomeres and tumors: Is cellular senescence more than an anticancer mechanism? **Trends Cell Biol** 5:293–297, 1995.
52. Yajima T, Yagihashi A, Kameshima H, Kobayashi D, Hirata K, Watanabe N: Telomerase reverse transcriptase and telomeric-repeat binding factor protein 1 as regulators of telomerase activity in pancreatic cancer cells. **Br J Cancer** 85:752–757, 2001.
53. Yamada M, Tsuji N, Nakamura M, Moriai R, Kobayashi D, Yagihashi A, Watanabe N: Down-regulation of TRF1, TRF2 and TIN2 genes is important to maintain telomeric DNA for gastric cancers. **Anticancer Res** 22:3303–3307, 2002.
54. Yokota T, Suda T, Igarasci M, Kuroiwa T, Waguri N, Kawai H, Mita Y, Aoyagi Y: Telomere length variation and maintenance in hepatocarcinogenesis. **Cancer** 98:110–118, 2003.

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COMMENTS

This is a relatively simple study that demonstrates a correlation between telemeric repeat binding factor 1 (TRF1) and tumor grade for gliomas. Although association does not prove causation, these findings support a general theme that loss of control over the ability of the genome to replicate with efficiency and stability is a characteristic of malignant gliomas. Many reports demonstrate significant genomic mutations in gliomas and that these mutations increase both over time and as tumors evolve into a higher grade. Most of the mutations are ignored or are considered not important (i.e., random). However, randomness of genomic mutations may be the result of loss of control over a stable mitotic process, and alterations in telomeres can provide one way in which this can occur. Altered telomeres may preclude accurate alignment of chromosomes in metaphase, and this can produce further mutations and the genomic chaos found in glioblastoma.

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Telemeres are tandem arrays of TTAGGG repeats placed at the end of linear chromosomes, which are involved in several essential biological functions. Telomeres shorten with successive cell divisions, resulting in progressive genomic instability. Telomere-specific deoxyribonucleic acid binding proteins, such as TRF1, have been suggested to be important in the maintenance of telemere function. In this study, expression of TRF1 was examined in a number of astrocytoma tumor specimens. The results of this relatively small study of 20 surgical specimens demonstrate that there is a decrease in the expression of TRF1 in the higher-grade tumors in comparison to low-grade tumors. Some of the results may be explained by age, in that those patients with low-grade tumors in this study tended to be younger and therefore had higher TRF1 expression. One would like to see a study with normal brain tissue either in regions surrounding the tumor or in patients undergoing surgery for other reasons to determine whether there really is a difference in the expression of TRF1 in tumor tissue in comparison to normal age-matched brain tissue. Nevertheless, there is some suggestion in these data that loss of TRF1 expression may play a role in the malignant progression of astroglial brain tumors.

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