Immunohistochemical TRFI Expression in Human Primary Intracranial Tumors

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Abstract. Background: The telomeric-repeat binding factor (TRFI) participates in a physiological homeostatic mechanism controlling telomere shortening by inhibiting telomerase activity: down-regulation of TRF1 expression results in telomere elongation and may be involved in cell immortalization. Patients and Methods: To determine the TRF1 expression by immunohistochemistry (IHC) in human brain tumors, a cohort of 20 consecutive flash-frozen surgical specimens (14 meningiomas and 6 anaplastic astrocytomas (M)) were collected. Results: Variable levels of TRF1 expression in 12 out of the 14 (87.5%) meningioma samples were observed. By contrast, no expression of TRF1 in tissue samples from AA (p = 0.008) was detected. Positive TRF1 cells were usually more differentiated (less atypical features) and Ki67 negative (inverse statistical association, x² = p < 0.001). Conclusion: We demonstrated, for the first time, that routine IHC techniques are capable of identifying TRF1 expression in intracranial tumors, which is heterogeneously expressed in meningiomas, but absent in M. Although these preliminary observations need confirmation from larger studies, the TRF1 status in intracranial tumors might become of prognostic value.

Malignant glial tumors (AA and glioblastomas) arise either from the progression of low-grade precursor lesions or rapidly in a de novo fashion. The transition from a low-grade astrocytoma to a glioblastoma is associated with a step-wise accumulation of genetic mutations (1). The molecular pathogenesis of malignant gliomas and the identification of genetic mutations that characterize these lesions has been the focus of major research recently. Genomic instability is a hallmark of malignant gliomas (2). It has been recently speculated that changes in the telomere domain can result in genetic disorder and genomic variability (3). Telomeres reside at the end of chromosomes and are required for the protection and replication of chromosome ends. In human somatic cells, telomeres shorten with successive cell divisions resulting in progressive genomic instability, altered gene expression and cell death (4), via p53-dependent and independent mechanisms (5, 6). Telomerase is a ribonucleoprotein that synthesizes telomeric repeats onto chromosome ends. Telomerase activation, alternative telomere maintenance mechanisms (ALT), or other adaptive responses, extend telomere length and cell survival (4-8). Telomerase or ALT activation is a necessary (9-11) and important event in the multi-stage carcinogenesis process (5, 12). Telomerase activity has been detected in many types of human cancers, including brain tumors (8, 11). In human gliomas, telomerase activity increases with malignancy (13-15). The telomeric-repeat binding factor (TRFI) participates in a physiological homeostatic mechanism controlling, in a negative feedback mechanism, the maintenance of telomere length by inhibiting the activity of telomerase (16). In fact down-regulation of TRF1 expression results in telomere elongation (16). Because in a previous study we reported a down-regulation of TRFI in gastrointestinal cancer, with respect to normal or inflammatory mucosa (17), TRFI may be involved in cell immortalization and carcinogenesis. The aim of the present study was to demonstrate the feasibility of using readily available IHC techniques for rapid characterization of TRF1 expression in different human brain tumor specimens.

Materials and Methods

Tumor specimen. Tumor specimens obtained from 20 consecutive patients who underwent craniotomies for microsurgical tumor resection at the Neurosurgical Clinic of the University of Messina, School of Medicine, Italy, comprised the cases in this investigation. A cohort of 20 flash-frozen surgical specimens were evaluated. Samples were placed in cryovials and immediately flash-frozen in liquid nitrogen in the operating room. According to histopathological examination with standard hematoxylin/eosin staining technique, the following diagnoses were obtained: meningioma (n = 14) and AA grade III of the WHO classification (n = 6). Specimens for the histological diagnosis and immunohistochemical evaluations were taken from the same general region of the tumor during surgery.
IHC analysis. TRF1 expression was assessed by using a goat polyclonal antibody anti-TRF1 (Santa Cruz Biotechnology, Santa Cruz, California w.d. 1:100) and the proliferation-related antigen Ki-67 by using a monoclonal antibody anti-Ki-67 (DAKO, Glostrup, Denmark w.d. 1:100). Briefly, surgical specimens were mounted in embedding medium for frozen tissue specimens, sectioned to 511 thickness with a 2800 Frigocut Cryostat (Reichert-Jung, Germany), mounted on glass slides and fixed in ice-cold acetone for 5 minutes. Each section was then rinsed 3 times in phosphate-buffered saline (PBS), incubated for 5 minutes in 3% hydrogen peroxide (H2O2) to inhibit endogenous peroxidase and then incubated overnight at 4 °C with the primary antibodies against TRF1. After rinsing 3 times in phosphate-buffered saline (PBS), 1:TJE sections were incubated (15 minutes at room temperature) with a biotinilated universal secondary antibody (Vestastain Universal Quick Kit, Vector Burlington, CA), rinsed 3 times in PBS, then labeled (15 minutes at room temperature) with streptavidin/peroxidase preformed complex (Universal Quick Kit, Vector Burlington, CA). Sections were stained in brown-red with 3-amino-9-ethylcarbazole (AEC, Vector), counterstained with hematoxylin and mounted on aqueous mounting medium. As a negative control the primary antibody was substituted with non-immune serum. Samples were then observed with a Zeiss photomicroscope.

The percentage of cells exhibiting positive nuclear staining reactions was determined for both TRF1 and Ki-67 from each case. The area that appeared to have the highest density of labeled nuclei 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 highpower field (HPF) (X200). The mean of the obtained values was determined for each case and rounded to 5% steps. The sections were independently evaluated by two of the authors blind to the patient's identity. We intentionally decided not to use the intensity of staining as a grading criterion because an "intensity" score is subjective and prone to variations among runs. These independent evaluations exhibited substantial interobserver correlation.

Statistical analysis. The student's t-test for independent variables was used when comparing TRF1 expression and Ki-67 in tumor samples grouped by histological grading. To determine whether any association existed between the status of TRF1 expression and cell proliferation as assessed by histological grading and Ki-67, a X2 analysis was performed and Fisher test was used. For each of these analyses, probability values of < 0.05 were designated as significant.

Results

TRF1 immunoreactivity was expressed with variable intensity and distribution by neoplastic cells in 12 cases out of 20 (60%). The percentage of cells exhibiting positive nuclear staining for TRF1 varied from 0 to 30% of tumor cells (mean 9.25 ± 11.73). While TRF1 expression varied greatly between individual meningioma specimens (mean 14.3 ± 11.6) (Figure 1), AA were invariably negative for TRF1 immunostaining. This difference was statistically significant (p = 0.008) as shown in Figure 2.

The percentage of Ki-67-positive cells ranged from 0 to 33% within all specimens with a mean of 14.6 ± 7.7% in meningiomas and 26.7 ± 11.7% in AA (p = 0.014) (Figure 2).

A different pattern of positivity was observed with respect to TRF-1 and Ki-67 expression. Positive TRF1 cells were usually more differentiated (less atypical features) and negative for Ki67, with respect to TRF1 negative cells.

For statistical analysis, TRF1 and Ki-67 were categorized as positive or negative when the percentage of 5 or 20 was used as cut-off, respectively and a significant inverse association was demonstrated (X2 = 16.8, P < 0.001; Fisher test, p = 0.018).

Discussion

To the best of our knowledge, this is the first report of the use of immunohistochemistry to investigate TRF1-expression in brain tumors. This pilot study demonstrated that the pathophysiological properties of the selected panel of intracranial tumors included variable levels of TRF1 expression in meningiomas and loss of expression in AA. This IHC techniques is based on commercially available antibodies for the rapid characterization of TRF1 expression in different human brain tumor specimens.

The human telomeric-repeat binding factor (TRF-1) is involved in a negative feedback mechanism that stabilizes telomere length (16, 18, 22). The physiologic binding of TRF1 to telomeres inhibits the action of telomerase, resulting in gradual and progressive telomeres shortening to the "mortality stages", the proliferative barriers that lead to a nondividing state and cell death (16, 19, 22) (Figure 3a). TRF1 accepts adenosine diphosphate (ADP)-ribosylation catalyzed by the tankirase-PARP [poly(adenosine diphosphate-ribose) polymerase] (23). The ADP-ribosylation of TRF1 diminishes its ability to bind to telomeric DNA, allowing telomerase to elongate telomeres and extending cellular life span (Figure 3b) (23-25). The mutation or deletion of TRF1 can result in telomere elongation and extend cell survival (16). Therefore the expression of TRF1 is considered a physiological homeostatic mechanism that controls the proliferative potential of normal cells (22) by inhibiting the activity of telomerase.

As in almost all tumors, malignant brain tumors are reported to be associated with higher telomerase activity than benign tumors, such as neurinomas, meningiomas (26) or normal brain (27). Telomerase expression has been associated with high proliferative index, grade of tumor, age, vascular endothelial proliferation (28), poor outcome (29, 30) and it increases with malignancy from low grade to high grade astrocytomas (15). In the light of the important role recently identified for telomerase in the process of tumorigenesis in particular in the progression of gliomas malignancy, the study of the molecular mechanisms controlling telomerase activity was the next logical research step and constituted the rationale of the present investigation. 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 analyzed TRF1 expression in a pilot panel of human brain tumors including benign meningiomas and malignant AA. Although these are very different tumors, TRF1 was expressed to a varying extent in meningiomas and was not detected in any of the AA samples.

Assessment of tumor proliferative activity by IHC methods that measure proliferation-associated nuclear proteins has
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Figure 1. Representative section from IHC analysis. Case of meningioma. TRFI antibody stains tumor cells (brown-red). The vacuolar aspects are due to freezing artifact (250x). TRFI expression was in 30% of cells and Ki67 in 15%

Figure 2. Column graph shows the differences in the TRFI and Ki67 expression according to the histopathological diagnosis. The percentage of TRFI expressing cells is reduced in the AA group (** p=0.008) and the percentage of Ki67 expressing cells is low in the meningioma group (*) p=0.014.

demonstrated prognostic promise in a number of central nervous system tumors. The Ki-67 antigen is a sensitive marker of the growth fraction. It is present in cells in the proliferative phases of the cell cycle (G1, S, and G2-M phases), but not in the resting G0 phase. The correlation between the Ki-67 and the histological grade has been demonstrated in a wide array of intracranial tumors, including astrocytomas (31) and oligodendrogliomas (31), meningiomas (32). In the present study we were able to demonstrate that TRF1 and Ki-67 are statistically inversely related and that they stain different populations of cell in different areas of the tumor; TRF1 usually stains cells with less atypical features with respect to those positive for Ki67.

Although we recognize the complexity of the problem, the large number of variables involved and the fact that our results are based on a small series of cases, these findings indicate that the pattern of TRF1-expressing tumor cells may be due to the loss of TRF1 expression capability. This may be the result of down-regulation of TRF1 expression in actively proliferating and more atypical neoplastic cells, as also reported in gastrointestinal tumors (17), probably representing a mechanism used by tumor cells to extend their proliferative potential, or an initial step in carcinogenesis.

These preliminary observations require confirmation from larger studies concerning the physiological or pathological role of TRF1 as cellular aging accelerator (telomere shortening and cell cycle checkpoints switching on), or cellular survival extender. The detailed understanding of events responsible for brain tumor growth may open new perspectives in the prognostic evaluation of intracranial tumors and is a prerequisite for the development of new therapeutic strategies.
Figure 3, Schematic view of telomere-telomerase homeostasis.
A: The physiologic binding of TRFI and Tankirase to telomeric TTAGGG-repeat arrays inhibits the action of telomerase, allowing the gradual and progressive telomeres shortening, to the "mortality stages", the proliferative barriers that lead to a non-dividing state and cell death.
S: TRFI and Tankirase function as acceptor for adenosine diphosphate (ADP)-ribosylation, catalyzed by poly-adenosine diphosphate-ribose polymerase (PARP). The ADP-ribosylation of TRFI diminishes its ability to binding to telomeric DNA, allowing telomerase activity in telomeric TTAGGG-repeat arrayS elongation and extending cellular life span.
References


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