

Relation between telomerase activity, hTERT and telomere length for intracranial tumours

L. MAES¹, L. VAN NESTE², K. VAN DAMME¹, J.P.O. KALALA³,
L. DE RIDDER¹, S. BEKAERT² and M. CORNELISSEN¹

¹Department of Anatomy, Embryology, Histology and Medical Physics, Section of Histology, Ghent University, ²Laboratory for Biochemistry and Molecular Cytology, Department of Molecular Biotechnology, FLTBW-Ghent University and ³Department of Neurosurgery, University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium

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Abstract. Human linear chromosomes are capped by specialized DNA-protein structures called telomeres. The present study analysed the telomerase activity, hTERT protein and telomere length in meningiomas and gliomas in relation to their WHO grading. Fifty-three freshly dissected tumour biopsies were analysed for telomerase activity, hTERT protein expression and telomere length. Telomerase activity was examined in 41 of the 53 biopsies. Telomerase activity was detected in 3 of 35 (8.6%) screened meningiomas (1 benign, 1 atypical and 1 malignant meningioma). For hTERT expression, 56.4% of meningiomas were positive with a mean labelling index (hTERT LI) of 31.3% (SD=26.5) for the hTERT positive meningiomas. The mean telomere length for meningiomas was 6.983 kb (SD=1.969). For gliomas, no active telomerase was detected in 2 low-grade astrocytomas, whereas three of the four screened glioblastomas were positive for telomerase activity. The only hTERT protein positive astrocytoma had a mean labelling index of 9.0%. On the other hand, the hTERT LI for glioblastomas was 53.6% (SD=28.0). The two low-grade astrocytomas had a telomere length of 14.310 and 9.236 kb. The anaplastic astrocytoma had a telomere length of 4.903 kb and the glioblastomas 5.767 kb (SD=2.042). The normal meningeal and neuronal tissue is negative for telomerase activity and hTERT. The length was ± 10.000 kb. These results indicate that telomere shortening may be a critical step in pathogenesis of atypical and malignant meningiomas and gliomas. Critical telomere shortening *in vitro* was shown to activate telomerase.

Introduction

Telomeres play an essential role in maintaining the stability and integrity of chromosomes (1,2). They consist of TTAGGG

tandem repeats (3). Telomeres gradually shorten with each cell division *in vitro* and telomere length is shown to decrease with age *in vivo* (2,4). This shortening of telomeres may be a mitotic clock for cells leading to senescence and apoptosis (5,6). Normal human cells shorten their telomeres until they reach a critical length (~4 kb). In some instances, by chromosomal instability, cells escape from apoptosis leading to indefinite life span by activating telomerase (7,8). Telomerase is a ribonucleoprotein enzyme complex that synthesizes telomeric repeats at the chromosomal ends (4). Two major components of the telomerase complex are hTERT (human telomerase reverse transcriptase) and an RNA template (9,10). If tumour cells turn on telomerase, the expression of this enzyme may lead to unlimited proliferation and immortality for tumour cells (11). Some results have already been published on telomerase and telomeres in intracranial tumours (12-14), showing a correlation between telomerase activity and histopathological grading. It has been reported that hTERT rather than telomerase activity could be used as a prognostic marker for progression and potential recurrence (15-17). Moreover, hTERT could be considered as an early signal towards indefinite life span (18). Telomere length can be seen as the result of the interaction between active telomerase and the telomeric DNA ends (19). This length is crucial for the protection of the chromosomes (1). In our previous studies, we found a lack of telomerase activity and low hTERT-protein expression in benign meningiomas in comparison to malignant tumours (18,20). In the present study, we examined the telomerase activity, the hTERT protein expression and the telomere length in intracranial tumours to see if there is any difference between benign, malignant and normal intracranial tissue and if tumours without telomerase activity also have attritions or aberrations in their telomere length.

Materials and methods

Surgical specimen. Biopsies of 53 intracranial tumours including 39 meningiomas [34 WHO (World Health Organization) grade I meningiomas, 3 atypical WHO grade II meningiomas and 2 malignant WHO grade III meningiomas] and 14 gliomas (2 low-grade WHO grade II astrocytomas, 1 anaplastic WHO grade III astrocytoma and 11 WHO grade

Correspondence to: Dr Lode Maes, Department of Anatomy, Embryology, Histology and Medical Physics, Section of Histology, Ghent University, De Pintelaan 185, B-9000 Gent, Belgium
E-mail: lode.maes@ugent.be

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IV glioblastoma multiforme) were collected from the University Hospital, St. Lucas Hospital and Maria-Middelares, Ghent, Belgium. There were 34 women and 19 men with a median age of 56.7 (range 31-81). The classification and grading of the tumours was performed by the pathologists based on the WHO criteria (21). Immediately after surgery, the tumour fragments were transferred into vials containing Minimal Essential Medium (MEM, Invitrogen, Merelbeke, Belgium) for transport. One part was used for histopathological diagnosis and a second part was conserved in liquid nitrogen for molecular analysis of telomerase activity and measurement of telomere length. This research was approved by the Ethics Committee of Ghent University, Belgium (project 2001/58).

Detection of telomerase activity. For telomerase activity analysis, the surgical specimens were immediately frozen in liquid nitrogen and stored until use. Protein extracts were prepared from frozen samples in 100-250 μ l ice-cold extraction buffer [10 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 1 mM ethylene glycol tetraacetic acid (EGTA), 0.1 mM benzamidine, 0.5% CHAPS, 5 mM β -mercaptoethanol, 10% glycerol], depending on the size of tumour sample. After 30 min incubation on ice, lysates were centrifuged at 13500 rpm for 20 min at 4°C, and 60-200 μ l from each supernatant was collected. Protein content in each lysate was measured using the Bradford assay (Bio-Rad Laboratories, Nazareth Eke, Belgium) and adjusted to 600 ng/ μ l. Aliquots of 40 μ l protein extracts were frozen in liquid nitrogen. Telomerase enzymatic activity was determined using the Telomeric Repeat Amplification Protocol (TRAP) of the TRAPEze Telomerase Detection Kit (Chemicon, Heule, Belgium). Briefly, 1.2 μ g protein extract was incubated in 50 μ l reaction mixture containing 5 μ l 10X TRAP reaction buffer, 1 μ l 50X dNTP Mix, 1 μ l TS primer (5'-AATCCGTC GAGCAGAGTT-3'), 1 μ l Primer Mix (RP primer 5'-GCGC GG[CTTACC]₃CTAACC-3', K1 primer and TSK1 template for internal control), and 2 units TaqDNA polymerase at 30°C for 30 min for telomerase-mediated extension of the TS primers. Reaction mixtures were subjected to 30 cycles using the following conditions: 94°C for 30 sec, 59°C for 30 sec and 72°C for 30 sec. Twenty-five microliters of each polymerase chain reaction (PCR) sample was analysed by electrophoresis on 10% polyacrylamide-urea gels and visualized with SYBR-Green I nucleic acid gel stain by means of an ultraviolet (UV) transilluminator. Telomerase activity is defined as an incremental ladder. The inclusion of an internal control, a synthetic DNA construct added to the samples and which is amplified with the TS and RP primers to generate a 36-bp product, aided the detection of false negatives, which can be the result from the presence of PCR inhibitors in the extracts. An immortal cell line derived from a glioblastoma multiforme showed telomerase activity and was used as positive control. As negative control lysis buffer replaced cDNA.

Immunohistochemical evaluation for hTERT. Paraffin-embedded tissue sections (4- μ m) from intracranial meningiomas were prepared for examination by immunohistochemical procedures. Slides were heated in sodium citrate

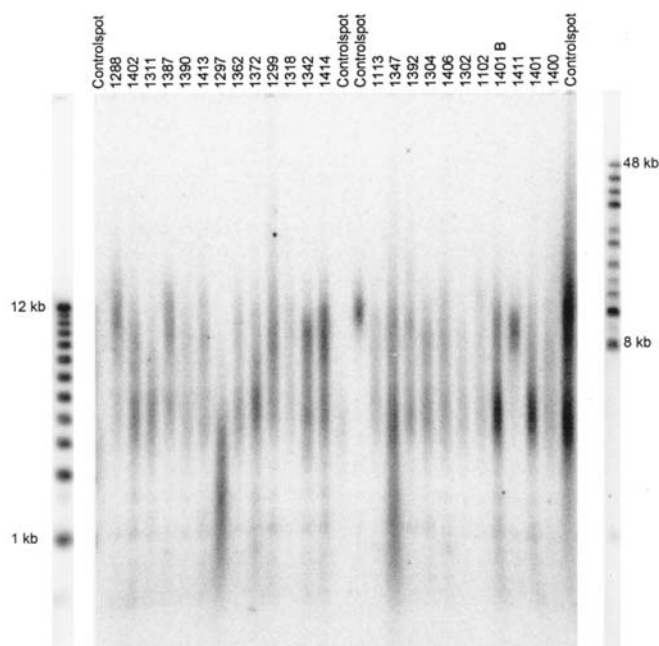


Figure 1. A blot after hybridisation with a telomere specific probe. The first and last lanes are DNA markers applied for molecular weight calibration [left, low molecular weight marker (LMW, 1-12 kb); right, high molecular weight marker (HMW, 8-48 kb)]. Average telomere length from a lysis of tumour cells is measured as mean telomere length (23).

buffer (10 mM, pH 6.0) for 30 min in a microwave oven at the highest power (700 W, Amana, Newton, IA, USA) for high temperature antigen unmasking. The endogenous peroxidase activity was blocked by 3% H₂O₂ (Eurolab, Leuven, Belgium) in distilled water for 10 min at room temperature. The standard streptavidin-biotin-peroxidase complex method was performed, employing antibodies against hTERT (1:50 dilution; clone 44F12; Novocastra, Newcastle upon Tyne, UK). hTERT expression was defined as the ratio of cells with immunoreactivity for 1000 tumour cells for biopsies and as positive/negative for cultures. Subsequently, samples were incubated with biotinylated rabbit anti-mouse, 1:200 for 30 min, followed by the third incubation with streptavidin-biotin-peroxidase complex, 1:200 for 30 min. Visualization of the complex was realized with diaminobenzidine (DAB) and sections were counterstained with haematoxylin. A glioblastoma multiforme, positive for hTERT, was used as positive control, whereas normal brain tissue and antigen-free buffer of the positive control as a negative control. hTERT percentages were calculated as mean percentage values by counting nuclei of tumour cells in three positive areas of a single tumour for thousand cells.

Telomere length analysis (Fig. 1). We followed standard methodology for telomeres sizing (22-24). Genomic DNA was double digested with respectively 5 U *RsaI* and 10 U *HinfI* for >3 h at 37°C and subsequently fragmented by Field Inversion Gel Electrophoresis (between 1 and 25 kb) on a 1% agarose gel. Samples were analysed in triplicate. Fractionated DNA was immobilized on positively charged nylon membranes after denaturation and neutralization. Southern blots were simultaneously hybridised overnight at 42°C with a P32

Table I. Telomerase activity, hTERT protein expression and telomere length in intracranial tumours (34 benign, 3 atypical and 2 malignant meningiomas; 2 low-grade and 1 anaplastic astrocytoma; 11 glioblastoma multiforme).

No.	Telomerase activity	hTERT labeling index (%)	Telomerase length (kb)
Benign meningiomas (WHO grade I)			
1	Neg	0.0	6.972
2	Neg	0.8	6.716
3	Neg	0.0	8.749
4	Neg	0.0	8.592
5	Pos	11.9	7.911
6	Neg	0.0	8.031
7	Neg	0.0	6.006
8	Neg	29.6	7.444
9	Neg	0.0	4.794
10	Neg	28	5.329
11	-	72.7	14.019
12	Neg	1.1	5.055
13	-	76.2	9.278
14	Neg	0.0	6.701
15	-	8.3	7.779
16	Neg	47.1	5.989
17	-	72.3	7.361
18	Neg	56.6	6.720
19	Neg	60.6	6.258
20	Neg	0.0	6.900
21	Neg	0.0	7.321
22	Neg	8.0	7.635
23	Neg	0.0	6.222
24	Neg	0.0	3.694
25	Neg	0.0	5.852
26	Neg	0.0	7.270
27	Neg	0.0	8.163
28	Neg	21.6	5.764
29	Neg	13.1	7.977
30	Neg	9.1	6.062
31	Neg	2.2	6.846
32	Neg	0.0	8.046
33	Neg	0.0	8.489
34	Neg	0.0	7.653
Atypical meningioma (WHO grade II)			
35	Neg	25.6	4.469
36	Neg	0.6	4.807
37	Pos	52.1	6.532
Malignant meningioma (WHO grade III)			
38	Neg	26.4	9.772
39	Pos	64.5	3.171
Astrocytoma LG (WHO grade II)			
40	Neg	0.0	14.31
41	Neg	9.0	9.236

Table I. Continued.

No.	Telomerase activity	hTERT labeling index (%)	Telomerase length (kb)
Anaplastic astrocytoma (WHO grade III)			
42	-	0.3	4.903
Glioblastoma multiforme (WHO grade IV)			
43	Neg	70.0	4.703
44	Pos	60.0	6.826
45	Pos	49.0	7.843
46	Pos	88.6	5.079
47	-	73.6	3.415
48	-	19.3	6.523
49	-	9.2	8.326
50	-	62.0	4.269
51	-	10.7	2.539
52	-	78.2	5.149
53	-	68.7	8.764
Telomere length in normal intracranial tissue (kb)			
	Meninges	11.3090	9.7589
	Brain tissue	8.833	10.074
		11.801	9.697

Neg, negative; Pos, positive; -, not available.

radiolabelled 5-mer synthetic oligonucleotide telomeric probe and radiolabelled high (8-48 kb) and low (1-12 kb) molecular weight DNA markers (Invitrogen). Corresponding molecular weights were calculated on the basis of DNA marker calibration curve.

Statistical analysis. All statistical analysis was carried out using SPSS for windows version 12.0 (SPSS Inc., Chicago, IL, USA). The categorized variables entered into the analysis were patients' histological subtype, telomerase activity, hTERT protein expression and telomere length. A Mann-Whitney test was used to analyse the association between histo-pathological grading, telomerase activity, hTERT protein expression and telomere length. The level of significance was set at $p < 0.05$.

Results

A general presentation for the results concerning telomerase activity, hTERT protein expression and telomere length is given in Table I. There were 53 intracranial tumours (34 benign meningiomas, 3 atypical meningiomas, 2 malignant meningiomas, 2 low-grade astrocytomas, 1 anaplastic astrocytoma and 11 glioblastomas).

Telomerase activity (Table I). Forty-one cases of intracranial tumours (30 benign, 3 atypical and 2 malignant meningiomas; 2 low-grade astrocytomas and 4 glioblastomas) were analysed

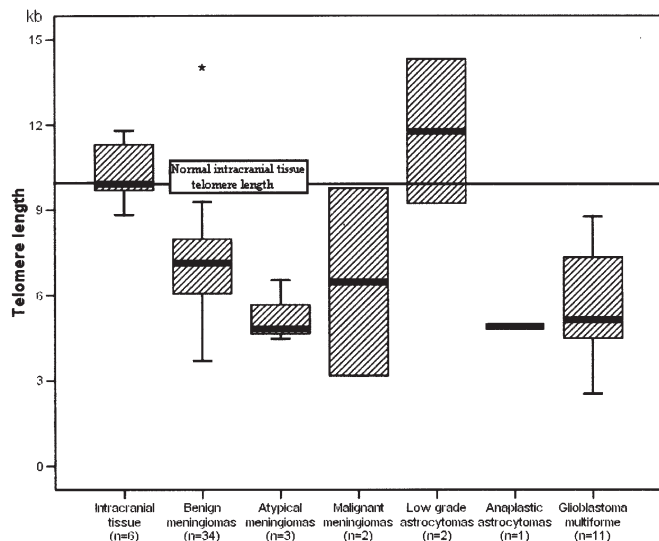


Figure 2. Telomere length in 53 intracranial tumours compared with normal intracranial tissue. Telomere length was determined by Southern blotting. The mean distribution of the different tumour types for telomere length lies under normal intracranial tissue with the exception of low-grade astrocytomas.

for telomerase activity. Telomerase activity was not examined for 12 of the 53 cases because no part of the biopsy was stored in the liquid nitrogen. Overall, telomerase activity was detected in 6 of 41 tumours (14.6%). One of 30 (0.03%) benign meningiomas, 1 of 3 (33.3%) atypical meningiomas and 1 of 2 (50%) malignant meningiomas expressed telomerase activity. Within the group of gliomas, neither 1 of the 2 low-grade astrocytomas and 3 of 4 screened glioblastomas possessed active telomerase. The number and percentage of intracranial tumours with telomerase activity increased in the higher histopathological grades. Thus, telomerase activity strongly correlated ($P < 0.001$) with the histopathological grading of the tumours by the WHO-classification.

Detection of hTERT-protein. The hTERT labelling indices for all intracranial tumour samples are presented in Table I. hTERT protein staining was seen in the nucleolar region of tumour cells. Heterogeneity and intensity of hTERT staining varied depending on the tumour. Positive cells were clustered together surrounded with non-stained cells. Twenty-two of 39 (56.4%) meningiomas were positive for the 44F12 antibody against hTERT. Within this group, there were 17 of 34 (50%) benign meningiomas, the 3 (100%) atypical meningiomas and all (100%) malignant meningiomas. The mean hTERT labelling index (LI) for all 22 hTERT positive meningiomas was 31.3% (SD=26.5). The mean hTERT LI for the 17 benign hTERT positive meningiomas was 30.5% (SD=27.6). The atypical meningiomas had a mean hTERT LI of 26.1% (SD=25.7) and the malignant meningiomas 45.5% (SD=26.9).

One of 2 low-grade astrocytomas was positive for hTERT with 9% of tumour cells labelled. The anaplastic astrocytoma had an hTERT expression of 0.3% of the tumour cells. All the glioblastomas were positive for hTERT and the mean hTERT LI was 53.6% (SD=28.0). The difference for

hTERT staining between hTERT positive benign meningiomas and glioblastomas is statistically significant ($P < 0.05$).

Telomere length measurement (Table I and Figs. 1 and 2). Telomere length appears as a smear on a Southern blot gel because the amount of TTAGGG repeats at the (sub)telomeric regions varies on the different chromosomes and the different cells. Also, the telomere length varies from cell to cell within a tumour.

We analysed the mean telomere length of 53 tumour biopsies of intracranial tumours. Also samples of meningeal and brain tissue from fresh corpses were analysed. These samples were collected from the Anatomical department of the university and included brain tissue and meninges. Samples were analysed twice to verify our results.

The mean telomere length of intracranial tissue from healthy persons was 10.245 kb (SD=1.11). This mean length was 10.534 kb (SD=1.096) for the meninges, 10.316 kb (SD=2.099) for grey matter and 9.885 kb (SD=0.266) for white matter.

The mean telomere length for the 39 meningiomas was 6.983 kb (SD=1.869). For the 34 benign meningiomas, we found a mean length of 7.165 kb (SD=1.724). The mean telomere length for the 3 atypical meningiomas was 5.269 kb (SD=1.107) and 6.472 kb (SD=4.668) for the malignant meningiomas.

For the low-grade astrocytomas, in one of the samples a mean telomere length was measured of 14.310 kb, while the telomere length for the other was 9.236 kb. The anaplastic astrocytoma had a mean length of 4.903 kb. For the 11 glioblastomas the mean telomere length was 5.767 kb (SD=2.042).

The telomere length was significantly shorter for the group of meningiomas and gliomas ($P < 0.001$), in comparison to the normal intracranial tissue, with the exception of one low-grade astrocytoma.

Discussion

In telomerase positive cells telomere length is balanced by telomere shortening due to cell division and telomere elongation by telomerase. In mortal cells, telomeres shorten during proliferation and hence can be considered as a marker for the replicative capacity of cells *in vitro* (22). This capacity keeps tumour cells proliferating and growing by the stabilization of their telomeres which is essential to maintain the unlimited dividing potential and to escape 'crisis' (25). At crisis, the telomeres are at critical length, and the integrity of the chromosomes declines with every subsequent cell division. Telomere length is maintained by telomerase activity and can be influenced in different ways and by various factors (26). From literature it is clear that telomerase activity can be regarded as a prognostic marker for malignancy (12,13,16). In contrast to malignant intracranial tumours, benign meningiomas seldom express telomerase activity.

As telomeres are essential for DNA replication and control of cell division (1), the regulation of their length has been a focus of research on tumorigenesis (13,14). It has been demonstrated that telomere shortening, which occurs at every successive cell division in somatic cells, is stopped in most tumours, which leads to immortalisation through the

activation of telomerase. The presence of telomerase activity indicates the capacity for unlimited cell proliferation (27).

In this study, we investigated the possible link between telomerase activity, hTERT expression and telomere length in intracranial tumours. The specificity for immunostaining against hTERT has been criticized although the sensitivity posed no problems (28). For them NCL-hTERT was a marker for nucleolin. Although, hTERT and nucleolin share the same intracellular distribution and their nucleolin demonstrates another molecular weight than that found by others.

We found a significant shortening of the telomeres in the group of meningiomas and gliomas as compared with normal intracranial tissue. Fig. 2 represents the attrition of telomeres for the different groups of tumours. A shortening is visible for the meningiomas in comparison to normal meninges. Moreover, a clear shortening is visible for the glioblastomas as compared to normal intracranial tissue and benign meningiomas. Cells from highly proliferating tumours such as glioblastomas present higher mitotic activity than benign slow proliferating tumours. As a result, the telomere will shorten quicker in glioblastomas than in benign meningiomas. A high expression of hTERT implies that telomeres could be stabilized or elongated by active telomerase. All glioblastomas expressed hTERT and 75% of the analysed samples had active telomerase. Only half of the benign meningiomas expressed hTERT and the labelling indexes were lower than the group of gliomas. Telomere ends of tumour cells from benign meningiomas shorten at ease because of their slower growth. The 'crisis' situation is leading to apoptosis (5,6). Only one benign meningioma had active telomerase (0.03%). Because the three atypical meningiomas had shorter telomeres than the benign meningioma, they seem to be in a further state of progression with higher proliferation and mitosis. hTERT was detected in all three atypical meningiomas and one had active telomerase. One of two malignant meningiomas had active telomerase and both expressed hTERT. The malignant meningioma with active telomerase had remarkable short telomeres as compared to normal intracranial tissue and the benign meningiomas. Chen *et al* hypothesized that telomerase activity can be regulated and temporary turned-off, as the telomeres are longer again (13). In our study, we found longer telomeres in one of the two low-grade astrocytomas. One of the lengths was even longer than the normal intracranial tissue. This was remarkable because there was no expression of hTERT or active telomerase. Our findings support the hypothesis that sufficient (hTERT-mediated) telomere stabilization is achieved late in tumourigenesis after extensive cell proliferation and telomere shortening has already taken place (29). In this respect, telomere maintenance or even elongation seem to be essential for the tumour to maintain its (indefinite) proliferative capacity and to continue further tumour progression and invasion (1,26). In normal somatic cells, when telomeres become critically shortened, these enter a growth arrest state. Longer telomeres provide the cell more mitotic potential. It is supposed that there is a mechanism that regulates telomerase activity and telomere length (30,31). When the length is long enough for proliferation, telomerase activity subsides. Because glioblastomas are high proliferating

tumours, they keep their telomere length steady with active telomerase. Telomerase and telomere length serve as indicators for the ability of each tumour to compensate for replicative telomere losses (32,33).

The anaplastic astrocytoma had short telomeres but hardly any expression of hTERT. This tumour could be in a further state of progression leading to active telomerase towards a classification of a secondary glioblastoma multiforme. Expression of hTERT does not mean activation of the telomerase complex but is one of the crucial steps towards the activation (17). Hiyama *et al* found a correlation between the length of telomeres and the prognosis in medulloblastomas (34). The author concluded that short telomeres are linked to a further state of progression and a more aggressive behaviour for these tumours. Also other studies on telomere length for intracranial tumours found a shortening of the telomeres for telomerase positive tumours (14,35). In our study, most tumours (96%) had shorter telomeres compared to normal intracranial tissue. Shorter telomeres in cancer tissue compared to normal tissue are indicative for extensive tumour cell proliferation. However, telomere stabilization by telomerase is inevitable at a critical point of telomere shortening to prevent the onset of crisis and senescence (26).

These results indicate that telomerase activation may be a critical step in the pathogenesis of gliomas and meningiomas. Shortening of the telomere length also indicates a high potential for malignant behaviour in these tumours. In our series, telomerase activity was detected in 2 of 5 specimens (40%) of atypical and malignant meningiomas. Compared to benign meningiomas, 1 out of 30 cases was positive for telomerase activity, this difference speaks for a late activation of telomerase in tumour progression.

Detectable telomerase activity and shortened telomere length are clinically relevant because the presence of these factors may suggest that the tumours contain a population of cells with the capacity for unlimited proliferation (13).

Telomere length was reduced in the high-grade tumours, whereas it was compatible with that of normal brain tissues in the low-grade astrocytomas, suggesting that telomerase activity with shortened telomeres correlates with the aggressive growth of high-grade intracranial tumours.

We conclude that telomere length cannot be used as a putative prognostic marker on its own but should be linked with hTERT expression and telomerase activity. There are signs for alternative mechanisms of telomere elongation (cases 11 and 40 in Table I), although most tumours have critically shortened telomeres.

These findings suggests that telomerase activity and hTERT, together with the telomere length can be an index of malignant potential or malignancy itself in intracranial tumours.

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