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Detection of the alternative lengthening of telomeres pathway in malignant gliomas for improved molecular diagnosis

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Abstract

Human malignant gliomas exhibit acquisition of either one of two telomere maintenance mechanisms, resulting from either reactivation of telomerase expression or activation of an alternative lengthening of telomeres (ALT) mechanism. In the present study, we analyzed 63 human malignant gliomas for the presence of ALT-specific extrachromosomal circles of telomeric DNA (C-circles) and measured telomerase expression, telomeric DNA content (Telo/Alu method), and telomeric repeat-containing RNAs (TERRA) levels. We also assessed histomolecular markers routinely used in clinical practice. The presence of C-circles significantly correlated with *IDH1/2* mutation, *MGMT* exon 1 methylation, low Ki-67 immunostaining, increased telomeric DNA content, absence of functional ATRX protein and level of *HTERT* gene expression. In multivariate analysis, we observed a trend to a correlation between elevated TERRA levels and increased survival. Interestingly, the C-circles assay allowed to detect ALT activation in glioblastomas exhibiting wild-type *IDH1/2* and ATRX expression. These results suggest that, after the correlations uncovered here have been confirmed on larger numbers of tumors, telomeric markers might be useful in improving diagnosis. They also point out to the utility of using the specific, sensitive and quantitative C-circle and Telo/Alu assays that can work with as few as 30 ng of tumor DNA.

Keywords: gliomas; telomeric markers; alternative lengthening of telomeres; C-circle assay; ATRX expression.

Introduction

Telomeres, which represent the ends of linear chromosomes of eukaryotic organisms and contain repeated non-coding TG-rich sequences, protect chromosome ends from DNA repair activities occurring following DNA damage. By limiting the replicative potential of cells, telomere length serves as a "mitotic clock" and telomere erosion acts as a barrier against tumorigenesis in healthy tissue [1]. Following cancer initiation, tumor cells must overcome the telomere-controlled replicative senescence barrier to be able to proliferate indefinitely. In most cases (around 90% of cancer types) this occurs through up-regulation of telomerase expression. However, some cancer cells maintain their telomeres through a telomerase-independent process termed ALT, for "Alternative Lengthening of Telomeres" [2]. ALT is thought to function principally by exchange of telomeric sequences from one telomere to the next (recombination). There is also good evidence that a sort of "rolling circle" mechanism could also lead to telomere amplification on a single telomere [3, 4]. Human gliomas are among the ~10% of cancer types that can survive either owing to telomerase or the ALT pathway [5]. Clinical studies indicate that 15 to 45% of human malignant gliomas develop the ALT pathway and that this is strongly associated with the low grade II which can exhibit up to 75% of ALT [6, 7].

Recent clinical studies have highlighted the existence of a strong correlation between the occurrence of the ALT pathway of telomere maintenance in several types of ALT cancers and the presence of mutations in the *ATRX* ("Alpha Thalassemia/mental Retardation syndrome X-linked") gene [8]. *ATRX* is a DNA helicase/ATPase of the SWI2/SNF2 family that binds repeated sequences of DNA, particularly the G-rich ones, and has been implicated mainly in chromatin remodeling [9].

C-circles are single- and double-stranded extrachromosomal DNA circles that, unlike other categories of extrachromosomal DNA species, are specific for the ALT pathway [10]. Potentially, C-circles represent the product of excision of over-elongated telomeres that have been previously amplified by homologous recombination between repeated telomeric sequences during ALT [3]. In the present study, we assessed for the presence of the ALT pathway, using the very sensitive and quantifiable C-circle assay, and other telomere markers in 63 grades II, III and IV gliomas. We next attempted to correlate these factors with clinical characteristics as well as with the occurrence of classical genetic/epigenetic markers.

Materials and methods

Ethics statement

Between 2007 and 2012, malignant glioma samples were obtained during therapeutic surgical management by the Neurosurgery Unit of Clermont-Ferrand hospital (France) and stored confidentially. These glioma samples were part of the “Tumorotheque Auvergne Gliomes” biobank (DC-2012-1584). This study obtained ethical approval from the ethics committees and the respective competent authorities. The authors state that they have complied with the World Medical Association Declaration of Helsinki regarding ethical conduct of research involving human subjects.

Patients and tissue collection

The clinical samples used in this study were taken from the material resected during the course of a standard diagnostic and treatment procedure. Immediately after surgery, the samples were snap-frozen and stored in liquid nitrogen.

Each sample was analyzed under a light microscope after hematoxylin-eosin staining for assessing necrosis and tumor cell percentage. Samples with less than 50% of tumor cells were excluded from this study. Histological diagnosis and grading of gliomas were based on WHO criteria used at the time of sample analyses of the present study [11]. Four non-tumoral control brain samples (healthy controls, mean age of 27.3, standard deviation \pm 2 years) were cadaveric specimens removed by autopsy 4-16 hr following accidental death (Brain and Tissue Bank of Maryland). These four non-tumoral control samples, identified by the Brain and Tissue Bank of Maryland as *corpus callosum* (n = 1) and frontal cortex (n = 3), corresponded to white matter enriched in astrocytes and oligodendrocytes, tissue from which gliomas originate. Tumor and control samples were processed by cryogenic grinding used for homogenization in powder and equally distributed into at least three vials, as described previously [12]. For each sample, two vials were used for DNA and RNA extractions.

Survival was calculated from the date of surgery. The extent of tumor resection was evaluated essentially based on two criteria: the surgical procedure report by the neurosurgeon and the presence of a persistent contrast-enhancement on the early post-surgery (48 h) CT scan or MRI. The degree of tumor resection was classified as: i) gross total resection (GTR) when tumor surgery was optimal and no contrast enhancement detected at 24/48 h post-surgery MRI, or ii) as partial resection (PR) when contrast enhancement was detected.

DNA sequencing for *IDH1/2* mutational analysis and methylation status of *MGMT* promoter

Genomic DNA was isolated from frozen tissues using the QIAamp DNA Mini Kit (Qiagen) and stored at -80°C. *IDH1* and *IDH2* genotyping and *MGMT* promoter methylation analyses were performed as previously described [12-14] and detailed in **Supplementary methods**.

Analysis of 1p/19q codeletion, *INA* and immunostaining

The analysis of 1p/19q codeletion, internexin neuronal intermediate filament protein alpha (*INA*) expression [15, 16] and immunostaining analyses were detailed in **Supplementary methods**.

Measurements of ALT-specific C-circles

The C-circle assay, which detects partially single-stranded telomeric (CCCTAA)_n DNA circles (C-circles) amplified by the Phi29 polymerase in the absence of dCTP, was performed as described previously [10] and detailed in **Supplementary methods**.

Detection of ALT-associated promyelocytic leukemia nuclear bodies (APBs) at the telomeres

APBs detection was conducted according to protocols previously described [5, 17] and detailed in **Supplementary methods**.

Telomeric DNA quantification by dot blot

Ten nanogramms of genomic DNA were transferred to a nylon membrane (Hybond N+; GE Healthcare) in a dot blot apparatus (Bio-Rad). After high-stringency washes, the membrane was hybridized overnight, in Church buffer, with a ³²P-labeled telomeric repeat [TTAGGG]₃ probe that had been end-labeled with 100 μCi ³²P-γATP at 37°C for 40 min using T4 polynucleotide kinase. After detection, the membrane was stripped and re-probed with an Alu-specific [GTGATCCGCCCGCCTCGGCCTCCCAAAGTG] ³²P-labeled probe, as above.

Measurement of *hTERT* expression

Total RNA was isolated from tumors using RNeasy Mini Kits (Qiagen). A reverse transcription reaction was performed on 500 ng RNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer's instructions. *hTERT* expression was measured using the nanofluidic digital PCR array platform (Fluidigm technology). The expression level of *hTERT* for each sample was calculated compared

to the three housekeeping genes *TBP*, *RPL13A* and *PPIA*, identified as valid control genes in human glioma [18] and using the $\Delta\Delta C_t$ method [19].

Measurement of telomeric DNA transcription (TERRA)

Two micrograms of tumor sample RNA were reverse transcribed using the Revertaid first strand cDNA synthesis kit from Thermo Scientific-Fermentas and quantified using specific primers as previously described [20] and detailed in **Supplementary methods**.

Cell lines and cell culturing

Cell lines and culturing are described in **Supplementary methods**.

Telomere length measurement of human cancer cell lines by telomere restriction fragment (TRF) analysis

Measurement of telomere length was performed by Southern blot analysis, also referred to as TRF (Telomere Restriction Fragment) analysis as already described [21, 22] and detailed in **Supplementary methods**.

Statistical analysis

Statistical analysis was performed using the Stata software, version 12 (StataCorp, College Station, TX, USA). Tests were two-sided, with a type I error set at $\alpha=0.05$. Baseline characteristics were presented as mean \pm standard deviation (sd) or as median [interquartile range] for each independent group for continuous data and as number of patients and associated percentages for categorical parameters. Comparisons of characteristics between groups were analyzed using Chi-squared or Fisher's exact test for categorical variables, and Student's t-test or Mann-Whitney test for quantitative variables when assumptions of t-test were not met ((i) normality verified by the Shapiro-Wilk test and (ii) homoscedasticity by the Fisher-Snedecor test). Correlation between quantitative parameters was assessed using correlation coefficient noted r (Pearson or Spearman according to statistical distribution). Multivariate analysis was considered to take into account adjustment on possible confounding factors according to univariate analysis and literature: age, WHO grade, surgery resection. Overall survival (OS) was defined as the interval from surgery to death, regardless of the cause of death. OS curves and estimates were constructed using the Kaplan–Meier method. The log-rank test was used in a univariate analysis to test the prognostic value of patient characteristics. Cox proportional hazards regression was used to investigate prognostic factors in multivariate situation by backward and forward stepwise analysis of the factors

considered significant in univariate analysis (entered into the model if $p \leq 0.15$) and according to clinically relevant parameters. The proportional hazard hypotheses were verified using Schoenfeld's test and plotting residuals. The interactions between possible predictive factors were also tested. Results were expressed as hazard ratios (HR) and 95% confidence intervals (CI).

Results

A total of 63 malignant glioma samples were analyzed; patient characteristics are outlined in **Supplementary Table 1**. According to the previous WHO glioma classification [11], this cohort comprised 23 GBM (mean age of 63.0 ± 9.5 years), 32 grade III (mean age of 57.4 ± 12.6 years) and 8 grade II (mean age of 40.1 ± 10.0 years). However, importantly, the definition of glioma sub-classes for our statistical analyses was based on criteria similar to those used in the new WHO classification [23], meaning that, for instance, grades II and III were grouped together but further classified according to their IDH1/2 and 1p/19q codeletion status. All patients underwent surgery. High-grade (III and IV) IDH WT glioma patients were treated by concurrent chemoradiation and adjuvant chemotherapy (temozolomide), while IDH -mutated glioma patients did not receive concurrent chemoradiation and were treated on a case-by-case basis with protocols selected by our neuro oncology staff (chemotherapy and/or radiation, not simultaneously).

Occurrence of the ALT mechanism of telomere maintenance

All 63 glioma samples were analyzed for the presence of ALT-specific C-circles (**Figure 1A, Supplementary Table 1**). We chose the so-called "C-circle assay" to measure ALT activity, a technique developed by Reddel and co-workers, because it is highly specific, highly sensitive and quantifiable and can work with as little as 30 ng of DNA [10]. The intensities of C-circle signal for cells from a telomerase positive cancer cell line, HeLa, as well as for a prototype of ALT positive cells, U2OS, an osteosarcoma-derived cell line, were measured under identical conditions. Two out of the 9 C-circle positive tumors had a higher signal than U2OS, both of which were *IDH1/2* WT GBM (**Supplementary Table 1**). Since the C-circle method for identification of the ALT mechanism is recent, we set out to detect in parallel, on a subset of samples, the presence of ALT-associated promyelocytic leukemia nuclear bodies (APBs) (**Figure 1B**). In the C-circle positive tumors, co-localizing signals were detected in the microscope (**Figure 1B**), but not in the ALT negative tumors (data not shown).

Nine out of the 63 samples analyzed (14.2%) were positive in the C-circle assay. The *IDH1/2* mutated and *IDH1/2* WT groups exhibited a statistically significant difference, with only 3 C-circle positive tumors

among the 49 IDH WT gliomas ($p=0.001$; **Table 1**). Six out of the 9 (66.7%) C-circle positive tumors *versus* only 8/54 (14.8%) C-circle negative tumors were *IDH*-mutated, pointing out to a statistically significant association between *IDH1/2* mutations and ALT ($p=0.001$, **Table 1**). In addition, C-circle positive patients were diagnosed at a younger age than C-circle negative patients ($p=0.04$, **Table 2**).

Median survival was statistically different between the patients negative (15.8 months) and positive (41.9 months) for C-circle, when considering the entire population ($p=0.03$, HR=0.28, 95% CI [0.09;0.90]). In addition, 5-year survival was 22% in C-circle negative *versus* 62% in C-circle positive tumors. Moreover, even if one of the 6 C-circle positive living patients died, the difference would remain statistically significant. When using multivariate model adjusted on the diagnosis age, histopathology grading and surgery resection, there was a trend for a significantly different OS depending on C-circle status ($p=0.09$, HR=0.35, 95% CI [0.10;1.17]). Therefore, C-circle status did not appear to be an independent prognostic factor in our series of 63 gliomas.

Thirty four/63 tumors (54%) exhibited methylation of *MGMT* exon 1. *MGMT* promoter methylation correlated not only with the occurrence of *IDH1/2* mutations ($p=0.001$) but also with the presence of C-circles ($p=0.02$; **Table 1**). This relation was less robust when considering the IDH mutated and IDH WT groups separately, but a trend was observed in the IDH WT group (39.1% *versus* 100%, $p=0.07$). Therefore, *MGMT* methylation correlates with the presence of the ALT mechanism.

***hTERT* expression**

The expression of *hTERT*, encoding the catalytic subunit of telomerase, exhibited a mean increase of 9.3-fold in the 43 tumors tested in comparison to four control brain samples (**Table 1**). Although *hTERT* expression was different between IDH mutated and IDH WT gliomas (5.0 ± 6.1 and 10.1 ± 7.6 respectively), this difference was not statistically significant ($p=0.12$, **Table 2**), most likely due to the small number of IDH mutated samples ($n=14$).

Overall survival was inversely correlated with *hTERT* expression in univariate (HR=1.08, [1.03 - 1.14], $p=0.001$) and multivariate (HR=1.08, [1.03 - 1.13], $p=0.003$) analyses. This correlation becomes more significant in the 1p/19q negative group (37/53; HR=1.09, [1.03 - 1.15], $p=0.001$), as expected from previous results [24], and even in the IDH WT group (36/49; HR=1.09, [1.03 - 1.15] $p=0.002$). Therefore, *hTERT* expression was an independent negative prognostic factor in the whole population. All 5 C-circle positive gliomas in which it was measured exhibited a very low, undetectable, rate of *hTERT* expression, whereas expression was higher in C-circle negative gliomas (10.5 ± 7.2 , $n=38$, $p<0.001$; **Table 1**), highlighting the

existence of a moderate inverse correlation between *hTERT* expression and the ALT mechanism ($r=0.31$, $p=0.04$).

Telomeric DNA content and telomeric DNA transcripts (TERRA)

In the current study, we had approximately 100 ng of tumor DNA available for each sample and this was not enough if we wanted to measure telomere length using telomere restriction fragment (TRF) analysis. To increase the sensitivity, we quantified telomeric DNA (TTAGGG repeats) with respect to an internal control (amount of Alu repeats), referred to as the Telo/Alu dot blot method (see Materials and methods), which we performed here using 10 ng of tumor DNA per sample (**Figure 2A; Supplementary Table 1; Supplementary results**). Using six different human cell lines, we confirm that the Telo/Alu dot blot signals were proportional to telomere length measured by TRF (**Figure 2B and Supplementary results**), and can be useful to approximate absolute values of telomere length, in kilobases.

No statistically significant difference in telomeric DNA content nor in TERRA expression [25, 26] was found in relation with *IDH1/2* mutational status (**Table 2**). On the other hand, a significant positive association was found between long telomeres and the presence of C-circles ($p=0.03$, 0.7 [0.54 – 0.87] *versus* 0.83 [0.70 – 1], **Table 1**). For instance, tumor #30 exhibited a 42.18 C-circle score and a 1.77 Telo/Alu ratio, the highest among all tumors measured (**Supplementary Table 1**). Finally, statistical analyses showed no correlation between telomeric DNA content/telomere length and survival (HR=1.16, [0.41 - 3.31], $p=0.78$).

Analysis of classical molecular markers in a context other than telomeric

IDH1/2 mutation status

Within the IDH WT group ($n=49$), overall survival was similar between GBM ($n=23$) and grade III gliomas ($n=25$), 1.03 and 1.32 year, respectively. Median survival between IDH WT ($n=49$) and mutated ($n=14$) populations was significantly different ($p=0.001$) with respectively 14.4 months [8.5 - 24.3] (5-year survival: 9%) for IDH WT and not reached at the moment for IDH mutated (5-year survival: 86%).

ATRX inactivation

In ALT positive tumors, inactivation of the ATRX gene product by mutation, insertion or deletion causes a lack of nuclear expression detected by immunolabeling, while tumors without ALT show robust nuclear labeling for ATRX [27, 28]. An absence of ATRX protein expression was found in 5/59 tested gliomas. This absence of

functional nuclear ATRX was linked to activation of the ALT pathway ($p=0.001$, **Table 1**), but also to the presence of an *IDH1/2* mutation ($p<0.001$, **Table 2**), but not to *MGMT* promoter methylation ($p=0.15$). Interestingly, ATRX was nuclear (and therefore presumably functional) in all 3 C-circle positive GBMs.

MGMT exon 1 methylation status

Thirty-four (54%) tumors had their *MGMT* promoter methylated (**Supplementary Table 1**). There was a strong correlation ($p=0.001$) between methylation and the occurrence of an *IDH1/2* mutation, since 13/14 (92.9%) *IDH1/2* mutated tumors (tumor #52 being the exception) exhibited *MGMT* methylation, while only 42.9% of *IDH1/2* WT tumors were *MGMT*-methylated. There was also a strong relation between overall survival and *MGMT* methylation with a univariate approach (HR=0.28, [0.15 - 0.53], $p<0.001$), but also after adjustment using a multivariate model (HR=0.31, [0.16 - 0.61], $p=0.001$).

1p/19q codeletion

All tumors were assessed for the presence of either 1p/19q codeletion ($n=38$) or INA immunostaining ($n=25$). Among the 10 positive tumors, 9 were *IDH*-mutated ($p<0.001$). In fact, patient #71 diagnosed as 1p/19q codeleted based on INA expression, turned out to be negative for 1p/19q codeletion following subsequent CNV analysis, which detected only partial codeletions on 1p and 19q chromosome arms (see Supplementary methods). *MGMT* promoter methylation in these 10 1p/19q codeleted tumors was 36.8% *versus* 17.0% in the 53 non codeleted tumors (median: 37.7% [32.3 – 44.7] *versus* 5.9% [1.7 – 29.5]), $p=0.004$).

P53 and Ki-67 immunostaining

P53 immunoreactivity was not correlated with any parameter, while the proliferation marker Ki-67 was found to be an independent prognostic factor. Indeed, a low Ki-67 immunostaining was associated with better survival ($p<0.001$). In addition, a low Ki-67 score is also associated with C-circle positivity ($p=0.02$, **Table 1; Supplementary Table 1**).

Discussion

In the present study, we have measured, in a small cohort of human malignant gliomas, basic telomeric parameters and evaluated their potential association with classical tumor markers and clinical outcome. Some of our conclusions confirm previous studies [7, 29, 30], while others represent novel findings, which will have to be

confirmed on a larger cohort. We have described a simple dot blot technique which, coupled with telomere length measurement in cell lines for calibration, allows to evaluate telomeric DNA content and telomere length in these tumors using as little as 30 ng of DNA. In parallel, the already described C-circle technique, which accurately and specifically measures ALT activity, can also be used with similar low amounts of tumor DNA. There are currently three main methods for detecting ALT in tumors. The first one is TRF Southern-blot analysis of telomere length profile, also used here (Fig. 2). In ALT cells, telomeres range from very short to extremely long in individual cells, with mean telomere length (> 20 Kb) being about twice of that of cells with positive telomerase activity or normal somatic cells (5–10 Kb). Therefore, ALT have long and heterogeneous telomeres as compared to the shorter and more homogeneous telomeres of ALT negative cells. TRF analysis currently uses around 3-5 µg of DNA per sample. The second method is that of the C-circles, used here, in which only 30 ng of DNA are needed. The third method does not use tumor DNA as the starting material. It consists in the detection, in tumor sections, of ALT-associated PML bodies (APBs), which are promyelocytic leukemia (PML) bodies that contain telomeric DNA and telomere binding proteins. This method, also used here (Fig. 1B), is labor-intensive, making screening of large numbers of tumors difficult. However, recently, a variation of this assay, in which telomere FISH was used to detect ultra-bright telomeric signals, was used to analyze more than 6,000 tumor samples, mostly in tissue microarray format studies [31].

An interesting finding of the present study was that all 3 ALT positive tumors detected among the 23 GBM were wild type for *IDH1/2*, while the remaining 6 ALT positive tumors detected among the 40 lower grades, were all mutated for *IDH1/2*. Importantly, GBM that were both C-circle/ALT positive and *IDH1/2* WT did not exhibit loss of ATRX function, unlike ALT positive grade II and III gliomas which were all *IDH1/2* mutated. This highlights the relevance to measure C-circles rather than ATRX protein expression to identify ALT positive tumors among IDH WT malignant gliomas. In addition, these observations lead to propose that ALT activity in GBM might not have the same causes and consequences as ALT activity in lower grades. Therefore, measuring ALT activity in all grades of gliomas will undoubtedly allow to distinguish sub-classes of gliomas. However, it should be stressed out again that larger numbers of analyzed cases will be needed in order to establish solid conclusions regarding the potential existence of two (or more) different sub-classes of ALT tumors.

Another new finding from the present study was that *MGMT* promoter methylation associated with the ALT pathway both in the IDH mutated and IDH WT groups. In fact, it has already been reported that *MGMT* promoter methylation associated with mutations in *IDH1/2* [32, 33] as well as with increased survival [33]. Our

data also reveal, in multivariate analysis, a highly significant correlation between OS and a low Ki-67 score ($p=0.004$, $HR=1.05$) as well as a trend to a correlation between OS and C-circle positivity ($p=0.09$, $HR=0.35$). Our conclusion that a low Ki-67 score is associated with C-circle positivity should be toned down by the fact that the Ki-67 value was unknown for 5/9 C-circle positive tumors, and therefore is based on a small number of cases.

As expected, there was a strong inverse correlation between *hTERT* expression and the presence of a functional ALT mechanism. In addition, high levels of *hTERT* expression correlated with decreased survival, especially in 1p/19q non-codeleted patients. This agrees with recent findings showing that the presence of mutations in the *hTERT* promoter of GBMs, associated with increased *hTERT* expression, correlated with decreased survival in 1p/19q non-codeleted patients [24]. We found a positive correlation between telomeric DNA content and the presence of C-circles, which was expected since ALT telomeres are much longer in average than telomerase-maintained telomeres [3]. However, we believe it would be relevant to detect the presence of high *versus* low telomeric DNA content by using just a simple technique (the Telo/Alu method) since telomere length might represent a marker of tumor radiosensitivity [34]. On the other hand, we found no positive correlation between high telomeric DNA content and the presence of an *IDH1/2* mutation. This was not surprising since a substantial number of tumors were *IDH1/2* mutated but ALT negative.

In summary, achievement of a molecular diagnostic of ALT activation using the C-circle assay provides several major advantages. First, this assay specifically, accurately and rapidly identifies ALT positive tumors using as little as few tens of tumor DNA. This will be particularly useful for GBMs since those, contrary to low grade gliomas, can be wild type for both *IDH1/2* and *ATRX* and yet develop the ALT pathway. Therefore, in these GBMs, routinely assessing *IDH1/2* mutations by sequencing and *ATRX* expression on tumor sections would not represent the right assay to detect ALT. Moreover, the C-circle assay reflects the overall ALT activity of the tumor, DNA being extracted from the whole chirurgical sample, contrary to the detection of ALT-associated PML bodies or FISH techniques, which both might accidentally analyze only a subset of tumor cells. In addition, FISH only indirectly assesses ALT activity, which is deduced from the signal measuring the amount of telomeric sequences, which might be misleading given that some tumors might exhibit long telomeres but yet be ALT negative. New ALT targeting therapies are currently under development [35] and it is therefore urgent to develop a reliable clinical test such as the C-circle assay to measure ALT activity in glioma tumors.

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Conflicts of interest

All authors declare that they have no conflict of interest.

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Legends of Figures

Fig. 1. Detection of ALT-specific C-circle by C-circle assay and of ALT-specific APBs by immunofluorescence-FISH. **(A)** Dot blot of the C-circle assay using a ^{32}P -labeled $(\text{TTAGGG})_3$ telomeric probe. All 64 samples were processed similarly, but a few examples only are shown here for illustration. Absolute C-circle values were measured by quantifying the signal after subtraction of the background signal on the nitrocellulose membrane. The vertical bars below represent the respective intensities of these values. The horizontal dashed line, which represents the C-circle value measured for HeLa cells (which are telomerase-positive), was arbitrarily chosen to establish a cut-off value below which tumors were considered ALT negative. Genomic DNA from an osteosarcoma-derived cell line, U2OS, a prototype of ALT positive cells, was used as a positive control for the C-circle reaction. **(B)** Detection of APBs by immunofluorescence using combined Telo-FISH analysis (red) and labeling with anti-PML antibodies (green). This is an example of a grade B oligodendroglioma (tumor #76) that was found to be ALT positive using the C-circle assay, representative of four analyzed tumors that were C-circle positive, two primary GBM and two grade B oligodendrogliomas.

Fig. 2. Quantification of total telomeric DNA content in patient tumors by dot blot analysis using the so-called Telo/Alu method (see Materials and methods). **(A)** Hybridization of the indicated tumor DNAs (10 ng per sample) with a ^{32}P -labeled $(\text{TTAGGG})_3$ telomeric probe (top panel) or with a ^{32}P -labeled Alu probe (bottom panel) that was used to normalize the telomeric signal to the total amount of genomic DNA. All 64 samples were processed similarly, but a few examples only are shown here for illustration. **(B)** Telomere length was measured in 6 cell lines using TRF analysis (30 μg per sample). Telomeric DNA content was measured in parallel in these 6 cell lines using the Telo/Alu method, as described above in A. This aimed at establishing a correlation between the total amount of telomeric DNA determined by the dot blot analysis and absolute telomere length measured in parallel using TRF analysis. A ^{32}P -labeled $(\text{TTAGGG})_3$ telomeric probe was used for both types of measurements. Horizontal dotted lines within the gel represent the upper and lower limits of each smear of telomeric tracts. The center of this smear is usually used to assess a telomeric mean value in kilobases of DNA. Note that the length of the smear is also important because it reflects the degree of scattering of all telomeres lengths around the mean value of the whole cell population.

Table 1: Statistical comparative analyses depending on C-circle status

	Total	Patients negative for C-circles (n=54)	Patients positive for C-circles (n=9)	p^(a)	p^(b)
Gender: male, n	38/63 (60.3%)	33/54 (61.1%)	5/9 (55.6%)	0.75	NP
Age, mean ± sd	57.1 ± 13.1	58.7 ± 11.7	47.6 ± 17.7	0.04	NP
IDH1/2 mutation	14/63 (22.2%)	8/54 (14.8%)	6/9 (66.7%)	0.001	NP
1p/19q codeletion or mutated INA	10/63 (15.9%)	9/54 (16.7%)	1/9 (11.1%)	1.00	0.99
Methylation MGMT>8%	34/63 (54.0%)	26/54 (48.2%)	8/9 (88.9%)	0.02	0.30
ATRX mutation	5/59 (8.5%)	1/51 (2.0%)	4/8 (50.0%)	0.001	0.02
P53 mutation	12/58 (20.7%)	8/49 (16.3%)	4/9 (44.4%)	0.08	0.14
Telomeric DNA content (Telo/Alu ratio), mean ± sd^(c)	0.75 ± 0.26	0.73 ± 0.22	0.89 ± 0.41	0.24	0.03
hTERT, mean ± sd^(c)	9.3 ± 7.5	10.5 ± 7.2 (n=38)	0 ± 0 (n=5)	<0.001	0.01
TERRA, mean ± sd ^(c)	2.53 ± 3.83	1.9 ± 1.1 (n=31)	8.6 ± 12.8 (n=3)	0.88	0.69
Ki-67, mean ± sd ^(c)	32.3 ± 14.8	34.2 ± 14.0 (n=38)	14.3 ± 10.5 (n=4)	0.02	0.11

^(a) Univariate analysis

^(b) Multivariate analysis adjusted on diagnosis age, histopathology grading, surgery type and IDH status

^(c) Quantitative levels, as explained in the material and methods section

NP: not performed

Complete data were available for all parameters (63/63), except for P53 mutation (58/63), Ki-67 (42/63), ATRX (59/63), hTERT (43/63) and TERRA (34/63).

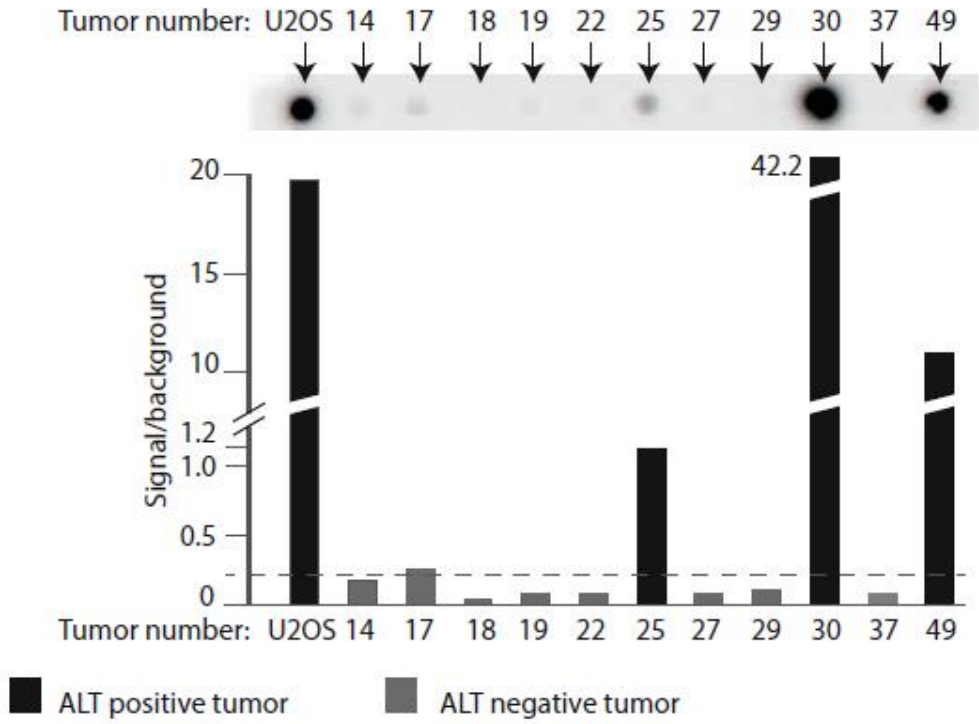
Table 2: Telomeric markers according to IDH status

	Total (n=63)	IDH mut. (n=14)	IDH WT (n=49)	P
ATRX mutated/non mutated, n (%)	5/59 (8.5)	5/12 (41.7)	0/47 (0.0)	<0.001
C-circle positive, n (%)	9 (14.3)	6 (42.9)	3 (6.1)	0.001
Telomeric DNA content (Telo/Alu ratio), mean \pm sd	0.75 \pm 0.26	0.70 \pm 0.25	0.76 \pm 0.26	0.46
hTERT expression, mean \pm sd	9.3 \pm 7.5	5.0 \pm 6.1	10.1 \pm 7.6	0.12
TERRA, mean \pm sd	2.53 \pm 3.83	5.29 \pm 8.88	1.94 \pm 1.13	0.68

Complete data were available for all parameters (63/63), except for P53 mutation (58/63), Ki-67 (42/63), ATRX mutation (59/63), hTERT expression (43/63) and TERRA (34/63).

Figure 1

A



B

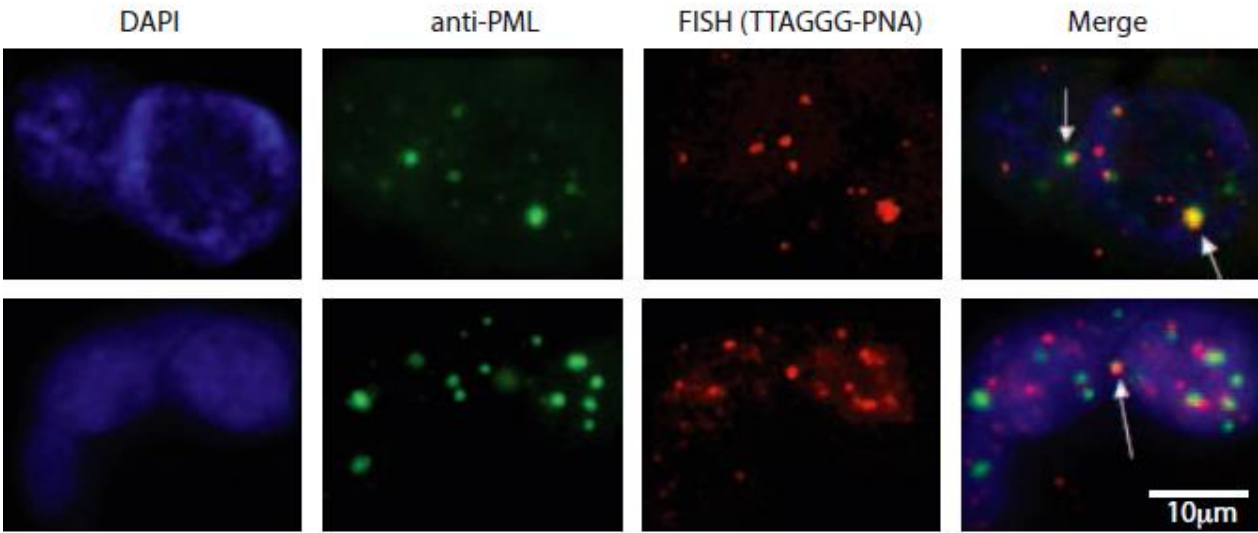
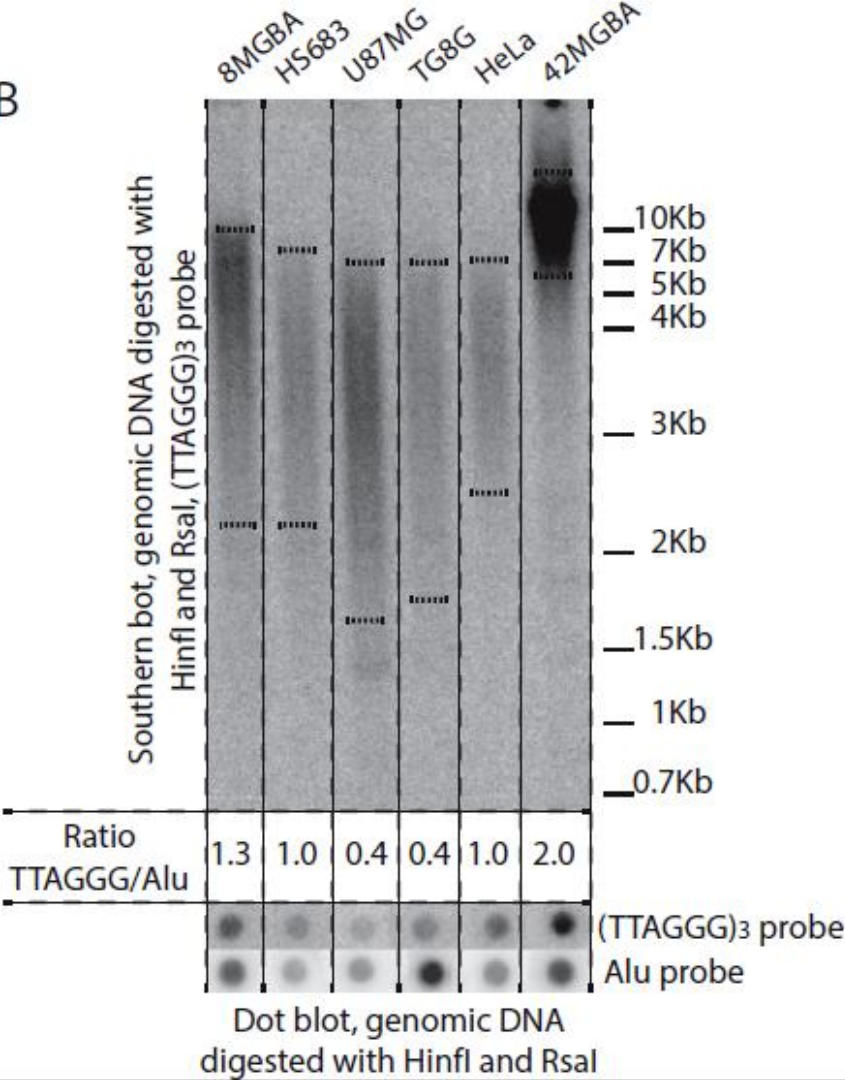
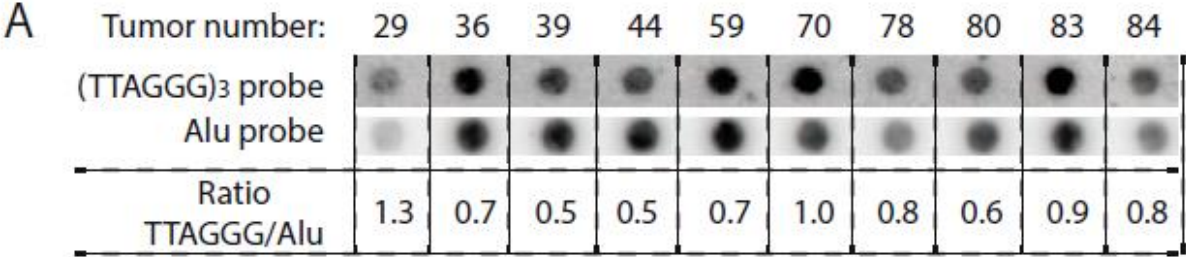


Figure 2



Supplementary Table 1: Molecular analyses results

Case number	WHO grades	1p/19q codel. ^a	IDH1	IDH2	MGMT mean methyl. (%) ^b	C-circle	C-circle quantif. ^c	Telomere length (Telo/Alu)	hTERT quantif.	TERRA quantif.	p53 ^d	Ki-67 (%)
6	IV	0	WT	WT	24.0	+	21.7	0.83	<0.01	NA	+	30
7	IV	0	WT	WT	18.7	-	0.16	0.72	NA	3.8 ± 0.8	+	40
9	IV	0	WT	WT	1.1	-	0.06	0.62	20.3 ± 3.1	0.9 ± 0.2	-	40
10	IV	0	WT	WT	2.2	-	0.09	0.92	NA	NA	-	30
13	IV	0	WT	WT	1.2	-	0.02	0.54	NA	NA	-	30
14	IV	0	WT	WT	1.1	-	0.18	0.69	10 ± 2.8	NA	-	40
16	IV	0	WT	WT	46.9	-	0	0.51	NA	NA	NA	NA
17	IV	0	WT	WT	49.2	-	0.27	1.12	NA	NA	-	30
18	IV	0	WT	WT	1.3	-	0.05	0.5	3.4 ± 1.6	NA	-	NA
19	IV	0	WT	WT	1.7	-	0.09	0.78	28.7 ± 5	NA	+	NA
22	IV	0	WT	WT	13.2	-	0.09	0.72	5.3 ± 2.9	NA	+	20
23	IV	0	WT	WT	1.3	-	0	0.75	6.1 ± 0.2	2.6 ± 0.3	-	NA
25	IV	0	WT	WT	29.8	+	1.18	0.4	NA	NA	+	NA
26	IV	0	WT	WT	19.5	-	0.02	0.82	4.7 ± 0.1	1.1 ± 0.3	-	40
27	IV	0	WT	WT	2.1	-	0.08	0.92	NA	NA	-	35
29	IV	0	WT	WT	0.8	-	0.12	1.31	0.3 ± 0.1	1.2 ± 0.3	-	NA
30	IV	0	WT	WT	24.6	+	42.18	1.77	<0.01	NA	-	NA
32	IV	0	WT	WT	2.4	-	0.03	0.43	8.4 ± 0.7	0.5 ± 0.1	-	30
34	IV	0	WT	WT	58.2	-	0.02	0.66	9.2 ± 6	0.7 ± 0.1	-	30
36	IV	0	WT	WT	1.6	-	0.01	0.69	19.8 ± 0.7	1.7 ± 0.2	NA	30
37	IV	0	WT	WT	1.5	-	0.08	0.34	NA	NA	-	50
39	IV	0	WT	WT	53.9	-	0.02	0.52	11.8 ± 3.4	1.4 ± 0.2	+	70
40	IV	0	WT	WT	1.3	-	0	0.53	NA	NA	-	30
41	II	0	WT	WT	5.4	-	0.25	0.64	<0.01	NA	-	5
42	III	1	R132C het	WT	31.8	+	0.74	0.49	<0.01	1.3 ± 0.2	+	NA

44	III	1	R132L het	WT	44.6	-	0.03	0.47	9.7 ± 1.2	1.2 ± 0.3	-	40
45	II	1	R132H het	WT	22.5	-	0.07	0.65	1.5 ± 0.2	NA	-	30
47	II	1	R132H het	WT	29.2	-	0.08	0.55	NA	NA	-	<5
48	III	1	WT	R172 M het	47.5	-	0.08	0.5	NA	NA	NA	40
49	II	0	R132H het	WT	30.2	+	11.8	0.74	<0.01	23.4 ± 4.4	-	8
50	II	0	R132C het	WT	40.9	+	11.5	0.7	NA	NA	-	NA
52	II	0	R132H het	WT	6.4	+	4.9	1	NA	NA	-	9
53	II	1	R132H het	WT	36.7	-	0.09	0.51	NA	NA	-	3
54	II	1	R132H het	WT	50.1	-	0.09	0.34	NA	NA	-	20
55	III	0	R132H het	WT	10.8	+	1.94	0.97	NA	NA	-	10
57	III	0	WT	WT	1.3	-	0.04	0.52	NA	NA	-	30
59	III	0	WT	WT	22.4	-	0.04	0.66	9.5 ± 0.1	1.9 ± 0.2	-	NA
61	III	0	WT	WT	33.5	-	0.03	0.49	11.5 ± 1.2	NA	-	40
62	III	0	WT	WT	0.9	-	0	0.86	NA	NA	-	50
63	III	0	WT	WT	1.4	-	0.01	0.43	8 ± 0.1	1 ± 0.2	NA	30
65	III	0	WT	WT	59.7	-	0.04	1.18	11.7 ± 2.7	NA	-	NA
66	III	0	WT	WT	48.9	-	0	0.58	6.7 ± 2	1.8 ± 0.4	+	30
69	III	0	WT	WT	33.7	-	0	0.76	19.9 ± 1.1	1.7 ± 0.6	-	NA
70	III	0	WT	WT	4.7	-	0.05	1.05	11.1 ± 2.7	4.1 ± 0.8	-	NA
71	III	1	WT	WT	2.1	-	0.04	1	8.8 ± 0.6	1.3 ± 0.1	-	NA
72	III	0	WT	WT	1.7	-	0.04	0.67	12.7 ± 1.6	NA	-	50
73	III	0	WT	WT	1.3	-	0.04	0.87	9.7 ± 3	1.5 ± 0.3	-	NA
74	III	0	WT	WT	0.9	-	0.03	0.97	NA	0.8 ± 0.1	-	NA

76	III	0	R132H het	WT	17.6	+	14.28	1.15	<0.01	1.2 ± 0.2	+	NA
77	III	0	WT	WT	5.1	-	0.13	0.87	9.8 ± 1	NA	-	30
78	III	1	R132H het	WT	50.9	-	0.13	0.79	15.2 ± 4.4	2 ± 0.5	-	40
80	III	0	WT	WT	29.2	-	0.03	0.57	5.4 ± 0.1	0.5 ± 0.1	+	NA
82	III	0	WT	WT	1.4	-	0.08	0.74	NA	4.8 ± 0.7	+	30
83	III	0	WT	WT	4.4	-	0.05	0.88	23.2 ± 6.8	2.1 ± 0.2	-	60
84	III	0	WT	WT	12.6	-	0.11	0.78	7.9 ± 0.9	3.3 ± 0.6	+	NA
85	III	0	WT	WT	2.0	-	0.02	0.93	6.5 ± 1.5	1.9 ± 0.5	-	NA
86	III	1	R132H het	WT	48.2	-	0.01	1	8.8 ± 1.1	2.7 ± 0.3	NA	30
87	III	0	WT	WT	1.8	-	0.01	0.62	7.9 ± 0.1	2.4 ± 0.2	-	40
88	III	0	WT	WT	37.0	-	0.07	0.7	6.2 ± 5	3.1 ± 0.1	-	NA
89	III	0	WT	WT	34.9	-	0.01	1.16	8.7 ± 2.5	0.9 ± 0.1	-	25
90	III	0	WT	WT	48.1	-	0	0.7	12.2 ± 1.2	2.5 ± 0.3	-	30
91	III	0	WT	WT	20.8	-	0.02	0.77	5.4 ± 1.2	1.6 ± 0.1	-	40
92	III	0	WT	WT	0.8	-	0.09	0.85	33.6 ± 4.1	2.9 ± 0.5	-	60

(a) 1p/19q codel. (codeletion 1p/19q): 1, present; 0, absent.

(b) *MGMT* mean methylation: mean methylation of CpG #74 to 78 expressed in % methylation of each CpG sites determined using pyrosequencing.

(c) The intensity of the C-circle signal was quantified with respect to the intensity of the P³² signal on the dot blot from which the background intensity of the nylon membrane had been subtracted. The mean value of the C-circle signal for HeLa cells, 0.23, was taken as a threshold for defining C-circle-positive tumors although two tumors, #17 and #41, with a score of 0.27 and 0.25, respectively, were not considered positive in this C-circle assay, because these values were too close to the HeLa cells signal. The mean value for 10 samples of U2OS cells in three different experiments was found to be 21.0.

(d) p53: mutated (+) or wild-type (-).

SUPPLEMENTARY DATA

Supplementary methods

DNA sequencing for *IDH1/2* mutational analysis and methylation status of *MGMT* promoter

Genomic DNA was isolated from frozen tissues using the QIAamp DNA Mini Kit (Qiagen) and stored at -80°C. *IDH1* and *IDH2* genotyping was performed by EpigenDx (Worcester, MA, US) using pyrosequencing. *MGMT* exon 1 methylation status was determined by EpigenDx (Worcester, MA, US) using the reproducible and sensitive bisulfite based-pyrosequencing strategy. Data were provided as % of methylation observed on the 5 CpG sites already described [1] as diagnostic CpG sites (cf PyroMark Q96 CpG *MGMT* kit from Qiagen: +34 from ATG (+65 from TSS), +39 from ATG (+70 from TSS), +42 from ATG (+73 from TSS), +46 from ATG (+77 from TSS) and +48 from ATG (+79 from TSS). The methylation status was defined according to the average methylation value of the 5 CpG sites of methylation, namely unmethylated when mean methylation was <8% and methylated when mean methylation was >8% [2].

Analysis of 1p/19q codeletion

The analysis of 1p/19q codeletion was performed using the Vysis 1p36/1q25 and 19q13/19p13 FISH Probe Kit (4N6020, Abbott), according to manufacturer's instructions. 1p/19q status was lacking for some patients. In these cases, internexin neuronal intermediate filament protein alpha (*INA*) expression was reported to be a surrogate marker of 1p/19q codeletion [3, 4]. For certain patients, in addition to FISH or *INA*, Copy Number Variation (CNV) measurements were performed afterwards, using the Cytoscan HD Affymetrix system. Among all patients analyzed using both CNV analysis and *INA* or FISH, patient #71 was the only one for whom there was a disagreement between the two methods. Immunohistochemical analyses were carried out on paraffin sections using antibodies directed against *INA* (see below).

Immunostaining

Immunohistochemistry for Ki-67 (clone MIB-1, dilution 1:150, Code no. M7240, Dako, Glostrup, Denmark), p53 (clone DO7, dilution 1:1000, Cat.#CM042C, Biocare Medical, Concord, CA, US), alpha-internexin (clone 2E3, dilution 1:300, Cat#MAB5224, Millipore Corporation, Billerica, MA, US) and ATRX (dilution 1:500, Cat#HPA001906, Sigma-Aldrich, Saint-Quentin Fallavier, France) were performed using the Benchmark Ventana system (Roche) as per the manufacturer's recommendations. Sections were semiquantitatively scored by light microscopy and percentage of stained tumor cells and staining intensity was evaluated. For p53, only

tumors with more than 70% of tumor cells demonstrating high intensity of p53 staining were considered as p53 mutated. For alpha-internexin, positive tumors were the ones with cytoplasmic staining observed in more than 10% of tumoral cells. For ATRX, a lack of nuclear positivity on tumoral cells was associated with an ATRX mutation.

Measurements of ALT-specific C-circles

The C-circle assay, which detects partially single-stranded telomeric (CCCTAA)_n DNA circles (C-circles) amplified by the Phi29 polymerase in the absence of dCTP, was performed as described previously [5]. Genomic DNA was digested with 4 U/ μ g *Hinf*I and *Rsa*I restriction enzymes and 25 ng/ μ g of Dnase-free RNase). Ten μ l of each sample (10 ng) was combined with 10 μ l 0.2 mg/ml BSA, 0.1% Tween, 1 mM each dATP, dGTP and dTTP, 1 \times Phi29 Buffer and 7.5 U Phi29 DNA polymerase (Thermo Scientific, Fermentas) and incubated at 30°C for 8 h, then at 65°C for 20 min. For quantification, the reaction products were diluted to 60 μ l with 2x SSC and dot-blotted onto a 2x SSC-soaked Hybond N⁺ nylon membrane (GE Healthcare). DNA was UV-cross-linked onto the membrane, which was then hybridized at 37°C with end-labeled ³²P-(CCCTAA)₃ and PerfectHyb Plus hybridization buffer (Sigma). Results were analyzed using a GE Storm phosphorimager and the ImageQuant software.

Detection of ALT-associated promyelocytic leukemia nuclear bodies (APBs) at the telomeres

APBs detection was conducted according to protocols previously described [6, 7]. Slides containing 8- μ m-thick sections from paraffin-embedded tumor samples were dewaxed and rehydrated by two washes in xylene for 5 min each, two washes in 100% ethanol for 5 min each, one wash in 70% ethanol for 5 min and finally immersed in distilled water to rinse off ethanol. Antigen retrieval was then performed by incubating the slides for 20 min at 100°C in a pressure cooker containing sodium citrate buffer at pH 6.0. The slides were then cooled and rinsed in PBS. Cells were then permeabilized by incubation with 0.5% Triton X-100 in PBS for 15 min. After three washes in 0.5% BSA, in PBS, they were blocked by incubation for 30 min in 7.5% BSA, 7.5% FBS and 0.1% Triton X-100 in PBS. Cells were incubated at room temperature for 1 h with an antibody against PML (1:50, Santa Cruz) and then with FITC-conjugated secondary antibody, at room temperature, for 1 h. Sections were then cross-linked with 4% formaldehyde and dehydrated. Telomere fluorescence *in situ* hybridization was done by denaturing slides together with the telomeric cyanin-3-conjugated (CCCTAA)₃ peptide nucleic acid (PNA) probe (Eurogentec) in 70% formamide, 0.25% blocking reagent (Roche), 10 mM Tris pH 7.2, for 5 min

at 75°C and hybridizing overnight at 37°C. Slides were washed in 70% formamide, 10 mM Tris pH 7.2, 0,1% BSA, counterstained with DAPI and mounted in Vectashield medium (Vector Laboratories). Images were captured on a Nikon epifluorescence microscope using NIS-Element D 3.1 image analysis software.

Measurement of telomeric DNA transcription (TERRA)

Two micrograms of tumor sample RNA were reverse transcribed using the Revertaid first strand cDNA synthesis kit from Thermo Scientific-Fermentas, with gene specific primers (see below) and random hexamers as primers for *GAPDH*. cDNAs were diluted to a final concentration of 10 ng/μl in sterile H₂O and amplified using a Bio-Rad Mini-Opticon instrument. Amplification was performed in 20 μl of reaction mix, containing 5 μl of diluted cDNA and 15 μl Mesa Green qPCR Mastermix Plus (Eurogentec), together with forward and reverse primers. Cycling conditions were 10 min at 95°C, followed by 45 cycles of 15 s at 95°C (denaturation) plus 1 min at 60°C (annealing/extension). At the end of the PCR run, melting curves of the amplified products were used to determine the specificity of the amplification reaction. Quantitative data of the samples were obtained using the BioRad CFX Manager software. All cDNA samples were assayed in triplicates. We chose the *GAPDH* as the endogenous normalizer because its expression was constant. The threshold cycle number for TERRA was normalized to *GAPDH*. TERRA expression was determined by the $\Delta\Delta C_t$ method. The primers used for PCR amplification of total TERRA transcription measurement were as previously described [8], namely Tel1b: 5'-CGGTTTGTGGTTGGGTTTGGGTTTGGGTTTGGGGT-3' and Tel2b: 5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'. The presented data represent the average \pm standard error for three replicates.

Cell lines and cell culturing

The following human cell lines were purchased from the American Type Culture Collection (ATCC) and the German Collection of Microorganisms and Cell Cultures (DSMZ) and were used to measure telomere length of cell populations, as described below. These were 8-MG-BA, a GBM cell line (DSMZ #ACC 432), Hs-683, an oligodendroglioma cell line (ATCC #HTB-138J3), 42-MG-BA, a GBM cell line (DSMZ #ACC 431), U-87 MG, a GBM astrocytoma cell line (ATCC #HTB-14), T98G, a GBM cell line (ATCC #CRL-1690), HeLa, an adenocarcinoma cell line (ATCC #CCL-2) and U2OS, an osteosarcoma, ALT-positive, cell line. Cells were cultivated in Minimum Eagle's medium (MEM) supplemented with 10% fetal bovine serum (FBS, PAA) in the

presence of 5% CO₂ in a 90%-humidified incubator or in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS in the presence of 5% CO₂.

Telomere length measurement of human cancer cell lines by telomere restriction fragment (TRF) analysis

To analyze telomere length by TRF [9], genomic DNAs were prepared from the cultured cell lines listed above, digested with *RsaI* and *HinfI* and separated in a 0.9% agarose gel (in TBE) run in TBE buffer overnight and, after denaturation, transferred and hybridized with a (TTAGGG)₃ ³²P-labeled telomeric probe. Following digestion of genomic DNA, telomere tracts of wild-type cells appear as a broad band which represents the average length of most chromosomes. Results were analyzed using a GE Storm phosphorimager and the ImageGauge software.

Supplementary results

Telomeric DNA content

Measurement of telomere length by Southern blot analysis, also referred to as TRF (Telomere Restriction Fragment) analysis [9] is currently considered as the gold standard for telomere length measurement [10]. However, when dealing with clinical samples for which the amounts of tumor DNA are often limited, TRF analysis is difficult because it requires a minimum of around 5 µg of DNA. In the current study, we had approximately 30 ng of tumor DNA available for each sample, once all the other analyses had been performed. To circumvent this problem, we quantified telomeric DNA (TTAGGG repeats) with respect to an internal control, namely the amount of Alu repeats, referred to as the Telo/Alu dot blot method (**Figure 2A**, **Supplementary Table 1**). A statistical study (see below) was conducted on the raw data obtained as explained above. However, in an attempt to determine an approximate absolute value for telomere length, we simultaneously measured telomere length by TRF and quantified telomeric DNA by the Telo/Alu dot blot method in cultured cells from six different human cell lines (**Figure 2B**). This strategy allowed us to confirm that the Telo/Alu dot blot signals were indeed proportional to telomere length measured by TRF. In addition, this could potentially be useful if approximate absolute values of telomere length, in kilobases, were needed for future glioma characterization.

No statistically significant difference in telomeric DNA content was found in relation with *IDH1/2* mutational status (**Table 1**). On the other hand, a significant positive association was found between long telomeres and the presence of C-circles ($p=0.03$, 0.7 [0.54 – 0.87] *versus* 0.83 [0.70 – 1], **Table 2**). For instance,

tumor #30 exhibited a 42.18 C-circle score and a 1.77 Telo/Alu ratio, the highest among all tumors measured (**Supplementary Table 1**). Finally, statistical analyses showed no correlation between telomeric DNA content/telomere length and survival (HR=1.16, [0.41 - 3.31], p=0.78).

Telomeric DNA transcripts (TERRA)

Telomeric DNA is transcribed into the non coding RNAs called TERRA [11]. In mammalian cells, TERRA molecules play an important role in regulating telomere structural maintenance and heterochromatin formation [12]. We therefore set out to measure TERRA levels in the 34 out of 63 gliomas for which enough material was available (**Supplementary Table 1**). A correlation between increased TERRA expression and better survival was found in multivariate analysis (p=0.07.). On the other hand, no statistically different value in TERRA levels was found between the IDH mutated and IDH WT gliomas (p=0.68, **Table 1**). Likewise, no statistically significant difference in TERRA expression was found between C-circle negative and positive tumors (**Table 2**).

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