The level of activity of the alternative lengthening of telomeres correlates with patient age in IDH-mutant ATRX-loss-of-expression anaplastic astrocytomas

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Abstract
All cancer cells need to maintain functional telomeres to sustain continuous cell division and proliferation. In human diffuse gliomas, functional telomeres are maintained due either to reactivation of telomerase expression, the main pathway in most cancer types, or to activation of a mechanism called the alternative lengthening of telomeres (ALT). The presence of IDH1/2 mutations (IDH-mutant) together with loss of ATRX expression (ATRX-lost) are frequently associated with ALT in diffuse gliomas. However, detection of ALT, and a fortiori its quantification, are rarely, if ever, measured in neuropathology laboratories. We measured the level of ALT activity using the previously described quantitative “C-circle” assay and analyzed it in a well characterized cohort of 104 IDH-mutant and ATRX-lost adult diffuse gliomas. We report that in IDH-mutant ATRX-lost anaplastic astrocytomas, the intensity of ALT was inversely correlated with age (p < 0.001), the younger the patient, the higher the intensity of ALT. Strikingly, glioblastomas having progressed from anaplastic astrocytomas did not exhibit this correlation. ALT activity level in the tumor did not depend on telomere length in healthy tissue cells from the same patient. In summary, we have uncovered the existence, in anaplastic astrocytomas but not in glioblastomas with the same IDH and ATRX mutations, of a correlation between patient age and the level of activity of ALT, a telomerase-independent pathway of telomere maintenance.

Keywords: Anaplastic astrocytoma, Secondary glioblastoma, Alternative lengthening of telomeres, IDH1/2 mutations, ATRX loss of expression

Introduction
Telomeres, first defined as structures located at the distal extremities of linear chromosomes with a specific function in preventing fusions between chromosome ends, are composed of 10 to 15 kb of repeated TTAGGG sequences [20, 27, 28]. Telomeres naturally erode with consecutive cell divisions, due to intrinsic mechanisms associated with the fixed 5’ to 3’ polarity of DNA replication, the so-called “end replication problem” [36]. Normally, telomerase, a reverse transcriptase enzyme with a built-in RNA template, compensates for this natural loss of telomeric sequences. However, due to the natural inactivation of telomerase, mainly by transcriptional mechanisms, telomeres of most somatic human tissues progressively shorten over time. This provokes a DNA damage-induced cell cycle arrest, which is the equivalent of replicative senescence in cultured cells [20, 31].

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Following cancer initiation, tumor cells must overcome the telomere-controlled replicative senescence barrier to be able to proliferate indefinitely. To do this, they need to reactivate a pathway of maintenance of functional telomeres to keep them at a minimal length compatible with minimal chromosome stability [7]. There are two major pathways of telomere maintenance mechanisms in tumor cells. One is reactivation of telomerase, hTERT, which occurs in ~ 85–95% of cancer types [16, 18], principally as the result, in diffuse gliomas, of the occurrence of mutations in hTERT promoter [23]. The second mechanism is the so-called ALT (alternative lengthening of telomeres) pathway [3]. ALT functions using recombination between the repeated telomeric DNA sequences on two different telomeres, or between a telomere and extra-chromosomal telomeric circles of DNA, or, else, by sister chromatid exchange of telomeric DNA. There is also good evidence that a sort of “rolling circle” mechanism could also lead to telomere amplification on a single telomere [33].

Human diffuse gliomas are among the ~ 5–15% of cancer types that can survive either owing to telomerase or the ALT pathway [16, 18]. The ALT pathway is prevalent in some glioma subtypes and strongly associated with astrocytomas and secondary glioblastomas (GBM). Clinical studies indicate that, all grades and subtypes considered, ~ 30% of gliomas develop the ALT pathway. In recent years, the molecular landscape of gliomas has been intensively studied [4, 37]. In diffuse gliomas, ALT activation has been associated with mutations in IDH1 or IDH2 and ATRX [32]. There are two main groups of IDH1/2 mutant gliomas: (i) astrocytomas exhibiting ATRX mutation, TP53 mutation and ALT activation, (ii) oligodendrogliomas harboring 1p/19q codeletion and hTERT activation, (iii) oligodendrogliomas harboring IDH1/2 mutations and the ALT pathway [3]. The IDH and ATRX mutations are associated with astrocytomas and secondary glioblastomas (grade III or IV) according to the WHO 2016 classification of nervous system tumors [25, 35]. When IDH1 R132H IHC was negative or unreliable, IDH1 and IDH2 mutational status was evaluated by Sanger sequencing as previously described [34]. Patient age was considered at the time of first surgery.

One hundred and four patients from the French nation-wide POLA network were included in this study. Inclusion criteria were the written consent of the patient for clinical data collection and genetic analysis according to the national and POLA network policies, an established diagnosis of high-grade gliomas (grade III or IV) according to the WHO 2016 classification of nervous system tumors [25, 35], availability of clinical data collection (gender, age at surgery, tumor location, contrast-enhancement on MRI, extent of surgical resection, post-operative treatment and outcome) and sufficient material for molecular studies available. The study was approved by the ethics committee of Hôpital Universitaire La Pitié-Salpêtrière.

Each sample was analyzed under a light microscope after hematoxylin-eosin staining for assessing necrosis and tumor cell percentage. Samples with less than 70% of tumor cells were excluded from this study. Automated immunohistochemistry (IHC) was performed for IDH1 R132H neomorphic enzyme, ATRX nuclear expression, Ki67 and p53 as previously described [11]. When IDH1 R132H IHC was negative or unreliable, IDH1 and IDH2 mutational status was evaluated by direct sequencing using the Sanger method as previously described [35]. The genomic profile and assessment of the 1p/19q codeletion status was determined based on SNP arrays, CGH arrays, or microsatellite marker analysis as previously described [19]. hTERT promoter mutation status was evaluated by Sanger sequencing as previously described [24]. Patient age was considered at the time of first surgery.

Materials and methods

Patients cohort from the French POLA network

One hundred and four patients from the French nation-wide POLA network were included in this study. Inclusion criteria were the written consent of the patient for clinical data collection and genetic analysis according to the national and POLA network policies, an established diagnosis of high-grade gliomas (grade III or IV) according to the WHO 2016 classification of nervous system tumors [25, 35], availability of clinical data collection (gender, age at surgery, tumor location, contrast-enhancement on MRI, extent of surgical resection, post-operative treatment and outcome) and sufficient material for molecular studies available. The study was approved by the ethics committee of Hôpital Universitaire La Pitié-Salpêtrière.
Measurements of ALT-specific C-circles

The C-circle (CC) assay detects partially single-stranded telomeric (CCCTAA)n, ALT-specific, DNA circles (C-circles) following amplification by the Phi29 polymerase in the absence of dCTP [17]. The previously described C-circle assay [17], used here to quantify ALT activity, has already been used in our laboratory [9, 13]. Figure 1 depicts this assay in its general outlines. Genomic DNA, prepared from fresh frozen tissue, was digested with 4 U/μg HinfI and RsaI restriction enzymes and 25 ng/μg of Dnase-free RNase. Ten μl of each sample (30 ng) was combined with 10 μl 0.2 mg/ml BSA, 0.1% Tween, 1 mM each dATP, dGTP and dTTP, 1X 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. The reaction products were diluted to 60 μl 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 Aldrich). Figure 1 also illustrates ALT-specific signals measured in tumor DNA samples. In addition to the tumor samples, each experiment contained both positive and negative control genomic DNAs from two cell lines, U2OS and HeLa, which are the prototypes of ALT positive and telomerase positive cells, respectively. Following measurement of the intensity of each spot (one spot representing one tumor), the C-circle value of each tumor was calculated relative to that measured in genomic DNA from the ALT positive U2OS cells, designated to be 100 arbitrary units (AU). All measurements were performed in duplicates.

\[\text{C-circle measurement to quantify telomeric recombination}\]

\[\text{products are detected with } ^{32}\text{P-(CCCTAA)3 probe under native conditions}\]

**Fig. 1** Measurement of the level of ALT activity levels in human diffuse gliomas. Top panels illustrate the principle of the ALT C-circle assay [17] and describe its general steps. ALT cells have very long telomeres that have been amplified mainly by homologous recombination that generates partially single-stranded extra-chromosomal circles. Genomic DNA prepared from tumor samples is then incubated with the Phi29 DNA polymerase that specifically amplifies this telomeric DNA. Middle panel illustrates ALT-specific signals measured in tumor DNA samples using this assay, which were detected here on dot blots hybridized with a telomeric 32P-labeled probe. Genomic DNAs from HeLa (telomerase positive) and U2OS (ALT positive) cells were also probed, representing negative and positive controls for the C-circle assay, respectively. These assays were systematically performed in duplicates and here dot blot 2, on the right, was loaded with the same tumor samples as dot blot 1, on the left, to insure for reproducibility. Bottom table illustrates examples of duplicate numbers obtained for each of the indicated tumors, real signals of which are represented in the middle panel above. The C-circle score was determined after calculating the intensity of the signal relative to that of the ALT positive U2OS cell line, designated to be 100 arbitrary units (AU). Note that the C-circle assays were performed on representative samples, including those from the two patient groups analyzed in the present study.
Results were analyzed using a GE Storm phosphorimager and quantified using ImageQuant software.

Detection of ALT by visualization of ultra-bright telomeric signals (Telom-FISH)
In the fluorescence microscope, promyelocytic leukemia nuclear bodies (PML) signals, detected with anti-PML antibody, are usually identified as corresponding to APB (ALT-associated PML bodies) when they co-localize with the FISH-labeled telomeres [18, 38]. However, recently, Heaphy et al. [15] found cases of fixed tumoral tissues in which ultra-bright Telo-FISH signals did not co-stain with PML. Since cultured cells lacking co-tissues in which ultra-bright Telo-FISH signals did not recently, Heaphy et al. [15] found cases of fixed tumoral tissues in which ultra-bright Telo-FISH signals did not co-stain with PML. Since cultured cells lacking co-tissues in which ultra-bright Telo-FISH signals did not

Statistical analysis
All analyses were performed using Stata software (Version 13, StataCorp, College Station, TX, USA) for a two-sided type I error of \( \alpha = 5\% \). Patients’ characteristics were expressed as mean ± standard deviation (SD) or median (interquartile range) for continuous data (assumption of normality assessed by using the Shapiro-Wilk test) and as numbers and associated percentages for categorical parameters. Correlation coefficients (Pearson or Spearman according to statistical distribution) were estimated to study relationships between quantitative parameters. As the statistical distribution of ALT activity was not Gaussian (for all patients and for each subgroup), the correlation coefficient estimated to study the relationships between ALT activity and other parameters was non parametric (Spearman). Quantitative variables were then compared between groups using Student t-test or non-parametric Mann-Whitney test if t-test assumptions were not met (normality and homoscedasticity analyzed using the Fisher-Snedecor test). For categorical parameters, the comparisons between groups were carried out with Chi-squared or Fischer’s exact tests. Overall survival and disease free survival were studied as a censored data. Therefore, comparisons were analyzed using log-rank test and Cox proportional-hazards regression. The proportional-hazard hypothesis was studied using Schoenfeld’s test and plotting residuals. Results were expressed as hazard ratios (HR) and 95% confidence intervals.

Results
Definition of diffuse glioma patients groups
Based on histomolecular characteristics described in the Materials and methods section, we selected, according to the WHO 2016 classification of brain tumors, 60 IDH-mutant anaplastic astrocytomas (AA) and 44 IDH-mutant GBM that all exhibited loss of ATRX. The percentages of p53 immunoreactivity and Ki67 positive tumor cells were also assessed. Measurements of these molecular markers in all 104 patients, plus the C-circle score (see below) and the presence of mutations in \( hTERT \) promoter, as well as all available clinical parameters are shown in Additional file 1: Table S1.

The level of ALT activity correlates with patient age in IDH-mutant ATRX-lost AA
Compared with telomerase positive cells, ALT cells have very long telomeres (up to 50 kb) that are generated by recombination-dependent DNA replication [33]. ALT
cells, but not telomerase positive cells, generate extrachromosomal telomeric single-stranded DNA called C-circles, which can be amplified using the Phi29 DNA polymerase [17] (Fig. 1). C-circle scores for all analyzed IDH-mutant ATRX-lost tumors, together with other molecular markers are shown in Table 1. In the AA population, we found a significant inverse correlation (−0.41, \( p < 0.001 \)) between the level of ALT activity and patient age, meaning that the younger the patient, the higher the ALT-associated C-circle value (Fig. 2a, bottom). In this group, ALT positive patients with ages comprised between 25 and 30 years old had tumors with a mean C-circle intensity of around 68 AU (arbitrary units; see Materials and methods), while ALT positive 66–70 years-old patients had tumors with a mean C-circle intensity of around 22 AU, all 5-year-classes in-between having intermediate, age proportional increasing values of C-circles (Fig. 2a, top). It was important to know whether the C-circle score/age correlation observed in the ALT positive AA also existed in the GBM with the same IDH-mutant ATRX-lost genotype. Interestingly, in the 44 analyzed GBM, there was no significant correlation between the level of ALT activity and patient age at the time of surgery (correlation of 0.01, \( p = 0.94 \); Fig. 2b). We note that, for these experiments, the correlation coefficient was very weak, close to zero, which led us to conclude that the lack of significant age/ALT intensity correlation in the GBM was not due to a lower statistical power analysis compared with the AA. When the entire cohort was considered \( (n = 104) \), a weak correlation between age and ALT activity level was found (correlation of −0.23, \( p = 0.02 \)).

In the two groups of patients, similar mean age values were present within the different quartiles, thus showing that the ALT intensity/age correlation found in AA was not biased by age distribution (Additional file 2: Table S2). In addition, the ALT intensity/age correlation found in AA was not biased by potentially large differences in the mean C-circle values between the two groups, because these were very similar, 40.95 ± 34.96 AU for AA vs 39.25 ± 33.05 AU for GBM.

Measurements of ALT activity in patients with relapse having evolved from lower grade or anaplastic astrocytoma to GBM

Given that the secondary GBM tumors are thought to derive from either low-grade or anaplastic astrocytomas, it was particularly remarkable that the patient age/ALT intensity correlation was observed in AA but not in secondary GBM. To better understand these mechanisms, we measured C-circle activity in matched samples from patients having evolved from astrocytoma to GBM. We identified 13 paired cases, among which only eight could be exploited, due to the fact that DNA extracted from paraffin-included tumors did not give signals in the C-circle assay under the conditions used for frozen tumor samples (data not shown). All relapsed tumors remained IDH-mutant, ATRX-lost and 1p/19q non-codeleted. In these experiments, we noted that all four patients with high ALT intensity (> 60 AU) nevertheless evolved to GBM (Additional file 3: Table S3). This suggested that an initial high level of C-circle in these four young patients (22 to 40 years old) did not prevent progression from AA to GBM. In some, but not all, patients, C-circle intensity remained rather low or rather high in GBM with respect to the first measurement in astrocytomas (Additional file 3: Table S3). Although these data are somewhat informative, they cannot elucidate the problem of knowing why AA and GBM differ in terms of age/ALT intensity correlation, which might nevertheless be possible if performed on a much larger number of patients with relapse.

Telomere length in somatic cells does not correlate with ALT intensity in tumors

Telomeres of human somatic cells erode with age. One might hypothesize that telomeres of somatic cells of younger patients are longer than those of older patients, which could be the reason why the ALT pathway is more intense in these patients. Therefore, we next set out to measure telomere length in peripheral blood mononuclear cells of patients chosen from several distinct classes of age and ALT characteristics (Additional file 4: Figure S1). These experiments clearly established that telomere length in healthy cells at the time of oncogenic transformation is not correlated with the intensity of the ALT mechanisms in the emerging tumor. Thus, for instance, among young AA patients, there was no difference in telomere length in mononuclear cells whether the tumors exhibited low, medium or high C-circle level. This was the same for older AA patients. In addition, there was no difference in telomere length of peripheral blood cells between AA and oligodendrogliomas patients (Additional file 4: Figure S1).

Correlations between the amount of ALT-specific C-circles and the presence of ALT-associated ultra-bright telomeric foci

In contrast to high score ALT tumors, for instance tumor #3240 (170 AU), a substantial number of tumors had a much lower score (Fig. 1; Table 1). An important issue was to determine whether these low to very low C-circle tumors developed true ALT mechanisms. To achieve this, we set out to visualize the presence of ultra-bright telomeric foci by Telo-FISH. Detection of these foci in the fluorescence microscope is now widely used to identify the very long and heterogeneous telomeres associated with ALT (see Materials and methods). Using this approach, we found that all nine fixed paraffin-embedded tumors in which ALT-specific C-
Table 1 Clinical parameters and molecular analyses of the tumors

| Tumor number | p53 | Ki67 | age at first symptoms | TERT promoter | C-circles (arbitrary units) |
|--------------|-----|------|-----------------------|---------------|-----------------------------|
| 3570         | 5%  | 7%   | 56                    | wt            | 20                          |
| 4080         | 50% | 10%  | 56                    | wt            | 6                           |
| 4140         | 50% | 70%  | 28                    | wt            | 82                          |
| 4827         | 60% | 5%   | 32                    | wt            | 55                          |
| 5595         | 30% | 12%  | 54                    | wt            | 91                          |
| 6637         | 80% | 10%  | 33                    | wt            | 87                          |
| 4185         | 40% | 15%  | 33                    | wt            | 80                          |
| 3552         | 70% | 10%  | 32                    | wt            | 21                          |
| 4644         | 60% | 5%   | 66                    | wt            | 31                          |
| 3635         | 3%  | 15%  | 43                    | wt            | 19                          |
| 3843         | 90% | 10%  | 29                    | wt            | 31                          |
| 3091         | 80% | 7%   | 68                    | wt            | 10                          |
| 4126         | 50% | 15%  | 61                    | wt            | 7                           |
| 4975         | 50% | 7%   | 55                    | wt            | 10                          |
| 3240         | 5%  | 15%  | NA                    | wt            | 170                         |
| 3402         | negative | 3%  | 38                    | wt            | 1                           |
| 4031         | 50% | 10%  | 47                    | wt            | 31                          |
| 4878         | negative | 15% | 24                    | wt            | 103                         |
| 5815         | negative | 12% | 45                    | wt            | 14                          |
| 3462         | 60% | 10%  | 40                    | wt            | 60                          |
| 4951         | 60% | 10%  | 43                    | wt            | 11                          |
| 5261         | 60% | 5%   | 38                    | wt            | 59                          |
| 5489         | 10% | 30%  | 48                    | C250T         | 69                          |
| 5546         | 80% | 10%  | 27                    | wt            | 99                          |
| 5841         | 60% | 10%  | 58                    | wt            | 34                          |
| 5895         | 100% | 5%  | 51                    | wt            | 19                          |
| 6013         | 60% | 6%   | 33                    | wt            | 16                          |
| 6095         | negative | 8%  | NA                    | wt            | 56                          |
| 6658         | 60% | 12%  | 33                    | wt            | 3                           |
| 3558         | 100% | 20% | 39                    | wt            | 22                          |
| 4056         | 50% | 15%  | 29                    | wt            | 5                           |
| 4289         | 90% | 10%  | 28                    | wt            | 24                          |
| 4572         | 90% | 8%   | 42                    | wt            | 49                          |
| 4840         | 75% | 8%   | 34                    | wt            | 61                          |
| 5238         | 80% | 8%   | 55                    | wt            | 21                          |
| 5744         | 50% | 12%  | 34                    | wt            | 83                          |
| 5987         | 80% | 15%  | 35                    | wt            | 90                          |
| 5443         | 80% | 12%  | 61                    | wt            | 12                          |
| 5448         | 50% | 12%  | 29                    | C228T         | 37                          |
| 5790         | 50% | 12%  | 47                    | wt            | 18                          |
| 5793         | 60% | 15%  | 43                    | wt            | 35                          |
| 5866         | 60% | 15%  | 40                    | wt            | 91                          |
| 6244         | 60% | 20%  | 45                    | wt            | 22                          |
Table 1 Clinical parameters and molecular analyses of the tumors (Continued)

| Tumor number | p53 | Ki67 | age at first symptoms | TERT promoter | C-circles (arbitrary units) |
|--------------|-----|------|-----------------------|---------------|---------------------------|
| anaplastic astrocytomas |     |      |                       |               |                           |
| 6309         | 60% | 12%  | 39                    | wt            | 102                       |
| 4569         | 90% | 15%  | 32                    | C250T         | 40                        |
| 2944         | 70% | 10%  | 57                    | wt            | 25                        |
| 5022         | negative | 12% | 66                    | wt            | 24                        |
| 5725         | 80% | 12%  | 53                    | wt            | 12                        |
| 6011         | 80% | 20%  | 39                    | wt            | 46                        |
| 5974         | 80% | 5%   | 34                    | wt            | 16                        |
| 5003         | 30% | 5%   | 33                    | C228T         | 109                       |
| 3684         | 25% | 7%   | 33                    | C228T         | 1                         |
| 4106         | negative | 10% | 31                    | wt            | 49                        |
| 5593         | 60% | 10%  | 34                    | wt            | 34                        |
| 5594         | 80% | 11%  | 59                    | wt            | 13                        |
| 6654         | 60% | 8%   | 52                    | wt            | 17                        |
| 6742         | 40% | 4%   | 48                    | wt            | 38                        |
| 5510         | 35% | 20%  | 28                    | C228T         | 45                        |
| 5912         | 80% | 12%  | 33                    | wt            | 0                         |
| 5398         | 80% | 12%  | 36                    | wt            | 21                        |

| Tumor number | p53 | Ki67 | patient age (years) | TERT promoter | C-circles |
|--------------|-----|------|---------------------|---------------|-----------|
| secondary GBM |     |      |                     |               |           |
| 3942         | 80% | 25%  | 37                  | wt            | 6         |
| 2592         | 40% | 10%  | 69                  | wt            | 41        |
| 3077         | 60% | 20%  | 25                  | wt            | 16        |
| 3007         | 80% | 30%  | 31                  | wt            | 82        |
| 3297         | 30% | 70%  | 59                  | wt            | 77        |
| 4034         | 30% | 10%  | 58                  | wt            | 7         |
| 4115         | 80% | 25%  | 55                  | wt            | 101       |
| 3845         | 80% | 8%   | 32                  | wt            | 32        |
| 3928         | negative | 30% | 41                  | wt            | 58        |
| 3933         | negative | 25% | 32                  | wt            | 88        |
| 3992         | 40% | 15%  | 34                  | wt            | 20        |
| 3959         | 90% | 15%  | 26                  | wt            | 13        |
| 3019         | 100%| 25%  | 29                  | wt            | 136       |
| 2835         | 20% | 8%   | 37                  | wt            | 52        |
| 3316         | 40% | 70%  | 24                  | wt            | 68        |
| 2695         | 60% | 60%  | 30                  | wt            | 38        |
| 3359         | 100%| 60%  | 41                  | wt            | 10        |
| 3451         | 40% | 3%   | 61                  | wt            | 40        |
| 4068         | 60% | 20%  | NA                  | wt            | 44        |
| 4082         | negative | 12% | 35                  | wt            | 35        |
| 4125         | 60% | 25%  | 36                  | wt            | 6         |
| 4145         | 30% | 12%  | 29                  | wt            | 29        |
| 4194         | 70% | 10%  | 33                  | wt            | 34        |
| 4245         | 15% | 12%  | 29                  | wt            | 24        |
| 4234         | 80% | 20%  | 30                  | wt            | 22        |
circles had been detected, in the corresponding frozen tissue, with a score equal or greater than 6 AU also contained ALT-specific ultra-bright Telo-FISH foci (Fig. 3). Two additional tumors with a C-circle score of 2 or 4 AU also contained ALT-specific ultra-bright Telo-FISH foci, but these foci were found to be smaller than in the other nine tumors examined (the tumor with the 2 AU score was in fact hTERT-mutated; data not shown). On the other hand, five oligodendroglioma tumors (with functional ATRX and a mutation in hTERT), measured with a C-circle score of 0 AU, did not exhibit ALT-specific ultra-bright Telo-FISH foci, but these foci were found to be smaller than in the other nine tumors examined (the tumor with the 2 AU score was in fact hTERT-mutated; data not shown). On the other hand, five oligodendroglioma tumors (with functional ATRX and a mutation in hTERT), measured with a C-circle score of 0 AU, did not exhibit ALT-specific ultra-bright Telo-FISH foci, but these foci were found to be smaller than in the other nine tumors examined (the tumor with the 2 AU score was in fact hTERT-mutated; data not shown). On the other hand, five oligodendroglioma tumors (with functional ATRX and a mutation in hTERT), measured with a C-circle score of 0 AU, did not exhibit ALT-specific ultra-bright Telo-FISH foci, but these foci were found to be smaller than in the other nine tumors examined (the tumor with the 2 AU score was in fact hTERT-mutated; data not shown).

High Ki67 levels correlate with ALT activity level in GBM
We also paid particular attention to p53 immunoreactivity and Ki67 levels. The correlation coefficient between p53 score and C-circle intensity was −0.11 (p = 0.41) for the 60 analyzed AA and −0.10 (p = 0.53) for the 44 analyzed GBM. On the other hand, interestingly, we found a moderate correlation between Ki67 levels and C-circle intensity in GBM (0.38, p = 0.01), but not in AA (correlation coefficient of 0.20, p = 0.12). However, the fact that Ki67 values were clearly lower in AA than in GBM (mean of 12.13 ± 8.96 for AA vs 22.27 ± 16.63 for GBM) prevented us from drawing conclusions concerning the presence or absence of correlation between Ki67 and C-circle intensity in AA (Additional file 5: Table S4).

Absence of relationships between survival and ALT activity level
There was no statistically significant correlation between ALT intensity (C-circle score) or patient age and overall survival both in AA and GBM patients (Additional file 6: Table S5). We also note that there was no significant difference in overall survival between AA and GBM patients (Additional file 6: Table S5). Therefore, the ALT intensity/patient age correlation we observe in the AA group does not result from the fact that younger patients have a higher level of ALT activity because they survive longer than older patients.

Discussion
The ALT pathway represents an alternative to telomerase in maintaining functional telomeres, which is essential for cell division and proliferation of tumor cells. ALT is prevalent in IDH-mutant ATRX-lost diffuse gliomas and we now propose, based on the present data, that its detection and quantification in pathology laboratories might be very useful. The major finding of the
present study is that in IDH-mutant ATRX-lost anaplastic astrocytomas (AA), the younger the patient with ALT positive tumor, the stronger the ALT-associated C-circle signal. IDH-mutant ATRX-lost GBM, with similar age distribution, failed to exhibit this correlation. Incidentally, we note that it was previously shown that in astrocytomas, GBM and osteosarcomas, ALT positive patients were younger than non-ALT patients [14, 18]. Most importantly, the ALT/age correlation we have found here concerns the intensity of ALT, not only its occurrence, contrary to these previous studies that examined ALT occurrence, but not its level of activity.

ATRX functions as an inhibitor of ALT in cancer cell lines [6, 29]. ATRX also seems to function as an inhibitor of ALT in vivo, as mutations in ATRX are associated with the ALT pathway in tumors. In addition, the few tumors of this study that exhibited both a mutation in hTERT promoter and ATRX were clearly ALT positive (Table 1), thus pointing out to ALT being epistatic to telomerase gain of function. The concomitant presence of these two mutations is rather unusual, but is nevertheless known to occur. Given the reliability and reproducibility of the C-circle assay as a molecular marker of ALT [17], the finding that most of these tumors (5/6 for AA, 1/2 for GBM) have a significant level of ALT activity should be kept in mind for further investigation.

It is not known yet why the intensity of ALT can largely differ from one tumor to the next, even within the same group. This could be due to the fact that C-circle activity might be highly dynamic and be affected by activation/deactivation of various pathways. Alternatively, different types of ALT mechanisms may exist within the same group of tumors. Our measurements of ALT intensity in AA patients with relapse and progression to GBM [30] seem to rule out the existence of an AA-specific type of ALT, with high intensity, no longer present in GBM. Differences in ALT intensities in a given patient at different times and tumor stages, noted

![Fig. 2](image-url) Correlation between the level of ALT activity, expressed as the percentage of radioactive signal with respect to the ALT signal recorded in genomic DNA from U2OS cells, designated to be 100 arbitrary units (AU), and patient age in anaplastic astrocytomas, AA (a, top and bottom left) and secondary glioblastomas multiform, GBM (b). Patient age was considered at the time of first surgery. a bottom right: According to statistical distribution, we arbitrarily defined two categories of anaplastic astrocytoma (AA) patients with C-circle score < or > 50 AU. This graphical representation also clearly shows the ALT intensity/patient age correlation.
in these patients with relapse (Table S3), are potentially meaningful and would deserve to be further examined in much larger numbers of patients. Technically, such an approach could be greatly facilitated by measuring C-circle levels in patient blood [17].

Telomere length in humans varies with time, progressively shortening as people age (see, for instance, reference [1]). Long telomeres might be more prone to ALT-based recombination than shorter telomeres. Excessively short telomeres might also be more prone to ALT-based recombination, which could then be used as a DNA repair pathway. We found no correlation between the level of ALT activity in the tumor and telomere length in the corresponding normal tissue. Therefore, it is unlikely that the intensity of the ALT pathway in the emerging tumor is dependent on telomere length of the healthy tissue that underwent oncogenic transformation.

The significance of the level of ALT activity as a clinical parameter was demonstrated in the present study by monitoring, in complement to C-circles, for the presence of ALT-specific ultra-bright Telo-FISH signals. All nine tumors in which a C-circle score > 6 AU was detected indeed exhibited ultra-bright Telo-FISH signals, thereby demonstrating that these low-C-circle score tumors are true ALT positive tumors.

An additional technical issue stems from the fact that our C-circle measurements were performed on a single sample from each tumor (albeit in duplicates) and, consequently, did not take into account intra-tumor heterogeneity. In future studies, at least some of the measurements of ALT intensity should ideally be performed on several samples from the same tumor.

Ki67 measurement represents a reliable cellular marker for cell proliferation [34]. As expected, we found

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**Fig. 3** Detection of ultra-bright telomeric foci by Telo-FISH in paraffin-embedded sections of tumors, together with detection of DNA by DAPI in the same section, as indicated (40X magnification). The upper chart indicates the mean number of telomeric foci per nucleus, as well as the percentage of nuclei with ultra-bright foci of an intensity over 40 pixels (px), the rate of C-circles and the diagnostic. C-circles values are expressed in arbitrary units (AU; see Materials and methods). The images are representative pictures of tumors, OD: oligodendroglioma; GBM IDHmt: IDH-mutant secondary glioblastoma; AA: anaplastic astrocytoma. Note that the photograph representing the OD tumor (picture a) was taken at a much higher exposure, as indicated, to show that the Telo-FISH signals are indeed present, but are much fainter than in the GBM and AA tumors. For both the OD tumor (a) and AA tumor (b) shown in the upper panels, lower magnifications are provided in the lower panels, as indicated (larger field).
that in GBM, Ki67 staining (higher than in AA) was correlated with ALT intensity. However, we note that in young AA patients a low Ki67 staining could co-exist with high ALT activity, thereby suggesting that the level of ALT activity in AA is unrelated to cell proliferation.

**Conclusions**

In summary, the present work demonstrates the importance and potential clinical significance of not only detecting, but also quantifying the ALT pathway, a telomere maintenance mechanism that functions when telomerase cannot be re-activated in the tumor. Using the C-circle assay, which requires only 30 ng of tumor DNA, we have shown that ALT intensity correlates with patient age in IDH1/2-, ATRX-mutated anaplastic astrocytomas (AA). This finding could certainly be used in a near future to delineate possible subtypes of ALT-positive AA based on the intensity of the ALT pathway by C-circle. On the other hand, it would be important to understand the correlation between ALT intensity and patient age at the molecular level. To do this, cells from patients with different ALT intensities could be derived for further molecular and genetic studies. The potential existence of different types of ALT would certainly open wide avenues of research, as well as design of targeted treatments, on the telomeric mechanisms that govern cell proliferation in cancer cells.

**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s40478-019-0833-0.

**Additional file 1:** Table S1. Clinical parameters and molecular analyses of the tumors.

**Additional file 2:** Table S2. Distribution of age within anaplastic astrocytomas (AA) and secondary glioblastoma (GBM) patients.

**Additional file 3:** Table S3. Measurement of ALT activity in patients with relapse.

**Additional file 4:** Figure S1. Telomere length in peripheral blood mononuclear cells from 25 patients using TRF analysis (S μg DNA per sample).

**Additional file 5:** Table S4. Ki67/C-circle correlation in GBM.

**Additional file 6:** Table S5. Patients survival data.

**Abbreviations**

AA: Anaplastic astrocytoma; ALT: Alternative lengthening of telomeres; APB: ALT-associated promyelocytic leukemia bodies; ATRX: α-thalassemia/mental/retardation X-linked; AU: Arbitrary units; GBM: Glioblastoma multiforme; DH: Dehydrogenase; IHC: Immunohistochemistry; MRI: Magnetic Resonance Imaging; TRF: Telomere restriction fragments

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