A Robust Assay for Alternative Lengthening of Telomeres in Tumors Shows the Significance of Alternative Lengthening of Telomeres in Sarcomas and Astrocytomas

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ABSTRACT

Purpose and Experimental Design: Telomeres of tumor cells may be maintained by telomerase or by alternative lengthening of telomeres (ALT). The standard ALT assay requires Southern analysis of high molecular weight genomic DNA. We aimed to establish and validate an ALT assay suitable for archived paraffin-embedded tumors and to use it to examine the prevalence and clinical significance of ALT in various types of tumors that are often telomerase negative.

Results: To assay for ALT, we detected ALT-associated promyelocytic leukemia (PML) bodies (APBs) by combined PML immunofluorescence and telomere fluorescence in situ hybridization. APBs are PML nuclear domains containing telomeric DNA and are a known hallmark of ALT in cell lines. The APB assay concurred with the standard ALT assay in 62 of 62 tumors and showed that 35% of 101 soft tissue sarcomas (STS), 47% of 58 osteosarcomas (especially younger patients), 34% of 50 astrocytomas, and 0% of 17 papillary thyroid carcinomas were ALT positive (ALT+). The prevalence of ALT varied greatly among different STS subtypes: malignant fibrous histiocytomas, 77%; leiomyosarcomas, 62%; liposarcomas, 33%; synovial sarcomas, 9%; and rhabdomyosarcomas, 6%. ALT correlated with survival in glioblastoma multiforme and occurred more often in lower-grade astrocytomas, but ALT+ and ALT− sarcomas were equally aggressive in terms of grade and clinical outcome.

Conclusion: The APB assay for ALT is suitable for paraffin-embedded tumors. It showed that a substantial proportion of STS, osteosarcomas, and astrocytomas, but not papillary thyroid carcinomas use ALT. APB positivity correlated strongly with survival of patients with astrocytomas.

INTRODUCTION

Telomeres consist of 4 to 15 kb of the repetitive DNA sequence TTAGGG and undergo progressive shortening during proliferation of normal human somatic cells (1). Telomere shortening eventually triggers senescence thus acting as a barrier to unlimited cellular proliferation (2). In cancer, the occurrence of cell death and the need for clonal evolution mean that the number of cell divisions required for most tumors to become clinically significant is greater than the proliferative capacity of normal somatic cells (3). The overwhelming majority of cancers evade this limitation by activating a telomere maintenance mechanism (TMM; refs. 4, 5). The dependence of most tumors on an active TMM may be useful for cancer diagnosis and treatment.

Cancers maintain the length of their telomeres with either telomerase (6, 7) or alternative lengthening of telomeres (ALT; ref. 8), with only a few having no known TMM (8–11). Approximately 85% of all human tumors have telomerase activity (TA; ref. 12), but the presence of ALT in tumors has not been examined extensively. An early study of ALT in tumors (8) found that 12% of various tumor types were ALT+. The prevalence of the two TMMs seems to vary among different tumor types. For example, only about 50% of sarcomas and thyroid carcinomas are TA+ (13) and two recent studies have found ALT to be common in osteosarcomas (66% ALT+; ref. 11) and glioblastoma multiforme (GBM; 25% ALT+; ref. 10).

It is likely that the study of ALT in cancer will be useful for clinical practice. In GBM the presence of ALT was found to correlate more strongly with long-term survival than the standard prognostic indicator, patient age (10). In osteosarcomas, however, ALT+ and TA+ tumors seemed to be equally aggressive and longer survival correlated well with the absence of both TMMs (11). ALT, like telomerase, may become an attractive drug target if it proves to be common in a number of tumor types, especially since repression of ALT in ALT+ immortal cell lines results in senescence and cell death (14, 15).

This study was motivated by the need for a practical method of screening routinely archived tumors for ALT. The mechanism of ALT is not fully understood, but it is telomerase independent and most likely involves recombination-mediated DNA replication (13, 16–19). Detection of ALT has been dependent on...
extracting genomic DNA of high molecular weight to test for the characteristic heterogeneous telomere length distribution of ALT cells by Southern analysis. Because it is sometimes necessary to study tumors archived as paraffin-embedded blocks, we investigated whether another hallmark of ALT cells, the presence of ALT-associated promyelocytic leukemia (PML) bodies (APBs; ref. 20), could be utilized as an assay for ALT.

ALT-associated PML bodies are PML bodies (21, 22) that contain telomere-related materials, including DNA with the telomeric (5′-TTAGGG-3′)ₙ sequence and the telomere-specific binding proteins, TRF1 and TRF2. In cell lines, the presence of APBs correlates precisely with the presence of ALT. APBs have been found in 21 of 21 ALT+ cell lines, 0 of 25 TA+ cell lines (including three with mean telomere lengths of 15-25 kb), and 0 of 5 mortal cell lines (20, 23, 24). The concordance between the ALT mechanism and the presence of APBs is strengthened by the finding that APBs are repressed when ALT is repressed (15, 18). Although APBs have been found in all ALT+ cell lines examined thus far, they are seen in only about 5% of cells within an asynchronously dividing ALT+ population (20), which suggests that they are mostly present during a specific phase of the cell cycle (23, 24). We show here that detection of APBs is a reliable method for detecting ALT in paraffin sections of human tumors and show its utility by surveying tumors of various types for the presence of ALT.

MATERIALS AND METHODS

Tumor Specimens. Tumor specimens were acquired with approval of the Ethics Committees or Institutional Review Boards of the Children’s Hospital Westmead, New South Wales, Australia and the other source institutions. Adult soft tissue sarcomas (STS) were from 68 patients of the MD Anderson Cancer Center, Houston, TX, where the 65 STS patients used for survival analysis were treated surgically between 1992 and 1999. Sixteen TA+ adult STS were from the University of Iowa, Iowa City, IA and their telomerase status has been reported (25). Thirty-three pediatric STS were from patients in Sydney, Australia (Royal Prince Alfred Hospital, Children’s Hospital Westmead and Douglass Hanly Moir Pathology). The 39 osteosarcoma patients used for survival analysis were all treated by one surgeon, diagnosed (1997-2002) with operable non-metastatic osteosarcoma, and received similar preoperative chemotherapy. Astrocytomas were from 50 patients of the Royal Hallamshire Hospital, Sheffield, United Kingdom and the University of Otago, Dunedin, New Zealand. The 32 GBM patients used for survival analysis were a subset of the patients described by Hakin-Smith et al. (10) for whom paraffin sections were available. Seventeen papillary carcinomas of the thyroid were from the Royal North Shore Hospital, St. Leonards, New South Wales, Australia.

Tumor samples from ALT+ (IIICF/c-EJ-ras) and TA+ (WM1175) cell lines grown in nude mice as described previously (20) were used as positive and negative controls, respectively, for the APB assay.

Detection of APBs. Frozen sections were cut 5 to 7 μm thick and fixed in 1:1 methanol/acetone. Paraffin sections were cut 8 μm thick and dewaxed. Surface decalcification was needed for some osteosarcoma paraffin sections; this did not affect results (data not shown). All subsequent treatment of frozen and paraffin sections was identical. Slides were rehydrated then microwave heated to 120°C in 90% glycerol, 10 mmol/L Tris (pH 10.5), 1 mmol/L EDTA and maintained at 110 to 120°C for 15 minutes. The slides were cooled and rinsed in PBS. PML was detected with anti-PML rabbit antibody from Chemicon (Temecula, CA) and anti-rabbit FITC-labeled goat antibody (Sigma, St Louis, MO). Sections were then cross-linked with 4% formaldehyde and hydrated. Telomere fluorescence in situ hybridization was done by denaturing slides together with the 5′-labeled Cy3 (5′-CCCTAA-3′)ₙ PNA probe (Applied Biosystems, Framingham, MA) 1 μg/ml in 70% formamide, 0.25% Blocking Reagent (Roche, Penzberg, Germany), 10 mmol/L Tris (pH 7.5), and 5% MgCl₂ buffer (82 mmol/L Na₂HPO₄, 9 mmol/L citric acid, and 25 mmol/L MgCl₂) for 3 minutes at 85°C and hybridizing for 3 hours at room temperature. Slides were washed in 70% formamide [0.01 mol/L Tris (pH 7.2)] then 0.05 mol/L Tris (pH 7.5), 0.15 mol/L NaCl, 0.05% Tween 20, counterstained with 4′,6-diamidino-2-phenylindole and mounted in 90% glycerol [buffered with 20 mmol/L Tris (pH 8.0)] containing 2.33% antifade compound 1,4-diazabicyclo[2.2.2]octane. Images were captured on a Leica DMLB epifluorescence microscope using SPOT image analysis software.

A set of criteria was used to determine the APB status of tumor sections. An APB was considered to be present if a focus of telomeric DNA was localized within (not adjacent or overlapping) a PML focus in the nucleus. To avoid false positives, we also required the telomeric DNA in the APB to have a more intense fluorescence than that of the telomeres on that slide (the working criterion we used with the Cy3 conjugated telomeric probe was to require that with the appropriate camera exposure for the telomeric DNA in the APB, the telomeres were not visible). The section was scored as positive for APBs if they were detected in ≥10 and in ≥25% of the cells in the section. To avoid artifacts, a cell was not considered to contain APBs if ≥25% of the colocalized foci occurred outside nuclei (correcting for ratio of nuclear to non-nuclear area). Slides were not scored as negative unless ≥2,000 tumor cell nuclei were examined. If a section fulfilled every criterion to be APB+ except for the requirement for ≥0.5% of cells to have PML/telomeric DNA colocalizations, but had at least four telomeric DNA foci inside an outer ring of PML (Fig. 1A) then it was scored as APB+ but was noted as having a low frequency of APBs.

Telomere Length and Telomerase Analysis. For tumor samples, ~ 100 mg of frozen tissue was homogenized in 200 μL CHAPS lysis buffer (7), incubated on ice for 20 minutes, and centrifuged at 18,000 × g at 4°C for 20 minutes before collecting 160 μL supernatant for the telomerase assay. Genomic DNA was prepared from the remainder. Telomerase activity was assayed using the telomere repeat amplification protocol (7) with modifications (18). Positive lysates were confirmed by heat inactivation of telomerase at 85°C for 10 minutes. Negative lysates were assayed at 10- and 100-fold dilutions to overcome

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11 L Colgin, P Bonnefin, and R Reddel, unpublished data.
the possibility of telomere repeat amplification protocol inhibitors and checked by spiking the lysate with 1 μg HeLa lysate. Telomere length was determined as previously described (18) by pulsed-field gel electrophoresis using 1.5 μg per well of genomic DNA that had been digested with Hin 1 and Rsa I restriction enzymes (Roche). Terminal restriction fragments (TRF) analysis for the astrocytomas only was done by conventional agarose gel electrophoresis as described (10).

ALT status was determined by calculating whether the mean, variance and semi-interquartile range of the TRF length distribution was >16 kb, 1,000 kb 2 and 4 kb, respectively. Tumors were classified as ALT+ when 2 of 3 or 3 of 3 of these criteria were met for unimodal or bimodal (e.g., Fig. 2A; STS 13 and 16) telomere length distributions, respectively. Statistical analysis of TRF length distributions was done using Telometric software (26). Each gel was standardized by inclusion of DNA from GM847 (ALT+) and HeLa (TA+) cell lines.

**Data Analysis.** All analyses were blinded with respect to previous results and patient data. Kaplan-Meier method was used to estimate and log-rank tests and hazard ratios were used to compare the distribution of overall survival times. Proportions were described with exact binomial 95% confidence interval (95% CI) and compared using Fisher’s exact test. Age distributions were compared with the t test if symmetrical and the Mann-Whitney test if asymmetrical. Mean and proportion differences with 95% CI were calculated as appropriate.

**RESULTS**

**APB Presence Corresponds to ALT in Human Tumors.** We tested 26 frozen STS specimens for ALT by both TRF Southern analysis and the APB assay. The frozen sections used for APB analysis were taken immediately adjacent to the region used for TRF analysis.

Results for the TRF analysis of all 26 STS are shown in Fig. 2A. We used the characteristic high mean, variance, and range of the ALT telomere length distribution to determine that STS 1-8 were ALT+ and STS 13-26 were ALT−. The ALT status of STS 9-12 was equivocal. Because we have not encountered equivocal telomere length distributions in cell lines, this result in tumors may arise from admixture of tumor and stromal cells or intratumoral spatial heterogeneity in either telomere length (27) or ALT status (this study). In order to assess how a mixed population of ALT+ and ALT− cells affected the determination of ALT status by TRF analysis, we analyzed different mixtures of DNA from ALT+ (GM847) and TA+ (HeLa) cell lines (Fig. 2B). The ALT status of the mixture could not be determined by our criteria (see MATERIALS AND METHODS) when 20% to 30% of the genomic DNA was from the ALT+ cell line (Fig. 2B, lanes 5 and 6). Thus, the heterogeneity of tumors may account for the TRF analysis sometimes giving equivocal results for ALT status. Telomerase activity was determined by the telomere repeat amplification protocol assay for all 26 STS and 4 of 26 (15%) were TA+ (data not shown). The TA+ STS were unequivocally ALT− by TRF analysis (Fig. 2A, STS 14, 18, 19, and 26).

Combined PML immunostaining and telomere fluorescence in situ hybridization on the same frozen sections identified 11 of 26 STS as APB+ and the remaining 15 were APB-. All eight of the STS determined to be ALT+ by TRF analysis were APB+ and all 14 of the STS found to be ALT− by TRF analysis were APB− (Fig. 2A). APB analysis allowed all of the STS to be objectively classified as ALT+ or ALT−, in contrast to the TRF analysis which was equivocal for 4 of 26 STS (Fig. 2A).

We also compared the APB assay in paraffin sections with the TRF assay for ALT in 40 grade 2 to 4 astrocytomas [33 GBM (WHO grade 4) and 7 grade 2-3 astrocytomas]. For this tumor set, the sections were taken from a different part of each tumor
than the portions used for TRF analysis. Both assays found 13 of 40 astrocytomas to be ALT+ (7 of 33 GBM and 6 of 7 grade 2-3 astrocytomas). There was exact concordance between the two assays in all 40 astrocytomas. Telomere repeat amplification protocol assay showed 13 of 40 astrocytomas to be TA+ (data not shown). All 13 TA+ astrocytomas were GBM and of these 12 were ALT/C0 and one was ALT+. GBM testing positive for both TA and ALT have been reported (10, 28).

**ALT is Common in Sarcomas and Astrocytomas.** We used the APB assay to test for ALT in paraffin sections of four major tumor types (i.e., osteosarcoma, STS, astrocytoma, and papillary carcinoma of the thyroid; Table 1; examples in Fig. 1), as we considered these more likely to have a substantial proportion of ALT+ tumors (10, 13). The assay detected ALT in 27 of 58 (47%) osteosarcomas, 35 of 101 (35%) STS (including the 26 STS in Fig. 2A), 17 of 50 (34%) astrocytomas, and none of the 17 papillary carcinomas.

STS include tumors derived from or resembling a diverse group of tissue types and the prevalence of ALT varied greatly between the subtypes (Table 1). Malignant fibrous histiocytomas (MFH), the most common STS subtype in adults (29, 30), had the highest prevalence of ALT, with 77% being ALT+. ALT was
Table 1  Prevalence of ALT in tumors

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>ALT+/total</th>
<th>% ALT+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteosarcoma</td>
<td>27/58</td>
<td>47</td>
</tr>
<tr>
<td>Total STS</td>
<td>35/101</td>
<td>35</td>
</tr>
<tr>
<td>MFH</td>
<td>17/22</td>
<td>77</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>8/13</td>
<td>62</td>
</tr>
<tr>
<td>Liposarcoma</td>
<td>3/9</td>
<td>33</td>
</tr>
<tr>
<td>Synovial Sarcoma</td>
<td>1/1</td>
<td>9</td>
</tr>
<tr>
<td>Rhabdomyosarcoma</td>
<td>2/35</td>
<td>6</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>2/2</td>
<td>NA</td>
</tr>
<tr>
<td>ASPS</td>
<td>1/4</td>
<td>NA</td>
</tr>
<tr>
<td>Chondrosarcoma</td>
<td>1/3</td>
<td>NA</td>
</tr>
<tr>
<td>Epithelioid sarcoma</td>
<td>0/2</td>
<td>NA</td>
</tr>
<tr>
<td>TA – set of STS</td>
<td>10/16</td>
<td>52*</td>
</tr>
<tr>
<td>MFH</td>
<td>8/10</td>
<td>73*</td>
</tr>
<tr>
<td>Leiomyosarcoma</td>
<td>1/3</td>
<td>NA</td>
</tr>
<tr>
<td>Liposarcoma</td>
<td>1/3</td>
<td>NA</td>
</tr>
<tr>
<td>Astrocytoma</td>
<td>17/50</td>
<td>34</td>
</tr>
<tr>
<td>Papillary carcinoma of the thyroid</td>
<td>0/17</td>
<td>0</td>
</tr>
</tbody>
</table>

NOTE: NA, not applicable.

*This separate set of STS were from a group of 24 STS of which 83% were telomerase negative (TA–) and included 12 MFH, of which 11 (92%) were TA– (25). Thus, 52% = 10/16 × 83% and 73% = 8/10 × 92%.

also common in leiomyosarcomas (62%) and liposarcomas (33%). Rhabdomyosarcomas (6%) and synovial sarcomas (9%) had a significantly lower prevalence of ALT compared with MFH (P < 0.0001 for each). Results from a separate set of 16 STS selected on the basis of being TA– (Table 1) supported the high prevalence of ALT in MFH.

**ALT and Tumor Aggressiveness.** Patient data were analyzed to determine if ALT is associated with tumor aggressiveness (Table 2). For both osteosarcomas and adult STS, ~50% of the high-grade tumors were ALT+. Thus, in sarcomas the prevalence of ALT was not reduced in the high grade tumors. In astrocytomas, we found ALT to be more prevalent in the lower grades, 88% compared with 24% in GBM (P = 0.001). Conversely, 0 of 7 grade 2 to 3 astrocytomas were TA+ compared with 13 of 34 (38%) of GBM, which is consistent with other reports that show the prevalence of TA in astrocytoma correlates with grade (31, 32).

Although 33% of the metastases in adult STS were ALT+, this was significantly less than the 67% of primary tumors that were ALT+ (P = 0.025). There was no significant difference between the prevalence of ALT in the recurrences (50%) and either the primaries or the metastases (P = 0.36 and P = 0.37, respectively). Whenever paired samples were available, metastases and recurrences always had the same ALT status as the primary tumor; for all tumor types, this totaled five metastases (2 of 5 ALT+) and two recurrences (1 of 2 ALT+).

**ALT and Outcome of STS.** The relationship between patient survival and ALT has not previously been investigated for STS. Patient survival data were available for all adult STS patients except the three patients with chondrosarcoma. The median survival for the entire group of 65 patients was 44 months, with no significant variation between the component STS subtypes (data not shown). Kaplan-Meier analysis (Fig. 3A and B) showed that there was no significant difference in survival between the ALT+ and ALT– patients in STS or the MFH subgroup [log-rank P = 0.87 and 0.90 respectively; hazard ratio, 1.0 (95% CI, 0.5-1.7) and 0.9 (95% CI, 0.3-3.0), respectively]. If the 2-fold longer median survival reported for ALT+ compared with ALT– GBM patients (10) had been present in STS, our sample size was sufficient to detect this (based on the 95% CIs described in Fig. 3A).

**ALT and Outcome of Osteosarcoma.** Kaplan-Meier analysis found no significant survival difference between the ALT+ and ALT– osteosarcoma patients (Fig. 3C; log-rank P = 0.59; hazard ratio, 1.6; 95% CI, 0.3-9.3). Although median follow-up time (28 months) was short, these results are consistent with recently published data (11) indicating that survival of patients with ALT+ osteosarcomas is the same or less than in the ALT– group.

We investigated if response to chemotherapy correlated with ALT status in osteosarcoma as there is currently no adequate predictor of osteosarcoma response that could facilitate tailoring of preoperative chemotherapy to increase survival (33). However, there was no significant difference between the proportion of ALT+ (6 of 17; 35%) and ALT– (8 of 24; 33%) osteosarcomas that responded (>90% necrosis) to chemotherapy (P = 1.00; mean difference of response = 2%, 95% CI, –33 to +29).

**ALT and Outcome of Glioblastoma Multiforme.** Because of the concordance between the assays for ALT, we expected the APB assay to be a prognostic indicator for patients with GBM as has been reported for ALT detected by TRF analysis (10). Kaplan-Meier analysis (Fig. 3D) showed that the patients with APB+ GBM had better survival than the APB– group (log-rank P = 0.001). The APB+ GBM patients had a 3-fold longer median survival (Fig. 3D; hazard ratio, 0.3; 95% CI, 0.1-0.6).

Table 2  Association of ALT with tumor grade and metastasis

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Total tumors</th>
<th>Grade</th>
<th>Tumor stage sampled</th>
<th>Metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Low*</td>
<td>High</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>27/58 (47%)</td>
<td>1/7</td>
<td>20/39 (51%)</td>
<td>27/57 (47%)</td>
</tr>
<tr>
<td>Adult STS</td>
<td>34/68 (50%)</td>
<td>3/5</td>
<td>8/16 (50%)</td>
<td>16/24 (67%)</td>
</tr>
<tr>
<td>Astrocytoma</td>
<td>17/50 (34%)</td>
<td>7/8†</td>
<td>10/42 (24%)</td>
<td>17/50 (34%)</td>
</tr>
</tbody>
</table>

Data are presented as number of tumors ALT+/total tumors in partition (% ALT+). Grading was not available for all tumors. Metastasis is not applicable (NA) for astrocytomas.

*Low or intermediate grade.
†1 of 2 grade 2 and 6 of 6 grade 3 astrocytomas were ALT+.
**ALT and Patient Age.** As illustrated in Fig. 4, there was a significantly lower age at diagnosis for the ALT+ osteosarcoma patients compared with the ALT− osteosarcoma patients (Mann-Whitney Test, \( P = 0.01 \)). This seemed to be due to a marked lack of ALT+ osteosarcomas diagnosed in patients >40 years (26 of 48 or 54% ALT+) compared with prevalence of ALT+ osteosarcomas diagnosed in patients <40 years (1 of 10 or 10% ALT+; \( P = 0.01 \)). However, Ulaner et al. (11) did not observe a significant difference in the mean age at diagnosis of ALT+ osteosarcomas; thus, this association needs to be tested in a larger tumor set.

ALT has been associated with a younger patient group in GBM (10). We also found that patients with ALT+ astrocytomas had a significantly lower mean age at diagnosis than that of patients with ALT− astrocytomas (Fig. 4; mean difference of age at diagnosis = 16.4 years; 95% CI, 9.0-23.7 years). If grade 2 and 3 astrocytomas (which can progress to GBM) are excluded from analysis, there was still a significantly lower mean age of the remaining ALT+ GBM (mean age of 40.1 years; 95% CI, 33.4-46.8 years for the ALT+ GBM compared with 54.6 years, 95% CI, 51.2-58.0 years for ALT− GBM; mean difference = 14.5 years; 95% CI, 6.3-22.7 years).

Due to significant differences in both the mean ages and prevalence of ALT in the different STS subtypes, they could not be combined and the sample sizes did not allow significant results when analyzed separately. No significant gender difference was found for the prevalence of ALT in osteosarcoma, STS, or astrocytoma (data not shown).

**Intratumoral Spatial Heterogeneity for ALT in Osteosarcomas.** Tumors are not genetically homogeneous (34); thus, it is possible that intratumoral spatial heterogeneity for ALT may occur and could affect interpretation of assays for ALT status. We looked for spatial heterogeneity for ALT in 10 primary osteosarcomas by assaying for APBs in paraffin-embedded samples from two separate sites but from the same time point. We found that 2 of 10 (20%) osteosarcomas had one site that was APB+ and the other APB−. Of the remaining osteosarcomas, five were APB− at both sites and three were APB+ at both sites. We classified an osteosarcoma as ALT+ if it was positive for APBs in at least one site. We similarly tested two separate paraffin blocks from 13 GBM (6 ALT− and 7 ALT+) but no spatial heterogeneity for ALT was found. However, this sample size is not big enough to significantly exclude heterogeneity levels <25% (95% CI, 0-25).

**DISCUSSION**

We have developed a robust assay for detecting ALT activity in archived tumor specimens that does not require extraction of high molecular weight genomic DNA for Southern analysis of TRF length that is currently relied on to detect ALT in tumors. Although we have previously shown that the presence of APBs is also a hallmark of ALT, detection of APBs in tumors had not been compared with TRF analysis and validated as an assay for ALT. In this study, we have shown an excellent correlation between the results of these two assays. Both in a set

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**Fig 3** Kaplan-Meier survival analysis of (A) STS, (B) STS subtype MFH, (C) osteosarcoma, and (D) GBM patients with ALT+ or ALT− tumors as determined by the APB assay. *Open circles or diamonds,* patients that exited the analysis for reasons other than death from disease. 95% CI for median survival are in parenthesis. **Comparison of the ALT+ and ALT− survival curves with the log-rank test showed no significant difference for any of the sarcoma groups but significantly longer survival for ALT+ GBM patients.**
Tumors

Values for ALT

Mean with 95% CI are not appropriate. Because the age distribution for osteosarcomas is skewed, the patients where there seems to be a younger average age of onset of ALT+ tumors in patients >40 to 45 years in contrast to astrocytoma

The APB assay and the TRF assay for every tumor sample where grade 2 to 4 astrocytomas there was an exact agreement between

Of frozen STS specimens and also in a set of paraffin sections of grade 2 to 4 astrocytomas there was an exact agreement between the APB assay and the TRF assay for every tumor sample where the TRF analysis yielded a result (total of 21 ALT+ and 41 ALT- tumors). In osteosarcoma patients there seems to be a lack of ALT+ tumors in patients >40 to 45 years in contrast to astrocytoma patients where there seems to be a younger average age of onset of ALT+ tumors. Because the age distribution for osteosarcomas is skewed, the means with 95% CI are not appropriate. Horizontal bar, median age values for ALT− and ALT+ groups (25 and 16 years, respectively).

The APB assay may be more appropriate than TRF analysis for assaying for ALT in tumors, as the former technique analyzes individual cells in contrast to Southern analysis which uses genomic DNA extracted from a mixture of cells in the tumor. This may be the reason we could determine the presence of ALT by APB status in four STS where the TRF pattern was equivocal (Fig. 2). In addition to frozen and paraffin sections, the APB assay can be applied readily to fine needle aspiration biopsy samples (data not shown). The absence of telomerase activity cannot be used to establish ALT status of tumors; a TA− tumor may not necessarily be ALT+ because some tumors do not contain cells that have a TMM (9) and conversely, a TA+ tumor cannot be assumed to be ALT− because it has been shown that some tumors utilize both TMMs (8, 10, 11).

We found evidence of intratumoral spatial heterogeneity for ALT in 2 of 10 (20%) osteosarcomas which is similar to reported frequencies of intratumoral spatial heterogeneity for TA of 7% to 38% (11, 27, 35). The lack of intratumoral spatial heterogeneity found for ALT in 15 osteosarcomas (11) or 13 GBM (this study) may be due to the sample sizes involved. Intratumoral heterogeneity may explain the observation that the proportion of APB+ nuclei was low in 7% of the ALT+ tumors. Alternatively, low frequencies of APBs have been observed in a minority of clones of an ALT+ cell line forced to express telomerase (36), suggesting that coexpression of TA and ALT in the same cells may sometimes partially mask ALT. The occurrence of intratumoral spatial heterogeneity for TMM may mean that several sites need to be tested for each tumor to reliably assess its TMM status. Where studies have not addressed this issue, the reported prevalence values for ALT in tumors may be underestimates.

We showed the utility of the APB assay by using it to investigate ALT in STS, osteosarcomas, papillary carcinomas of the thyroid and grade 2 to 4 astrocytomas. Although less than half of all papillary carcinomas of the thyroid have TA (13) compared with 85% of cancers overall (12), none of the 17 papillary carcinomas were ALT+. This may reflect less need for a TMM in a cancer with a low cell turnover (37, 38). The prevalence of ALT in STS has not previously been investigated in detail. Guillem et al. (39) reported that 4 of 22 TA− STS were ALT+ and a recent study (40) found 6 of 18 STS to be ALT+. We found the prevalence of ALT in STS to be 35 of 101 (35%), and that ALT is common among some types of STS: 77% of MFH, 62% of leiomyosarcomas, and 33% of liposarcomas were ALT+. The prevalence of ALT in the 22 MFH of 77% was supported by the independent set of TA− STS in which 8 of 10 MFH were ALT+. We showed ALT to be significantly less common in other types of STS: only 9% of synovial sarcomas and 6% of rhabdomyosarcomas were ALT+. The APB assay also showed ALT to be common in osteosarcomas and grade 2 to 4 astrocytomas (47% and 34%, respectively), which is consistent with recent reports using TRF analysis to test for ALT (10, 11).

The reason for the differing proportions of ALT+ tumors in various tumor types is unknown. We speculated previously that the higher frequency of ALT in cell lines of mesenchymal origin may be due to a tighter repression of telomerase in normal mesenchymal cells than in epithelial cells (13). Similarly, the cells that give rise to astrocytomas may have tight repression of telomerase given that TA is undetectable in normal brain tissue (31, 41) and normal human astrocyte cells (42, 43). However, different sarcoma subtypes have widely varying proportions of ALT+ tumors. Sarcoma subtypes can be classified into two groups: one group with near-diploid karyotypes with few chromosome rearrangements that often include a specific translocation and another group with complex karyotypes which are characteristic of severe genetic and chromosomal instability (44). A recent report suggested an association between ALT and complex karyotype STS (40). Our data are partly consistent with this, as we found ALT to be common in osteosarcomas, MFH, leiomyosarcomas, and nonmyxoid liposarcomas that typically have complex karyotypes, and rare in synovial sarcomas which typically have simple karyotypes (44). However, embryonal rhabdomyosarcomas typically have complex karyotypes, but we found only 2 of 15 (13%, data not shown) to be ALT+. It has been proposed that the ALT mechanism causes chromosome instability because of chromosome end-to-end fusion events resulting from the subset of telomeres within ALT cells that are very short (45). If such a causal association exists between ALT and complex karyotypes, then most or all ALT+ tumors would have complex karyotypes, but because ALT activity is not the only source of chromosomal instability some types of tumors with complex
karyotypes will be found to be ALT−. Thus, our data are consistent with ALT being one cause of complex karyotypes.

The APB assay was also useful for investigating associations between ALT and clinical data. Our data support the view that the ALT TMM can support fully malignant sarcomas (46). It was recently reported that ALT+/TA− osteosarcomas were just as aggressive as TA+ osteosarcomas in terms of clinical outcome and that they were also able to metastasize (11). Our results for osteosarcomas were consistent with this, and we show for the first time a similar pattern in STS. We found no difference between the survival rates of patients with ALT+ STS and the ALT− group, and there was no reduction in the proportion of ALT+ STS in the high-grade group or in the group with local recurrence. Although a significant proportion of STS metastases were ALT+ (9 of 27), the prevalence of ALT was lower in the metastases than in the primary tumors. It is possible that cancers utilizing ALT may take longer than TA+ tumors to acquire properties such as the ability to metastasize. There is growing evidence that telomerase has cancer promoting properties independent of its role in telomere maintenance (47), but the data for human tumors presented here clearly indicate that ALT+ tumors may nevertheless be highly malignant.

This study has shown for the first time that the prevalence of ALT+ is significantly higher in grade 2 to 3 astrocytomas compared with GBM (grade 4 astrocytomas). Grade 2 to 3 astrocytomas slowly progress to GBM over 5 to 10 years (these GBM are referred to as “secondary” in contrast to “primary” GBM which arise de novo in the absence of a preexisting low grade lesion; ref. 48). This suggests an association between ALT and secondary GBM, which is strengthened by the observation of Hakin-Smith et al. (10) that ALT is associated with younger GBM patients. In our data set the mean age of ALT+ GBM was 40 years compared with 55 years for ALT− GBM, possibly reflecting the reported mean ages of secondary and primary GBM patients of 39 and 55 years, respectively (49). This is of interest because secondary GBM have genetic alterations (48) that are distinct from those of primary GBM and which conceivably may facilitate activation of the ALT mechanism.

In vitro studies showing that inhibition of TA or ALT results in apoptosis or senescence (4, 5) support the notion that TMMs may be a useful drug target for cancer treatment. Telomerase inhibitors are already entering clinical trials, but Telomere maintenance (47), but the data for human tumors presented here clearly indicate that ALT+ tumors may nevertheless be highly malignant.

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In vitro studies showing that inhibition of TA or ALT results in apoptosis or senescence (4, 5) support the notion that TMMs may be a useful drug target for cancer treatment. Telomerase inhibitors are already entering clinical trials, but they are not expected to be effective against ALT+ tumors; thus, it may become necessary to assay for ALT before commencing treatment. Furthermore, for GBM and possibly other types of tumors, TMM assays may be very useful for assessing prognosis. We have shown here that whenever ALT was clearly present or absent according to TRF analysis the APB assay gave a concordant result. The APB assay seems to be superior to TRF analysis when there is intratumoral heterogeneity and is very useful for testing archives of paraffin-embedded specimens.

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