Short Communication

Pilocytic Astrocytomas Have Telomere-Associated Promyelocytic Leukemia Bodies without Alternatively Lengthened Telomeres

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Telomere maintenance by either telomerase activity or the recombination-mediated alternative lengthening of telomeres (ALT) mechanism is a hallmark of cancer. Tumors that use ALT as their telomere maintenance mechanism are characterized by long telomeres of great heterogeneity in length and by specific nuclear structures of co-localized promyelocytic leukemia protein and telomere DNA, called ALT-associated promyelocytic leukemia bodies (APBs). Recent advances have revealed a direct role for APBs in telomere recombination in ALT-positive cells. In this study, we investigated the possibility that APBs could occur before the long 'alternatively' lengthened telomeres arise, particularly in low-grade tumors. We measured APBs, telomere length, and telomerase activity in 64 astrocytomas inclusive of grade 1–4 tumors. Almost all grade 1–3 tumors (93%) were APB-positive using published criteria. Grade 2–3 APB-positive tumors also had long telomeres and were confirmed as ALT positive. However, grade 1 tumors lacked long telomeres and were therefore classified as ALT negative, but positive for telomere-associated promyelocytic leukemia bodies (TPB). This is the first report of a TPB-positive but ALT-negative tumor, and suggests that low-grade tumors have the foundation for recombinational telomere repair, as in ALT. Further work is warranted to characterize the TPB-positive phenotype in other early malignancies, as well as

to determine whether TPBs predispose to telomere maintenance by ALT. (Am J Pathol 2010; 177:2694–2700; DOI: 10.2353/ajpath.2010.100468)

The proliferative life span of somatic cells is controlled by telomere length.¹ Telomeres are repeatedly shortened with each cell division until the telomere is too short, and the cell ceases proliferation and goes into senescence.^{2,3} Acquisition of a telomere maintenance mechanism allows malignant cells to evade senescence and continue proliferation.⁴ Known telomere maintenance mechanisms include telomerase⁵ and the alternative lengthening of telomeres mechanism (ALT) that describes the elongation of telomeres by any telomerase-independent mechanism.⁶ Tumors that use ALT are distinguished by their low, or lack of, detectable telomerase activity,⁷ increased recombination at telomeres,⁸ long and heterogeneous telomere length distributions by terminal restriction fragment (TRF) Southern blotting,^{9–13} and the presence of a specific type of aggregate that contains the promyelocytic leukemia (PML) protein and telomere DNA called ALT-associated PML bodies (APBs) in 5 to 10% of asynchronized dividing cells.^{14,15} The prevalence of the ALT telomere maintenance mechanism has been studied less extensively than telomerase, which is the predominant telomere maintenance mechanism used in over 85% of tumors collectively.¹⁶ The estimated frequency of ALT tumors varies from 25 to 34% in grade 2–4 astrocytomas, 35% in smooth muscle sarcomas, 47

Supported by the Cancer Society of New Zealand. J.G.-G. was supported by a grant from the Pathological Society of Great Britain and Northern Ireland, and elective grants from The British Division of the International Academy of Pathology and Cancer Research UK.

Accepted for publication August 5, 2010.

The authors have no conflicting financial or personal interests.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

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to 66% in osteosarcomas, 11% in melanomas, 9% in neuroblastoma, and 0% in papillary thyroid and lung carcinomas.^{13,17–20}

Measurement of APBs, the co-localization of PML and telomere DNA by fluorescence *in situ* hybridization, is a “robust” assay for identification of ALT tumors that can be used in paraffin-embedded tissues.¹⁷ To date, all ALT tumors¹⁷ and ALT cell lines (with two exceptions, VA13-C3-c16 and AG11395 cells) have APBs,^{14,21,22} and APBs are absent in normal cells and absent or rare in telomerase-positive cells.^{14,23} The exact function of APBs is unclear.²⁴ Recent evidence suggested that APBs are involved in DNA recombination of ALT cells.²⁵ Alternative lengthening of telomere-associated PML bodies can contain other proteins involved in DNA repair, recombination, and replication.^{9,12,14,26} The frequency of APBs increased in ALT cells following DNA damage²⁷ and in murine cells engineered to be deficient in DNA methyltransferases (DNA methyltransferase 1^{−/−}, and DNA methyltransferase 3a/3b^{−/−}).²⁸ Further, evidence suggests that APBs function to increase telomere length variability by promoting telomeric recombination in ALT cells.^{15,25} Murine cells were proposed to gain an ALT mechanism due to deregulated telomere length by DNA methyltransferases²⁸ or telomerase deficiency (Terc^{−/−} mice).^{29–32} Another suggestion is that APBs may function to “trim” overlengthened telomeres based on the finding that although rare, APBs can present in telomerase-positive cells if telomere lengthening occurs.²³

Whether APBs are present in low-grade tumors before the appearance of the “alternatively” lengthened telomere phenotype is unknown. Astrocytomas are well suited for this investigation, having no telomere maintenance mechanism in the lowest-grade 1 tumor by telomere length and telomerase activity analyses,³³ but with ALT present in higher-grade tumors.^{17,18,34} We investigated whether APBs are present in grade 1 astrocytomas. Grade 1 astrocytomas were of interest as grade 2–4 tumors use ALT, although the most aggressive grade 4 tumors are predominately telomerase positive.^{17,18} Grade 1 astrocytomas (predominantly pilocytic astrocytoma) occur in children³⁵ and show an unpredictable behavior compared to that of adult astrocytomas that usually progress from grades 2 and 3 to grade 4 tumors.³⁶ Instead, pediatric astrocytomas can be aggressive with recurrent growth, stability, or progression after years of dormancy.^{37,38} Approximately, 50% of pediatric grade 1 astrocytomas will progress if incompletely resected.³⁸

In this study measurement of APBs, telomere length, and telomerase activity were performed using 64 astrocytomas inclusive of grade 1–4 tumors. We show that APBs are present in the least aggressive pilocytic astrocytoma, but unlike grade 2–4 astrocytomas are present without alternatively lengthened telomeres.

Materials and Methods

Patient Population and Tumor Collection

The inclusion criterion for the study was a diagnosis of a primary astrocytoma, and 64 tumors were included (25

grade 1, 7 grade 2, 12 grade 3, and 20 grade 4 tumors). All tumors were collected in New Zealand, and all were tumor debulking or biopsy samples. Forty-eight tumors had paraffin-embedded blocks and frozen tissue, and 16 tumors (all grade 1 astrocytomas) had only paraffin-embedded blocks available. Consultant pathologists performed the histopathological tumor diagnoses. The study had ethical approval, and individual consent was obtained from individuals 16 years of age and older, and parental consent obtained for patients under the age of 16.

Measurement of APBs

Measurement of co-localized bodies of the PML protein and telomere DNA, in cellular nuclei, were performed on paraffin embedded tumors according to the APB assay described previously.¹⁷ The measurement of APBs included four positive controls (two ALT-positive osteosarcoma cell lines, G-292 and Saos-2,¹³ and two adult astrocytoma ALT-positive tumors previously analyzed)³⁹ and six negative controls (two telomerase positive glioblastoma cell lines T98G and U118-MG, two telomerase-positive adult astrocytomas,³⁹ and two histologically normal tumor-adjacent brain tissue samples). The PML protein was detected using a goat anti-PML antibody (N-19; Santa Cruz Biotechnology, Santa Cruz, CA), and a secondary donkey anti-goat IgG antibody labeled with Alexa Fluor 488 (Invitrogen, Carlsbad, CA). Telomere DNA was detected by fluorescence *in situ* hybridization with a Cy3-labeled PNA probe (5′-CCCTAACCCTAAC-CCTAA-3′; Applied Biosystems, Framingham, MA). Cellular nuclei were stained using 4,6-diamidino-2-phenylindole. Cells were imaged using confocal microscopy (Zeiss LSM510; Carl Zeiss, Thornwood, NY), and images analyzed by Zeiss LSM Image Examiner software version 3.2.0.115 (Carl Zeiss). Tumors were deemed ALT positive using the criteria described in Henson et al, namely, at least two co-localized PML/telomere-DNA fluorescent signals in an individual cell nucleus, which were large in size (>0.9 μm), in sharp focus, with a clear peak signal, and present in greater than 0.5% of total tumor cells (400 tumor cells were measured for APBs).¹⁷

Telomere Length and Telomerase Activity Analyses

The analysis of telomere length was made by the TRF assay and used the TeloTAGGG Telomere Length Assay Kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s instructions. The criteria for ALT by TRF length was as previously described¹⁷: mean TRF length greater than 16 kb, with a wide range in TRF lengths (typically <3–> 50 kb). Measurement of telomerase activity was made by the telomeric repeat amplification protocol (TRAP) method, and used the TeloTAGGG Telomerase PCR ELISA Plus kit (Roche Applied Science, Mannheim, Germany) according to the manufacturer’s instructions. The TRF and TRAP assays were performed on the 48 tumor biopsy samples with frozen tissue available.

Survival and Statistical Analyses

The survival times (post brain tumor diagnosis) were compared between astrocytoma grades by the Kaplan-Meier method, and the difference between two medians evaluated by the log-rank test with 95% CI. The age at brain tumor diagnosis was compared between astrocytoma grades and used the Mann-Whitney test with 95% CI. A $P < 0.05$ was taken as a significant difference.

Results

Clinicopathologic Features and Patient Survival

Grade 1–4 astrocytomas from 64 individuals were included in the study. Individuals with grade 1 astrocytoma were younger and had improved survival compared to those with higher-grade astrocytomas. The median age for individuals with a grade 1 tumor was 6 years of age, compared to 23 years for grade 2, 28 years for grade 3, and 52.5 years for individuals with grade 4 tumors ($P = 0.001$, $P < 0.0001$, $P < 0.0001$, respectively by Mann-Whitney comparison 95% CI).

Patient survival data were available for 43 individuals: grade 1 ($n = 13$), grade 2 ($n = 5$), grade 3 ($n = 9$), and grade 4 ($n = 16$). All individuals with grade 1 tumors were still alive 1 to 10 years following tumor diagnosis, with post-4-year survival data for eight children. Patient survival was greater for individuals with grade 1 and 2 tumors compared to those with grade 3 ($P < 0.001$ and $P = 0.02$, respectively) and grade 4 astrocytoma ($P < 0.0001$ and $P = 0.0011$, respectively), consistent with that expected based on the tumor grade classifications.⁴⁰

Grade 1 Astrocytomas Are APB Positive

The measurement of APBs used the same protocol and criteria established for detection of APBs in adult astrocytomas¹⁷ and was performed on all 64 astrocytomas, four ALT-positive controls, and six ALT-negative controls (inclusive of two samples of normal tumor-adjacent brain tissue). Sixty-nine percent of astrocytomas (44 of 64), and the four positive controls were APB positive ($> 0.5\%$ APB-positive cells). An example of an APB-positive grade 1 astrocytoma is shown in Figure 1A. The percentage of APB-positive tumors is listed in Table 1, and the distribution of APB measurements for each tumor grade in Figure 1B. Ninety-two percent (23 of 25) of grade 1 tumors were APB positive, and the percentage of APB-positive cells ranged from 2 to 15.4%, with a median of 7.7% for APB-positive tumors. All grade 2 and all but one grade 3 (median 6%, range 3 to 14% APB-positive cells), and only three grade 4 astrocytomas were APB positive. Twenty tumors were APB negative. The APB-negative tumors included two grade 1, one grade 3, and 17 of 20 (85%) grade 4 astrocytomas. The predominance of APB-positive tumors in grades 2 and 3, with a reduced frequency in grade 4 astrocytomas was consistent with that reported for adult astrocytomas.¹⁷

Figure 1. Grade 1 astrocytomas are positive for APBs. **A:** Measurement of APBs in astrocytomas. APBs (indicated with an **arrow**) had co-localized PML (green) and telomere DNA (red) fluorescent signals in nuclei (not shown) on analysis of paraffin-embedded samples at $\times 630$ magnification. The criteria for an APB-positive cell included at least two co-localized areas greater than $0.9 \mu\text{m}$ in size (profile, **bottom**). Scale bar = $20 \mu\text{m}$. **B:** Distribution of APB measurements among grade (G) 1–4 astrocytomas. The percentage of APB-positive cells is plotted against tumor grade. The criteria for APB-positive tumors included APBs greater than 0.5% of cells, as indicated by a dashed line. The median APB measurement is represented with a solid horizontal bar.

Of further interest, small co-localized PML telomere DNA signals ($< 0.3 \mu\text{m}$) were found in both normal tumor-adjacent brain samples in astrocytes and endothelial cells (see Supplemental Figure S1 at <http://ajp.amjpathol>).

Table 1. Telomere Maintenance Mechanisms in 64 Astrocytomas

Tumor type	<i>n</i>	ALT+ by APB (%)	ALT+ by TRF (%)	TEL+ (%)
Grade 1 astrocytoma	25	23 (92%)*	0†	0†
Grade 2 astrocytoma	7	7 (100%)*	5 (71%)	0
Grade 3 astrocytoma	12	11 (92%)	11 (92%)	1 (8%)
Grade 4 astrocytoma‡	20	3 (15%)	4 (20%)	11 (55%)

n, number of tumors.

*percentage of tumors positive for APBs (>0.5% APB-positive cells), 'alternatively' lengthened telomeres (mean TRF length >16 kb), or telomerase activity (TEL) (TRAP assay).

†Some tumors did not show 'alternatively' lengthened telomeres by TRF length analysis and were reclassified as TPB positive.

‡Measurement made on 9/25 samples with frozen tissue available.

§Five tumors were negative for all assays (TPB/APB, ALT by TRF length, and telomerase activity), and one tumor was ALT positive by TRF length, but APB and telomerase activity negative.

org). In astrocytomas small "APB-like" bodies were present in endothelial cells of most tumors (56 of 64), and in astrocytes of all telomerase negative tumors, and few telomerase positive tumors (2 of 12). Small APB-like bodies were present in 0.5 to 1% of astrocytes and 1 to 2% of endothelial cells and were not measured as APBs due to the size exclusion criterion set for consistency with reports of the large "donut"-shaped APBs well characterized in ALT tumors.^{14,17} Small APBs co-existing with large APBs have been reported in ALT cells, but small APBs have not been reported previously for nonmalignant cells.^{13,27}

Alternatively Lengthened Telomeres Are Absent in Grade 1 Astrocytomas

Telomere lengths were measured using the standard TRF assay on 48 tumors for which frozen tissue was available to identify tumors with alternatively lengthened telomeres. Telomere length was measured by TRF length, and tumors with a mean TRF length, greater than 16 kb were classified as ALT positive.¹⁷ Based on the criteria of Henson et al,¹⁷ for determining ALT by TRF analysis, 19 tumors (40%) had TRF profiles consistent with being ALT positive. The percentage of ALT-positive tumors, based on TRF profiles, are listed in Table 1 for each tumor grade. The distribution of mean TRF lengths is shown in Figure 2B relative to tumor grade. Five of the seven grade 2, and all but one grade 3 astrocytoma were ALT positive (median TRF length 21.5, range 17–31.25 kb for ALT-positive tumors). All grade 1 and 80% of grade 4 astrocytomas were ALT negative by TRF measurement. Grade 1 astrocytomas had mean TRF lengths below 13 kb (median 8.5, range 6.5–13 kb), which were not heterogeneous in length by TRF measurement. An example of TRF profiles to demonstrate the ALT-negative phenotype of grade 1 astrocytomas is shown in Figure 2A. Grade 4 astrocytomas that were ALT negative had mean TRF lengths below 8.5 kb (median 5.1, 25–75th percentile 4.4

Figure 2. Grade 1 astrocytomas are ALT negative by telomere length analysis. **A:** Measurement of ALT in astrocytomas by TRF length. Determination of mean TRF lengths followed standard procedures for the TRF assay using agarose gel electrophoresis. A TRF profile with long telomeres (mean TRF length >16 kb) that typically ranged widely in length (approximately <3–>50 kb) was classified as ALT positive, and was present in grade (G) 2–4 tumors (highlighted by asterisks). Shorter telomeres with less length variation were classified as ALT negative, and presented in grade (G) 1 and 4 astrocytomas. Molecular weight marker, kb. **B:** Distribution of mean TRF length measurements among grade (G) 1–4 astrocytomas. The mean TRF length is plotted against tumor grade. The criteria for an ALT-positive status included a mean TRF length above 16 kb, as indicated by a dashed line. The median TRF measurement is represented with a solid horizontal bar.

to 8.4 kb). In summary, all APB-positive grade 1 astrocytomas and two grade 2 astrocytoma, were ALT negative by TRF length. Due to the absence of ALT, we modified the nomenclature for these tumors to describe them as TPB (telomere-associated PML body) positive and APB negative.

Telomerase-Positive Tumors

Telomerase activity was measured using the standard TRAP method for the 48 tumors with frozen material avail-

able. Twelve tumors (25%) were telomerase positive. The percentage of telomerase positive tumors is listed in Table 1 for each tumor grade. Fifty-five percentage of grade 4 tumors (11 of 20), and one grade 3 astrocytoma were telomerase positive; however, all grade 1–2, and 12 of 13 grade 3 astrocytomas were negative for telomerase activity. All telomerase-positive tumors were negative for ALT by both the APB assay and telomere length measurement. Consistent with the report of Tabori et al,³³ all grade 1 astrocytomas in this study were negative for the ALT and telomerase telomere maintenance mechanisms.

Discussion

Astrocytomas present as a wide range of neoplasms that differ in histological features, location in the brain, age group typically affected, and clinical course. The pilocytic astrocytoma is a low-grade circumscribed neoplasm (World Health Organization grade 1/4) that is the major astrocytoma in young children,³⁵ while the infiltrative higher grade (World Health Organization grades 2, 3, and 4) astrocytomas accounted for about 80% of primary brain tumors.³⁶ Genetic changes in astrocytoma show typical patterns with increasing malignancy, and different pathways appear to converge in the primary or secondary grade 4 astrocytoma (The Cancer Genome Atlas, cancergenome.nih.gov). While telomerase has been reported as the major telomere maintenance system in grade 4 astrocytomas,^{17,18} we investigated the telomere maintenance system in lower-grade astrocytomas, including pilocytic astrocytoma. In our series, pilocytic astrocytomas do indeed contain APBs. However, these pilocytic astrocytomas did not fit with the classical ALT phenotype for APB-positive tumors because we found no “alternatively” lengthened telomeres as determined by Southern blotting, and hence we subsequently called the “APB-like” bodies TPBs. Our results suggest a progression from a TBP-positive phenotype in grade 1 to an ALT-positive telomere maintenance mechanism in grade 2–3 and the telomerase telomere maintenance mechanism in grade 4 tumors, indicating that TPBs may be present before the alternatively lengthened heterogeneous telomere phenotype emerges.

In this study, the lack of concordance between the APB and TRF length assays used to identify ALT-positive tumors was specific to grade 1 and some grade 2 astrocytomas. All TPB-positive tumors had greater than 5% of cellular nuclei positive for TPBs (with two exceptions) corresponding to the frequency of APBs found in ALT-positive cell lines.¹⁴ All grade 2–4 astrocytomas (with two exceptions) that were APB-positive tumors had alternatively lengthened telomeres. This concordance between the two ALT assays is consistent with that reported for ALT-positive tumors.¹⁷ All telomerase-positive tumors were TPB and APB negative, as were the normal-adjacent brain tissues consistent with that reported.¹⁴ The APB assay can define ALT status in tumors with equivocal TRF profiles.¹⁷ However, equivocal TRF profiles do not explain the TPB-positive/APB-negative phenotype found in our study, as all mean TRF lengths were lower

than 14 kb and did not show a heterogeneous TRF length profile. It is possible that the TBP-positive phenotype is associated with a deregulated telomere length on individual chromosomes, which is not detected in a larger cell population assayed by TRF length. Our results suggest tumors with no reported telomere maintenance mechanism may have a TBP-positive phenotype; however, this will not be the case for all tumors with no telomere maintenance mechanism as papillary thyroid carcinomas were APB negative,^{17,18,33} and five of our grade 4 astrocytoma series were negative for all three telomere maintenance mechanism assays. Since all high-grade tumors are proposed to acquire telomere maintenance it is unclear why some grade 4 astrocytomas were negative for both ALT and telomerase activity. According to Hakin-Smith et al,¹⁸ 46% of grade 4 astrocytomas were negative for ALT and telomerase activity, with affected individuals associated with poor survival similar to those with telomerase-positive tumors. It is unclear whether high-grade tumors negative for ALT and telomerase activity use an uncharacterized telomere maintenance mechanism. In yeast, telomerase-negative mutant cells can maintain telomeres by amplification of Y' subtelomeric elements without acquiring long heterogeneous telomeres in addition to an ALT-like mechanism.^{41,42} Alternatively, tumors that are assayed as negative for telomerase activity may have telomerase activity below the accurate detection limit of the standard TRAP assay or contain TRAP assay inhibitors.⁴³ In this study, the limitations of the TRAP assay were minimized by measuring telomerase activity in different dilutions (2 to 5) of each cell lysate.

The presence of TPBs in grade 1 astrocytomas is consistent with these tumors having a foundation for telomere recombination, which could be considered a possible pre-ALT phenotype. The function of TPBs is unclear. Stabilization of APBs in ALT cells results in telomere clustering and promotion of telomere–telomere recombination, suggesting that large APBs directly contribute to ALT.²⁵ Since the TPB were similarly large, compared to APBs, and TPBs like APBs may be associated with increased telomere recombination and DNA damage.^{25,27}

We do not show direct evidence that TPBs lead to ALT-positive tumors. Recurrent tumors were not available in our series to assess whether the TPB-positive tumors progressed to tumors with alternatively lengthened telomeres, as all grade 1 astrocytomas were resected and, to date, no tumors have relapsed. According to Tabori et al,³³ a recurrent tumor following a low-grade astrocytoma can have a reduced telomere length compared to the primary tumor. This seems inconsistent with grade 1 tumors predisposing to ALT. Telomere length reduction, however, can precede an acquisition of ALT, as murine cells with defective telomere maintenance show a reduced telomere length before lengthened telomeres at later passages, attributed to the acquisition of an ALT mechanism.^{28–32} Transition from a TPB to an APB-positive phenotype may require the acquisition of mutations in proteins that maintain telomere stability, such as the tumor protein p53.^{44,45} Mutations in the p53 gene are absent in grade 1 astrocytomas,³³ but have an increased

incidence in grade 3 and 4 ALT-positive astrocytomas compared to telomerase-positive tumors.³⁹ An alternative explanation for TBP based on the findings of Pickett et al,²³ is that TBP are generated in circumstances where telomeres are overlengthened. Therefore, TBP may arise in grade 1 astrocytomas with telomere lengthening events but where telomere lengthening is not sufficient to counterbalance the normal causes of telomere attrition.

Using the APB assay, we identified "small" co-localized areas of PML and telomere DNA in nonmalignant brain tissue and astrocytomas. The 'small' PML telomere DNA aggregates are visible using confocal microscopy at $\times 630$ magnification, but were not readily detected using conventional fluorescent microscopy at lower magnification ($\times 200$) (unpublished observations). This could explain why the small telomere associated PML phenomenon has not been reported for normal cells^{14,17}; however, it has been identified in ALT cells in addition to the large donut APBs that have been characterized in ALT-positive tumors.²⁷ The function of small APB-like bodies is unknown but is proposed to be generated during recombination as "new" sites of linear extra chromosomal telomere repeat DNA or sites where telomere lengthening can occur.²⁷ In normal cells there is evidence for a recombination-mediated mechanism to maintain short telomeres,⁴⁶ and in murine telomerase-deficient cells, progressive telomere shortening eventuated in telomere lengthening and increased APBs at later passages.^{29–32} It is possible, therefore, that an ALT-like mechanism exists in normal cells to preserve telomere structure. In this study, only tumor-adjacent normal brain tissue was analyzed, and small APB-like bodies could reflect a peritumoral phenomenon. A much greater characterization of small APB-like bodies is required for comparison to the larger tumor associated TPB and APBs.

In summary, we report a TBP-positive phenotype is present in grade 1 astrocytomas without alternatively lengthened telomeres. Further work is required to determine whether TPBs are present in other low-grade tumors and to elucidate whether TPBs provide a foundation for increased susceptibility to ALT-positive tumors. The prevalence of small telomere-associated PML bodies in normal tissues suggests aspects of ALT may be involved in a normal cellular process. For astrocytoma patients, the presence of TPBs suggests that the foundation for ALT may exist well before tumor development, a finding that could have consequences if therapeutic telomerase inhibitors are used for tumor treatment.

Acknowledgments

We thank Roger Reddel for his comments on the manuscript and for providing the telomere probe used in this study. We thank Helen Morrin and the Cancer Society Tissue Bank in New Zealand for input and assistance on this project.

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