Telomere Content and Risk of Second Malignant Neoplasm in Survivors of Childhood Cancer: A Report from the Childhood Cancer Survivor Study

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Abstract

Purpose: Shorter constitutional telomere length has been associated with increased cancer incidence. Furthermore, telomere shortening is observed in response to intensive chemotherapy and/or ionizing radiation exposure. We aimed to determine whether less telomere content was associated with treatment-related second malignant neoplasms (SMN) in childhood cancer survivors.

Experimental Design: Using a nested case–control design, 147 cancer survivors with breast cancer, thyroid cancer, or sarcoma developing after treatment for childhood cancer (cases) were matched (1:1) with childhood cancer survivors without a SMN (controls). Cases and controls were matched by primary cancer diagnosis, years since diagnosis, age at the time of sample collection, years of follow-up from childhood cancer diagnosis, exposure to specific chemotherapy agents, and to specific radiation fields. We performed conditional logistic regression using telomere content as a continuous variable to estimate ORs with corresponding 95% confidence intervals (CI) for development of SMN. ORs were also estimated for specific SMN types, i.e., breast cancer, thyroid cancer, and sarcoma.

Results: There was an inverse relationship between telomere content and SMN, with an adjusted OR of 0.3 per unit change in telomere length to single-copy gene ratio (95% CI, 0.09–1.02; P = 0.05). Patients with thyroid cancer SMN were less likely to have more telomere content (OR, 0.04; 95% CI, 0.00–0.55; P = 0.01), but statistically significant associations could not be demonstrated for breast cancer or sarcoma.

Conclusions: A relation between less telomere content and treatment-related thyroid cancer was observed, suggesting that shorter telomeres may contribute to certain SMNs in childhood cancer survivors.

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Telomere Content and Second Cancers in Pediatric Cancer Survivors

Translational Relevance
Shortened constitutional telomeres are a well-established risk factor for the development of primary malignancies. This association is thought to derive from genomic instability due to excessive telomere shortening, predisposing cells toward malignant transformation. In this study, we hypothesized that second cancers may also be associated with shortened telomeres. To investigate this hypothesis, we obtained biologic specimens from the Childhood Cancer Survivor Study and performed a matched case–control analysis of telomere content between survivors with and without second malignant neoplasm (SMN). Our results suggest an association between shortened telomeres and SMN that is primarily driven by thyroid SMN. This study represents the first report of telomere content analysis in childhood cancer survivors. As approximately 1 out of 3 cancer survivors with SMN develops additional cancers, our results suggest a genetic predisposition for cancer related to telomere biology, whether due to inherited short telomeres, therapy-related telomere shortening, or other factors affecting telomere homeostasis.

thus, progressive telomere shortening serves as a molecular clock for cellular replicative aging. In the absence of cellular senescence or apoptosis, short telomeres lose their protective capacity, permitting chromosomal fusions and breakage–fusion–breakage cycles that may result in genomic instability and a potential for malignant transformation (12). Therefore, telomere shortening that naturally occurs with age has been proposed as a mechanism for the increased cancer risk observed in older populations (14). Indeed, the relation between risk for de novo cancers and estimates of average constitutional telomere length suggest that shortened telomeres may be a marker for cancer susceptibility (15–17).

In addition to the effect of aging upon telomere length, chemotherapy and ionizing radiation can significantly impair telomere maintenance and function in human cells (18). In fact, exposure to ionizing radiation has been shown to result in persistent irreparable damage to telomeric DNA (19). Thus, cancer therapy–induced telomere dysfunction may predispose cancer survivors to development of additional cancers. In the current study, we tested the hypothesis that less telomere content would be associated with SMNs among childhood cancer survivors.

Materials and Methods
Participants
The study population was drawn from the CCSS: a multiinstitutional retrospective cohort of more than 5-year survivors of leukemia, brain tumor, Hodgkin lymphoma, non-Hodgkin lymphoma, Wilms tumor, neuroblastoma, soft-tissue sarcoma, or bone tumor diagnosed before the age of 21 years and between 1970 and 1986 (20). All participating institutions obtained local institutional review board approval for the CCSS protocol and participants provided informed consent for data collection, medical record abstraction, and banking of a biologic specimen. The current study was approved by the Baylor College of Medicine Institutional Review Board, where the quantitative PCR (qPCR) analysis was performed.

Study design
Using a nested case–control design, CCSS participants with a confirmed SMN cases were compared with survivors who had not developed a SMN (controls). We restricted this analysis to three SMN types, breast cancer, thyroid cancer, and sarcomas, thus capturing the most common SMNs observed in this population. To be eligible for the current study, subjects must have had a buccal cell DNA sample available in the CCSS biorepository and should not have received hematopoietic cell transplantation (HCT). Subjects with buccal cell samples procured before the development of their first SMN (n = 41) were prioritized. However, to ensure adequate sample size, we did include subjects with samples procured after first (n = 96) and second SMN diagnosis (n = 10). Among the 10 subjects with a second SMN, those with breast cancer as a first SMN (n = 5) had developed breast (n = 4) and thyroid (n = 1) as second SMNs; those with sarcoma as first SMN (n = 3) had developed sarcoma (n = 2) and thyroid (n = 1) second SMNs; those with thyroid cancer as first SMN (n = 2) had developed melanoma (n = 1) and sarcoma (n = 1) second SMNs.

In selecting the appropriate control group for this study, our goal was to ensure that both cases and controls had equal opportunity for developing a SMN. Accordingly, controls comprised CCSS participants matched (1:1) to cases by primary diagnosis, age at time of sample collection (±10 years, with 87 (59.2%) pairs falling within ±2 years, 33 pairs (22.4%) falling between 2+ and 5 years, and 27 pairs (18.4%) falling within 5+ years), number of years between primary cancer diagnosis and sample collection (exceeded the latency between primary cancer diagnosis and development of SMN for the index case by a mean of 4.4 ± 6.7 years [with 79 (53.7%) pairs falling within ±2 years, 56 pairs (38.1%) falling between 2+ and 5 years, and 12 pairs (8.2%) falling within 5+ years], exposure (yes/no) to specific classes of chemotherapeutic agents (anthracyclines, alkylators, epipodophyllotoxins, other, or none), and exposure (yes/no) to specific radiation fields (chest/spine, brain/neck/head, abdomen/pelvis, other, or none). The case–control pairs were generated by density sampling and, as with cases, HCT recipients were excluded from consideration as controls.

Source of DNA and methods of isolation
Estimations of constitutional telomere length may be made using DNA extracted from blood, buccal cell samples, or fibroblasts as a representative cell population for the organism as a whole (21, 22). In individuals with an underlying defect of telomere biology, these three cell types

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have demonstrated significant intra-individual correlation when telomere content is measured by qPCR (21). However, because telomere length may vary between cell populations, only subjects with DNA from mouthwash samples were included in this study, to minimize potential intra-individual variability (see Supplementary Methods and Supplementary Figs. S1 and S2). Samples were collected using methods described previously (23), and genomic DNA was isolated from buccal cells using Qiagen kits. DNA was quantified using a NanoDrop spectrophotometer (Thermo Scientific) after vortexing to ensure accurate and uniform concentration. Samples were stored at –80°C until time of use.

qPCR for telomere content

We used a qPCR technique to measure the telomere content in buccal cell DNA. The data generated are a relative rather than absolute quantification, with the value corresponding to the amount of telomere DNA amplified relative to a single-copy gene (36B4). Because this technique estimates telomere DNA quantity relative to the quantity of a single-copy gene, we refer to the output of this measure as "telomere content," rather than "telomere length." The qPCR technique, as first described by Cawthon and colleagues (24), does correlate with telomere length measured by the Southern blot analysis of telomere restriction fragments (25), and was selected to accommodate the large number of samples and the limited quantity and quality of DNA available (see Supplementary Methods). Following PCR amplification, wells with threshold cycle \( C_t \) values greater than 0.5 from the middle value of each triplicate or those wells failing to amplify were excluded, so that the \( C_t \) values ascribed to the remaining two wells were averaged for the telomere content calculation. Case and control samples were intermixed at random so that the investigators were blinded to the case/control status at the time of telomere content measurement and calculation.

Statistical analysis

To account for matching, we compared cases and controls with respect to the distributions of potential confounders using the Generalized Estimating Equation (26) when the outcomes were continuous or binary, and the bootstrap method (27) when the outcomes were from three or more categories (Tables 1 and 2). The Generalized Estimating Equation (26) was also used to compare cases and controls with respect to mean telomere content (Table 3). ORs and corresponding 95% confidence intervals (CI) for developing an SMN per unit change in telomere content (a continuous variable) were estimated by conditional logistic regression, adjusting for age at diagnosis of primary cancer, sex, race/ethnicity, smoking status, and family history of cancer in a first-degree relative. ORs were first estimated for all SMNs, and then by specific SMN type (Table 3). The same analyses were repeated using only the case–control pairs with the case sample drawn before SMN diagnosis (Table 4), and then only pairs whose cases were exposed to ionizing radiation. All tests were two-sided, with a two-sided \( P \) value of < 0.05 considered statistically significant. All statistical analyses were performed using SAS software version 9.2 (SAS Institute).

Results

A total of 159 SMN cases (breast cancer: \( n = 75 \), thyroid cancer: \( n = 49 \), and sarcoma: \( n = 35 \)) and 153 matched controls were identified. Two cases and two controls were excluded because of failure to amplify in all three wells or because the difference between all three values in the triplicate was greater than 0.5, leaving 157 cases and 151 controls. Exclusion of cases without matched controls resulted in a final analysis set of 147 case–control pairs. The 147 cases included 68 cases with breast cancer, 48 with thyroid cancer, and 31 with sarcoma. The clinical characteristics of the cases and controls are summarized in Table 1, and the distribution of potential confounding factors not included in the matching criteria are shown in Table 2, with corresponding \( P \) values. Significant differences between cases and controls were noted in the sex distribution, primarily reflective of the number of women developing breast cancer SMN, and smoking status, with more ">1 pack/week for 5+ years" smokers among controls than cases.

Telomere content was obtained for the 294 samples. All unknown \( C_t \) values were within the range of the standard curve, with correlation coefficients of \( \geq 0.98 \). The average coefficient of variation for telomere \( C_t \) values was 0.58% (0.02%–1.86%), and for 36B4 was 0.39% (0.004%–1.28%). Paired analysis of telomere content indicated no significant difference in mean telomere content between the cases and controls for all SMNs (\( P = 0.71 \)), or when analysis was conducted by type of SMN (breast cancer, thyroid cancer, or sarcoma; Table 3).

We conducted a multivariable analysis with telomere content as a continuous explanatory variable, adjusting for age at diagnosis of primary disease, sex, race, family history of cancer in a first-degree relative, and smoking status. For all SMNs, cases had less telomere content than controls (adjusted OR, 0.3; 95% CI, 0.09–1.02; \( P = 0.05 \)), a finding that seemed to be primarily driven by observations in cases with thyroid cancer (OR, 0.04; 95% CI, 0.00–0.55; \( P = 0.01 \)). This effect was not observed in the sarcoma and breast cancer SMN subgroups (Table 3).

We then examined only pairs in which the case sample was taken before their SMN diagnosis (41 pairs; Table 4). In this group, for all SMNs, the adjusted OR was 0.08 (95% CI, 0.01–1.11; \( P = 0.06 \)), suggesting that the SMN risk associated with less telomere content may not have been the result of additional treatment exposure for the SMN. For this subanalysis, the population was too small to conduct the analysis for individual SMN types.

To understand the relation between telomere content and SMN among patients exposed to radiation, we restricted the analysis to case–control pairs with a history of exposure to radiation (116 pairs). In this group, for all SMNs, the adjusted OR was 0.38 (95% CI, 0.09–1.70; \( P = 0.21 \)).
Within the individual SMN types, the observed difference between cases and controls exposed to radiation was only significant for those pairs including cases with thyroid cancer as a SMN (OR, 0.06; 95% CI, 0.00–0.92; \( P = 0.04 \)). A similar analysis restricted to case–control pairs exposed only to chemotherapy was uninformative due to small sample size.

**Discussion**

In this study, we demonstrated an association between less telomere content and SMNs in childhood cancer survivors, an observation primarily driven by subjects with secondary thyroid cancer. The three SMN types included in the current study, breast cancer, thyroid cancer, and sarcoma, are among the most prevalent SMNs in childhood cancer survivors and are associated primarily with exposure to radiation, and to a lesser extent, chemotherapy (28–33), factors that are known to affect telomere maintenance. By selecting controls matched to cases by primary diagnosis and specific therapeutic exposures, we were able to account for the risk associated with these variables in the development of SMN.

Evidence exists for telomere shortening and downregulation of telomerase activity after chemotherapy or radiation exposure \textit{in vitro} (18), with effects that are irreversible (19). These results have also been noted \textit{in vivo}, as both telomere shortening and reduced telomerase activity were noted in individuals who had received chemotherapy, compared with samples taken before the exposure (34). In addition, when compared with age-matched healthy controls, those who received chemotherapy had significantly shorter telomeres, suggesting that chemotherapeutic agents may accelerate the natural shortening that occurs with the aging process (35). Exposure to ionizing radiation also impairs telomere maintenance \textit{in vivo}, as evidenced in Chernobyl workers exposed to low-dose ionizing radiation where...
telomeric shortening was seen even 20 years after exposure, further suggesting that the effect of radiation-induced telomere loss is prolonged (36). In a prospective study of patients with Hodgkin lymphoma, those with shorter telomeres, more complex chromosome rearrangements, and in vitro radiation sensitivity were more likely to develop SMN compared with those who did not demonstrate those characteristics (37). Therefore, individuals with constitutionally short telomeres or telomere damage after chemotherapy or radiation exposure may be at increased risk for developing SMN.

Our study suggests a relation between SMN and telomere content for all SMNs. Cases and controls were closely matched by age, diagnosis, and therapeutic considerations to allow each group an equal opportunity to develop an SMN. Of note, significant differences in both sex distribution and tobacco exposure between the two groups may have acted as potential confounders, although adjustments for these factors were included in our statistical analyses. In considering specific SMN diagnoses, the association between telomere content and thyroid cancer, breast cancer, and sarcoma varied among the three subtypes examined. A statistically significant association was observed between less telomere content and thyroid cancer. Risk for secondary thyroid cancer (in particular the papillary variant) has been associated primarily with radiation exposure, although associations with chemotherapy have also been described (28, 32). This risk exhibits a dose–response relationship, as demonstrated previously in the larger CCSS cohort, of which this study is a subset (28). Interestingly, constitutional telomere shortening has been described in subjects with familial papillary thyroid carcinoma, when compared with those with sporadic thyroid cancers (38, 39). Moreover, evidence of damage to telomeric DNA, such as increased number of telomeric fusions and spontaneous telomeric interactions, was observed in familial papillary thyroid cancer subjects when compared with healthy subjects and patients with spontaneous thyroid cancer, suggesting an underlying genomic instability predisposing these patients to cancer (40). These findings support the existence of abnormalities in telomere maintenance specific to thyroid cancer with hereditary predisposition, which may be a

<table>
<thead>
<tr>
<th>Table 2. Distribution of childhood cancer survivors with and without SMN for criteria not used in matching</th>
</tr>
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<tbody>
<tr>
<td>Survivors with SMN (n = 147)</td>
</tr>
<tr>
<td>n</td>
</tr>
<tr>
<td>Sex of patient</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Race</td>
</tr>
<tr>
<td>NonHispanic White</td>
</tr>
<tr>
<td>Other</td>
</tr>
<tr>
<td>Family history of cancer in a 1st degree relative</td>
</tr>
<tr>
<td>Yes</td>
</tr>
<tr>
<td>No</td>
</tr>
<tr>
<td>Smoking exposure by baseline</td>
</tr>
<tr>
<td>&gt;1 pack/wk × 5+ years</td>
</tr>
<tr>
<td>Had smoked, but ≤1 pack/wk × 5+ years</td>
</tr>
<tr>
<td>Never</td>
</tr>
<tr>
<td>Mean</td>
</tr>
<tr>
<td>Age at diagnosis of primary disease, y</td>
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</table>

<table>
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<tr>
<th>Table 3. Association between telomere content and SMN, cases versus controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMN Number of case–control pairs</td>
</tr>
<tr>
<td>Cases</td>
</tr>
<tr>
<td>All SMN</td>
</tr>
<tr>
<td>Breast cancer</td>
</tr>
<tr>
<td>Thyroid cancer</td>
</tr>
<tr>
<td>Sarcoma</td>
</tr>
</tbody>
</table>

*Adjusted for sex, race, family history, smoking status, and age at diagnosis of the primary disease.
contributing factor to the significant association between secondary thyroid cancer and shortened telomeres observed in our study population.

Our results fail to demonstrate a relation between telomere content and secondary breast cancer. Findings with regard to telomere length and risk for de novo breast cancer have been inconsistent. Although most retrospective studies show an association with shorter telomeres, this association loses significance when the question is examined prospectively, suggesting that treatment effect may play a role in observed telomere shortening (41, 42). Therefore, the absence of an association with regard to secondary breast cancer is perhaps not surprising. We observed a statistically nonsignificant positive association between telomere content and secondary sarcoma. Of the three SMN types considered in the current study, the sarcoma population was the most limited, both by sample size and heterogeneity, as samples represented both soft-tissue sarcomas and bone sarcomas such as the Ewing sarcoma or osteosarcoma. Recently, a case–control study showed longer leukocyte telomeres to be associated with an increased risk for soft-tissue sarcomas (43). In contrast, another case–control study examining constitutional telomere length and risk for de novo osteosarcoma demonstrated a statistically significant association with shortened telomeres among females (44). Osteosarcoma is often associated with chromosomal aneuploidy (45), suggesting a predisposing underlying chromosomal instability. However, no significant correlations with polymorphisms in telomere biology genes have been found thus far (44). In addition, the matched controls for our sarcoma SMN group consisted of a disproportionately high number of heavy smokers compared with the study population as a whole, which may have confounded the analysis, as smoking has been shown to shorten telomere length with each pack-year smoked (46). Although attempts were made to adjust for smoking status, an imbalance of tobacco exposure may in part explain the observation of less mean telomere content among the sarcoma cases. Furthermore, restricting the sarcoma population to those whose sample was collected before SMN reversed the direction of the association to what was observed in the breast and thyroid cancer populations.

The association between telomere content and the subsequent development of SMN is most reliable when the sample is taken before the diagnosis of the SMN. One limitation to our study was that a significant proportion of our SMN cases had samples taken after their SMN diagnosis, raising the concern for further reduction in telomere content as a result of additional chemotherapy rather than as an observation that preceded SMN diagnosis. We examined this possibility by conducting a subanalysis among the cases that had samples collected before SMN diagnosis, demonstrating a maintained association between less telomere content and SMN that was concordant with our hypothesis. Such an association would be further strengthened if there were a measurable trajectory of telomere attrition before the development of SMN, as was shown by Chakraborty and colleagues to occur preceding development of therapy-related myelodysplasia (47).

Additional limitations include the relatively small cohort size, and the small amount of DNA available for analysis, which precluded our ability to validate our qPCR findings by Southern blot analysis by probing for telomere DNA. Similarly, although it is the shortest telomere end, rather than the average telomere length, that contributes to chromosomal instability (48), the limited amount of DNA available also prohibited performance of single telomere length analysis. There may be additional confounders potentially affecting the telomere length, such as presence of gingival inflammation at the time of sample procurement, that we were unable to control for in our analysis due to lack of information. For example, we were unable to determine the influence of familial cancer syndromes on our data set, as information regarding p53 or BRCA status, for example, was also not available. However, we did control for family history of cancer in a first-degree relative; furthermore, previous estimates of the proportion of familial cancer syndromes within the CCSS cohort, based upon self-reported family history, are low (9).

To our knowledge, this study represents the only investigation of an association between telomere content and SMNs in childhood cancer survivors. Our findings in this unique cohort suggest value in further study of shortened telomeres as a predisposing factor to development of SMN.

### Table 4. Association between telomere content and SMN, cases versus controls, with case sample drawn before SMN diagnosis

<table>
<thead>
<tr>
<th>SMN</th>
<th>Number of case–control pairs</th>
<th>Mean telomere content ± SD</th>
<th>Unadjusted OR</th>
<th>Adjusted OR*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>Controls</td>
<td>P</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>All SMN</td>
<td>41</td>
<td>41</td>
<td>0.56 ± 0.20</td>
<td>0.65 ± 0.33</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>22</td>
<td>22</td>
<td>0.57 ± 0.15</td>
<td>0.65 ± 0.29</td>
</tr>
<tr>
<td>Thyroid cancer</td>
<td>13</td>
<td>13</td>
<td>0.53 ± 0.28</td>
<td>0.69 ± 0.41</td>
</tr>
<tr>
<td>Sarcoma</td>
<td>6</td>
<td>6</td>
<td>0.57 ± 0.17</td>
<td>0.58 ± 0.28</td>
</tr>
</tbody>
</table>

*Adjusted for sex, race, family history, smoking status, and age at the time of diagnosis of the primary disease.

bUnable to estimate ORs due to the small sample size.
Prospective validation of our results may elucidate whether subjects with SMN had short telomeres preceding their first diagnosis of cancer or had inappropriately rapid germline telomere attrition in response to chemotherapy or radiation. It is worth noting that the CCSS cohort is still relatively young, with the oldest subjects now in their fifth decade of life. Therefore, the effects of further age-related telomere shortening may yet be forthcoming, so that revisiting this question in 5 to 10 years may provide a larger sample size and a stronger statistical comparison. Current recommendations for SMN surveillance are based upon known risk factors, such as patient demographics and therapeutic exposures (49). Methods for predicting risk for SMN in childhood cancer survivors have been proposed on the basis of statistical modeling that incorporate clinical and demographic variables (50). Prospective confirmation of our findings may lead to incorporation of telomere length measurements into such predictive algorithms in the future, thus targeting surveillance to those at highest risk for developing SMNs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Authors’ Contributions

Conception and design: M.M. Gramatges, M.F. Okcu, J.P. Neglia, L.C. Strong, G.T. Armstrong, S. Bhatia
Development of methodology: M.M. Gramatges, M.F. Okcu
 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.P. Neglia, L.L. Robison
 Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.M. Gramatges, Q. Liu, Y. Yasui, M.F. Okcu, L.L. Robison
 Writing, review, and/or revision of the manuscript: M.M. Gramatges, Y. Yasui, M.F. Okcu, J.P. Neglia, L.C. Strong, G.T. Armstrong, L.L. Robison, S. Bhatia
 Study supervision: J.P. Neglia, L.C. Strong, G.T. Armstrong

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