1. STUDY TITLE: *Telomere Length and Second Malignancy in Pediatric Cancer Survivors*

2. WORKING GROUP AND INVESTIGATORS

2.1 Working Group: Genetics (primary) and Second Malignancy (secondary)

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3. BACKGROUND AND RATIONALE

A childhood cancer survivor's risk for developing a second malignant neoplasm (SMN) is 5.8 to 6.4 fold greater than the risk for cancer in the general population (1, 2), comprising close to a 10% 30-year cumulative incidence (2). SMNs also account for nearly 20% of deaths in survivors (3). Higher risks for SMN have been associated with primary cancer diagnosis, age at time of primary cancer diagnosis, female gender, and exposure to specific chemotherapeutic agents and radiotherapy (3, 4). Currently, there are no recommendations for screening survivors for genetic susceptibility for SMN, though siblings of survivors with SMN have been found to have a greater risk for cancer than the general population (2). This proposal tests the novel hypothesis that shortened germline telomere length may play a role in the development of SMN in pediatric cancer survivors exposed to radiation.

Telomeres are repetitive DNA-protein structures localized to chromosome ends, protecting chromosome integrity by preventing end-to-end fusions and the loss of proximal coding terminal base pairs. Telomere length averages between 8 and 12 kbp and shortens with DNA replication (5). Telomere length in epithelial or hematopoietic cells serves as a convenient proxy for germline telomere length (6). Telomere length is genetically determined, varies between individuals and cell populations, and is inversely correlated with age (5, 7-9). Telomere shortening may result from environmental exposures such as chemotherapy or radiation (10). When compared to individuals with no history of cancer, cancer patients who had received more than 50 Gy of therapeutic radiation demonstrated a high proportion of telomere loss (11). Exposure to high doses of irradiation impairs telomere maintenance, resulting in generalized shortening and telomere loss of integrity (12). Ongoing telomere attrition may permit unmasking of radiation-induced mutations, perhaps conveying a proliferative advantage as a step toward carcinogenesis and SMN.

Critically short telomeres are recognized as DNA damage by p53 and Rb mediated checkpoints, which initiate cellular senescence or apoptosis (13-16). Failure of the checkpoint system can result in chromosome end-to-end joining and breakage during anaphase, resulting in genomic instability and potential malignant transformation in the setting of an uncontrolled proliferative capacity (17, 18). Short germline telomeres confer chemo-sensitivity both clinically and *in vitro* (19, 20), and have been associated with various cancers (21). Specifically, lymphocyte and/or buccal cell telomere shortening have been associated with bladder, small cell lung, renal, basal cell, esophageal, and papillary thyroid cancers (22-28). In regards to second cancers, a prospective analysis of Hodgkin's lymphoma patients demonstrated shorter telomeres, complex chromosome rearrangements, and *in vitro* radiation sensitivity in the cohort that developed second cancers (29). In addition, Hodgkin's patients who developed a SMN demonstrated higher rates of baseline, induced, and unrepaired DNA damage (30). Accelerated telomere shortening has also been associated with the development of therapy-related myelodysplasia or acute myleogenous leukemia following autologous transplant for lymphoma (31). Though the mechanism for shortened blood or epithelial cell telomeres and cancer incidence is not well understood,

the relationship is presumably multifactorial, resulting from increased likelihood for mutation gains or losses in an already strained checkpoint system.

Similarly to hematopoietic cell telomeric DNA, epithelial cell telomeric DNA derived from buccal samples also shortens progressively with age (6, 32). Compared with lymphocytes, buccal cells provide a more efficient means for collecting high quality DNA, with the convenience of a non-invasive sample collection method and reduced risk to the patient (33). In assessing telomere length, understanding the cell type being measured is important, as variability in telomere length has been observed between tissues and cell types; for example, telomere length in white blood cell subsets vary both at baseline and in their observed attrition rate with aging (5, 34, 35).

The goal of this research proposal is to investigate the relationship between telomere length and development of radiation-related second cancers in patients enrolled in the Childhood Cancer Survivor Study. Although longer telomeres have also been associated with specific cancers including melanoma and non-Hodgkins lymphoma, and, with some conflicting evidence, renal cell carcinoma, lung cancer, and breast cancer (36-41), the majority of the literature associates cancers with shortened germline telomeres. **Due to the effect of chemotherapy and radiation on both DNA repair mechanisms and telomere length, combined with evidence associating shortened telomere length with second cancers, we hypothesize that very short telomeres will be associated with a higher incidence of SMN among survivors.** We will control for factors known to affect development of SMN, including length of time since primary diagnosis, primary diagnosis, chemotherapeutic agents received, and exposure to radiation, by matching second cancer cases with a control group comprised of survivors without SMN. In addition, because another factor likely to affect telomere length in this population will be the subject's age (as telomere length decreases with age), we will match cases and controls by their age at the time of buccal sample donation. We will exclude all subjects who have received stem cell transplantation, as this procedure is highly likely to result in significant telomere shortening, and there is a risk of donor cell contamination of the buccal sample, although the sample should contain DNA from primarily epithelial cells.

We have powered this study to detect small differences in telomere length between subjects with and without SMN. The size of this difference exceeds the intra-individual variation observed with the assay used for this analysis. **Our goal is to elucidate a potential underlying genetic contributing factor to incidence of SMN.** If larger clinically significant differences are detected, telomere length has the potential to serve as a screening tool to identify individuals at risk for SMN in the survivor population. If our hypothesis proves correct, we will not be able to determine whether subjects with short telomeres and SMN have shortened telomeres that preceded their diagnosis of cancer, or have responded to chemotherapy with inappropriately rapid germline telomere attrition. To answer this question would require a prospective study design.

We plan to conduct this proposal as follows:
We will compare telomere length in DNA collected from buccal samples in CCSS survivors with and without SMN, matched as described above.

4. SPECIFIC AIM/OBJECTIVES/RESEARCH HYPOTHESES

4.1 Primary Aim:
To investigate the relationship between telomere length and the three most commonly occurring radiation-related SMNs (breast, thyroid and sarcoma) collectively, in childhood cancer survivors.

Secondary Aim:
To investigate the relationship between telomere length and specific radiation-related SMNs in childhood cancer survivors.

4.2 Objectives:

4.2.1 *Primary*: To compare buccal DNA relative telomere length from childhood cancer survivors with and without a radiation-related SMN.

4.2.2 *Secondary:* To compare buccal DNA relative telomere length from childhood cancer survivors with and without secondary breast cancer.

4.2.3 *Secondary:* On an exploratory basis, to compare buccal DNA relative telomere length from childhood cancer survivors with and without secondary thyroid cancer.

4.2.4 *Secondary:* On an exploratory basis, to compare buccal DNA relative telomere length from childhood cancer survivors with and without secondary sarcoma.

4.3 Hypothesis:

4.3.1 Subjects with radiation-related SMN will have significantly shortened telomeres, when compared with matched controls that are survivors of childhood cancer without SMN.

5. ANALYSIS FRAMEWORK

5.1.1 Outcome of Interest: The primary outcome of interest is buccal DNA telomere length in relation to risk for SMN in childhood cancer survivors.

5.2 Study Population:
By searching the CCSS database, we have identified 5790 buccal cell samples from childhood cancer survivors (from the online CCSS database). Within this population, 2286 individuals have a documented SMN (personal communication with S. Bhatia). Excluding non-melanoma skin cancer and meningioma (due to absence of data if these cancers were observed to occur in a previous radiation field), as well as less common SMN diagnoses, 469 buccal and Oragene samples from survivors experiencing the more common second malignancies remain **(Table 1).**

Table 1: CCSS Second Malignancy Samples

Diagnosis	Typecode	Sub-Diagnosis	Buccal Cells (total number of cases)	Oragene (total number of cases)	Total
Leukemia	1	Lymphoid	4	2	
	2	Myeloid	7	3	
	3	Other	2	1	
	4	Unspecified	1	0	
		Total	14	6	20
Lymphoma	17	Hodgkins	5	5	
	18	NHL	9	6	
	19	Unspecified	0	0	
		Total	14	11	25
CNS	5.1	Astrocytoma	10	8	
	5.2	Other glial	2	0	
	6	Medulloblastoma	3	2	
	8	Other CNS	5	3	
	9	Unspecified	1	0	
		Total	21	13	34
Breast	10	Breast	137	79	216
Thyroid	12	Thyroid	62	38	100
Sarcoma	13	Osteosarcoma	7	2	
	14	Ewing's	1	0	

	15	Other bone tumors	3	1	
	16	Unspecified bone	1	0	
	20	Rhabdo	1	1	
	21	Other SSC	34	18	
	22	Unspecified SSC	4	1	
		Total	51	23	74
Total SMN Buccal/Oragene Samples available			299	170	469

The primary comparison includes samples from within a cohort of 299 available mouthwash samples (out of 469 buccal samples including both Oragene and mouthwash) identified from survivors with SMN, and matched mouthwash samples selected from survivors without SMN.

5.2.1 Power Analysis:

Assuming a case-control population with a normal distribution, calculations were made to determine the sample size required to observe a range of differences in mean telomere length (RTL) between 0.1 and 0.3, based upon an expected standard deviation (SD) of telomere length of 0.5 from literature on telomere length from buccal DNA ($\alpha = 0.05$ and $\beta = 0.2$) (23, 32)**.** The SD of the difference of telomere length between the matched pairs, conservatively assuming a low correlation reflected by $\rho = 0$, would then be 0.71. For a range of mean detectable differences, the sample size for a paired analysis is calculated from a SD of differences = 0.71, $\alpha = 0.05$ and $\beta = 0.2$ **(Table 2)**.

We propose to match subjects in the SMN group 1:1 with survivors who have not developed SMN by:

- Primary diagnosis
- Number of years since primary cancer diagnosis
- Age at time of sample donation (decade)
- Exposure to specific therapeutic agents (anthracyclines, alkylators, topoisomerase inhibitors, radiation, or none of the above)

Table 2: Power Analysis

Mean Detectable Difference	Survivors without SMN	Survivors with SMN	Total samples
0.10	398	398	796
0.15	178	178	356
0.20	101	101	202
0.25	65	65	130
0.30	46	46	92

Because length of time since primary cancer is independently associated with cumulative incidence of SMN, we will match subjects both by age at time of sample donation as well as by the number of years since the primary cancer diagnosis to adjust for length of follow up. Also, because both primary diagnosis and specific therapy exposures have been shown to be predictive of SMN, we will include these factors in our case control matching as well (2). We will power this study to detect a relatively small difference in telomere length of 0.20, less than 50% of the expected SD of telomere length, between subjects with and without SMN. Smaller differences have less expected clinical relevance. In order to detect this difference with a 1:1 control: case ratio, allowing for the matching described above, we would require 101 samples from the SMN group and 101 samples from the without SMN group, for an overall total of **202** patient samples for **each planned comparison**. Samples from the **without** SMN group will be matched 1:1 to samples in the SMN group by primary cancer diagnosis, number of years since primary cancer diagnosis, age at time of sample donation, and exposure to chemotherapy or radiation.

5.2.2 Inclusion Criteria:
All individuals who are childhood cancer survivors enrolled in the CCSS, with or without a second cancer diagnosis, with a mouthwash sample available.

5.2.3 Exclusion Criteria:
Subjects without SMN and:

- Exposure to stem cell transplantation

Subjects with SMN and:

- Exposure to stem cell transplantation
- Subjects with samples drawn over one month after beginning therapy for SMN

5.2.4 Requested Specimens: SMN
To achieve homogeneity while exploring both hematologic and oncologic SMN, we are requesting the following specimens from subjects with the most common SMNs, so that we are able to analyze enough samples within each SMN category to detect a difference in RTL of 0.2. **Ideally, these specimens should have been taken within one month of diagnosis of SMN to remove the confounder of the effect of additional chemotherapy on RTL (Table 3).**

Table 3: Samples Requested

SMN Diagnosis	Typecodes	Number of Buccal Samples
Sarcoma	13,14,15,16,20,21,22	51
Thyroid	12	62
Breast	10	101
Total		**214**

5.2.5 Requested Specimens: Matched controls
We are requesting an additional **214** samples matched 1:1 to the SMN specimens by the criteria outlined above.

The total number of samples requested, including cases and controls, is **428**.

5.2.6 Requested Variables
Gender
Race
Ethnicity
Annual household income
Family history of malignancy
Cancer diagnosis
Second cancer diagnosis(es) (if applicable)
Age at first cancer diagnosis (and second, third, fourth, if applicable)
Age at time of sample donation, and timing from SMN diagnosis, if applicable
Tobacco exposure history
Chemotherapy exposures (yes/no), including anthracyclines, alkylators, topoisomerase inhibitors
History of radiation exposure including fields irradiated, if possible

5.3 Methods:

5.3.1 Experimental Outline:
We will first compare telomere length in 214 samples with radiation-related SMN to 214 matched controls. We will next compare telomere length in 101 samples with breast cancer as a SMN to 101 matched controls. Lastly, we will compare telomere length in 62 samples with thyroid cancer as a SMN to 62 matched controls, and 51 samples with sarcoma as a SMN to 51 matched controls. These final two comparisons will be made on an exploratory basis as the statistical analysis is insufficiently powered.

5.3.2 Laboratory Methods:
Relative Telomere Length (RTL) Analysis: Extracted DNA from mouthwash samples will be analyzed for RTL using qPCR. **From each sample, 20 to 30 ng of DNA is required to perform the analysis.** Samples will be quantified and run in triplicate on a high-throughput real-time PCR system. RTL will be calculated in each sample from the telomere repeat to single copy gene ratio, using a modified version of the method described by Cawthon et al (42), and as previously performed by Dr. Gramatges (43). Telomere and single gene copy amplifications will be run with a SYBR detector and a negative control (water), as well as a standard curve on each plate. Amplification plots and dissociation curves will be analyzed for each run. PCR plates will be loaded with a liquid handling robot for precision and accuracy.

This qPCR technique has been validated to correlate with the telomere restriction fragment analysis performed by southern blot (44-46), considered to be the accepted gold standard for measuring telomere length; however, the southern blot technique has certain disadvantages, requiring a minimum of 1 ug of purified, intact DNA, and is not designed for large sample numbers (47). Other forms of telomere length analysis include fluorescence in situ hybridization, which uses an oligonucleotide or peptide nucleic acid probe to directly label the telomere sequence and requires a metaphase spread. Flow cytometry FISH offers telomere length measurement within immunologically characterized populations, but also requires live cells. **Given the high throughput nature of this proposal, the authors believe that the qPCR technique for relative telomere length analysis is best suited to perform analyses involving larger sample numbers, where the DNA may be of limited quantity and quality, such as this proposed comparison.** It should be noted that this technique does not produce an absolute quantitation of telomere length, and is best used when comparing two distinct populations in a case: control scenario.

5.3.3 Statistical Methods:
First, the three targeted radiation-related SMNs (breast, thyroid and sarcoma) will be analyzed as a group. We will examine the results analyzing RTL as both a continuous and categorical variable while accounting for the matched design. RTL will be compared, as a continuous variable, between matched cases and controls using a matched pairs t-test or nonparametric analogue. We will also divide the data set of RTL values into quartiles and perform conditional logistic regression to obtain odds ratios for odds of second cancer for each quartile, relative to the first, or shortest, quartile. Significance will be determined using the Wald Chi-square test, and corresponding 95% Wald confidence intervals will be reported for the odds ratios. Analyses will include adjustment for gender and race. We will then analyze each SMN diagnosis (sarcoma, breast, and thyroid cancer) separately, though of note only the breast cancer cases have sufficient sample sizes to achieve adequate power to detect our desired difference. For this reason, thyroid cancer and sarcoma will be analyzed on an exploratory basis.

5.3.4 Potential Pitfalls and Alternative Strategies
For this exploratory analysis, study power was designed to detect a large difference in RTL of 0.2 between the two groups. A smaller difference of less than 0.2 may not be detected with adequate power. If smaller differences are observed, we may request an additional samples to permit detecting a difference in relative telomere length as small as 0.1 between the groups. Some intra-individual variation exists in the qPCR assay, which we will minimize by analyzing samples in triplicate, utilizing a high-throughput liquid handling robot plate loader, and intermixing samples in a minimal number of runs. Dr. Gramatges is proficient in use of the real-time PCR instrument and software as well as the ep-motion robot, and this equipment is readily available to her within the Feigin Center building at Texas Children's Cancer Center (TXCCC).

6. PRELIMINARY DATA

6.1 *Relative Telomere Length in Oragene Compared with Mouthwash Samples*
The Childhood Cancer Survivor Study collects buccal DNA samples using two methods, by mouthwash and by use of an Oragene kit (which is a saliva collection). Given the known variability in telomere length from cell type to cell type, we have determined if the RTL from each of these collection methods are equivalent. We compared the RTL in samples collected from individuals using each of these methods. Our results indicated that some intra-individual variability does exist between the two collection methods.

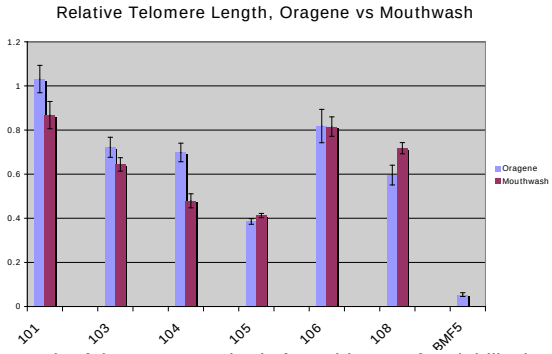


Figure 1: Comparison of Oragene to mouthwash telomere length in six individuals, with varying degrees of correlation. Samples from healthy subjects were compared to a sample from a subject with a diagnosis of dyskeratosis congenita, a short telomere syndrome (labeled BMF5).

We then compared the cell types collected from each of these two methods for evidence of variability in the cell type collected from mouthwash *vs*. saliva, to potentially explain the intra-individual differences observed in RTL between the two collection method groups.

6.2 *Cell Types in Mouthwash Samples vs. Oragene Samples*

We collected samples from twenty subjects using three different methods of collection, buccal brush, mouthwash, and saliva, noting that the Oragene kit collects saliva that is spit directly into the collection device. Slides were then prepared and stained using a Wright-Giemsa stain. Cell types were then counted in three different fields under 20x. Our data indicates that mouthwash samples demonstrate less variability in the cell type collected, when compared with the Oragene samples, and are composed almost entirely of epithelial cells, *vs*. saliva samples, which contain a relatively large proportion of lymphocytes. These findings were recently confirmed by the Oragene manufacturer, Genotek (*posted to website on March 31st).*

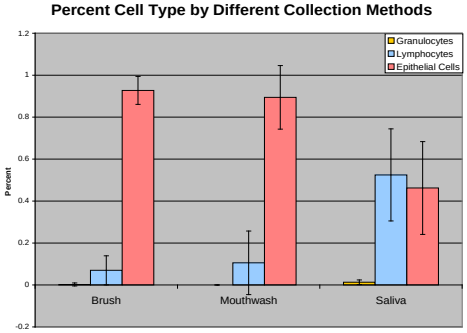


Figure 2: Comparison cell types collected from three different methods, buccal brush, mouthwash, and saliva. Saliva demonstrated the highest degree of variability from sample to sample, with the mean percentage of epithelial cells and lymphocytes approximately equal.

From the results of this preliminary analysis, we have concluded that the difference in RTL observed between Oragene and mouthwash collection methods is likely due to a combination of intra-individual assay variability and variability in the cell type collected by each of the two methods used by the CCSS. We have determined that that less cell type variability is obtained from the mouthwash *vs*. Oragene, with results approximating that of a buccal swab.

6.3 *Relative Telomere Length Assay:* We will utilize the relative telomere length (RTL) quantitative PCR (qPCR) technique. Dr. Gramatges has over three years of experience with this technique, primarily while a clinical postdoctoral fellow at Stanford, and has published her previous research data on RTL in breast cancer (43). She has further validated this technique at TXCCC (**Figures 3 and 4)**. Here, we demonstrate reduced RTL in lymphoblastoid cell lines (LCLs) from children with clinical dyskeratosis congenita, a short telomere syndrome, with both known and unknown mutations **(Figure 3)**. We have

also performed this assay in seven healthy individuals at two time points, demonstrating that the intra-individual variability of this assay falls between 0.02 and 0.12 **(Figure 4).**

Figure 3: Relative Telomere Length in LCLs, Normal (Wild Type) Compared with Dyskeratosis Congenita

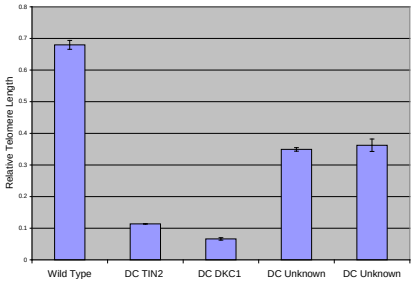
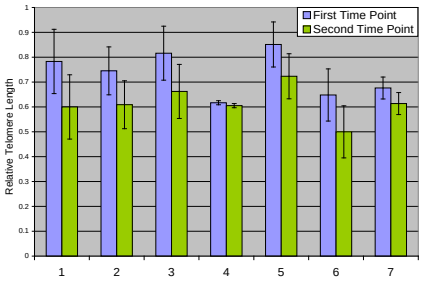


Figure 4: Relative Telomere Length in Epithelial Cells from 7 Healthy Donors at Two Consecutive Time Points



6.4 Preliminary Data Conclusions

From these preliminary results, we have determined that for this proposal, DNA collected using either the mouthwash method or the Oragene method should be requested, but not both, as RTL from DNA collected from each of these two methods is not comparable given the differences in cell type collected. **Therefore, given that DNA from mouthwash collections is predominantly from epithelial cells, *vs.* the Oragene method which collects a variably mixed population of epithelial cells and lymphocytes, we will request only samples obtained through the mouthwash collection method, thereby achieving the highest consistency and comparability of RTL results in evaluating the sample type with the purest cell population.** We have also calculated our sample estimate with the power to detect a minimum difference in relative telomere length of 0.2, given that a difference of 0.1 falls within the intra-individual variability of the assay, and we seek to identify a clinically significant difference.

7. SPECIAL CONSIDERATIONS

Dr. Gramatges receives research funding through an NIH-supported K12 grant, which has been awarded as a part of her academic appointment at Baylor College of Medicine beginning in July of 2009. This 3-year mentored training grant includes 75% protected time for research inclusive of the Concept described in this proposal. Dr. Gramatges' career interests are in telomere biology clinical and translational research pertaining to genetic risks for long-term effects of childhood cancer, as well as risks for cancer predisposition and acute therapy-related toxicities.

8. FUTURE DIRECTIONS

If a positive association between shortened telomeres and second malignancy is observed, we would then consider requesting sibling samples from the SMN samples in our cohort, to better elucidate the mechanism for short telomeres in the SMN population, inherited *vs.* individual response to chemotherapy. We would then propose a prospective trial for telomere length evaluation at the time of initial cancer diagnosis, followed by serial samples at defined time intervals, to again investigate whether short telomeres preceded the original cancer diagnosis, or are an individualized response to cancer treatment, perhaps exploring heritable factors such as polymorphisms in the telomerase enzyme. If patients who develop SMN are found to have significantly shorter germline telomeres, this finding would suggest potential for screening the survivor population for those individuals in need of increased surveillance. Based on the data of Chakraborty et al (31), in addition to predicting overall risk for second cancer, an increase in attrition rate may also predict timing of second cancer. Our objective would be to better understand the role of baseline telomere length in predicting both initial and second cancer, as well as the effect of telomere attrition rate on the development of cancer.

Lastly, we will explore additional long-term effects of cancer therapy for association with underlying telomere shortening. Grade 3 or 4 toxicity heart failure and coronary artery disease carry a relative risk

of 15.1 and 10.4 respectively in childhood cancer survivors compared with siblings (48). Both conditions have been associated with significant telomere shortening (49-52). Similarly, pulmonary fibrosis has also been associated with significant telomere shortening (53). Lastly, childhood cancer survivors may be at risk for premature osteopenia and osteoporosis (54). Shorter telomere length has been demonstrated in elderly women with decreased bone mineral density (55). The association between short telomere and osteoporosis is thought to be secondary to defects in telomere maintenance resulting in impairment of osteoblast differentiation (56). In conclusion, telomere length may predict which individuals in an already at-risk population are at highest risk for developing severe long-term outcomes.

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Figure 3: Mock Tables for Analyses: Distribution of Telomere Length by Selected Variables

Variable	CCSS Population			
	RTL in Survivors without SMN +/- SD		RTL in Survivors with SMN +/- SD	
Gender				
Male				
Female				
Race				
Caucasian				
Asian				
Black				
Other				
Ethnicity				
Hispanic				
Non-Hispanic				
Family History of Malignancy				
No				
Yes				
Chemotherapy Exposure (Y/N)				
None				
Alkylating Agent				
Anthracycline				
Epipodophyllotoxin				
Radiation Exposure				
No				
Yes				
Primary Tobacco Exposure				
None				
>1 pack/week x 5 years				
Socio-economic status (income)				
<40,000/year				
40,000 – 80,000/year				
>80,000/year				
Primary Diagnosis				
Leukemia				
Lymphoma				
CNS				
Bone Tumor				
Soft Tissue Sarcoma				
Wilms				
Neuroblastoma				