CCSS Study Analysis Concept Proposal
Genetics Working Group

1. Study Title: Identifying genetic mechanisms of Second Malignant Neoplasm development

2. Working Group and Investigators: This proposed publication will be within the Genetics Working Group.

Proposed Investigators include:

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3. Background and rationale:

There is a critical need for experimental models in which to study second malignant neoplasm (SMN) pathogenesis and prevention (1, 2). To model radiotherapy-induced malignancies, we irradiated *Nf1*$^{+/-}$ mice and found that similar to individuals with Neurofibromatosis I (NF1), *Nf1* mutant mice are sensitized to radiation-induced tumorigenesis (3). Our mouse model, which recapitulates clinical SMN induction, is a valuable experimental system in which biochemical and genetic mechanisms can be investigated in a translationally relevant manner. Understanding the genetic and biochemical mechanisms underlying SMN development will be critical to developing effective strategies for reducing the rate of this complication in cancer survivors.

We are performing genome wide analyses of our radiation-induced tumors to identify genetic mechanisms driving radiation-induced tumorigenesis, and are correlating our findings to human SMN specimen. In our initial analysis we are identifying tumor suppressor genes showing loss of heterozygosity (LOH) in radiation-induced tumors generated by our mouse model, an analysis that is facilitated by the fact that these mice harbor strain specific polymorphisms allowing us identify loci of heterozygosity throughout the genome (3, 4). We have begun to validate the significance of a few identified genes in our mouse model in human radiation-induced tumors. Our objective is to determine whether LOH of candidate tumor suppressor genes occurs in human SMNs. This validation will facilitate future studies of SMN development, prevention and treatment in our mouse model by focusing efforts on genes most commonly altered in human SMNs.

4. Specific Aims/Objectives/research hypotheses:

Based on findings in our animal model (3), we hypothesize that loss of tumor suppressors promote SMN development similarly in our mouse model and human SMNs. We also hypothesize that LOH of the genes of interest is common to all radiation-induced SMNs. We do not specifically hypothesize that specific histologies harbor particular frequencies of LOH. Major mechanisms by which tumor suppressor proteins can be reduced or lost include LOH and reductions in transcript levels. The objective of this proposal is to determine whether evidence of these mechanisms in genes identified in our mouse model is present in human SMNs.

Aim 1. **Determine whether loss of heterozygosity in tumor suppressor genes identified in the *Nf1* mutant mouse model occurs in SMNs**. We will determine whether LOH of tumor suppressor genes have occurred in SMNs by performing Taqman-based SNP genotyping on DNA isolated from formalin-fixed, paraffin embedded samples of SMNs. The genotyping analysis program SDS3.0 will be used to generate allelic discrimination plots of tumor and control normal tissue DNA samples. Our initial analysis will focus on the five most commonly altered tumor suppressor genes identified in our mouse model.

Aim 2. **Determine whether transcript levels of candidate tumor suppressor genes are reduced in SMNs**. We will isolate mRNA from matched normal and tumor tissues from formalin-fixed, paraffin embedded samples of SMNs and perform PCR-based quantitation of candidate tumor suppressor transcripts. These targets will be the same as those assessed in Aim 1.

5. Analysis Framework:

a. Outcome of interest: The initial outcomes of interest are 1) SNP genotypes in target tumor suppressor genes in SMN samples and 2) the mRNA levels of corresponding transcripts in SMN samples. Change in SNP genotype from heterozygous to homozygous will be considered evidence of LOH. We expect that LOH will be common to all radiation-induced SMNs, hence there is no comparison group. Baseline LOH frequencies for loci of interest are expected to be extremely low. Reduction of transcript levels by at least half will be considered significant. Contingent on additional funding, unbiased comprehensive analyses of SMN samples could include an unbiased, global assessment of LOH by SNP genotyping, copy number variation (CNV), and expression analyses.

b. Subject population: We request unstained, fixed sections of SMN samples accrued by the Biopathology Center of the CCSS. The specific SMN histologies requested are: breast (n=48), meningioma (n=23), CNS (n=18), sarcoma (n=10). The specific clinical data we request are: whether the SMN arose in an irradiated field, and if so, what estimated dose did the area receive, birthdate of the patient, date when the specimen was removed from patient, dates when radiotherapy and chemotherapy for initial primary were completed, histologic type and grade. The numbers of samples requested are based on the numbers of samples available through the Biopathology Center.

c. Exploratory variables: Our initial analysis will focus on the five most commonly altered tumor suppressor genes identified in our mouse model that have homologous human genes. The primary goal is to determine whether LOH of target genes are present in SMNs. We hypothesize that radiation-induced tumors demonstrate an increased incidence of LOH of the target genes is as compared to background rates of LOH, which we estimate to be extremely low, less than 1%. We will test whether the incidence of LOH in radiation-induced tumors exceeds the background rates of 1% and 5%, with 5% representing the worst case scenario with regard to background LOH. We will also calculate 95% confidence intervals around our estimates.

Power Analysis:
With 48 samples (Breast cancers), there is 80+% power for the null hypothesis of an LOH frequency of =1% or less with alpha=5% if the true frequency of LOH is 8% or above.

With 48 samples, there is 80+% power for the null hypothesis of an LOH frequency of =5% or less with alpha=5% if the true frequency of LOH is 16% or above.

With 99 samples (all SMN samples requested), there is 80+% power for the null hypothesis of an LOH frequency of =1% or less with alpha=5% if the true frequency of LOH is 5% or above.

With 99 samples, there is 80+% power for the null hypothesis of an LOH frequency of =5% or less with alpha=5% if the true frequency of LOH is 12% or above.

Similarly, for meningioma (n=23), CNS (n=18), sarcoma (n=10) samples:

with 23 samples, a frequency of =1% can be rejected with 80+% power if the true frequency is 13%;
with 23 samples, a frequency of =5% can be rejected with 80+% power if the true frequency is 28%;

with 18 samples, a frequency of =1% can be rejected with 80+% power if the true frequency is 16%;
with 18 samples, a frequency of =5% can be rejected with 80+% power if the true frequency is 29%;

with 10 samples, a frequency of =1% can be rejected with 80+% power if the true frequency is 28%; and
with 10 samples, a frequency of =5% can be rejected with 80+% power if the true frequency is 39%.

d. Specific table

Proposed Data Summary Table

	Breast cancers	Meningiomas	CNS tumors	Sarcomas
Total number of cases	48	23	18	10
Number of cases in which SMN arose in irradiated field				
Median age at diagnosis				
Median age at completion of chemotherapy/radiotherapy				
Median age at SMN diagnosis				
Number of tumors heterozygous at SNP1				
Number of tumors with LOH at SNP1				
Number of tumors with reduced Target1 transcript				
Number of tumors heterozygous at SNP2				
Number of tumors with LOH at SNP2				
Number of tumors with reduced Target2 transcript				

Number of tumors heterozygous at SNP3				
Number of tumors with LOH at SNP3				
Number of tumors with reduced Target3 transcript				
Number of tumors heterozygous at SNP4				
Number of tumors with LOH at SNP4				
Number of tumors with reduced Target4 transcript				

References

1. Bhatia S, Sklar C. Second cancers in survivors of childhood cancer. Nat Rev Cancer. 2002 Feb;2(2):124-32.
2. Friedman DL, Whitton J, Leisenring W, Mertens AC, Hammond S, Stovall M, et al. Subsequent neoplasms in 5-year survivors of childhood cancer: the Childhood Cancer Survivor Study. J Natl Cancer Inst. 2010 Jul 21;102(14):1083-95.
3. Nakamura JL, Phong C, Pinarbasi E, Kogan SC, Vandenberg S, Horvai AE, et al. Dose-dependent effects of focal fractionated irradiation on secondary malignant neoplasms in Nf1 mutant mice. Cancer Res. 2011 Jan 1;71(1):106-15.
4. Chao RC, Pyzel U, Fridlyand J, Kuo YM, Teel L, Haaga J, et al. Therapy-induced malignant neoplasms in Nf1 mutant mice. Cancer Cell. 2005 Oct;8(4):337-48.