CCSS STUDY ANALYSIS CONCEPT PROPOSAL

SUBSEQUENT MALIGNANCIES AND GENETICS WORKING GROUPS

1. **STUDY TITLE: Secondary Cancers among NF1 Cancer Survivors**

2. **WORKING GROUP AND INVESTIGATORS:** Second Malignancies Working Group and Genetics Working Group

Proposed Investigators include:

Name	email	Institution
Smita Bhatia	sbhatia@peds.uab.edu	University of Alabama at Birmingham (UAB)
Purnima Singh	psingh@peds.uab.edu	University of Alabama at Birmingham (UAB)
Lennie Wong	flwong@coh.org	City of Hope
Michael Fisher	fisherm@email.chop.edu	Children's Hospital of Philadelphia
Bruce Korf	bkorf@uabmc.edu	University of Alabama at Birmingham (UAB)
Joseph Neglia	jneglia@umn.edu	University of Minnesota
Greg Armstrong	Greg.Armstrong@stjude.org	St. Jude Children's Research Hospital
Les Robison	Les.Robison@stjude.oeg	St. Jude Children's Research Hospital
Jean Nakamura	Jean.Nakamura@ucsf.edu	University of California, San Francisco
Yutaka Yasui	Yutaka.Yasui@stjude.org	St. Jude Children's Research Hospital
Rebecca Howell	rhowell@mdanderson.org	MD Anderson Cancer Center

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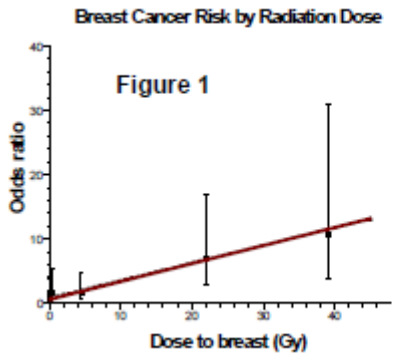
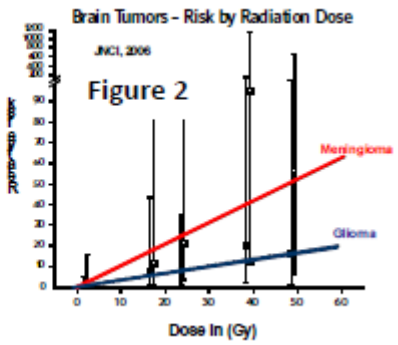
4. **BACKGROUND AND RATIONALE**

SMNs are potentially life-threatening late complications in childhood cancer survivors: Among children with cancer, the cumulative incidence of SMNs approaches 20% at 30 years after diagnosis of the primary neoplasm, representing a 6-fold increased risk, when compared with the general population (1, 2). SMNs are a leading cause of non-relapse late mortality (3). Unique associations with specific therapeutic exposures have resulted in the classification of SMNs into two distinct groups: chemotherapy-related myelodysplasia/ acute myeloid leukemia (t-MDS/AML) and radiation-related solid SMNs. Solid SMNs have a strong and well-defined association with radiation (4-6). Radiation induces SMNs within the radiation field (6). Radiation-induced solid SMNs account for the largest burden of SMNs (6). The latency for radiation-induced solid SMNs usually exceeds 10 years (7). The risk is highest when radiation exposure occurs at a younger age, and increases with increasing doses of radiation (**Fig. 1, 2**) (7-12). The mean age at diagnosis of SMNs has been reported to be 29 years, representing a significant cause of morbidity and premature mortality among the adolescents and young adults (AYAs) – who have been increasingly recognized to be a vulnerable population, malignancies of the breast, thyroid, brain, bone, soft tissue and skin are commonly observed. (6, 7, 9, 10, 13).





Primary neoplasms among individual with NF1: The prevalence of primary

neoplasms in NF1 ranges from 5% to 29% (14), and is 4-6 times the prevalence in the general population (15, 16). Individuals with NF1 have a predisposition to the development of cutaneous neurofibroma (frequency: >99%), internal nerve sheath tumor (intraneural and plexiform: up to 60%), MPNST (8-13%), OPG (15-20%), malignant glioma (0.8%), leukemia (MDS, JMML: <1%); pheochromocytoma (0.1-13%), rhabdomyosarcoma (1-6%), gastrointestinal stromal tumors (GIST: 5-30%), and breast cancer (8.4% by age 50 years) (17, 18).

SMNs among individuals with NF1: Previous small case series indicate that NF1 patients with a primary neoplasm may be at increased risk of SMNs (when compared with the general population). The prevalence of SMN was 11% among the 64 NF1 registrants with primary neoplasms in the CHOP registry; it was 75% among NF1 patients treated for a primary embryonal cancer (19). With the exception of one patient, all others had received prior chemotherapy and/or radiation. In another case series of NF1 individuals, the risk of SMNs after exposure to radiation was reported to be 3-fold higher when compared with the risk among those not exposed to radiation (20).

However, whether the risk of SMNs in NF1 individuals is in excess of that observed in non-NF1 cancer patients is unclear. Furthermore, whether the risk of SMNs in NF1 patients exposed to specific genotoxic agents is in excess of NF1 patients not exposed to radiation or chemotherapy is unknown. Nonetheless, radiation is generally avoided for non-malignant tumors in NF1, being reserved only as a last resort measure, due to concern about radiation-induced malignant transformation; this is despite the fact that radiation results in improved tumor control (compared to chemotherapy) for sporadic OPG)/LGG. Furthermore, in NF1 patients with MPNST, radiation use is largely based on regional preferences, because of lack of conclusive evidence regarding the risk of SMNs in this setting, despite the knowledge that radiation (when compared to surgery) provides superior nerve-sparing/ improved functional outcomes and is standard therapy in sporadic MPNSTs. Similarly, alkylators are used sparingly in NF1 patients, because of the potential for increasing the risk of therapy-related leukemia, thus limiting treatment options for OPG. Using large cohorts of individuals with and without NF1 who have survived a primary neoplasm, we will describe the magnitude of excess risk of SMNs within the context of therapeutic exposures and help elucidate the etiology of SMNs in this setting, thus providing guidance in the management of patients with NF1 at risk for SMNs. ***We hypothesize that NF1 patients with a primary neoplasm are at increased risk of SMNs, compared with non-NF1 patients with childhood cancer, and that radiation and alkylating agents increase the risk of SMNs in NF1 patients.***

Pathogenesis of NF1 Cancer Survivors at high risk for SMNs: The pathogenesis of SMNs in the setting of NF1 (with and without exposure to genotoxic agents) has not been explored. Epigenetic events including *de novo* promoter methylation of tumor-suppressor genes and genomic imprinting are implicated in the pathogenesis of SMNs (30-32). ***We hypothesize that treatment for a primary malignancy in NF1 leads to aberrant DNA methylation (hyper- or hypomethylation) in cancer associated genes that ultimately lead to development of SMNs in survivors. SNPs at CpG sites can also cause aberrant methylation of cancer predisposition genes. The epigenotype-genotype associations will allow for causal inferences in biological pathways contributing to SMNs among children with NF1.***

SIGNIFICANCE

To describe the magnitude of risk of SMNs in individuals with NF1: This proposal addresses an important but understudied problem relevant to individuals with NF1 and by extension to the larger population of childhood cancer survivors. Utilizing the rare and valuable resources available through the SPORE, the CCSS and the CHOP NF1 Registry, we will perform an extensive examination in individuals with NF1 to examine demographic and therapeutic factors influencing SMN risk.

Epigenetic markers to identify NF1 Cancer Survivors at high risk for SMNs: The proposed study will take a preliminary step toward examining the epigenetic factors contributing to the pathogenesis of SMNs in NF1 patients. Whereas genetic mutations and chromosomal defects permanently alter the genome, epigenetic alterations can potentially be pharmacologically reversed to restore gene function altered as a result of primary disease or its treatment (33). Identification of those at the greatest risk of developing SMNs through discovery of novel biomarkers will aid in the implementation of targeted interventions, alterations in therapeutic strategies, and the potential use of epigenetic therapy to lessen the impact of these late effects.

SPECIFIC AIMS

Using a retrospective cohort study design

Aim 1. To describe the magnitude of risk of SMNs in individuals with NF1.

Aim 1.1 *Compare SMN rate in the NF1+ cohort (CCSS + CHOP) with SMN rate in the non-NF1 cohort (CCSS)*

Aim 1.2 *Compare SMN risk in NF1+ cohort exposed and not exposed to radiation and/or chemotherapy (CCSS+CHOP)*

Aim 2: Determine if gene-specific DNA methylation status is associated with SMNs in children with and without NF1 and primary neoplasia, by conducting genome-wide DNA methylation profiling.

5. ANALYSIS FRAMEWORK

- ***Outcome of interest:*** The primary outcomes of interest are:
    - Aim 1: SMNs developing after the diagnosis of primary cancer
    - Aim 2: DNA methylation patterns associated with SMNs

- ***Exposure of interest:*** The primary exposure of interest is NF1 status prior to development of the Outcomes of Interest in childhood cancer survivors.

- ***Subject population:***

  Aim 1: The cohorts will consist of survivors of primary neoplasms at age ≤21 years with NF1 (n=125 from CCSS; n=450 from NF registry at CHOP) and survivors of primary neoplasms at age ≤21 years without NF1 (n=24,000 from CCSS)

- **Definition of NF1 for CCSS patients (reviewed independently by two pediatric oncologists):**

  "Yes" to the question "Have you ever been told by a doctor that you have...Neurofibromatosis (Type 1)" [Q1a(j) in the Expansion Baseline Survey]
  OR,
  "Not Sure" to the question "Have you ever been told by a doctor that you have...Neurofibromatosis (Type 1)" [Q1a(j) in the Expansion Baseline Survey] *AND* "Yes" to the question "to the best of your knowledge, were you born with... large or multiple birthmarks (any 1 larger than a quarter, or 6 larger than a dime") *AND* diagnosed with astroglial tumor, malignant nerve sheath tumor, rhabdomyosarcoma, or leukemia.

- **Definition of NF1 at the CHP NF Clinic**
  A clinical diagnosis is the most common (simple and effective) way that patients are diagnosed with NF1. Criteria for clinical diagnosis: child has 2 or more of the following:
    - 6 or more café-au-lait spots, at least 0.5 cm
    - 2 or more neurofibromas on or under the skin, or 1 plexiform (deep tissue) neurofibroma
    - axillary (armpit) or inguinal (groin) freckling
    - optic pathway glioma, also called a visual pathway tumor
    - 2 or more Lisch nodules
    - bone changes such as bowing of the long bones
    - a close relative (parent, child, or sibling) with a confirmed diagnosis of NF1

In the event of ambiguity with the clinical diagnosis, a genetic test is performed (to detect NF1 mutations).

Aim 2: Genomic DNA will be procured from a primary cohort of 24 children with SMNs after a primary neoplasm in NF1 patients, 24 patients with NF1 and a primary neoplasm but no SMNs, 24 patients without NF1 but with SMNs after a primary neoplasm and 24 patients without NF1 and a primary neoplasm and no SMN. These samples will be obtained from the following sources: CCSS and UAB NF registry (PI: Bruce Korf).

	NF+ and primary cancer	**NF- and primary cancer**
SMN+ (any histology)	N=24	N=24
SMN -	N=24	N=24

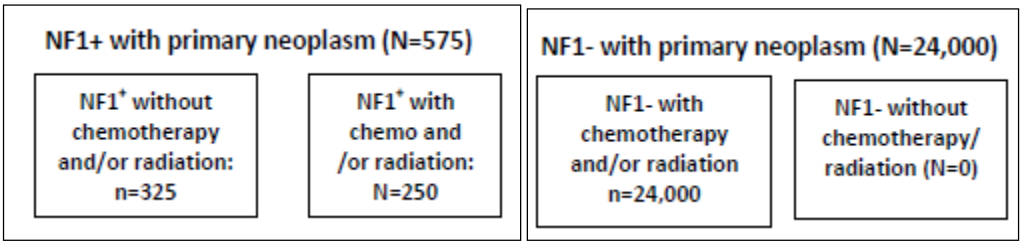
The patients will be matched on race/ethnicity, primary cancer diagnosis, age at primary cancer diagnosis, time since primary cancer diagnosis to procurement of biological specimen, sex and exposure to radiation (any site) and exposure to chemotherapy (yes/no).

- ***Exploratory variables:*** The following information will be requested:
  - Primary cancer diagnosis
  - Subsequent malignant neoplasms
  - Age at primary cancer diagnosis
  - Age at diagnosis of SMN
  - NF1 yes/no
  - Date of death (and cause)
  - Gender
  - Race/ethnicity
  - Treatment history, including:
    - Radiation yes/ no (any field)
    - Radiation field (prescribed radiation, irrespective of SMN field)
    - Prescribed radiation does to each field
    - Chemotherapy: yes/ no
    - Cumulative dose of alkylating agents, topoisomerase II inhibitors, anthracyclines, or platinum exposures.

- ***Statistical analysis:***

Aim 1: The cohorts will consist of survivors of primary neoplasms with NF1 (NF1+cohort) and survivors of primary neoplasms without NF1 (non-NF1cohort) – Figure 3).

Figure 3: NF1+ cohort and Non-NF1 cohort



The clinical outcome of interest, SMN, will be treated as a dichotomized variable. Clinical variables and SMN will be characterized by NF1 status, using tabulation, distribution/ density estimation to document distribution of clinical variables and outliers, and a series of questions will be asked. Statistical analyses will be performed by F Lennie Wong, PhD (City of Hope).

Aim 1.1 Compare SMN risk in the NF1+cohort with SMN risk in non-NF1 cohort

The effect of NF1 status on the development of all subsequent SMNs will be examined by fitting Cox proportional hazards regression models, adjusted for clinical/ demographic factors, and chemotherapeutic and radiation variables. The association between SMNs and NF1 status, as well as the clinical/ demographic characteristics will be determined by estimating the hazard ratio (HR) and its 95% confidence interval; significance of HR will be assessed by Wald test. Variables with p value <0.25 in univariate analysis will be entered into a multivariable regression model. Backward stepwise regression will be used to develop the final multivariable model; possible interactions, especially those involving NF1 status, will be examined. Fit of model will be assessed by regression diagnostics procedures.

Radiation: Each individual in the two cohorts will be assigned an indicator (yes/ no) variable depending upon exposure to radiation. This variable will be included in the analysis examining the risk of developing any SMN (irrespective of site). When examining the risk of homogenous subgroups of SMNs (e.g., brain tumors), an indicator variable for site-specific radiation (cranial radiation in this case) exposure will be assigned to each individual in the cohort. Maximum radiation dose will be included in the analysis.

Chemotherapeutic agents: For the association between chemotherapeutic agents and SMNs, we will explore individual chemotherapeutic agents as cumulative doses of exposures/m^2 body surface area, as well as a cumulative alkylating agent, topoisomerase II inhibitor, anthracycline, or platinum exposure.

Radiation and chemotherapeutic agent doses will be treated as continuous variables to assess dose-response in terms of linear and non-linear relationships. They will also be treated as categorical variables to identify thresholds for carcinogenic doses in clinical settings.

Sample size and Power: Assuming the incidence of SMN in NF1+ survivors to be 0.11 and the incidence of SMNs in the non-NF1 survivors to be 0.056, a cohort of 24,575 survivors (n=575 NF1+ and n=24,000non-NF1) will provide 80% power to detect a HR of 1.55 at Type I error probability of 0.05.

Aim 1.2 Compare SMN risk in NF1+cohort exposed and not exposed to radiation and/or chemotherapy

Among NF1 individuals, the relation between radiation/ specific chemotherapies and SMN risk will be examined similarly using Cox proportional hazards regression.

Sample size and Power: Assuming a Type I error of 0.05, 250 NF1+ individuals exposed to radiation and/or chemotherapy and 325 NF1+ individuals not exposed to chemotherapy or radiation will provide 80% power to detect a HR of 2.04, assuming an incidence of SMNs among the exposed group to be 15% and among the unexposed group to be 7.7%

Aim 2

Genome-wide DNA methylation profiling: We will characterize the DNA methylation patterns by performing an epigenome wide association analysis by using an Infinium MethylationEPIC BeadChip. The Chip features >850,000 CpGs in enhancer regions, gene bodies, promoters, and CpG islands at single nucleotide resolution. Genomic DNA will be temperature denatured and bisulfite converted using the Zymo Research EZ-96 DNA Methylation-Gold Kit. Prior to conversion, all gDNA samples will be quantitated using the Invitrogen Quant-iT fluorescent assay for normalization. Converted DNA will be amplified, fragmented, precipitated and re-suspended in preparation for hybridization to BeadChips. DNA to beadchip hybridization will be accomplished with the aid of Tecan robotics, as will DNA extension and staining. Array chips will be scanned utilizing the iScan reader. Methylation data will be extracted and compiled using GenomeStudio software (Illumina) and extracted (un-normalized) data as well as raw scan data will be analyzed. Preprocessing of the array data will be performed in the Methylumi and ChAMP R-packages.

After extensive quality filtering, batch normalization, and chemistry correction, we will run linear regression models at each locus with CpG DNA methylation to test for association between DNA methylation levels and SMNs (case/control). We will conduct DNA methylation association analysis for children with NF1, without NF1, and all children (with and without NF1), respectively. The p-values for the disease term in our regression models will be used to establish the significance of the association at each locus.

Sample size and Power: Assuming that the mean difference in methylation levels at a disease susceptible locus between cases and controls is 21%, the estimated power of our epigenome-wide association test is 86% based on

24 cases with NF1 and SMN and 24 controls with NF1 but no SMN, and 94% based on 48 cases and 48 controls for children with and without NF1 together (using a significance level of $1x10^{-8}$ estimated by Bonferroni correction).

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