1) Study Title
Genome-Wide Association Study (GWAS) of Late-occurring Intestinal Obstruction

2) Working Group and Investigators
This study will be performed with the assistance of the Childhood Cancer Survivor Study (CCSS) Genetics Working Group. Secondary oversight will be provided by the CCSS Epidemiology/Biostatistics and Chronic Disease Working Groups.

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3) Background/Significance
Intestinal obstruction is a common and serious surgical problem. In the United States, intraperitoneal adhesions are the primary underlying etiology for intestinal obstruction, necessitating over ~500 procedures per 100,000 person-years[1] and contributing to over 80% of emergency general surgery morbidity and mortality.[2] Childhood cancer survivors have previously been shown to be at elevated risk of gastrointestinal complications,[3] including intestinal obstruction requiring surgical intervention, compared to siblings.[4] This risk is increased in the settings of abdominal or pelvic tumors, surgery as cancer treatment, and radiotherapy as cancer treatment.[4]

Among cancer survivors, acute treatment with abdominal surgery and/or radiotherapy may result in late intestinal obstruction from bowel adhesions[5] and/or fibrosis.[6] Despite contributing to substantial surgical disease, the biological underpinnings of peritoneal adhesions are still incompletely understood.[7] The formation of adhesions to the peritoneum is nearly ubiquitous after surgery; however, only 5% of patients become symptomatic.[7] The reasons for this are unknown. In different individuals, intra-abdominal scar tissue may form at varying rates and varying degrees of regulation, akin to individuals who scar normally and those who form keloids, which is a

condition associated with genetic variants.[8] Important insight into the biology of adhesions may be attained with knowledge of genetic variants associated with this condition.[9]

Existing data implicate inflammation and dysregulation of the peritoneal fibrinolysis pathway in pathological adhesion formation.[10,11] In 2005, Katada and colleagues investigated the role of the inflammatory pathway in adhesion formation in a murine model. The authors observed elevated cyclooxygenase 2 (COX-2) levels and activation of downstream molecules (i.e. p38 mitogen-activated protein [MAP] kinase) in traumatized intestine.[12] The increased expression of the *COX-2* gene was demonstrated among fibroblasts from human tissue samples.[13] Furthermore, administration of a MAP kinase inhibitor was associated with decreased adhesion formation.[12] Modulating the same pathway with dexamethasone administration has been shown to reduce adhesions and inhibit MAP kinase.[14]

Other studies have interrogated the fibrinolytic pathway and its effect on peritoneal adhesions. Saed and colleagues studied intra-operatively obtained tissue specimens from human patients and observed that tissue plasminogen activator (tPA) and plasminogen activator inhibitor (PAI) levels were higher in normal fibroblasts, compared to peri-adhesion fibroblasts.[15] Corroborating evidence includes that HMG-CoA reductase inhibitors (statins) reduced post-surgical adhesion formation in a murine model and induced changes in tPA and PAI, thereby upregulating local fibrinolysis.[16] Administration of medications that affect coagulation/fibrinolysis homeostasis, such as enoxaparin, has been shown to reduce adhesions in a mouse model.[17] Experiments performed by Rout and colleagues note mixed results regarding fibroblast phenotype in the setting of regulatory protein transforming growth factor ß1 (TGF-ß1) or a hypoxic environment.[18,19] Similarly, the genes for neurokinin receptor, substance P, and intracellular and vascular adhesion molecules have been potentially implicated in the formation of peritoneal adhesions.[20,21]

A second mechanism of intestinal obstruction among cancer survivors is fibrosis, which may have a predilection for bowel exposed to radiotherapy. The most likely mechanism by which radiotherapy causes intestinal obstruction entails full thickness damage to the bowel wall.[22] Fibrosis as a response to radiotherapy may vary depending on several factors, including an individual's efficiency and accuracy of DNA repair, resilience to oxidative stress, and degree of fibroblast proliferation. As with post-surgical adhesion formation, not all patients treated with radiotherapy progress to develop intestinal obstruction.

Pathways potentially implicated in radiation fibrosis have largely been studied in the lung and skin. Like adhesions, pulmonary fibrosis is mediated through the fibrogenic TGF-ß/Smad pathway, and in a murine model exposed to radiation, treatment with an LDH inhibitor (hypothesized to prevent activation of the TGF-ß pathway) reduced degree of pulmonary fibrosis.[23] An exome analysis of participants exposed to radiotherapy as breast or prostate cancer treatment, comparing those who developed vs. did not develop fibrosis, likewise identified genes (*FBN2, FST, GPRC5B, NOTCH3, PLCB1, DPT, DDIT4L and SGCG*) associated with the pro-fibrotic TGF- ß and retinoic acid pathways, which may contribute to radiation-induced fibrosis.[24] One additional pathway potentially involved in radiation-induced fibrosis involves the *ataxia-telangiectasia-mutated* (*ATM*) gene, which modulates the DNA-damage checkpoint.[25] Similarly, variants genes involved

in mitigating damage from reactive oxygen species, such as *TXNRD2* may contribute to fibrosis.[26] Finally, one recent report specific to intestinal fibrosis related to radiotherapy illustrates how microRNA may play a role in intestinal smooth muscle fibrosis (potentially mediated by the TGF- ß and other pathways).[27]

While radiotherapy-induced fibrosis has largely been studied in extra-intestinal tissue, the pathogenesis of intestinal fibrosis has been extensively studied on a genetic level among patients with Crohn's disease.[28] In a genome-wide analysis of DNA methylation and gene expression, Sadler and colleagues compared resected specimens of colon from patients with Crohn's associated fibrosis with those from control patients. The authors observed decreased expression of hypermethylated WNT2B, prostacyclin synthase, and prostaglandin D2 synthase among fibrotic samples.[28] Additionally, adhesion formation may also occur after radiotherapy, and adhesion formation has been experimentally shown to be radiation time- and dose-dependent.[29]

Knowledge regarding genetic variants associated with IOS would be useful in guiding clinical decisions for children undergoing cancer treatment, risk-stratifying cancer survivors who underwent predisposing treatments, and better understanding the biology of intestinal obstruction. The purpose of the present study is to identify genetic variants associated with IOS, including with possible modification from specific disease and treatment-related risk factors.

4) Specific Aims (S.A.)

S.A. #1. To identify genetic variants associated with the development of late intestinal obstruction requiring surgery (IOS) in a candidate gene analysis. The key pathways include the candidate genes which may affect intraperitoneal adhesions in the setting of surgery and intestinal fibrosis in the setting of radiotherapy. The included candidate genes are based on a MEDLINE review of studies with replicated results, in which other groups have reported genetic associations between the outcomes of interest and DNA repair (ERCC5), ataxia telangiectasia mutated (ATM), TGF-ß/SMAD4 (NEDD4), expression of GnRH, cholesterol metabolism, and steroidogenesis (FOXL2). Further information is displayed in Tables 1A-C.

Hypothesis #1: Inherited genetic variation in one or more of the above candidate genes predisposes to late IOS, and this risk is modified by prior abdominal surgery and/or prior abdominal radiotherapy.

Table 1A. Single nucleotide polymorphisms (SNPs) associated with radiation fibrosis

SNP	***Pathway***	***Sample size***	***Citation***
rs1047768	DNA repair (*ERCC5*)	Replicated in 120 individuals with head/neck cancer treated with radiotherapy (outcome: dermal fibrosis or xerostomia)	Borchiellini 2017[30]
rs1801516	Ataxia telangiectasia mutated (*ATM*)	Meta-analysis of 5456 individuals treated with radiotherapy from 17 cohorts (outcome: late	Andreassen 2017[31]

		radiation toxicity including telangiectasia, fibrosis, and rectal toxicities)	

PubMed search: (("single nucleotide polymorphism"[tiab] or "snp"[tiab]) or (“genome wide”[tiab] OR “GWAS”[tiab] OR “whole genome sequencing”[tiab] OR "WGS"[tiab])) and "fibrosis"[tiab] and ("radiation"[tiab] or "radiotherapy"[tiab])

Table 1B. Single nucleotide polymorphisms (SNPs) associated with adhesion formation

SNP	***Pathway***	***Sample size***	***Citation***
N/A	-	-	-

PubMed search: (("single nucleotide polymorphism"[tiab] or "snp"[tiab]) or (“genome wide”[tiab] OR “GWAS”[tiab] OR “whole genome sequencing”[tiab] OR "WGS"[tiab])) and ("adhesion"[tiab] or "adhesions"[tiab] or "adhesive"[tiab])

- No useable published articles.

Table 1C. Single nucleotide polymorphisms (SNPs) associated with adhesion formation

SNP	***Pathway***	***Sample size***	***Citation***
rs8032158 rs2271289	TGF-ß/SMAD4 (*NEDD4*)	GWAS with replication cohort in Japanese population. Replicated in Chinese Han population.	Nakashima 2010,[8] Zhao 2016[32]
rs1511412	Expression of GnRH, cholesterol metabolism, and steroidogenesis (*FOXL2*)	GWAS with replication cohort in Japanese population.	Nakashima 2010,[8] Lu 2015,[33] Lu 2018[34]

PubMed search: (("single nucleotide polymorphism"[tiab] or "snp"[tiab]) or (“genome wide”[tiab] OR “GWAS”[tiab] OR “whole genome sequencing”[tiab] OR "WGS"[tiab])) and ("keloid"[tiab] or "hypertrophic scar"[tiab])

S.A. #2. To identify genetic variants associated with the development of late intestinal obstruction requiring surgery (IOS) in a genome-wide association study (GWAS). Specifically, our aim is to identify and distinguish genetic variants that modify:

- The effect of prior abdominal surgery on risk of late IOS
- The effect of prior abdominal radiotherapy on risk of late IOS
- The risk of late IOS independent of prior treatment exposures or tumor location

Hypothesis #2: Inherited genetic variation predisposes to late IOS, and this risk is modified by prior abdominal surgery and/or prior abdominal radiotherapy.

5) Analysis Framework

a) Outcomes of Interest

Primary outcome of interest: Late (occurring ≥5 years after cancer diagnosis) intestinal obstruction requiring surgery (IOS; baseline #I-11 or FU2007 #J-14) developed by

survivors (Original Cohort), who have available genotyping data as below. Survivors with the primary outcome (IOS) occurring at the same age as abdominal/pelvic surgery will be excluded. Those survivors who are included in the St. Jude LIFE cohort (which will be used for the replication cohort) will be excluded from the derivation analysis.

b) Subject Population

Survivors who have a minimum amount of DNA available for genotyping, which includes 5739 survivors diagnosed 1970-1986.

c) Exploratory Variables of Interest

- Demographic
  - Age (continuous and categorical; Baseline #A1; ExpBaseline #A1)
  - Sex (categorical; Baseline #A2; ExpBaseline #A2)
  - Ancestry (categorical: Caucasian vs. non-Caucasian)
- Cancer variables
  - Abdominal or pelvic tumor site (binary; defined by ICD-9-O topography codes)
  - Type of cancer
  - Development of recurrence or second malignant neoplasm (binary; ExpBaseline #L1, LTFU 2003 #R1, LTFU 2005 #B1, LTFU 2007 #P1)
- Treatment variables (within 5 years of cancer diagnosis)
  - Abdominal or pelvic surgery (binary; MRAF for any abdominal/pelvic surgery; or splenectomy, ExpBaseline #I18, LTFU 2007 #J18)
  - Chemotherapy (binary)
    - Alkylating agent (binary)
      - Cyclophosphamide equivalent dose (CED; categorical: 0, 1-3999, 4000-7999, $\geq$8000mg/m^2)[35]
    - Anthracycline (binary)
      - Doxorubicin equivalent dose (categorical: 0, <250, $\geq$250 mg/m^2)[36]
    - Platinum agent (continuous)
      - Total platinum dose[37]
    - Other categories of chemotherapy
  - Radiotherapy (categorical)
    - Maximum total dose (categorical: 0, <10, 10-19, 20-29, 30-39, 40-49, >49 Gy)
    - Maximum total dose to abdomen/pelvis (categorical: 0, <10, 10-19, 20-29, 30-39, 40-49, >49 Gy)
- Congenital abnormality variables
  - Abdominal wall hernia (binary; Baseline #P1; ExpBaseline #Q3ap; LTFU 2000 #5p; LTFU5 #W3ap)
- Comorbidities
  - Inflammatory bowel disease (binary; Baseline #H11, #H14; ExpBaseline #H4; LTFU 2007 #I9; LTFU5 #I9)
- CCSS GWAS genotyping data using the Illumina HumanOmni5Exome microarray

d) Statistical Methods

Methodology

In this GWAS for association with IOS, we propose an evaluation of candidate single nucleotide polymorphisms (SNPs) in order to identify regions of the genome that may be biologically associated with peritoneal adhesion formation or intestinal fibrosis. For each given locus, we will capture a minimum of 20kb upstream and 10kb downstream using current haplotype software to determine the relevant haplotype tagging SNPs (htSNPs) and haplotype blocks in order to adequately map variability. We restrict our analysis to individuals of European ancestry. Standard quality control will be performed on the IOS GWAS data, excluding individuals with > 1% missingness, outlying per-sample heterozygosity, cryptic relatedness (pi-hat > 0.2), and gender discordances. Similarly, SNPs with > 1% missingness, Hardy-Weinberg Equilibrium (HWE) $P < 10^{-6}$ among non-IOS individuals, minor allele frequency of < 1%, and in non-autosomal chromosomes will also be excluded.

Gene effects will be tested independently and with interaction with abdominal/pelvic radiotherapy and abdominal/pelvic surgery treatment variables. Piecewise exponential models stratified by abdominopelvic tumor type with the following non-genetic factors were built from the previous clinical paper[4] on IOS: abdominal/pelvic surgery, chemotherapy, radiation dose to abdomen/pelvis, primary cancer, sex, race/ethnicity, age at diagnosis, year of diagnosis, and attained age as cubic splines. Main effects of candidate SNPs (S.A. #1) will then be added to the above model to assess the adjusted genetic effects on IOS rates. To assess potential difference in SNP effect by abdominal/pelvic surgery or radiation to abdomen/pelvis, interactions between SNPs and abdominal/pelvic surgery or radiation to abdomen/pelvis will be checked. As such, we will initially evaluate for SNPs associated with the primary outcome of IOS in the following groups separately: 1) the overall cohort of survivors with available genetic data, 2) survivors who were treated with abdominal/pelvic surgery, 3) survivors who were treated with abdominal/pelvic radiotherapy, 4) survivors who were treated with abdominal/pelvic surgery and radiotherapy, and 5) survivors who were treated with neither abdominal/pelvic surgery nor radiotherapy. Our main analysis following the initial analysis will be a single-candidate SNP association analysis with IOS, combining the groups as appropriate. The appropriateness will be based on the results of the initial group-specific analysis, i.e., we will combine the groups for increased power unless there is evidence in the initial analysis results that the groups in question differ with respect to genetic associations (indicating different pathways to IOS with different genetic factors contributing). Additionally, a secondary analysis with an agnostic approach (S.A. #2) will be undertaken.

Bioinformatics analysis will be conducted using existing data repositories to assist interpretations of the GWAS findings, especially chromatin state/conformation and eQTL databases, considering possible links to the biological pathways discussed in the Background section. Results may also inform future basic laboratory validation of SNPs found to be associated with IOS.

Preliminary query: In the preliminary query, among a total of 5193 survivors, 134 (2.6%) developed the primary endpoint of IOS, with further stratification by cancer treatment

status with abdominal/pelvic surgery or abdominal/pelvic radiotherapy summarized in the Table below. In the SJLIFE cohort, 34 survivors developed the primary endpoint of IOS.

Table 2. Preliminary query

Abdominal/pelvic surgery	Abdominal/pelvic RT	N	Intestinal obstruction
Yes	Yes	815	66
Yes	No	614	27
No	Yes	607	18
No	No	3157	23

Technical plans for validation

For specific aim #1, the candidate gene analysis will first be performed in the combined CCSS and SJLIFE cohorts. Any SNPs found to be statistically significant will subsequently be separately evaluated for magnitude and direction of association in the CCSS cohort and the SJLIFE cohort.

For specific aim #2, the GWAS findings from the CCSS cohort above for association with IOS will be replicated using the SJLIFE cohort of childhood cancer survivors using the same exact models and treatment stratification, utilizing its whole genome sequencing data. When the whole genome sequencing of the Expansion Cohort of CCSS becomes available in time for this project, we will utilize it for replication.

Functional studies. N/A

Other statistical considerations:

Assuming a multiplicative model, IOS prevalence of 2.5 % (134/5193) and significance threshold of $P = 5 \times 10^{-8}$, our sample size has ~50% and ~86% power to detect SNPs of MAF 0.20 contributing to genotype relative risk of 2.00 and 2.25, respectively.[38] There will be diminished power for each sub-analysis undertaken within groups stratified by treatment.

e) Examples of Tables/Figures

Table 1A. Characteristics of survivors who develop IOS and those who do not develop IOS (including CCSS GWAS cohort and the St. Jude LIFE replication cohort).

			By Abdominal/Pelvic Surgery			
			Abdominal/Pelvic Surgery		No Abdominal/Pelvic Surgery	
Characteristic	*IOS (n=)*	*No IOS (n=)*	*IOS (n=)*	*No IOS (n=)*	*IOS (n=)*	*No IOS (n=)*
Cohort						
CCSS						
SJLIFE						
Diagnosis						
CNS tumor						
Leukemia						
Lymphoma						
Nephroblastoma						
Neuroblastoma						
Bone tumor						
Soft tissue sarcoma						
Female						
Race/ethnicity						
Non-Hispanic white						
Non-Hispanic black						
Hispanic						
Other						
Age at diagnosis, years						
0-3						
4-9						
10-14						
15-20						
Year of diagnosis						
1970-1974						

1975-1979	
1980-1986	
Abdominal/Pelvic surgery	
None	
1	
2 or more	
Abdominal/Pelvic tumor	
Any chemotherapy	
Maximum total dose to abdomen/pelvis from all radiotherapy, Gy	
0	
0.1-9.9	
10-19.9	
20-29.9	
30-39.9	
40-49.9	
≥50	

CNS, central nervous system; *IOS*, Intestinal obstruction requiring surgery

Table 1B. Characteristics of survivors who develop IOS and those who do not develop IOS (including CCSS GWAS cohort and the St. Jude LIFE replication cohort).

	By Abdominal/Pelvic Radiotherapy*			
	≥20 Gy		<20 Gy	
Characteristic	*IOS (n=)*	*No IOS (n=)*	*IOS (n=)*	*No IOS (n=)*
Cohort				
CCSS				
SJLIFE				
Diagnosis				
CNS tumor				

Leukemia
Lymphoma
Nephroblastoma
Neuroblastoma
Bone tumor
Soft tissue sarcoma
Female
Race/ethnicity
Non-Hispanic white
Non-Hispanic black
Hispanic
Other
Age at diagnosis, years
0-3
4-9
10-14
15-20
Year of diagnosis
1970-1974
1975-1979
1980-1986
Abdominal/Pelvic surgery
None
1
2 or more
Abdominal/Pelvic tumor
Any chemotherapy

CNS, central nervous system; *IOS*, Intestinal obstruction requiring surgery
*Note: could also stratify by ≥50 Gy vs. <50 Gy

Table 2. Top SNPs associations identified in the GWAS of late IOS

Location, nearest gene, SNP information	Population	Cohort	Genotyping status	Controls/cases	RAF Controls	RAF Cases	P_{M-H}	HR (95% CI)[a]	P_{Cox}
	Overall	Pooled CCSS SJLIFE							
	Abd Surgery	Pooled CCSS SJLIFE							
	No abd surgery	Pooled CCSS SJLIFE							
	≥20 Gy	Pooled CCSS SJLIFE							
	≥50 Gy	Pooled CCSS SJLIFE							

CCSS, Childhood Cancer Survivor Study; P_{Cox}, multivariable Cox regression p-value; P_{M-H}, Mantel-Haenszel p-value; *RAF*, risk allele frequency; *SJLIFE*, St Jude Lifetime cohort;
[a]Adjusting for abdominal/pelvic surgery, radiotherapy dose to abdomen/pelvis, and age at diagnosis.

Figure 1. Manhattan plot for the SNPs identified in the GWAS of late IOS
Figure 2. Quantile-Quantile plot to evaluation inflation of test statistics

Table 3. Interaction between GWAS significant SNPs and risk factors on IOS

SNP (mapped gene)	*HR*[a]	*95% CI*	P
SNP1			
SNP1*abdominal/pelvic surgery			
SNP1*radiotherapy≥20Gy			
SNP2…			

[a]HR for interaction term, adjusting for age and cohort

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