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ORIGINAL RESEARCH

Gene-Level Analysis of Anthracycline-Induced Cardiomyopathy in Cancer Survivors

A Report From COG-ALTE03N1, BMTSS, and CCSS

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ABSTRACT

BACKGROUND Anthracyclines are highly effective in treating cancer, albeit with increased cardiomyopathy risk. Although risk is attributed to associations with single nucleotide polymorphisms (SNPs), multiple SNPs on a gene and their interactions remain unexamined.

OBJECTIVES This study examined gene-level associations with cardiomyopathy among cancer survivors using whole-exome sequencing data.

METHODS For discovery, 278 childhood cancer survivors (129 cases; 149 matched control subjects) from the COG (Children's Oncology Group) study ALTE03N1 were included. Logic regression (machine learning) was used to identify gene-level SNP combinations for 7,212 genes and ordinal logistic regression to estimate gene-level associations with cardiomyopathy. Models were adjusted for primary cancer, age at cancer diagnosis, sex, race/ethnicity, cumulative anthracycline dose, chest radiation, cardiovascular risk factors, and 3 principal components. Statistical significance threshold of 6.93×10^{-6} accounted for multiple testing. Three independent cancer survivor populations (COG study, BMTSS [Blood or Marrow Transplant Survivor Study] and CCSS [Childhood Cancer Survivor Study]) were used to replicate gene-level associations and examine SNP-level associations from discovery genes using ordinal logistic, conditional logistic, and Cox regression models, respectively.

RESULTS Median age at cancer diagnosis for discovery cases and control subjects was 6 years and 8 years, respectively. Gene-level association for *P2RX7* (OR: 0.10; 95% CI: 0.04-0.27; $P = 2.19 \times 10^{-6}$) was successfully replicated (HR: 0.65; 95% CI: 0.47-0.90; $P = 0.009$) in the CCSS cohort. Additional signals were identified on *TNIK*, *LRRK2*, *MEFV*, *NOBOX*, and *FBN3*. Individual SNPs across all discovery genes, except *FBN3*, were replicated.

CONCLUSIONS In our study, SNP sets having 1 or no copies of *P2RX7* variant alleles were associated with reduced risk of cardiomyopathy, presenting a potential therapeutic target to mitigate cardiac outcomes in cancer survivors.

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ABBREVIATIONS AND ACRONYMS

ATP = adenosine triphosphate

CVRF = cardiovascular risk factor

EF = ejection fraction

HF = heart failure

IL = interleukin

LD = linkage disequilibrium

P2RX7 = purinergic receptor P2X7

QC = quality control

SNP = single nucleotide polymorphism

Anthracyclines are a class of highly effective chemotherapeutic agents that are used in the treatment of a wide range of cancer types. Despite the high efficacy, a dose-dependent risk of cardiomyopathy compromises the use of these agents to their fullest potential.[1-3] Childhood cancer survivors exposed to anthracyclines at a younger age have a higher risk of developing cardiomyopathy, up to 15 times greater than that observed in the general population.[4,5] The interindividual variability in anthracycline-induced cardiomyopathy risk among cancer survivors suggests a role for genetic susceptibility. Several candidate gene and genome-wide association studies implicate the role of genetic variants,[6] including our previously published findings.[7-9]

Important gaps in knowledge, however, remain to address design and analytic limitations of prior studies and fully describe the role of genetic susceptibility beyond individual variant effects.[6,10] Next-generation sequencing and agnostic approaches to discovery comprehensively interrogate the genome. Traditional genetic association studies focus on single nucleotide polymorphisms (SNPs). Considering multiple SNPs on a gene and their interactions at the gene level as the unit of analysis yields additional power to discover associations otherwise missed when examining marginal effects of individual SNPs.

In this study, we harness technological and analytic powers of genetic association to examine the relation between SNP set interaction effects within genes and anthracycline-induced cardiomyopathy in cancer survivors. Our gene-level approach aimed to identify associations in the discovery sample for further replication in three independent populations of cancer survivors. Associations of individual variants with cardiomyopathy identified in discovery were also assessed in replication samples.

METHODS

DISCOVERY SET. Participants. Study participants comprised 278 childhood cancer survivors (cases: 129 survivors with cardiomyopathy; control subjects: 149 survivors without cardiomyopathy) from the COG (Children's Oncology Group) study ALTE03N1 (Genetic Analysis in Identifying Late-Occurring Complications in Childhood Cancer Survivors; NCT00082745) that used a matched case-control design to understand the pathogenesis of cardiomyopathy. The Pediatric Central Institutional Review Board of the National Cancer Institute and local institutional review boards approved the study. Written informed consent and/or assent was obtained from patients and parents/legal guardians.

Cases with cardiomyopathy and control subjects were identified from individuals diagnosed with cancer who were 21 years of age or younger. For each case, 1 to 3 control subjects with no signs or symptoms of cardiomyopathy were randomly selected from childhood cancer survivors at participating institutions, matched on primary cancer diagnosis, year of diagnosis (±10 years), race/ethnicity, and duration of cardiomyopathy-free follow-up equal to or greater than the case. All participants provided blood or saliva as a source of germline DNA.

Cardiomyopathy phenotype. Documentation of cardiomyopathy was provided by participating sites. Cases fulfilled American Heart Association criteria for cardiac compromise[11] of presenting with signs and/or symptoms (dyspnea, orthopnea, fatigue, edema, hepatomegaly, and/or rales) or, in the absence of signs or symptoms, with echocardiographic features of left ventricular dysfunction (ejection fraction [EF] ≤40% and/or fractional shortening ≤28%). We categorized the cases into severe cardiomyopathy (symptomatic cardiac dysfunction with an EF ≤40% or fractional shortening ≤25%) and mild cardiomyopathy (all other cases) (Supplemental Table 1).

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DNA isolation and whole-exome sequencing analysis. Genomic DNA was isolated from peripheral blood or saliva and whole-exome sequencing with 100× coverage was performed. Variants were called using HaplotypeCaller followed by variant recalibration and were annotated using ANNOVAR[12] and GEMINI[13] to identify mutational hot spots. Exonic, nonsynonymous SNPs were retained, and additional filtering criteria were applied (Supplemental Appendix). Standard quality control (QC) measures for genotype data were employed using PLINK.[14] Of the 234,394 successfully genotyped autosomal SNPs, 6,180 SNPs with missing genotype >5% were removed, and 91 SNPs with $P < 1 \times 10^{-6}$ in Hardy-Weinberg equilibrium were excluded. We focused only on common variants for this analysis, excluding 193,933 SNPs with minor allele frequency <5%. We excluded 22 individuals from the analysis for the following reasons: 1 individual had >5% missing genotype data, another individual failed the relatedness test (identity by descent value >0.1875), and 20 individuals were excluded based on results of the heterozygosity test (inbreeding coefficient |F| >0.1). Population stratification was assessed using multidimensional scaling with PLINK; the first 3 principal components were adjusted for in the analysis.[14] Post-QC, 34,190 SNPs were available for exome-wide association analysis, with a total genotyping call rate of 99.98%. Additional exclusions were made in the gene-level data analysis, which involved removing SNPs not mapped to a gene (n = 40) and genes with a single-sequenced SNP (n = 4,511). The final analytic set included 29,639 SNPs mapped to 7,212 genes in the discovery set.

REPLICATION SETS. Three independent populations were used to assess gene-level and individual variant associations identified from the gene-level analyses in the discovery sample.

Replication set 1. Replication set 1 used nonoverlapping cases (n = 32) and matched control subjects (n = 173), enrolled in the COG study ALTE03N1 following the enrollment of the discovery set.

Replication set 2. Replication set 2 used the BMTSS (Survivor Study).[15] BMTSS is a cohort study examining health outcomes in individuals who had received a blood or marrow transplant between 1974 and 2014 across the age spectrum (2 to 70 years of age) and had survived ≥2 years. Cases (n = 135) comprised study participants who reported whether a health care provider had diagnosed them with heart failure (HF), along with age at diagnosis. Control subjects (n = 262) comprised BMTSS participants who did not report a diagnosis of HF and were matched (≤2 per case) on race/ethnicity, type of transplant, primary cancer diagnosis, year of cancer diagnosis, and duration of cardiomyopathy-free follow-up (equal to or greater than the matched case).

Replication set 3. Replication set 3 comprised a cohort of 5,589 non-Hispanic White participants from the CCSS (Childhood Cancer Survivor Study) who survived ≥5 years following diagnosis of a primary childhood cancer before reaching the age of 21 years.[16] Using previously described methodology to define HF,[17,18] only those outcomes graded as severe (grade 3: self-reported cardiomyopathy or HF, plus medications), life-threatening (grade 4: requiring heart transplantation), or fatal (grade 5) were included, yielding 229 cases.

Genotyping. SNPs from the discovery set that crossed the study significance threshold, along with SNPs in high linkage disequilibrium (LD) ($r^2 \geq 0.9$, with the index SNP identified using the HaploReg tool),[19] were selected for replication. Assays were designed using Fluidigm D3 in the COG and BMTSS replication sets. For the CCSS cohort, genotyped (Illumina HumanOmni5Exome array) and imputed data[20-22] were available for replication for non-Hispanic White survivors. Genotyping and QC details for all replication samples are presented in the Supplemental Appendix.

Therapeutic exposures. Therapeutic exposures were abstracted from medical records at participating sites. Anthracycline exposure across all populations was calculated by multiplying the cumulative dose (mg/m^2 of body surface area) of individual anthracyclines (doxorubicin, daunomycin, epirubicin, or idarubicin) by a drug cardiotoxicity factor[23] and summing the results. Participants were considered to have received chest radiation if the heart was in the radiation field. Self-report of diabetes, hypertension, or dyslipidemia was used to create a binary cardiovascular risk factors (CVRFs) indicator of any vs none.

STATISTICAL ANALYSIS. Discovery. In the statistical analysis, we used a 2-step approach (**Table 1**). In the first step, we used the machine learning method of logic regression to identify gene-level SNP-SNP interactions.[24-26] Logic regression searches for Boolean combinations (AND, OR, and NOT) of binary indicators (ie, SNPs) within each set (ie, gene). These binary indicators of SNPs are obtained by creating 2 dummy variables for the heterozygous and homozygous variant genotypes (Supplemental Appendix). We explored 2 biologically plausible forms of SNP set interactions: SNP *intersection*, where disease risk is elevated only if *all* SNPs in the SNP set carry their respective high-risk genotypes

TABLE 1 Gene-Level Analysis Steps

Analysis Step	Variable/Association of Interest	Model
Step 1: Summarize gene effects	SNP set interaction within gene 2 trees 10 leaves (SNPs); 5 SNPs/tree	Logic regression with logit link
Step 2: Test gene-level effects	Gene-level association	Ordinal logistic regression model Model adjusted for age at diagnosis, sex, race/ethnicity, primary cancer diagnosis, chest radiation, cardiovascular risk factors, anthracycline dose, 3 principal components

SNP = single nucleotide polymorphism.

(ie, SNP 1 *and* SNP 2 *and* SNP 3), and SNP *union*, where disease risk may be elevated in 1 of several independent ways, which could include a SNP intersection or an individual SNP carrying the high-risk genotype (ie, [SNP 1 *and* SNP 2] *or* SNP 3). High-risk genotypes may be homozygous or heterozygous for the minor risk/variant allele. For each gene, the logic regression model was fit using the logit link and a random seed. Optimal model size was derived using 10-fold cross-validation up to a maximum size of 2 trees and 5 leaves/tree. Models were fit using the optimal tree/leaf size 100 times, and the best solution based on a lower deviance was selected as the final model producing the gene-level SNP set.

In the second step, we fit ordinal logistic regression models of the cardiomyopathy outcome, using no cardiomyopathy as reference, and used the gene-level SNP sets identified from step 1 as independent variables. SNPs were coded using additive encoding (0, 1, or 2 indicating the number of risk alleles). The models were adjusted for cumulative anthracycline dose (continuous variable), chest radiation (yes/no), CVRFs (yes/no), primary cancer diagnosis, age at primary cancer diagnosis, sex, race/ethnicity, and 3 principal components. We used Bonferroni correction to account for multiple testing based on the number of genes included in the analysis (7,212 genes), resulting in an overall P value threshold of 6.93×10^{-6}.

REPLICATION ANALYSES. SNP interaction sets identified from discovery were manually constructed and tested in replication cohorts. Associations with individual SNPs were also examined. SNP sets were binary coded (0/1), and individual SNPs were coded using additive encoding (0/1/2). Cluster plots of associated SNPs were examined visually to exclude false associations due to genotyping errors (Supplemental Figure 1). Statistical significance threshold of 5% was used for all associations. All models adjusted for age at cancer diagnosis, sex, chest radiation, anthracycline dose, and CVRFs.

Replication set 1. Ordinal logistic regression models measured the association of SNP sets and individual SNPs with cardiomyopathy (no cardiomyopathy as reference vs mild vs severe). Models were adjusted for cumulative anthracycline dose (continuous variable), chest radiation (yes/no), CVRFs (yes/no), primary cancer diagnosis, age at primary cancer diagnosis, sex, and race/ethnicity.

Replication set 2. Conditional logistic regression models were used to measure associations between a binary HF outcome and SNP sets as well as individual SNPs. Models were adjusted for cumulative anthracycline dose (continuous variable), chest radiation (yes/no), CVRFs (yes/no), age at primary cancer diagnosis, and sex (race/ethnicity was not included in the model because the cases and control subjects were matched on this variable).

Replication set 3. Time-to-event main effect analyses were conducted using Cox proportional hazards regression models. Time from age at primary cancer diagnosis to the age at onset of HF for a case and time from primary cancer diagnosis to age at study participation for a control subject were used as the time axis. Models were adjusted for cumulative anthracycline dose (continuous variable), chest radiation (yes/no), CVRFs (yes/no), age at primary cancer diagnosis, and sex (race/ethnicity was not included in the model because the cohort included only non-Hispanic White patients).

We presented descriptive summaries of demographic and clinical characteristics for all cohorts as mean ± SD, median (range) for continuous variables, and count (percentage) for categorical variables. Results from ordinal and conditional logistic regression models were presented as OR with 95% CIs, and Cox proportional hazards model as HRs with 95% CI. All statistical analyses were performed using R version 3.6.0 (R Foundation for Statistical Computing) and Stata14 (StataCorp). Data that support the findings of this study are available on request from the corresponding authors. The data are not

publicly available due to privacy or ethical restrictions.

RESULTS

DISCOVERY SET. Patient characteristics. The median age at primary cancer diagnosis was 7 years for cases (range: 0-21 years) and control subjects (range: 0-20 years) (**Table 2**, Supplemental Table 1). Cases received higher cumulative anthracycline exposure (median dose, 340 mg/m^2 [range: 0-760 mg/m^2] vs 164 mg/m^2 [range: 0-600 mg/m^2]; $P < 0.001$); were more likely to have received chest radiation (35.7% vs 24.8%; $P = 0.09$); and were more likely to have a CVRF (37.2% vs 8.7%; $P < 0.001$).

Gene-level associations. The following 6 genes were significantly associated with cardiomyopathy risk (**Table 3**): *TNIK* (TRAF2 and NCK interacting kinase; OR: 4.58; 95% CI: 2.47-8.49; $P = 1.34 \times 10^{-6}$); *NOBOX* (NOBOX oogenesis homeobox; OR: 7.21; 95% CI: 3.23-16.1; $P = 1.43 \times 10^{-6}$); *LRRK2* (leucine-rich repeat kinase 2; OR: 0.19; 95% CI: 0.09-0.39; $P = 6.62 \times 10^{-6}$); *P2RX7* (purinergic receptor P2X7; OR: 0.10; 95% CI: 0.04-0.27; $P = 2.19 \times 10^{-6}$); *MEFV* (MEFV innate immunity regulator, pyrin; OR: 0.08; 95% CI: 0.03-0.24; $P = 4.07 \times 10^{-6}$); and *FBN3* (fibrillin-3; OR: 4.59; 95% CI: 2.42-8.71; $P = 3.05 \times 10^{-6}$).

REPLICATION. Patient characteristics for the 3 replication sets are summarized in **Table 2**.

Genotyping results and final replication SNP list. A total of 19 SNPs from the genes identified in the discovery set, along with an additional 17 SNPs in high LD ($r^2 \geq 0.9$) with the index SNP, were used to replicate their association with cardiomyopathy risk across the 3 replication samples.

CCSS replication set. As shown in **Table 4**, the SNP set on *P2RX7* gene was successfully replicated in the CCSS cohort (HR: 0.65; 95% CI: 0.47-0.90; $P = 0.009$).

Significant SNP-level associations were as follows: rs2525702 [*NOBOX*] ($P = 0.039$), rs3761863 [*LRRK2*] ($P = 0.035$), rs3751143 [*P2RX7*] ($P = 0.03$), and rs1231123 [*MEFV*] ($P = 0.006$), rs224213 [*MEFV*] ($P = 0.008$), and rs224208 [*MEFV*] ($P = 0.009$) (Supplemental Table 2).

COG and BMTSS replication sets. Two SNPs (rs3751144 and rs11878432) were not genotyped because they did not meet assay design parameters. Post-genotyping, 2 triallelic SNPs (rs2699503 and rs3751143) with 0% call rate and 14 additional samples with low genotype call rates (<95%) were excluded from the final analysis (COG: 1 case, 4 control subjects; BMTSS: 2 cases, 7 control subjects). Overall, 32 SNPs in 588 patients (COG: 31 cases, 169 matched control subjects; BMTSS: 133 cases, 255 matched control subjects) were included in this analysis. Individual SNPs on the *P2RX7* gene were associated with cardiomyopathy in the COG set (rs2230911/rs3751142; $P = 0.05$). Three SNPs were significantly associated with cardiomyopathy in the BMTSS set: rs3796295 [*TNIK*] ($P = 0.05$), rs7133914 [*LRRK2*] ($P = 6.25 \times 10^{-7}$), and rs224222 [*MEFV*] ($P = 0.012$) (**Table 4**).

DISCUSSION

We identified susceptibility loci on the *P2RX7* gene in association with cardiomyopathy risk in childhood cancer survivors using a gene-level analysis, and we successfully replicated the finding in an independent cancer survivor population. The *P2RX7*gene has been previously linked to cardiac disorders in nononcology populations.[27-30] Associations from additional genes *TNIK*, and *LRRK2, NOBOX*, and *MEFV* genes, with potential links to cardiac disease, [31-36] were replicated at the individual SNP level. The added value of an interaction gene-level analysis is the emergence of evidence implicating new genes that were not detected before but are supported by apparent biological links to disease. Measures of SNP-disease associations such as ORs reported from single-SNP-based analyses are typically in the range of 1.1 to 1.5, which, despite the high statistical significance, are of minimal clinical and public health importance.[37] By contrast, our gene-level analysis yielded ORs with substantially larger magnitudes indicating strong associations with potentially enhanced clinical relevance.

Our main finding showed that the union of complementary SNPs, having 1 or no copies of variant alleles on *P2RX7*, yielded a reduced risk of cardiomyopathy. *P2RX7* encodes the P2X7 receptor belonging to a family of purinoceptors primarily activated by extracellular adenosine triphosphate (ATP) (**Central Illustration**). Prolonged stimulation of P2X7 with ATP promotes ATP-dependent lysis of macrophages through the formation of cell membrane macropores permeable to large molecules, resulting in inflammatory cell death. P2X7-induced inflammasome activation is primarily mediated through generation of T helper type 17 (Th17) lymphocytes and intracellular interleukin (IL)-1β, IL-18, and IL-6 proinflammatory cytokines inducing the pathological inflammatory response.[38,39] Specific cardiovascular effects of P2X7 activation involve promoting renal and vascular dysfunction, predisposing to hypertension, endothelial dysfunction and atherosclerotic plaque formation and rupture, and

TABLE 2 Demographic and Clinical Characteristics of the Discovery and Replication Cohorts

	COG Discovery (N = 278)				COG Replication (N = 205)		
	Mild Cases (n = 80)	Severe Cases (n = 49)	Control Subjects (n = 149)	*P* Value[a]	Mild Cases (n = 18)	Severe Cases (n = 14)	Control Subjects (n = 173)
Age at primary cancer diagnosis, y							
Mean ± SD	7.5 ± 5.7	8.3 ± 5.5	7.9 ± 5.9	0.29	9.1 ± 5.1	8.1 ± 5.3	8.1 ± 5.6
Median (IQR)	6 (0-21)	8 (0-20)	7 (0-20)		7.8 (1.5-17.9)	8.8 (0.6-17.4)	7 (0.04-20)
Sex							
Female	39 (48.7)	32 (65.3)	69 (46.3)	0.07	9 (50.0)	11 (78.6)	85 (49.1)
Race/ethnicity							
White	62 (77.5)	40 (81.6)	124 (83.2)	0.72	11 (61.1)	7 (50.0)	144 (83.2)
Asian	3 (3.7)	1 (2.0)	4 (2.7)		3 (16.7)	2 (14.3)	8 (4.6)
Black	6 (7.5)	1 (2.0)	7 (4.7)		2 (11.1)	4 (28.6)	10 (5.8)
Hispanic	7 (8.8)	7 (14.3)	12 (8.1)		2 (11.1)	1 (7.1)	9 (5.2)
Multiracial	2 (2.5)	0 (0)	2 (1.3)		–	–	2 (1.2)
Primary diagnosis							
ALL	12 (15.0)	5 (10.2)	18 (12.1)	0.62	5 (27.8)	1 (7.1)	50 (28.9)
AML	3 (3.7)	8 (16.3)	12 (8.1)		0 (0.0)	4 (28.6)	14 (8.1)
Brain tumors	5 (6.2)	1 (2.0)	9 (6.0)		1 (5.6)	0 (0.0)	8 (4.6)
Ewing sarcoma	12 (15.0)	7 (14.3)	19 (12.7)		1 (5.6)	1 (7.1)	16 (9.2)
Hodgkin lymphoma	5 (6.2)	9 (18.4)	16 (10.7)		0 (0.0)	2 (14.3)	17 (9.8)
NHL	9 (11.2)	4 (8.2)	14 (9.4)		3 (16.7)	5 (35.7)	19 (10.9)
Neuroblastoma	7 (8.7)	1 (2.0)	10 (6.7)		1 (5.6)	0 (0.0)	13 (7.5)
Osteosarcoma	9 (11.2)	4 (8.2)	16 (10.7)		3 (16.7)	0 (0.0)	13 (7.5)
Sarcoma	10 (12.5)	5 (10.2)	22 (14.8)		4 (22.2)	1 (7.1)	14 (8.1)
Wilms	7 (8.7)	3 (6.1)	11 (7.4)		0 (0.0)	0 (0.0)	6 (3.5)
Other[b]	1 (1.2)	2 (4.1)	2 (1.3)		0 (0.0)	0 (0.0)	3 (1.7)
Year of primary cancer diagnosis							
≤1990	26 (32.5)	22 (44.9)	51 (34.2)	0.009	8 (44.4)	4 (28.6)	44 (25.4)
1991-2000	44 (55.0)	11 (22.5)	61 (40.9)		5 (27.8)	6 (42.9)	83 (47.9)
>2000	10 (12.4)	16 (32.7)	37 (24.9)		5 (27.8)	4 (28.6)	46 (26.6)
Length of follow-up, y							
Mean ± SD	10.2 ± 8.4	9.6 ± 9.2	15.8 ± 8.7	<0.001	10.5 ± 10.1	6.8 ± 8.6	12.5 ± 7.9
Median (IQR)	8.58 (0.3-35.1)	7.46 (0.22-30.3)	14.44 (1.9-38.9)		7.3 (0.3-27.6)	3.0 (0.1-29.8)	11.5 (1.4-41.1)
Cumulative anthracycline exposure, mg/m^2							
Mean ± SD	312.5 ± 134.1	310.7 ± 141.9	185.8 ± 167.4	<0.001	276.6 ± 128.8	282.8 ± 165.6	200.9 ± 170.2
Median (IQR)	349 (0-630)	300 (0-760)	164 (0-600)		300 (0-480)	275 (0-575)	175 (0-750)
Chest radiation							
Yes	26 (32.5)	20 (40.8)	37 (24.8)	0.09	5 (27.8)	2 (14.3)	31 (17.9)
Cardiovascular risk factor							
Yes	23 (28.7)	25 (51.0)	13 (8.7)	<0.001	3 (16.7)	7 (50.0)	11 (6.4)

Values are n (%) unless otherwise indicated. [a]Univariate chi-square tests and analyses of variance were used for categorical and continuous variables, respectively, to compare mild cases and severe cases with control subjects. [b]Other primary diagnoses: COG (Children's Oncology Group): myelodysplastic syndrome, germ cell tumor, spindle cell carcinoma, pancreatoblastoma, mesenchymal chondrosarcoma, clear cell sarcoma of kidney, small cell-type malignant tumor; BMTSS (Bone Marrow Transplant Survivor Study): multiple myeloma, chronic lymphocytic leukemia, plasma cell disorders; CCSS (Childhood Cancer Survivor Study): bone tumor, other leukemia.

ALL = acute lymphoid leukemia; AML = acute myeloid leukemia; NHL = non-Hodgkin lymphoma.

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cardiomyocyte apoptosis in ischemic hearts and eventual cardiac dysfunction.[27,38-40]

In animal models, a significant cardioprotective role of P2RX7 antagonism has been repeatedly demonstrated through reduction of the inflammatory response and cardiovascular disease severity.[27-29] Studies of *P2RX7* knockout mice reported cardioprotective effects, including prolonged heart transplant survival, prevention of coronary vasculopathy and atherosclerosis progression, and reduction in systolic blood pressure, heart rate, cardiac enzymes, and anti-inflammatory cytokines in

TABLE 2 Continued

BMTSS Replication (N = 397)		CCSS Replication (N = 5,589)	
Cases (n = 135)	Control Subjects (n = 262)	Cases (n = 229)	Internal Control Subjects (5,360)
Age at primary cancer diagnosis, y			
45.3 ± 15.5	41.2 ± 17.2	11.5 ± 5.6	7.7 ± 5.9
47.0 (6-71)	43.5 (2-70)	13.0 (0-20)	6.0 (0-20)
Sex			
58 (43.0)	118 (45.0)	134 (58.5)	2,743 (51.2)
Race/ethnicity			
105 (77.8)	207 (79.0)	229 (100)	5,360 (100)
8 (5.9)	14 (5.3)	–	–
8 (5.9)	14 (5.3)	–	–
11 (8.1)	21 (8.0)	–	–
3 (2.2)	6 (2.3)	–	–
Primary diagnosis			
8 (5.9)	16 (6.1)	24 (10.5)	1,652 (30.8)
31 (22.9)	61 (23.3)	2 (0.9)	99 (1.9)
–	–	1 (0.4)	675 (12.6)
–	–	21 (9.2)	145 (2.7)
19 (14.1)	35 (13.4)	77 (33.6)	671 (12.5)
65 (48.1)	127 (48.5)	33 (14.4)	422 (7.9)
–	–	4 (1.8)	404 (7.5)
–	–	34 (14.9)	267 (5.0)
–	–	21 (9.2)	481 (9.0)
1 (0.7)	1 (0.3)	12 (5.2)	505 (9.4)
11 (8.1)	22 (8.4)	–	39 (0.7)
Year of primary cancer diagnosis			
4 (2.9)	8 (3.0)	–	–
40 (29.6)	92 (35.1)	–	–
91 (67.4)	162 (61.8)	–	–
Length of follow-up, y			
4.9 ± 5.9	12.2 ± 6.5	23.8 ± 10.2	32.7 ± 6.0
2 (0-22)	11 (0-27)	26 (0-43)	33 (14-46)
Cumulative anthracycline exposure, mg/m^2			
253.4 ± 154.8	203.5 ± 127.7	232 ± 221	100.1 ± 160.8
250 (30-800)	200 (20-600)	230.4 (0-917.8)	0 (0-1,120)
Chest radiation			
74 (54.8)	151 (57.6)	115 (50.2)	1,248 (23.3)
Cardiovascular risk factor			
102 (75.6)	145 (55.3)	191 (83.4)	1,847 (34.5)

ischemic hearts.[27,28] In a mouse model of diet-induced systemic inflammation, similar protective effects were observed in *P2RX7* knockout mice, including enhanced tissue homeostasis and cardiac function preservation.[29] Human studies include a recent whole-exome sequencing of inherited hypertrophic cardiomyopathy, which identified a single nonsynonymous exonic germline mutation of *P2RX7* segregating in cases, but not in unaffected family members or control subjects.[30]

The *P2RX7* SNPs identified and replicated in our gene-level analysis include loss-of-function SNP rs3751143 and rs3751144, in strong LD with loss-of-function rs2230911, and rs208294 gain-of-function SNP (Supplemental Table 3).[41] At the SNP level, rs3751143 was previously reported to be significantly associated with a decreased risk of ischemic heart disease and stroke in smokers and individuals with hypertension.[42] It is a nonsynonymous SNP leading to impairments in several functions of P2X7 receptor, including the ability of the channel to undergo dilation and release of proinflammatory cytokines from macrophages.[43,44] The rs208294 gain-of-function SNP is located in the extracellular domain

TABLE 3 Gene-Level Associations With Anthracycline-Induced Cardiomyopathy in the Discovery Cohort

Chromosome	Gene	Significant Tree	Odds Ratio (95% CI)	*P* Value[a]
3	*TNIK*	((((not rs2291900_1) OR rs3796295_2) OR (not rs3796295_1)) AND (not rs17857452_1))	4.58 (2.47-8.49)	1.34×10^{-6}
7	*NOBOX*	(rs2525702_1 OR (not rs727714_1))	7.21 (3.23-16.1)	1.43×10^{-6}
12	*LRRK2*	((rs3761863_2 OR (not rs10878245_1)) OR (((not rs7308720_1) OR (not rs3761863_1)) AND (not rs11176013_2)))	0.19 (0.09-0.39)	6.62×10^{-6}
12	*P2RX7*	((((not rs208294_1) OR rs208294_2) OR (not rs3751143_1)) AND (not rs3751144_2))	0.10 (0.04-0.27)	2.19×10^{-6}
16	*MEFV*	((rs224213_1 AND (not rs224208_2)) OR ((not rs224222_2) AND ((not rs224208_1) OR rs224213_2)))	0.08 (0.03-0.24)	4.07×10^{-6}
19	*FBN3*	((rs11878432_2 OR (not rs12974280_1)) AND (rs35002391_1 OR rs3813774_1))	4.59 (2.42-8.71)	3.05×10^{-6}

OR and AND in the column Significant Tree represent Boolean combinations. [a]*P* value significance threshold was 6.93×10^{-6}.

of the P2X7 receptor and is associated with increased ATP-dependent calcium influx.[45] The LD rs2230911 loss-of-function is evidenced by P2X7 receptor reduced pore formation that is restored with up-regulation of its expression.[46] In silico analyses generated by the Human Protein Atlas revealed medium expression levels of *P2RX7* in cardiomyocytes.[47] The variant effect predictor analysis showed that rs208294 and rs3751143 are labeled "deleterious" by SIFT and "probably" and "possibly" damaging by PolyPhen, respectively. Additionally, rs3751143 and rs3751144 have a CTCF binding site with enhancer (Supplemental Table 4). There were no eQTL in the heart atrial appendage or left ventricle for the *P2RX7* SNPs. The combination of these SNPs with either loss-of-function or gain-of function give rise to various haplotypes that can modify the *P2RX7* gene function.[48] The observed combination identified from our gene-level approach in our survivor data sets was associated with a reduced risk of cardiomyopathy.

Clinical trials of P2X7 antagonist compounds from major pharmaceutical companies have been tested in other chronic inflammatory conditions, including chronic obstructive pulmonary disease, rheumatoid arthritis, and Crohn's disease.[49-51] Limited serious adverse events were reported with prolonged use of these antagonists for up to 6 months. This provides the advantage of blocking downstream effects of P2X7 activation and inflammasome activation. Development of targeted P2X7 agents have been more recently facilitated by the ability to crystallize P2X7, potentially enhancing its clinical efficacy as a therapeutic target for a variety of diseases, including in cardiovascular disease.[52-56] The influence of *P2RX7* variants on cardiovascular disease risk combined with the safety profile and relative tolerability of several P2X7 inhibitors reported from human clinical trials positions them as viable therapeutic targets for the prevention of anthracycline-induced cardiomyopathy.

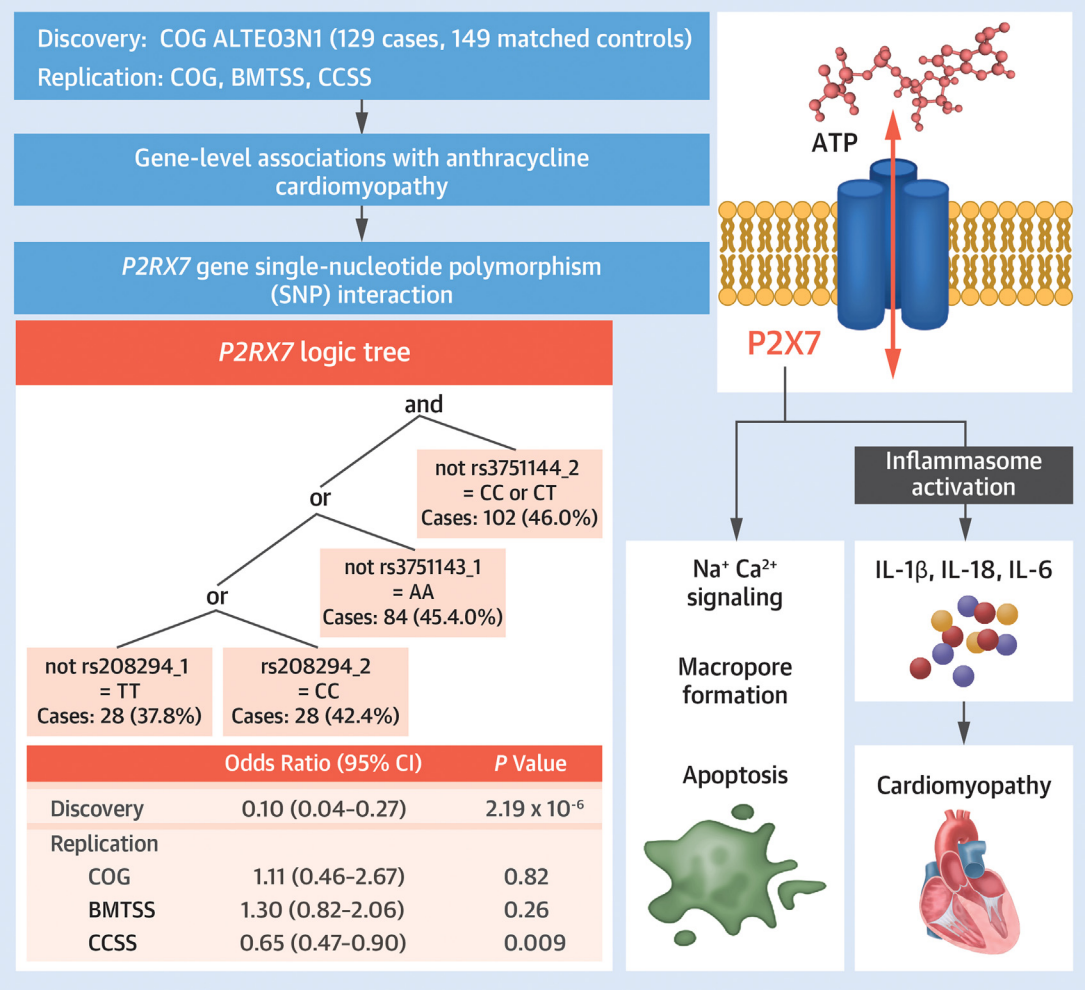
In a genome-wide association study using the CCSS and COG cohorts for discovery and replication, respectively, we previously identified variants on the *ROBO2* gene to be associated with high-dose anthracycline-related cardiomyopathy.[57] *ROBO2* is involved in the Slit-Robo signaling pathway that promotes cardiac fibrosis. In the present study, using the COG

TABLE 4 Gene-Level Associations With Anthracycline-Induced Cardiomyopathy Across the Replication Sets

Chr	Gene	Significant Tree	COG Discovery OR (95% CI); *P* Value[a]	COG Replication OR (95% CI); *P* Value[b]	BMTSS Replication OR (95% CI); *P* Value[b]	CCSS Replication HR (95% CI); *P* Value[b]
3	*TNIK*	((((not rs2291900_1) OR rs3796295_2) OR (not rs3796295_1)) AND (not rs17857452_1))	4.58 (2.47-8.49); 1.34×10^{-6}	0.97 (0.35-2.68); 0.95	0.98 (0.41-1.13); 0.14	0.87 (0.65-1.16); 0.34
7	*NOBOX*	(rs2525702_1 OR (not rs727714_1))	7.21 (3.23-16.1); 1.43×10^{-6}	1.10 (0.46-2.63); 0.83	0.80 (0.50-1.28); 0.36	0.90 (0.68-1.20); 0.48
12	*LRRK2*	((rs3761863_2 OR (not rs10878245_1)) OR (((not rs7308720_1) OR (not rs3761863_1)) AND (not rs11176013_2)))	0.19 (0.09-0.39); 6.62×10^{-6}	0.44 (0.17-1.14); 0.09	0.96 (0.48-1.92); 0.91	0.77 (0.53-1.12); 0.17
12	*P2RX7*	((((not rs208294_1) OR rs208294_2) OR (not rs3751143_1)) AND (not rs3751144_2))	0.10 (0.04-0.27); 2.19×10^{-6}	1.11 (0.46-2.67); 0.82	1.30 (0.82-2.06); 0.26	0.65 (0.47-0.90); 0.009
16	*MEFV*	((rs224213_1 AND (not rs224208_2)) OR ((not rs224222_2) AND ((not rs224208_1) OR rs224213_2)))	0.08 (0.03-0.24); 4.07×10^{-6}	1.55 (0.16-14.8); 0.70	1.34 (0.46-3.96); 0.59	1.13 (0.42-3.06); 0.80
19	*FBN3*	((rs11878432_2 OR (not rs12974280_1)) AND (rs35002391_1 OR rs3813774_1))	4.59 (2.42-8.71); 3.05×10^{-6}	1.01 (0.32-3.21); 0.98	1.49 (0.75-2.96); 0.25	1.13 (0.79-1.60); 0.51

OR and AND in the column Significant Tree represent Boolean combinations. [a]*P* value significance threshold was 6.93×10^{-6}. [b]*P* value significance threshold was 0.05.

CENTRAL ILLUSTRATION ***P2RX7* Gene Cardiac Effects**



Sharafeldin N, et al. J Am Coll Cardiol CardioOnc. 2023;5(6):807–818.

The *P2RX7* gene association was identified and replicated in an independent cohort of childhood cancer survivors. The figure provides a graphical representation of the single nucleotide polymorphism (SNP) union of SNPs and SNP complements on *P2RX7* gene identified through logic regression and its effects on the P2X7 receptor. P2X7 activation by extracellular adenosine triphosphate (ATP) results in the influx of sodium and calcium ions, macropore formation, leading to inflammatory cell death and proinflammatory cytokine release promoting vascular dysfunction predisposing to hypertension, cardiomyocyte apoptosis, and eventual cardiac dysfunction. IL = interleukin.

data set for discovery, we used a gene-level approach and identified the *P2RX7* gene, where the inflammatory pathway promotes cardiomyopathy. We want to highlight the differences in the cardiomyopathy/HF cases in the 2 data sets: COG captures cardiomyopathy events that develop earlier in the survivorship trajectory (median latency: 8.6; range: 0.2-35.1), whereas CCSS captures later occurring events (median latency: 26; range: 0-43) (Table 2). We speculate that using COG as the discovery data set in the current study identified pathways that are more prominent in the early phases of anthracycline-related cardiomyopathy (inflammatory pathway), whereas using CCSS for discovery, we identified pathways that are more prominent in the later phases (profibrotic pathway). In addition, findings from the same cohorts using different methodological approaches provide further insights into the mechanistic underpinnings of anthracycline-related cardiomyopathy in childhood survivors.

Additional discoveries were identified in our study using a gene-level approach with plausible links to cardiovascular disease, emphasizing the importance of searching for SNP set interaction effects, in addition to the standard single-SNP analyses. *TNIK* gene is an essential activator of the Wnt signaling pathway and is related to STRIPAK and STRIPAK-like complexes, which are associated with several conditions including cardiac disease.[31-34] The *LRRK2* gene is linked to Parkinson disease; however, early clinical trials of LRRK2 inhibitors for Parkinson disease and phenome-wide association studies reported potential adverse cardiovascular outcomes, such as cardiac conduction disorders, atrial fibrillation, and flutter, related to LRRK2 expression.[35,36] The *MEFV* gene is associated with the regulation of innate immunity and inflammatory response linked to a common etiological pathway with cardiovascular disorders.[58] The *NOBOX* gene, although mainly linked to ovarian failure, is highly expressed in the porcine heart, suggesting a potential role in heart tissue.[59]

STUDY LIMITATIONS. It is important to consider the limitations of this study when interpreting and fully understanding these findings. To manage the large computational demand of the logic regression search, we had to limit the search for SNP set interactions to only 2 trees or SNP combinations and fix the size within each tree to 5 SNPs per gene. Our approach, however, has higher power compared with a pairwise interaction test. Our use of Boolean logic through logic regression likely captured a subset of existing cis-regulatory modules; however, a more comprehensive modeling would be required for gene-gene interactions. Although we based our gene-level significance threshold on the number of genes tested, our top gene signals surpassed a more conservative significance threshold based on the number of SNPs. We visually inspected the genotype clusters of the genotyped SNPs using the Fluidigm assay to ensure that spurious associations did not result from genotype errors. Despite the limitations associated with the use of logic regression, searching for SNP set interactions is a step toward addressing the complexity of genetic associations compared with a marginal assessment of individual SNP effects on disease.

There are distinct strengths and limitations for each of the independent replication sets. The COG replication set was closest to the discovery set with respect to the study design, the phenotype characterization, and population characteristics. The BMTSS replication set differed in outcome ascertainment and population characteristics, yet yielded itself to significant replication of SNPs on 50% of the discovery genes, underscoring the robustness of our findings. However, SNPs that failed genotyping in both COG and BMTSS precluded the full assessment of SNP set (gene-level) effects. The CCSS replication cohort presented differences in analytic and statistical methodologies, yet carried the advantage of a large sample and allowed us to fully test replication for the SNP sets identified in discovery. Our choice of replication sets aligns with the genetic replication philosophy, which involves using independent sets with a similar but not identical study base. This approach helps confirm that the initial discovery associations are not due to subtle biases, most notably population stratification bias.[60,61] Finally, using animal models and/or cell cultures to generate experimental functional evidence remains ideal for replication purposes.[60]

CONCLUSIONS

In summary, we identified gene-level associations with anthracycline-induced cardiomyopathy in cancer survivors. The replicated *P2RX7* gene is linked to cardiovascular protective effects. In addition, several SNP-level associations were supported by biological plausibility. This study demonstrates that leveraging technological and analytical approaches can provide new and potentially important associations, which can inform future prevention and intervention strategies.

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PERSPECTIVES

COMPETENCY IN PATIENT CARE AND PROCEDURAL SKILLS:

In this comprehensive gene-level genetic association study of anthracycline-induced cardiomyopathy, discovery and replication of biologically plausible findings in independent cohorts of cancer survivors implicated the *P2RX7* gene.

TRANSLATIONAL OUTLOOK:

Identifying genetic determinants of anthracycline-induced cardiomyopathy paves the way for a personalized management of cardiomyopathy in cancer survivors, in addition to providing insights into potential therapeutic targets.

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KEY WORDS anthracycline-induced cardiomyopathy, childhood cancer survivors, gene-level association, purinergic receptor P2X7, whole exome sequencing

APPENDIX For an expanded Methods section and a supplemental figure and tables, please see the online version of this paper.