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ORIGINAL REPORT

Hyaluronan Synthase 3 Variant and Anthracycline-Related Cardiomyopathy: A Report From the Children's Oncology Group

Xuexia Wang, Wei Liu, Can-Lan Sun, Saro H. Armenian, Hakon Hakonarson, Lindsey Hageman, Yan Ding, Wendy Landier, Javier G. Blanco, Lu Chen, Adolfo Quiñones, Daniel Ferguson, Naomi Winick, Jill P. Ginsberg, Frank Keller, Joseph P. Neglia, Sunil Desai, Charles A. Sklar, Sharon M. Castellino, Irene Cherrick, ZoAnn E. Dreyer, Melissa M. Hudson, Leslie L. Robison, Yutaka Yasui, Mary V. Relling, and Smita Bhatia

Author affiliations appear at the end of this article.

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X.W., W.L., and C.-L.S. contributed equally to this work as lead authors.

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S.B. has full access to all the data in the study and takes responsibility for the integrity of the data and accuracy of the data analysis.

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Corresponding author: Smita Bhatia, MD, MPH, Department of Population Sciences, City of Hope, Duarte, CA 91010; e-mail: sbhatia@coh.org.

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ABSTRACT

Purpose
The strong dose-dependent association between anthracyclines and cardiomyopathy is further exacerbated by the co-occurrence of cardiovascular risk factors (diabetes and hypertension). The high morbidity associated with cardiomyopathy necessitates an understanding of the underlying pathogenesis so that targeted interventions can be developed.

Patients and Methods
By using a two-stage design, we investigated host susceptibility to anthracycline-related cardiomyopathy by using the ITMAT/Broad CARe cardiovascular single nucleotide polymorphism (SNP) array to profile common SNPs in 2,100 genes considered relevant to de novo cardiovascular disease.

Results
By using a matched case-control design (93 cases, 194 controls), we identified a common SNP, rs2232228, in the hyaluronan synthase 3 (*HAS3*) gene that exerts a modifying effect on anthracycline dose-dependent cardiomyopathy risk ($P = 5.3 \times 10^{-7}$). Among individuals with rs2232228 GG genotype, cardiomyopathy was infrequent and not dose related. However, in individuals exposed to high-dose (> 250 mg/m^2) anthracyclines, the rs2232228 AA genotype conferred an 8.9-fold (95% CI, 2.1- to 37.5-fold; $P = .003$) increased cardiomyopathy risk compared with the GG genotype. This gene-environment interaction was successfully replicated in an independent set of 76 patients with anthracycline-related cardiomyopathy. Relative *HAS3* mRNA levels measured in healthy hearts tended to be lower among individuals with AA compared with GA genotypes ($P = .09$).

Conclusion
Hyaluronan (HA) produced by *HAS3* is a ubiquitous component of the extracellular matrix and plays an active role in tissue remodeling. In addition, HA is known to reduce reactive oxygen species (ROS) –induced cardiac injury. The high cardiomyopathy risk associated with AA genotype could be due to inadequate remodeling and/or inadequate protection of the heart from ROS-mediated injury on high anthracycline exposure.

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INTRODUCTION

Anthracyclines are one of the most effective classes of chemotherapeutic agents currently available for cancer diagnosed across the age spectrum. The therapeutic potential of anthracyclines, however, is limited because of their strong dose-dependent relation with progressive and irreversible cardiomyopathy leading to congestive heart failure.[1-3] This dose-dependent risk is modified by the coexistence of cardiovascular disease risk factors such as hypertension and diabetes.[4,5] An interindividual variability in cardiomyopathy risk has been observed, such that cumulative anthracycline exposure as low as 150 mg/m^2 results in cardiomyopathy in some patients, although exposure as high as 1,000 mg/m^2 is tolerated without cardiomyopathy by others.[6]

This study aimed to investigate reason(s) for observed interindividual variability by identifying single nucleotide polymorphisms (SNPs) that might modify the association between anthracycline exposure and risk of cardiotoxicity. Specifically, we used the ITMAT/Broad CARe (IBC) cardiovascular SNP array, which profiles SNPs in 2,100 genes considered

relevant for cardiovascular disease in the general population,[7] to identify interaction of SNPs known to increase the risk of cardiovascular disease in the general population with anthracycline exposure. We used a two-stage design; SNPs surpassing a prespecified threshold for statistical significance in the discovery stage were validated in the replication stage by using an independent set of cases with anthracycline-related cardiomyopathy. Functional significance of validated SNPs was evaluated by measuring gene expression levels in heart samples procured from the National Disease Research Interchange (Philadelphia, PA).

PATIENTS AND METHODS

Study Participants

Discovery set. The discovery set was drawn from a Children's Oncology Group study (COG-ALTE03N1) that aims to understand the pathogenesis of cardiomyopathy in childhood cancer survivors. COG member institutions (Appendix Table A1, online only) enrolled patients after obtaining approval from local institutional review boards. Written informed consent/assent was obtained from patients/parents/legal guardians. Cases and controls were identified from individuals diagnosed with cancer at age 21 years or younger. Cases consisted of individuals who developed cardiomyopathy and were alive at study participation. For each case, one to four controls were randomly selected from the same COG childhood cancer survivor cohort by using the following matching criteria: cancer diagnosis, year of diagnosis ($\pm$ 5 years), race/ethnicity, and duration of cardiomyopathy-free follow-up for controls to exceed time from cancer diagnosis to cardiomyopathy for the corresponding case. In all, 401 individuals (130 cases; 271 controls) participated in this study. All participants provided a biologic specimen (blood, 89%; buccal cells/saliva, 11%) for DNA.

Anthracycline-exposed participants had normal cardiac function before anthracycline exposure. Cases fulfilled American Heart Association criteria for cardiac compromise by presenting with symptoms (dyspnea, orthopnea, and/or fatigue) and/or signs (edema, hepatomegaly, and/or rales) of cardiac compromise, and echocardiographic evidence of left ventricular dysfunction, or, in the absence of symptoms/signs, had echocardiographic features of left ventricular dysfunction (ejection fraction [EF] $\leq$ 40% and/or fractional shortening [FS] $\leq$ 28%; Data Supplement).

Replication set. An independent set of 76 patients (all ages and all racial/ethnic backgrounds) diagnosed with cardiomyopathy after anthracycline exposure were drawn from a single institution (Data Supplement).[5] The study was approved by the institutional review board, and written informed consent/assent was obtained. Eligible cases met American Heart Association criteria and echocardiographic cutoffs for cardiac compromise.

Therapeutic Exposures

Lifetime anthracycline exposure was calculated by multiplying the cumulative dose (in milligrams per meter squared) of individual anthracyclines by a factor that reflects the drug's cardiotoxic potential (Data Supplement)[8] and then summing the results. Total dose of radiation with heart in field ("chest radiation") was computed in Gy.

Genotyping and Quality Control

Discovery set. Genomic DNA was isolated from peripheral blood (QIAamp/Qiagen kits; Valencia, CA) and buccal cells/saliva (Puregene/Oragene kits; Minneapolis, MN). Genotyping was performed on the Illumina IBC cardiovascular SNP array (San Diego, CA) by the Center for Applied Genomics at The Children's Hospital of Philadelphia. The IBC cardiovascular SNP array[7] uses a "cosmopolitan" approach to determine tagging SNPs for loci of interest to cover genetic diversity in populations of different ancestry.[9]

Quality control for genotype data was performed with PLINK.[10] Of the 401 study participants, 399 (99.5%; 129 cases, 270 controls) met call rates of more than 95%. No duplicated samples or sample contamination was identified. The multidimensional scaling method[10] was used to cluster individuals in the discovery set into non-Hispanic whites and "others." To control for potential population stratification, 112 individuals in the other category were filtered out, retaining 287 non-Hispanic whites (93 cases, 194 controls) in the discovery stage analysis. After adjusting for the overall genomic control inflation factor (λ = 1.23), type I error appeared to be under control (Data Supplement). The total genotyping rate exceeded 98.9%. Of the 43,293 autosomal SNPs in the IBC SNP array, 988 that failed a missingness threshold for missing fraction more than 0.05 and 7,267 that failed a frequency threshold for a minor allele frequency less than 0.01 were removed. A check for Hardy-Weinberg equilibrium resulted in exclusion of 108 SNPs with *P* value less than .000001. The final data set retained 34,912 (81%) autosomal SNPs.

Replication set. Genomic DNA was extracted from peripheral blood and buccal cells/saliva by using Qiagen kits (Valencia, CA). Genomic DNA from formalin-fixed paraffin-embedded bone marrow biopsies or unstained bone marrow slides was extracted by using QuickExtract formalin-fixed paraffin-embedded DNA Extraction Solution (Epicentre Biotechnologies, Madison, WI). Significant SNP(s) identified in the discovery stage were genotyped by using Sequenom iPLEX SNP chemistry on a MassARRAY system (San Diego, CA; call rate, 93.4%).

Gene Expression Analysis

Gene expression analysis was performed on heart samples procured from the National Disease Research Interchange (Philadelphia, PA). Tissue procurement, processing, genotyping, and gene expression analyses are detailed in the Data Supplement. Significant SNPs validated in the replication stage were genotyped with TaqMan genotyping assays (Applied Biosystems, Carlsbad, CA). Total RNA from heart tissue samples was reverse transcribed and amplified by using one-step QuantiTect SYBR Green reverse transcriptase polymerase chain reaction kits (Qiagen, Valencia, CA). Relative hyaluronan synthase 3 (*HAS3*) mRNA levels were obtained after normalization to reference gene (*ACTB*).[11]

Statistical Analyses

Discovery stage. Discovery stage was designed as a genome-wide association study to examine the main effects of SNPs and gene-environment (anthracycline) interactions. Conditional logistic regression techniques (model 1; equation 1) were used in R (http://www.r-project.org/) for each SNP that passed quality control, minor allele frequency, and Hardy-Weinberg equilibrium filters.

$$\text{logit}(p) = \text{matched set} + \text{agedx} + \text{sex} + \text{RT} + \text{anthracycline} + \text{SNP} + \text{SNP}\times\text{anthracycline} \quad (1)$$

where p is the probability of cardiomyopathy in a patient conditional on matched set, agedx is the age at diagnosis of primary cancer (continuous variable), sex (male/female) of participants, RT is the chest radiation dose (continuous variable), anthracycline is the cumulative anthracycline dose in milligrams per meter squared (continuous variable), and SNP is the genotype for each SNP in additive coding.

The repeated sliding-window procedure of Purcell et al[10] estimated 10,000 independent tests, taking into consideration linkage disequilibrium (LD) among SNPs. This allowed a *P* value less than 5×10^{-6} to serve as the threshold for the whole genome significance test, after accounting for multiple testing.[12] Odds ratio (OR) estimates were interpreted as approximate relative risk estimates because of the relative rarity of cardiomyopathy.

Replication stage. Replication stage used a case-only design to verify significant gene-environment interactions identified in the discovery stage. Cumulative anthracycline exposure was dichotomized as low to moderate dose ($\leq$ 250 mg/m^2) and high dose ($>$ 250 mg/m^2) on the basis of previous observations by us and others, of a significantly increased risk of cardiomyopathy associated with anthracycline dose exceeding 250 mg/m^2.[1,13] We treated the binary variable of anthracycline exposure as a dependent variable and used logistic regression techniques to conduct a gene-environment interaction analysis (model 2; equation 2).

$$\text{logit}(p\_\text{anth\_exp}) = \text{agedx} + \text{sex} + \text{RT} + \text{race/ethnicity} + \text{SNP} \quad (2)$$

where p_anth_exp is the probability of being in the high-dose anthracycline group (anth_exp 0 is the low-to-moderate dose [$\leq$ 250 mg/m^2] and anth_exp 1 is the high dose [$>$ 250 mg/m^2]).

On the basis of the significantly increased risk of cardiomyopathy in anthracycline-exposed adult patients with coexisting diabetes or hypertension,[5] we repeated the analysis after including these comorbidities.

Final model. By using the combined data from the discovery and replication sets, we used model 2 to test for gene-environment interactions for significant SNP(s) identified in the discovery stage.

Gene expression. Log-transformed mRNA levels were compared across genetic variants by using the *t* test.

RESULTS

Discovery Stage

Demographic and clinical characteristics. Cases received higher cumulative anthracycline exposure (median dose: 300 *v* 152 mg/m^2; $P < .001$) compared with the controls. Among the cases, median EF was 40% (range, 10% to 56%), and median FS was 23% (range, 5% to 33%; Table 1).

Controls had no signs or symptoms of cardiac compromise at study participation. Of the 194 controls, 155 had normal echocardiographic features (median EF, 66; median FS, 37). Six of the 39 controls without echocardiograms did not receive any cardiotoxic exposure, and exclusion of the 33 anthracycline-exposed controls without echocardiograms did not alter results (Data Supplement); we opted to include them in the analysis.

Risk of cardiomyopathy. In a multivariable conditional logistic regression analysis that included age at diagnosis of primary cancer, sex, and chest radiation dose in the model, among low-to-moderate-dose ($\leq$ 250 mg/m^2) and high-dose ($>$ 250 mg/m^2) anthracycline-exposed individuals cardiomyopathy risk was 7.3 (95% CI, 1.5 to 35.8; $P = .01$) and 49.5 (95% CI, 9.2 to 268.1; $P < .001$) times higher, respectively (P for trend $< .001$), than among unexposed patients.

Genome-wide association and gene-environment interaction analysis. No main-effect association was observed between any of the SNPs examined and cardiomyopathy. However, one SNP (rs2232228) in the *HAS3* gene on chromosome 16 exceeded the multiple-comparison-corrected threshold for significant SNP*anthracycline interaction ($P = 5.3 \times 10^{-7}$; Fig 1; Data Supplement).

The main effect association between SNP rs2232228 genotype and cardiomyopathy risk is provided in Table 2. In addition, the modifying effect of rs2232228 genotype on the dose-dependent association between anthracycline and cardiomyopathy risk is shown graphically (Fig 2) and in a tabular format (Table 2). At low-to-moderate-dose anthracycline exposure, the risk of cardiomyopathy did not differ significantly by rs2232228 genotype (GG, GA, or AA; Fig 2). However, among individuals with AA genotype exposed to higher doses of anthracyclines, cardiomyopathy risk increased substantially with anthracycline exposure (anthracycline exposure $>$ 450 mg/m^2: OR, 56.5). Although in individuals with GG genotype, cardiomyopathy risk was not increased at any anthracycline dose, the odds of developing cardiomyopathy were approximately 1 at all doses, and at cumulative anthracycline doses exceeding 450 mg/m^2, the risk was not increased (OR, 0.6; Fig 2). Furthermore, as delineated in Table 2, among individuals exposed to high-dose ($>$ 250 mg/m^2) anthracyclines, the presence of AA genotype conferred an 8.9-fold (95% CI, 2.1- to 37.5-fold; $P = .003$) increased cardiomyopathy risk when compared with individuals with GG genotype.

Replication Stage

Demographic and clinical characteristics. Median cumulative anthracycline exposure was 300 mg/m^2, and median EF was 39% (Table 1).

Gene-environment interaction. Compared with cases with GG genotype, the odds for cases with GA and AA genotype of being in the high-dose anthracycline group were 3.6 (95% CI, 0.9 to 15.0; $P = .07$) and 4.5 (95% CI, 1.1 to 18.7; $P = .04$) times higher, respectively (Table 3). After inclusion of the presence or absence of hypertension and diabetes in the model, the odds of cases with AA genotype for being in the high-dose anthracycline group were 4.9 times higher (95% CI, 1.1 to 22.8; $P = .04$).

Final Model

We combined cases from the discovery and replication sets to test gene-environment interaction at SNP rs2232228. Cases with AA genotype had 3.7 (95% CI, 1.3 to 10.2; $P = .01$) times higher odds of being in the high-dose anthracycline group than those with GG genotype (Table 3).

Gene Expression

The relative *HAS3* mRNA levels in heart samples with homozygous A genotype (n = 16; geometric mean, 1.9) tended to be lower than the relative *HAS3* mRNA levels for heart samples carrying the GA genotype (n = 9; geometric mean, 7.8; $P = .09$; Data Supplement). Because of the small number (n = 3), hearts with GG genotype were not included in the analysis.

DISCUSSION

We used the high-density IBC cardiovascular SNP array to study the association between anthracycline-related cardiomyopathy and 34,912 SNPs in 2,100 carefully curated genes known to be associated with de novo cardiovascular disease.[7] Among individuals with a homozygous G genotype on SNP rs2232228, cardiomyopathy risk did not demonstrate any dose-dependent increase. However, in individuals with AA genotype, cardiomyopathy risk increased substantially as anthracycline exposure increased, such that among individuals exposed to high-dose anthracyclines, the presence of AA genotype conferred an 8.9-fold increased cardiomyopathy risk when compared with the GG genotype. This significant gene-environment interaction at SNP rs2232228 was successfully replicated by using an independent set of cases with anthracycline-related cardiomyopathy.

The *HAS3* gene, located on chromosome 16, encodes for an enzyme that produces low-molecular-weight hyaluronan (HA). HA is a ubiquitous component of extracellular matrix (ECM)[14] and plays a dynamic role in ECM organization following injury by providing a matrix to support cell migration and adhesion.[14-20] HA is especially enriched in matrices undergoing remodeling. Anthracyclines injure heart muscle through induction of cardiomyocyte apoptosis, which is then replaced by fibrosis.[21] Anthracycline-related injury is directly linked to the amount of anthracyclines in the heart.[22] The ECM provides a scaffold for alignment of cardiomyocytes, fibroblasts, endothelial cells, and vasculature after injury.[23] In fact, cardiac fibroblasts play a pivotal role in the repair and remodeling of the heart that occur following myocardial infarction, serving as a central mediator of cardiac remodeling by using the ECM as a scaffold.[24] An example of

Table 1. Characteristics of the Study Population in the Discovery and Replication Sets

Characteristic	Discovery Set					Replication Set	
	Cases (n = 93)		Controls (n = 194)			Cases (n = 76)	
	No.	%	No.	%	*P**	No.	%
Race/ethnicity†					Matched		
Non-Hispanic whites	93	100	194	100		45	59.2
Hispanics	0	0	0	0		21	27.6
Other	0	0	0	0		10	13.2
Age, years							
At primary cancer diagnosis					0.43		
Median	6.9		6.3			48	
Range	0-20.2		0-20.6			13-68	
At study participation					0.35		
Median	19.4		18.5			55	
Range	0.4-41.7		3.5-49.2			16-71	
Females	53	57.0	100	51.5	.74	44	58
Primary diagnosis†					Matched		
Hodgkin lymphoma	11	11.8	17	8.8		14	18.4
Non-Hodgkin lymphoma	11	11.8	15	7.7		34	47.4
Bone tumors	22	23.7	32	16.5		0	0
Soft tissue sarcoma	9	9.7	10	5.2		0	0
ALL	12	12.9	62	32.0		5	6.6
AML	8	8.6	20	10.3		10	13.2
Other	20	21.5	38	19.6		11	14.5
Year of primary cancer diagnosis†					Matched		
1990 or before	45	48.4	55	28.3		9	11.8
1991-2000	33	35.5	97	50.0		51	67.1
2001-2008	15	16.1	42	21.7		16	21.1
Length of follow-up, years†					.10		
Median	10.0		11.3			4.0	
Range	0.1-35.1		0.9-41.0			0.5-22.5	
Cumulative anthracycline exposure, mg/m^2					<.001		
Median	300		152			300	
Range	0-630		0-825			60-649	
0‡	7	7.5	43	22.2		0	0
1-100	2	2.2	31	16.0		1	1.3
101-150	6	6.5	22	11.3		8	10.5
151-200	4	4.3	13	6.7		6	7.9
201-250	9	9.7	21	10.8		12	15.8
251-300	20	21.5	15	7.7		22	29.0
> 300	45	48.4	49	25.3		27	35.5
1-250	21	22.6	87	44.9		27	35.5
> 250	65	69.9	64	33.0		49	64.5
Exposed to radiation to chest	23	24.7	22	11.3	.01	47	61.8
Dose, Gy§					.3		
Median	36		35			12	
Range	12-54		7.5-55.8			12-50	
Age at cardiomyopathy diagnosis, years							
Median	19.4		N/A			55	
Range	0.4-41.7					16-71	
Ejection fraction (%)							
Median	40		66‖			39	
Range	10-56		55-81			13-50	
HAS3 genotype¶					.42		
GG	13	14.0	38	19.6		14	18.4
GA	50	53.8	91	47.2		32	42.1
AA	30	32.2	64	33.2		30	39.5

Abbreviations: ALL, acute lymphatic leukemia; AML, acute myelogenous leukemia; N/A, not applicable.
*P values were obtained from conditional logistic regression or generalized linear model taking into consideration the matched set.
†Because of variation in the number of controls per case, the percentage of controls and cases in each category of a specific matching variable may not be identical.
‡Fifteen patients with no exposure to anthracyclines received radiation to chest (five cases, 10 controls).
§Among those who received radiation to chest.
‖Ejection fraction available for the 35 controls with anthracycline exposure.
¶One individual missed genotype call at SNP rs2232228 in the discovery data set.

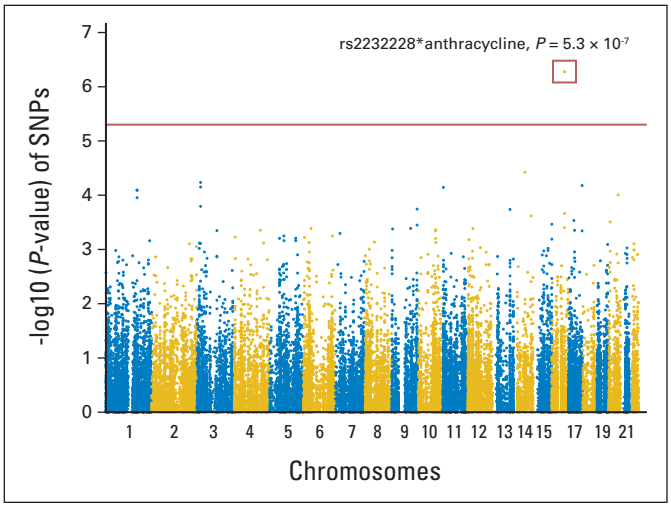

Fig 1. Results for test for a trend in the gene-environment (anthracycline) interaction between cardiomyopathy and each single nucleotide polymorphism (SNP) measured in the genome-wide association study. *P* values are shown for each SNP measured among the 93 cases with cardiomyopathy and 194 controls. Analyses are based on 34,912 SNPs (80.64%) on the ITMAT/Broad CARe SNP array. A result above the horizontal red line indicates strong evidence of association at $P < 5 \times 10^{-6}$.

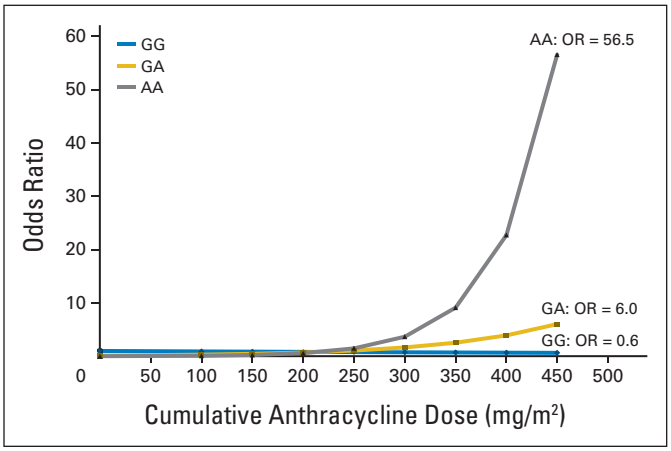

Fig 2. Risk of cardiomyopathy by anthracycline dose and genotype status (AA, GA, GG). Odds ratios (ORs) were calculated based on model 1, treating anthracycline dose as a continuous variable (reference group: GG genotype with no anthracycline exposure).

the role of HA in an injured myocardium is evidenced by the observation that HA accumulates after myocardial infarction in rats.[25] These observations suggest that ECM is involved in cardiac remodeling after anthracycline-related injury. The extent of remodeling and repair is possibly modulated by variability in HA production in the ECM, consistent with the genotype-dependent cardiomyopathy risk observed in this study.

Anthracyclines are cardiotoxic per se but gain further toxicity after one-electron reduction with reactive oxygen species (ROS) overproduction. Variants of genes involved in ROS production (nicotinamide adenine dinucleotide phosphate oxidase)[26] contribute to anthracycline-induced toxicity. HA has antioxidant activity[27] and interacts specifically with the CD44 receptor on cardiomyocytes,[28] maintaining integrity of the cardiomyocytes during ROS damage by stimulating cell proliferation[29] as well as preventing activation of death receptors, thus maintaining survival and function.[30] Recent in vitro data support the ability of HA to reduce ROS-mediated cardiac injury and activate the damage surveillance system.[31] Our genotype-phenotype analysis suggests a trend toward higher *HAS3* mRNA expression in heart samples with *HAS3* rs2232228 GA genotype (minimal risk of anthracycline-related cardiomyopathy) as compared with hearts with AA genotype (high cardiomyopathy risk at high doses of anthracyclines). Taken together, these data suggest that lower cardiac *HAS3* mRNA expression (AA genotype) may result in decreased synthesis of the antioxidant HA, supporting our findings of the high cardiomyopathy risk in individuals with AA genotype.

The SNP rs2232228 on exon 2 of *HAS3* (67701078 bp) resides on chromosome 16 and appears to be in a region of low LD. Our data contain seven SNPs in *HAS3*, but the LD between rs2232228 and the other six SNPs was not strong (the largest r^2 was 0.42 between rs2232228 and rs8047014); gene-environment interaction for rs8047014 was not significant. We also imputed the entire chromosome 16 by using 1,000 genome SNPs as reference. No gene-environment interaction was identified in the analysis based on SNPs with imputed r^2 more than 0.5. Finally, we examined the HapMap CEU data (Utah residents with Northern and Western European ancestry) within 1 Mb of rs2232228 and identified only two SNPs, both with weak LD (rs8082856 $r^2 = 0.56$; rs9332431 $r^2 = 0.579$).

The case-only design used to replicate significant findings is well established as an efficient and valid method for evaluating gene-environment interactions.[32] A positive (> two-fold) association implies a relevant gene-environment interaction.[33] This study demonstrated that cases homozygous for the A allele of

Table 2. Main and Modifying Effects of *HAS3* rs2232228 Genotypes on Dose-Dependent Risk of Anthracycline-Related Cardiomyopathy in Discovery Set

Cumulative Anthracycline Exposure (mg/m²)	*HAS3* rs2232228 Genotype Status	Risk of Cardiomyopathy		
		OR	95% CI	*P*
	Main Effect of *HAS3* Genotype			
All exposures*				
	GG	1.0		—
	GA	2.0	0.8 to 4.6	.12
	AA	1.8	0.7 to 4.7	.20
	Modifying Effect of *HAS3* Genotype†			
0-250‡	GG	1.0		—
	GA	0.5	0.2 to 1.8	.3
	AA	0.2	0.1 to 0.8	.03
> 250‡	GG	1.1	0.3 to 4.8	.9
	GA	5.2	1.6 to 17.4	.007
	AA	9.9	2.4 to 40.9	.002
	Patients Exposed to High-Dose Anthracyclines§			
> 250	GG	1.0		
	GA	4.7	1.4 to 16.2	.02
	AA	8.9	2.1 to 37.5	.003

Abbreviation: OR, odds ratio.
*ORs were obtained from conditional logistic regression adjusting for age at diagnosis, sex, chest radiation dose, and anthracycline exposure (continuous).
†Reference group: 0-250 mg/m² anthracycline exposure and rs2232228 GG genotype.
‡ORs were obtained from conditional logistic regression adjusting for age at diagnosis, sex, and chest radiation doses.
§Reference group: rs2232228 GG genotype for the anthracycline exposure level of > 250 mg/m².

Table 3. Analysis of Gene-Environment Interaction for Among Cases Only

HAS3 rs2232228 Genotype Status	Cumulative Anthracycline Exposure (mg/m^2) ≤ 250 No.	≤ 250 %	> 250 No.	> 250 %	OR*	95% CI	*P*
Replication set							
GG	8	29.6	6	12.2	1.0		
GA	11	40.8	21	42.9	3.6	0.9 to 15.0	.07
AA	8	29.6	22	44.9	4.5	1.1 to 18.7	.04
Combined discovery† and replication set							
GG	12	25.0	12	10.5	1.0		
GA	23	47.9	56	49.1	2.6	1.0 to 6.9	.05
AA	13	27.1	46	40.4	3.7	1.3 to 10.2	.01

Abbreviation: OR, odds ratio.
*ORs were calculated based on multivariable logistic regression adjusting for age at diagnosis, sex, chest radiation dose, and race/ethnicity (white *v* other).
†Cases of cardiomyopathy in the discovery set with no anthracycline exposure (n = 7) were excluded from this analysis.

rs2232228 in *HAS3* had a 3.7-fold increased odds of being in the high-dose anthracycline group, suggesting a valid gene-environment interaction in the replication stage.

For logistical reasons, we used a prevalent case-control study design. Prevalent case-control studies are vulnerable to the underestimation of effect size for genotypes associated with both increased disease risk and disease-associated lethality.[34,35] Applying this hypothetical scenario to this study, the lack of association between the GG genotype and cardiomyopathy risk could possibly represent a false-negative finding (ie, the GG genotype could be associated with an increased risk of cardiomyopathy and cardiomyopathy-related death), making the GG-associated cardiomyopathy cases unavailable for enrollment onto our study. But because there is no published data to support high cardiomyopathy-related lethality associated with the G allele of rs2232228, we believe that the lack of association between the GG genotype and the risk of cardiomyopathy is not affected by the study design. Furthermore, the replication set included all consecutive patients with cardiomyopathy (alive and deceased) from a cohort of hematopoietic cell transplantation recipients at a single institution. The frequency of GG genotype among cases in the discovery set (14%) and replication set (18%) was comparable ($P = .3$). Thus, successful replication of significant findings identified in the discovery set indicates that survival bias is likely not a significant issue with the prevalent cases and controls in the discovery set.

Of note, although the discovery set was limited to non-Hispanic white survivors of childhood cancer, the replication set included cases drawn from survivors of childhood and adult-onset cancer from all racial/ethnic backgrounds. The clear dose-response relation between anthracycline exposure and cardiomyopathy in both pediatric[1] and adult populations[5] suggests shared mechanisms for anthracycline-induce toxicity. Moreover, the successful replication of the finding in a clinically and demographically diverse population speaks to the robustness of the association between the common variants in rs2232228 in *HAS3* gene and anthracycline-related cardiomyopathy.

We used the IBC cardiovascular SNP array with a carefully curated yet comprehensive list of genes enriched for their association with de novo cardiovascular disorders. Our choice of the IBC array was based on our clinical observation that cardiovascular risk factors (diabetes, hypertension) interact with anthracycline exposure in cancer survivors to increase the risk of heart failure.[36] Thus, the main focus of this study was to identify the interaction of SNPs on the IBC array with anthracycline exposure and not to examine the main effects of these SNPs.

The IBC cardiovascular SNP array does not include genes that regulate anthracycline metabolism or disposition.[1,26,37] Thus, genes implicated in anthracycline-related cardiomyopathy such as *CBR3*[1] and *SLC28A3*[37] are not included on the IBC array. However, some SNPs from *ABCC1*[38] and *ABCC2*[26,39] genes are included, but the smallest *P* values did not meet the cutoff (5×10^{-6}) for our discovery stage (Data Supplement). As opposed to 10,000 independent associations examined in this study, these previous studies used a limited candidate gene approach with a higher likelihood of identifying associations without the penalty of a lower α level due to multiple testing.

In this study, we provide evidence that SNP rs2232228 in the *HAS3* gene alters the risk of anthracycline-related cardiomyopathy among patients exposed to high-dose anthracyclines. Our investigation establishes a foundation for understanding the functional significance of SNP rs2232228 and sets the stage for validation in a prospective cohort.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Conception and design: Leslie L. Robison, Mary V. Relling, Smita Bhatia
Financial support: Mary V. Relling, Smita Bhatia
Administrative support: Wendy Landier, Smita Bhatia
Provision of study materials or patients: Jill P. Ginsberg, Joseph P. Neglia, Sharon M. Castellino, Melissa M. Hudson, Smita Bhatia
Collection and assembly of data: Hakon Hakonarson, Lindsey Hageman, Yan Ding, Wendy Landier, Javier G. Blanco, Jill P. Ginsberg, Frank Keller, Joseph P. Neglia, Sharon M. Castellino, Irene Cherrick, Melissa M. Hudson, Mary V. Relling, Smita Bhatia
Data analysis and interpretation: Xuexia Wang, Wei Liu, Can-Lan Sun, Saro H. Armenian, Lu Chen, Adolfo Quiñones, Daniel Ferguson, Naomi Winick, Joseph P. Neglia, Sunil Desai, Charles A. Sklar, ZoAnn E. Dreyer, Yutaka Yasui, Mary V. Relling, Smita Bhatia
Manuscript writing: All authors
Final approval of manuscript: All authors

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Affiliations

Xuexia Wang, University of Wisconsin-Milwaukee, Milwaukee, WI; Wei Liu and Yutaka Yasui, University of Alberta; Sunil Desai, Stollery Children's Hospital, Edmonton, AB, Canada; Can-Lan Sun, Saro H. Armenian, Lindsey Hageman, Yan Ding, Wendy Landier, and Smita Bhatia, City of Hope, Duarte; Lu Chen, University of Southern California, Los Angeles, CA; Hakon Hakonarson and Jill P. Ginsberg, Children's Hospital of Philadelphia, Philadelphia, PA; Javier G. Blanco, Alfo Quiñones, and Daniel Ferguson, The State University of New York at Buffalo, Buffalo; Charles A. Sklar, Memorial Sloan-Kettering Cancer Center, New York City; Irene Cherrick, Upstate Medical University, Syracuse, NY; Naomi Winick, University of Texas Southwestern Medical Center, Dallas; Zoann E. Dreyer, Baylor College of Medicine, Houston, TX; Frank Keller, Children's Healthcare of Atlanta and Emory University School of Medicine, Atlanta, GA; Joseph P. Neglia, University of Minnesota Medical School, Minneapolis, MN; Sharon M. Castellino, Wake Forest University Health Sciences, Winston-Salem, NC; and Melissa M. Hudson, Leslie L. Robison, and Mary V. Relling, St. Jude Children's Research Hospital, Memphis, TN.

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Appendix

Table A1. Participating Children's Oncology Group Institutions

A.B. Chandler Medical Center-University of Kentucky
A.I. duPont Hospital for Children
Advocate Hope Children's Hospital
All Children's Hospital
Allan Blair Cancer Centre
Baptist Children's Hospital
British Columbia's Children's Hospital
Brooklyn Hospital Center
C.S. Mott Children's Hospital
Cancer Research Center of Hawaii
CancerCare Manitoba
Cedars-Sinai Medical Center
Children's Healthcare of Atlanta, Emory University
Children's Hospital and Clinics, Minneapolis and St. Paul
Children's Hospital London Health Sciences
Children's Hospital Los Angeles
Children's Hospital Medical Center, Akron, Ohio
Children's Hospital Oakland
Children's Hospital of Eastern Ontario
Children's Hospital of Michigan
Children's Hospital of Philadelphia
Children's Hospital of the Greenville Hospital System
Children's Hospital of the King's Daughters
Children's Medical Center Dayton
Children's Memorial Medical Center at Chicago
Children's National Medical Center, Washington, DC
Children's of New Orleans/Louisiana State University Medical Center Community Clinical Oncology Program
Cincinnati Children's Hospital Medical Center
City of Hope National Medical Center
Connecticut Children's Medical Center
Cook Children's Medical Center
Dana-Farber Cancer Institute and Children's Hospital
Driscoll Children's Hospital
East Tennessee Children's Hospital
East Tennessee State University
Eastern Maine Medical Center
Emanuel Hospital-Health Center
Hackensack University Medical Center
Helen DeVos Children's Hospital
Hospital Sainte-Justine
Hospital for Sick Children
Hurley Medical Center
Indiana University-Riley Children's Hospital
Inova Fairfax Hospital
IWK (Izaak Walton Killam) Health Centre
Kaiser Permanente Medical Group, Inc., Northern California
Kalamazoo Center for Medical Studies
Kingston General Hospital/Kingston Regional Cancer

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Table A1. Participating Children's Oncology Group Institutions (continued)

Kosair Children's Hospital
MD Anderson Cancer Center
Maimonides Medical Center
Mayo Clinic and Foundation
McGill University Health Center-Montreal Children's Hospital
McMaster University
Medical College of Georgia Children's Medical Center
Memorial Sloan-Kettering Cancer Center
Methodist Children's Hospital of South Texas
Miami Children's Hospital
Michigan State University
Midwest Children's Cancer Center
Nationwide Children's Hospital
Nemours Children's Clinic-Jacksonville
Nevada Cancer Research Foundation-Community Clinical Oncology Program
New York Medical College
Newark Beth Israel Medical Center
Primary Children's Medical Center
Princess Margaret Hospital for Children
Rady Children's Hospital San Diego
Rainbow Babies and Children's Hospital
Royal Children's Hospital, Brisbane
Royal Children's Hospital, University of Melbourne
Sacred Heart Children's Hospital
Sacred Heart Hospital
Saint Barnabas Medical Center
Saint Peter's University Hospital
Saskatoon Cancer Center
Scott and White Memorial Hospital
Seattle Children's
South Carolina Cancer Center
St. John Hospital and Medical Center
St. Joseph's Hospital and Medical Center
St. Jude Children's Research Hospital-Memphis
St. Vincent Children's Hospital-Indiana
St. Vincent Hospital-Wisconsin
Stanford University Medical Center
State University of New York at Stony Brook
Stollery Children's Hospital
State University of New York Upstate Medical University
Swiss Pediatric Oncology Group-Geneva
Tampa Children's Hospital
Texas Children's Cancer Center at Baylor College of Medicine
Texas Tech University Health Sciences Center-Amarillo
The Children's Hospital, Denver, CO
The Children's Hospital of Southwest Florida Lee Memorial Health System
The Children's Mercy Hospital
The University of Chicago Comer Children's Hospital
Tulane University Medical Center
University of California at Los Angeles David Geffen School of Medicine
University of Alabama
University of Florida
University of Iowa Hospitals and Clinics
University of Kansas Medical Center
University of Minnesota Cancer Center
University of Mississippi Medical Center Children's Hospital
University of Missouri-Columbia
University of New Mexico School of Medicine
University of North Carolina at Chapel Hill
University of Oklahoma Health Sciences Center

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Table A1. Participating Children's Oncology Group Institutions (continued)

University of Pittsburgh
University of Texas Health Science Center at San Antonio
University of Vermont College of Medicine
University of Wisconsin-Children's Hospital Madison
University of Texas Southwestern Medical Center
Vanderbilt Children's Hospital
Virginia Commonwealth University Health System
Wake Forest University School of Medicine
Washington University Medical Center
West Virginia University Health Sciences Center-Charleston
Winthrop University Hospital
Women's and Children's Hospital, Adelaide
Yale University School of Medicine