Hyaluronidase synthase 3 *(HAS3)* variant and Anthracycline-related Cardiomyopathy – A Report from the Children's Oncology Group

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Online only Materials

eMethods

Discovery set: Validation of Cases with Cardiomyopathy

At enrollment, an echocardiogram report documenting the presence of cardiomyopathy was submitted for all cases. In addition, the study required documentation of the presence or absence of symptoms at the time of diagnosis of cardiomyopathy, so that the cases could be categorized according to American Heart Association (AHA) criteria as having symptomatic cardiomyopathy, if they had symptoms (dyspnea, orthopnea and/or fatigue) and/or signs (edema, hepatomegaly, and/or rales) of cardiac decompensation. Conversely, patients were classified as having asymptomatic cardiomyopathy if they had echocardiographic features of left ventricular dysfunction as evidenced by ejection fraction ≤40% and/or fractional shortening ≤28%, in the absence of signs or symptoms suggestive of cardiac decompensation. Thus, for each case of cardiomyopathy, the following report was submitted by the participating site:

Symptomatic

- Diagnosis of cardiomyopathy based on clinical criteria: dyspnea, pulmonary edema, peripheral edema, orthopnea, fatigue, or hepatomegaly
- Echocardiogram at time of diagnosis of cardiomyopathy

  Date of diagnosis: Date of report:

Asymptomatic

- Echocardiogram with ejection fraction ≤40% and/or fractional shortening ≤28%
- Absence of symptoms of cardiomyopathy, such as dyspnea, pulmonary edema, peripheral edema, orthopnea, fatigue, or hepatomegaly

  Date of diagnosis: Date of report:

Replication set: Validation of Cases with Cardiomyopathy

Medical records maintained at City of Hope (COH) were the primary source of data, and were used to abstract details regarding cardiac dysfunction. The COH Long-term Follow-up (LTFU) Program follows patients who have undergone hematopoietic cell transplantation (HCT). The following protocol is used to ensure complete follow-up after HCT. If the date of last medical visit at COH is not recent, or if there are any gaps in patients' history within the window of interest, a standard protocol is used to identify and contact physicians who are treating patients outside COH to obtain relevant details regarding patient health. If the physician is not available or unable to provide recent information, the patient is contacted to obtain this information. The human subjects committee at COH approved the protocol. Informed consent was provided according to the Declaration of Helsinki. Case definition of congestive heart failure (CHF) was per the American Heart Association (AHA)/ American College of Cardiology (ACC) guidelines; this required clinician documentation of patient symptoms (dyspnea, orthopnea and/or fatigue) and signs (edema, hepatomegaly and/or rales) consistent with CHF. Diagnostic echocardiogram reports were used to document extent of cardiac compromise.

Genomic Inflation Factor

The overall genomic control inflation factor was first proposed by Devlin and Roeder in 1999 and is a ratio of the median observed test statistic to its theoretical expected value.[1] It is used widely as a divisor of the test statistic to adjust for inflation of test statistic caused by various potential biases including population stratification.[2]

Population stratification, a recognized issue in genetic association studies, emerges when there is systematic difference in allele frequencies among study subjects due to ancestry. Unrecognized population stratification can lead to both false-positive and false-negative findings and can obscure true association signals if not corrected appropriately; controlling population stratification has become a routine in GWAS.

Although we have matched case-control status with race/ethnicity as one of the matching criteria, and filtered non-Hispanic white individuals with principal component analysis in our quality control procedure, there may be fine-scale population structures within ethnic groups or population admixture that cannot be accounted for by matching. In the presence of these population structures, there will be many more (false) positive signals of association than we would expect by chance. Therefore, we wanted to use the overall genomic control inflation factor to further correct population stratification. Using this popular simple method, we can correct the overall inflation of the test statistics and prevent excess false positives created by potential biases.

Gene expression analysis

Tissue procurement and processing: Twenty-eight heart samples were procured from the National Disease Research Interchange (Philadelphia, PA). Tissue procurement protocols included the following criteria: no current diagnosis of cancer, myocardial infarction, and/or congestive heart failure, no evidence of sepsis, and no history of chemotherapy and/or radiation within the last year. Postmortem to tissue recovery interval was ≤10 hours. Heart samples were frozen immediately after recovery and stored in liquid nitrogen until further processing. Cardiac DNA and RNA were isolated by following standardized procedures.

HAS3 rs2232228 was genotyped with TaqMan genotyping assays (Applied Biosystems, Carlsbad, USA). Genotyping runs included appropriate controls for each HAS3 rs2232228 genotype combination. Genotyping controls were verified by direct DNA sequencing with gene-specific primers.

Total RNA from heart (10 ng) was reverse transcribed and amplified by using one-step QuantiTect SYBR Green RT-PCR kits (QIAGEN, Valencia, CA). *HAS3* primers were 5'-AGCCTTGGCTACCGAACTAA-3' (forward) and 5'-TATAACCGTGGCAATGAGGA-3' (reverse). *ACTB* primers were 5'-GGACTTCGAGCAAGAGATGG-3' (forward) and 5'-AGCACTGTGTTGGCGTACAG-3' (reverse). *HAS3 mRNA* levels were obtained after normalization to the reference gene (*ACTB*) according to the approach described by Simon.[3] The following equation was used: $MNE=E_{ref}^{Ct(ref,mean)}/E_{target}^{Ct(target,mean)}$, where MNE stands for mean normalized expression; E_{ref} is the efficiency of reference gene; E_{target} is target gene efficiency; $Ct_{ref,mean}$ is the mean CT value of the reference gene; and $Ct_{target,mean}$ stands for the mean Ct value of the target gene. *HAS3* mRNA values were expressed relative to the *HAS3* mRNA content of heart sample 21, which was given an arbitrary value of 100.

eResults

Semsei et al (***Cell Biol Int 2012***) examined the association between 9 polymorphisms in the *ABCC1* gene and left ventricular dysfunction. The *ABCC1* rs3743527TT genotype and rs3743527TT-rs246221TC/TT genotype combination were associated with lower LV fractional shortening.[4] The *ABCC1* gene was included on the IBC array, and the smallest p-value of main effect among all SNPs on *ABCC1* in our study was 0.0136 (SNP rs246214), and the smallest p-value for a gene-environment interaction effect among all SNPs on *ABCC1* was 0.071 (rs8053266). Both these p-values are far less than the significant cutoff ($5x10^{-6}$) of our Discovery stage. Among the 1,350 SNPs annotated in gene *ABCC2* (http://www.ncbi.nlm.nih.gov/gene; also known as MRP2), there are only a dozen in the IBC array; of these, ten were retained in our final analysis. The smallest p-value of main effect among all SNPs on *ABCC2* was 0.056 (at rs4148391), and the smallest p-value of gene-environment interaction effect was 0.095 (at rs4148399). Both failed to pass the significant cutoff ($5x10^{-6}$). Wojnowski et al (***Circulation, 2005)***[5] had examined the association between SNP (rs8187710) in *ABCC2* gene and doxorubicin-induced cardiotoxicity (using a candidate gene approach), and demonstrated a relation with acute, but not with chronic heart failure. Elens et al[6] examined the functional defect caused by the 4544G>A SNP (rs8187710) by transfecting human embryonic kidney cell (HEK293) with full-length ABCC2 cDNA coding the wildtype or the variant *ABCC2* allele, and reported that C1515Y amino acid substitution caused by the 4544G>A SNP in *ABCC2* impairs its ATPase activity and is associated with a higher accumulation of ABCC2 substrates (***Elens et al., Pharmacogenet Genomics, 2011***). Although this SNP (rs8187710) was present on the IBC array, both the main effect (p=0.298) and gene-environment interaction effect (p=0.162) association between the SNP rs8187710 in the *ABCC2* gene and cardiomyopathy were not statistically significant in our study. We believe that the lack of association in our current study is in part due to the fact that (as opposed to 10,000 independent associations examined in the current study), the Semsie study (***Cell Biol Int 2012***) and the Wojnowski study utilized a limited candidate gene approach (9 and 206 SNPs, respectively) and did not correct for multiple comparisons, with a higher likelihood of identifying associations, without the penalty of significant reduction in power due to multiple testing (as was the case in our study).

eTable 1: Calculation of Cumulative Anthracycline Exposure

Name of Anthracycline	Conversion factor to Doxorubicin Isotoxic Dose
Doxorubicin	**1**
Daunomycin	**0.833 (5/6)**
Idarubicin	**5**
Epirubicin	**0.67 (2/3)**
Mitoxantrone	**4**

eTable 2: Risk of Cardiomyopathy with and without 33 controls without echocardiographic details

	Exclude 33 controls		Include 33 controls	
	OR (95% CI)	**P value**	**OR (95% CI)**	**P value**
Gender				
Males	1.0		1.0	
Females	0.79 (0.41-1.54)	0.49	0.84 (0.45-1.59)	0.60
Age at Primary Cancer Diagnosis				
Per year increase	0.97 (0.90-1.05)	0.44	0.97 (0.90-1.04)	0.40
Chest Radiation				
No	1.0			
Yes	5.88 (1.76-19.62)	0.004	5.09 (1.67-15.47)	0.004
Cumulative Anthracycline Exposure (mg/m^2)				
0 mg/m^2	1.0		1.0	
1-250	6.15 (1.49-25.41)	0.01	5.03 (1.25-20.25)	0.02
251+	34.76 (7.63-158)	<0.0001	33.63 (7.58-149)	<0.0001

Odds ratios were obtained using multivariate conditional logistic regression.

e**Table 3. Model fit statistics**

Criterion	Without Covariates	With Covariates
AIC	196.413	131.821
SC	196.413	153.757
-2 Log L	196.413	119.821

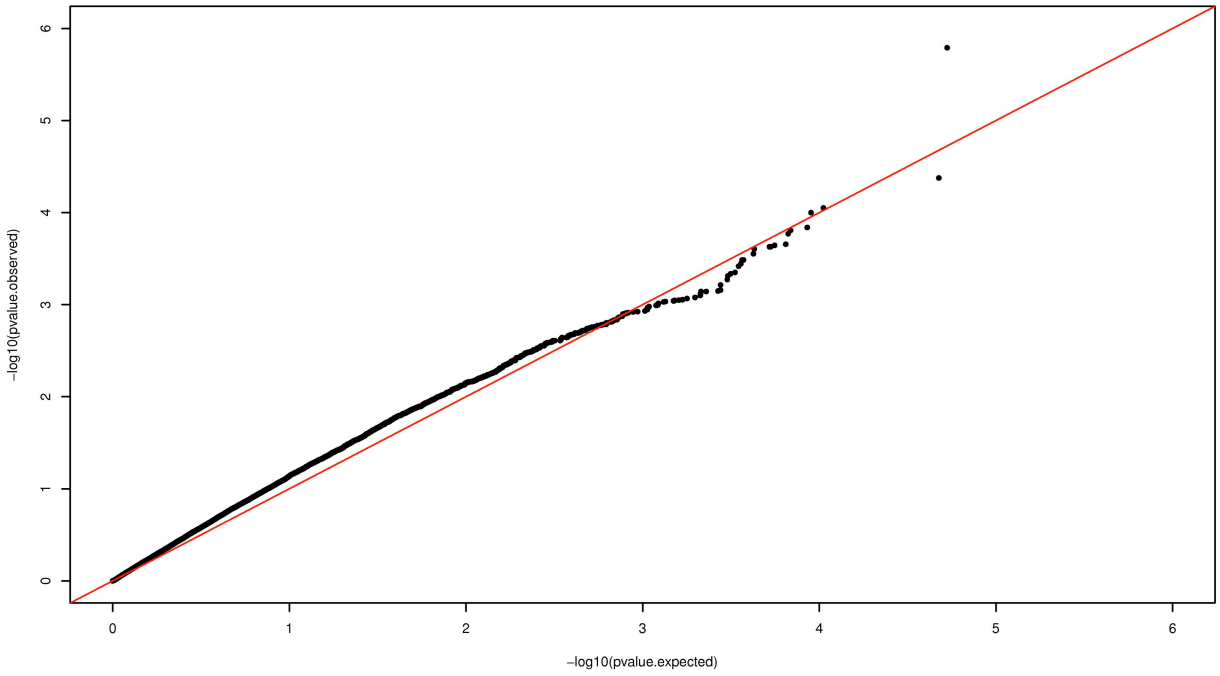
eTable 4. Testing Global Null Hypothesis: Beta=0

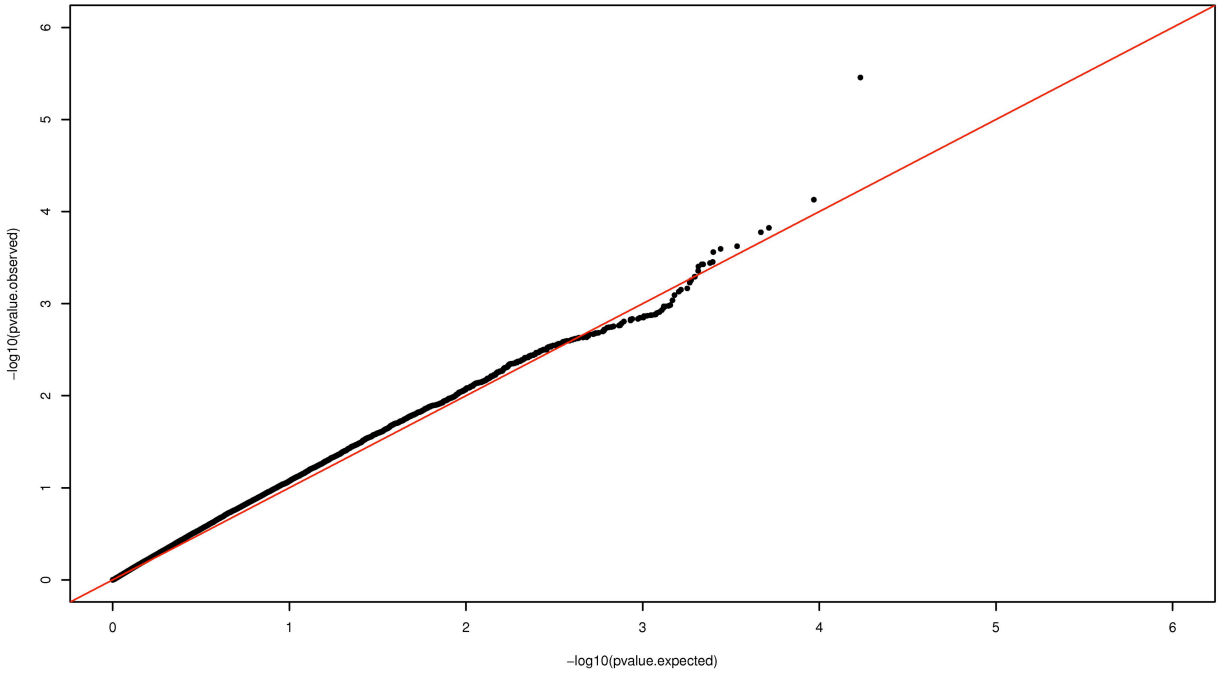
Test	Chi-Square	DF	Pr > Chi Sq
Likelihood Ratio	76.5915	6	<.0001
Score	55.2771	6	<.0001
Wald	37.1423	6	<.0001

eTable 5. Analysis of Conditional Maximum Likelihood Estimates

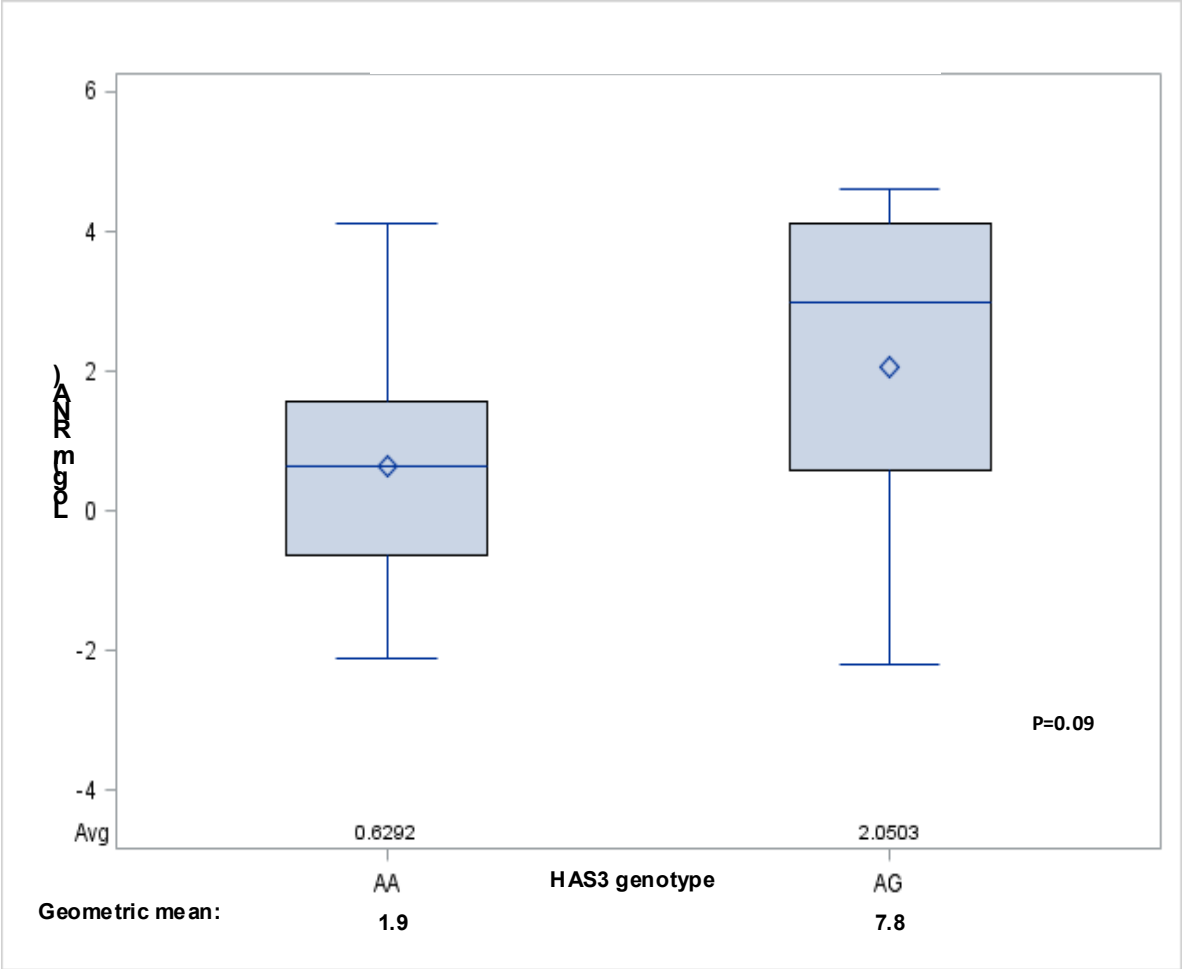
Parameter	DF	Estimate	Standard Error	Wald Chi-Square	Pr > Chi Sq
SEX	1	-0.0624	0.3461	0.0325	0.85688316
Age at diagnosis	1	-0.0393	0.0376	1.0927	0.29588328
Radiation to heart	1	0.000661	0.000182	13.1410	0.00028890
Anthracycline dose	1	-0.00099	0.00163	0.3700	0.54302528
***HAS3* genotype**	1	-2.0890	0.5289	15.6010	0.00007822
Anthracycline**HAS3***	1	0.00962	0.00192	25.1507	0.00000053

eFigure 1. Q-Q plots for the gene environment (anthracycline) interaction, before and after adjusting for the genomic control inflation factor





eFigure 2. Distribution of geometric mean of mRNA expression by *HAS3* genotype



References

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