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Exome array analysis identifies *ETFB* as a novel susceptibility gene for anthracycline-induced cardiotoxicity in cancer patients

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ABSTRACT

Background

Anthracyclines are widely used chemotherapeutic drugs that can cause progressive and irreversible cardiac damage and fatal heart failure. Several genetic variants associated with anthracycline-induced cardiotoxicity (AIC) have been identified, but they explain only a small proportion of the interindividual differences in AIC susceptibility.

Patients and Methods

In this study we evaluated the association of low-frequency variants with risk of chronic AIC using the Illumina HumanExome BeadChip array in a discovery cohort of 61 anthracycline-treated breast cancer patients with replication in a second independent cohort of 83 anthracycline-treated pediatric cancer patients, by using gene-based tests (SKAT-O).

Results

The most significant associated gene in the discovery cohort was *ETFB* (electron transfer flavoprotein beta subunit) involved in mitochondrial β-oxidation and ATP production (P=4.16x10^{-4}) and this association was replicated in an independent set of anthracycline-treated cancer patients (P=2.81x10^{-3}). Within *ETFB* we found that the missense variant rs79338777 (p.Pro52Leu; c.155C>T) made the greatest contribution to the observed gene association and it was associated with increased risk of chronic AIC in the two cohorts separately and when combined (OR=9, P=1.95×10^{-4}, 95%CI=2.83–28.6).

Conclusions

We identified and replicated a novel gene, ETFB, strongly associated with chronic AIC independently of age at tumor onset. Although experimental verification and further studies in larger patient cohorts are required to confirm our finding, we demonstrated that exome array data analysis represents a valuable strategy to identify novel genes contributing to the susceptibility to chronic AIC.

KEYWORDS

Long-term cancer survivors, anthracycline, chronic cardiotoxicity, low-frequency variants, predictive genes

KEY MESSAGE

Previous efforts made to understand the interindividual variability in AIC risk have focused exclusively on common variants mainly through a candidate gene strategy. In this study, we evaluated the association of low-frequency variants at genome-wide level by exome-array analysis. We identified and replicated *ETFB* as a novel gene strongly associated with risk of chronic AIC in cancer patients.

INTRODUCTION

Anthracyclines are highly effective chemotherapeutic agents used in a wide range of cancers, including hematopoietic and solid tumors. However, a prominent dose-limiting side effect of treatment with anthracyclines is cardiac damage [1]. Anthracycline-induced cardiotoxicity (AIC) may occur during treatment (acute) or can be delayed, being diagnosed within the first year of treatment (early onset) or many years after completion of therapy (late onset) [2]. Both chronic forms are characterized by an irreversible left ventricle (LV) dysfunction that can be progressive and, which, in some cases leads to heart failure and death [3]. In addition to clinical cardiotoxicity, chronic AIC can manifest as asymptomatic cardiotoxicity [4]. The cardiac effects of anthracycline chemotherapy are highly variable between individuals, suggesting a genetic component to AIC susceptibility, apart from well-known risk factors [5]. Several studies [6–20] have identified genetic variants associated with AIC, including genetic polymorphisms in genes involved in anthracycline transport and metabolism (e.g., *SLC28A3* [12, 13]*, ABCB1* [12]*, ABCC1* [10, 12]*,* and *CBR3* [7, 9, 11, 20]) or genes involved in the mechanism of oxidative stress-mediated AIC (e.g., NADPH oxidase multi-enzyme complex: *NCF4* [6, 14]*, RAC2* [14] and *CYBA* [14]). Because all previous efforts have been focused on the identification of common susceptibility variants (minor allele frequency (MAF) ≥5%) and the vast majority [6–18] via a candidate gene approach; it is plausible that analyses of low-frequency (MAF<5%) variants could explain additional interpatient variability in susceptibility to AIC. To investigate this hypothesis, we performed a genome-wide association analysis using the Illumina HumanExome Beadchip, which is enriched for low-frequency coding variants

(>80% variants with MAF ≤1%) [21], in Spanish breast cancer patients treated with anthracyclines with replication in an independent cohort of anthracycline-treated pediatric cancer patients.

MATERIALS AND METHODS

Patients

Discovery cohort: 71 patients with pathological confirmed locally advanced breast cancer and treated at the *San Carlos* University Hospital, (Madrid, Spain) were included. These patients were aged older than 18 years at cancer diagnosis and were enrolled in a neoadjuvant phase II randomized clinical trial [22], as previously described [23]. Patients were randomly assigned to receive four cycles of either neoadjuvant doxorubicin (75 mg/m^2) (39 patients) or neoadjuvant docetaxel (100 mg/m^2) (32 patients) every 3 weeks. After surgery, patient treatment assignment was crossed-over to receive four cycles of the opposite drug.

Replication cohort: 83 anthracycline-treated patients aged less than 30 years and treated at the *La Paz* University Hospital or *Niño Jesús* University Hospital in Madrid or at the University Clinic of Navarra in Pamplona were included. Details of the replication cohort have been described elsewhere [24]. All patients were treated with doxorubicin, daunorubicin or epirubicin as part of their chemotherapy protocol.

Patients in both cohorts received anthracyclines as part of their chemotherapy protocol, had normal cardiac function before anthracycline chemotherapy and

had echocardiographic evaluations (prechemotherapy and postchemotherapy). Patients were excluded if they had a personal history of cardiac disease or were treated with concomitant (neo) adjuvant use of trastuzumab, because of its well-known association with cardiotoxicity. Written informed consent was obtained from adult patients and from the parents or legal guardians of children. The study was approved by the ethics committees of each participating hospital.

Patient medical records were reviewed by oncologists and cardiologists. Demographic, clinical and therapeutic information extracted from medical records included demographics, disease characteristics, chemotherapy, diagnostic echocardiograms to document baseline and follow-up cardiac function and any cardiac compromise and its severity, and any symptoms or signs consistent with chronic AIC.

AIC definition. AIC in breast cancer patients was defined as early or late-onset (i) cardiac failure grade 3–5 using the CTCAE 4.0 scoring system (grade 3: severe symptoms at rest or with minimal activity or exertion, intervention indicated; grade 4: life-threatening consequences, urgent intervention indicated; 5: death) [25] (ii) asymptomatic decrease of left ventricular ejection fraction (LVEF) ≥10%. Control patients were defined as those having no symptoms or signs of cardiac complications and normal echocardiograms (with a LVEF>60% at both baseline and follow-up and with a decline in LVEF≤5%) during and after therapy. Pediatric cases were required to have early/late-onset LV dysfunction evidenced by symptoms/signs of severe mitral valve insufficiency, pericardial effusion, LV hypertrophy or pulmonary hypertension. The criteria for determining a symptomatic event were established by pediatric cardiologists.

Asymptomatic pediatric cases had shortening fraction (SF) ≤ 27% any time after anthracycline treatment completion. Pediatric controls had normal echocardiograms (SF≥35%) during and after anthracycline therapy.

To rule out acute AIC, only echocardiograms obtained 30 days or more after an anthracycline dose were considered.

Methods

To determine the role of low-frequency variants, patients from the discovery cohort were genotyped for the 247,870 variants on the Illumina HumanExome-12v1_A Beadchip (Illumina, San Diego) array according to the manufacturers´ recommended protocols. We conducted gene-based tests using the optimized sequence kernel association test (SKAT-O) [26, 27] and considering only genes with at least 3 genotyped variants and age at diagnosis as covariate. Genes with a $P<5x10^{-4}$ in the discovery cohort were assessed in the replication cohort (with available genetic data for the Illumina HumanExome array [24]), using the same statistical methods. While the SKAT-O does not provide any parameter estimates, sensitivity analyses for individual variants within replicated genes were applied. The impact of selected variants on protein structure or function was assessed using *in silico* predictions. Details of genotyping, statistical analyses and *in silico* prediction are provided in the **Supplementary Material**.

RESULTS

The main patient´s demographic and clinical characteristics of the discovery and replication cohorts are shown in Table 1.

We carried out gene-based analysis to investigate the role of low-frequency variants in chronic AIC using the (SKAT-O) test. The most significantly gene associated with chronic AIC in the discovery cohort was *ETFB* (electron transfer flavoprotein beta subunit), which encodes a cardiac protein involved in mitochondrial β-oxidation and ATP production [28] (P=4.16x10^{-4}) (**Table 2**). Given that cardiac mitochondria are preferential targets of anthracyclines, we assessed the association of *ETFB* in an independent cohort of 83 anthracycline-treated pediatric cancer patients and this association was replicated (P=2.81x10^{-3}) (**Table 2**).

Examination of variants within *ETFB* revealed that the low-frequency variant rs79338777 (p.Pro52Leu; c.155C>T) made the greatest contribution to the observed association. The minor T allele of rs79338777 was more common in cases in the discovery cohort (MAF_{CASES}=13% *v* $MAF_{CONTROLS}$=2%) (**Table 3**). Consistently, the T risk allele was more common in cases than in controls in the replication cohort (MAF_{CASES}=18% *v* $MAF_{CONTROLS}$=7%) (**Table 3**).Combined analysis revealed that rs79338777 was significantly associated with chronic AIC (OR=9, P=1.95×10^{-4}, 95%CI=2.83–28.6) (**Table 3**). Overall, it was found that the T risk allele for rs79338777 to be 3.55 times more frequent in cases than in controls (MAF_{CASES}=16% *v* $MAF_{CONTROLS}$=5%).

In order to evaluate the impact of the missense variant rs79338777 (p.Pro52Leu) on ETFB protein structure or function, we applied four *in silico* prediction algorithms. Interestingly, rs79338777 was classified as pathogenic by the consensus classifier Predict-SNP and SIFT, and as possibly damaging by PolyPhen-2.

DISCUSSION

Genetic variation has been shown to influence susceptibility to AIC; however, the contribution of rare and low-frequency variants to the interindividual variation in AIC occurrence remains unexplored. Exome chip arrays constitute a cost-effective alternative to whole-exome sequencing and have proven their capacity to identify low-frequency and rare variants associated with complex diseases [29–32]. In the present study, we have identified and replicated *ETFB* as a novel gene associated with chronic AIC susceptibility in anthracycline-treated cancer patients by exome-array analysis.

ETFB is the β subunit of the heterodimer electron transfer flavoprotein (ETF) protein located in the inner mitochondrial membrane. ETF acts as an electron acceptor of energy production from amino acid and fatty acids that transfers electrons to the main respiratory chain via the ETF ubiquinone oxidoreductase (ETF-QO) and subsequent ATP production [33]. Fatty acids are the main energy substrate of the heart and alterations in mitochondrial fatty acid oxidation occur in different forms of heart disease including heart failure, ischaemic heart disease and diabetic cardiomyopathy [34]. Anthracycline therapy is known to inhibit long chain fatty acid oxidation and transport across mitochondrial membrane [35] and mitochondrial dysfunction (decrease ATP production, direct damage to the mitochondria and mitochondria dependent apoptosis) along with oxidative stress have been proposed as major contributors to anthracycline mediated myocardial injury [35,36]. Analysis of protein expression in doxorubicin-treated adult rat cardiomyocytes revealed differential downregulation of *ETFB* [38]. In addition, proteomic analyses of

cardiac proteins from mice treated with doxorubicin showed elevated oxidative modifications of cardiac proteins, including ETF-QO, and these oxidative modifications altered their enzymatic activity [39], thus compromising ATP production in cardiac mitochondria. Taken together, these findings indicate that *ETF* is an important target in anthracycline-mediated mitochondrial dysfunction. The fact that *ETFB* was associated to chronic AIC in both breast and pediatric cancer patients points to mitochondrial dysfunction as a molecular mechanism of AIC independent of age at tumor onset. Within *ETFB,* we found that the variant allele of rs79338777, which has a predicted pathogenic effect according to two of the *in silico* prediction algorithms used, including the consensus classifier, was significantly associated with higher risk of developing chronic AIC in the combined analysis.

Strengths of this study include that it was based on a well-characterized series of uniformly anthracycline-treated patients with extensive patient, tumor and therapy-related information and the notable long-term follow-up of patients, which is critical for a clear distinction between controls and cases of chronic AIC. The main limitation of the present study is the relatively small sample size of individual cohorts.

Although further analysis in larger cohorts of patients and functional characterization of the precise role of the *ETFB* gene in chronic AIC are required, this study demonstrates that exome-array genotyping is a valuable approach to identify novel genes that contribute to chronic AIC susceptibility.

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Table 1. Patient clinical characteristics								
	Discovery cohort (*N*=71)				**Replication cohort (*N*=83)**			
	Controls (*N*=53)		Cases (*N*=18)		Controls (*N*=52)		Cases (*N*=31)	
Characteristic	*N*	%*	*N*	%*	*N*	%*	*N*	%*
Age at diagnosis (years)								
Median	49		59.5		5.1		10.4	
Range	27-73		36-72		1.4-16.9		1.2-21.1	
Sex								
Female	53	100	18	100	23	44	7	23
Male	-	-	-	-	29	56	24	77
Primary diagnosis (tumor type)								
Breast cancer								
Ductal	42	79	13	72	-	-	-	-
Lobular	8	15	4	22	-	-	-	-
Others	3	5.7	1	5.6	-	-	-	-
Pediatric cancer								
Leukemia	-	-	-	-	45	87	12	39
Osteosarcoma	-	-	-	-	3	5.8	9	29
Ewing Sarcoma	-	-	-	-	4	7.7	10	32
Tumor grade								
1	1	1.9	-	-	-	-	-	-
2	36	68	14	78	-	-	-	-
3	16	30	4	22	-	-	-	-
Radiotherapy**	25	47	6	33	-	-	2	6.5
Anthracycline type								
Doxorubicin	53	100	18	100	44	85	29	94
Epirubicin	-	-	-	-	5	9.6	-	-
Daunorubicin					8	15	1	3.2
Cumulative anthracycline dose (mg/m^2)								
Median	298.6		298.4		134		360	
Mean	282.9		298.1		189.2		362.7	
Range	150-375		200-588		49.2-562		105-780	
Follow-up (years)								
Median	4.76		5.74		8.55		10	
Range	2-16		1.19-10.07		1-24.1		1-27.5	

* Percentages are computed based on the total number of non-missing values. a Radiotherapy includes mediastinal and mantle radiation and total body irradiation. b Cumulative anthracycline dose was calculated using doxorubicin equivalents.

Table 2. Association results for *ETFB* with chronic AIC in cancer patients

Gene	Chr.	Start	End	Discovery cohort (*N*=61)			Replication cohort (*N*=83)	
				Number of variants	*P*	P_{FDR}	Number of variants	*P*
ETFB	19	51,848,546	51,869,541	4	4.16×10^{-4}	0.77	4	2.81×10^{-3}

Positions are based on Genome Reference Consortium Human Build 37 (GRCh37/hg19). Associations between *ETFB* and risk of chronic AIC were assessed using SKAT-O considering only genes with at least 3 genotyped variants and including important clinical covariates (discovery cohort: age at diagnosis; replication cohort: age at diagnosis, cumulative anthracycline dose and bleomycin concomitant therapy)

Table 3. Association of variant rs79338777 (*ETFB*) with chronic AIC

Variant	Gene	Discovery cohort (*N*=61)			Replication cohort (*N*=83)			Overall combined logistic regression (*N*=144)		
		MAF cases	MAF controls	MAF	MAF cases	MAF controls	MAF	*P*	OR	95%CI
rs79338777 (C>T)	*ETFB*	0.13	0.02	0.04	0.18	0.07	0.11	1.95×10^{-4}	9	2.83–28.6

Covariates for the logistic regression in the combined analysis were age at diagnosis, cumulative anthracycline dose and whether patients had breast o pediatric tumors. Abbreviations: MAF, minor allele frequency; OR, odds ratio; CI, confidence interval.

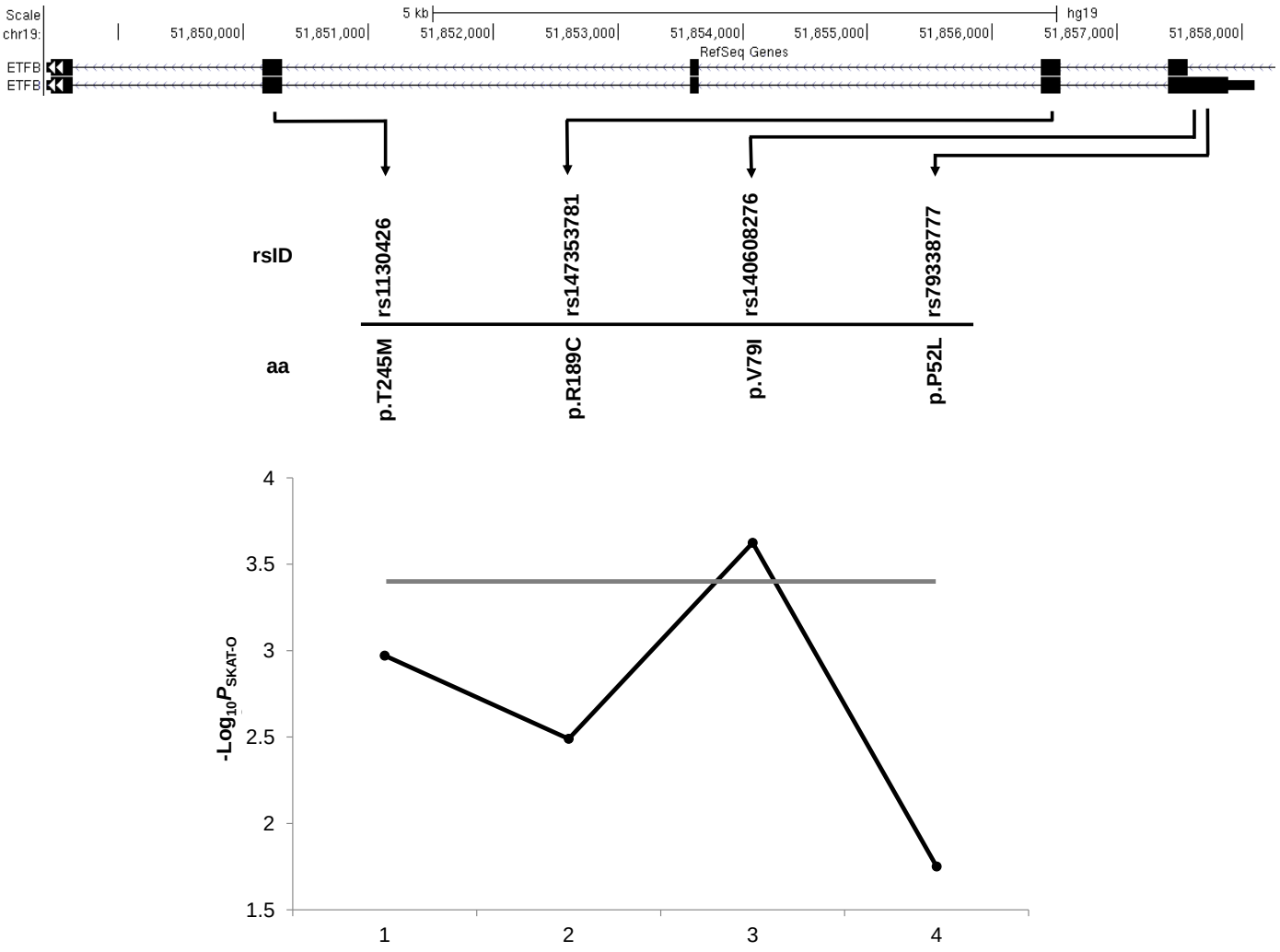

Figure 1. Contribution of individual *ETFB* variants on statistical significances for the *ETFB* gene in the discovery cohort. Top: genomic location of *ETFB* displayed in the USCS Genome Browser. Exon location and amino acid substitution of each of the 4 coding polymorphic variants included in the Illumina HumanExome BeadChip array are depicted. Bottom: *P*-values for the *ETFB* association in SKAT-O gene-based tests after removing one variant at a time and recalculating the association. Grey line indicates the *P*-value for the *ETFB* association with chronic AIC including all 4 coding variants (P=4.16x10^{-4}).