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Genetic Variation in the Alternative Splicing Regulator, RBM20, is associated with Dilated Cardiomyopathy

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Abstract

BACKGROUND—Dilated cardiomyopathy (DCM) is a leading cause of heart failure and death. The etiology of DCM is genetically heterogeneous.

OBJECTIVES—We sought to define the prevalence of mutations in the RNA splicing protein, RBM20, in a large cohort with DCM, and to determine if genetic variation in *RBM20* is associated with clinical outcomes.

METHODS—Subjects included in the GRADE (Genetic Risk Assessment of Defibrillator Events) study were at least 18 years of age, had an ejection fraction of $\geq 30\%$, and an implantable cardioverter-defibrillator (ICD). The coding region and splice junctions of *RBM20* were screened in DCM subjects; two common polymorphisms in *RBM20*, rs942077 and rs35141404, were genotyped in all GRADE subjects.

RESULTS—1465 subjects were enrolled in the GRADE study and 283 with DCM were screened for RBM20 mutations. The mean age of subjects with DCM was 58 ± 13 years, 64% were male and the mean follow up was 24.2 ± 17.1 months after ICD placement. RBM20 mutations were identified in eight subjects with DCM (2.8%). Mutation carriers had a similar survival, transplantation rate, and frequency of ICD therapy compared to non-mutation carriers. Three of

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eight subjects (37.5%) with *RBM20* mutations had atrial fibrillation (AF) whereas 19 (7.4%) subjects without mutations had AF ($p=0.02$). Among all GRADE subjects, rs35141404 was associated with AF (minor allele OR 0.62, 95% CI 0.44–0.86, $p=0.006$). In the subset of GRADE subjects with DCM, rs35141404 was associated with AF (minor allele OR 0.58, $p=0.047$).

CONCLUSIONS—Mutations in *RBM20* were observed in approximately 3% of subjects with DCM. There were no differences in survival, transplantation rate, and frequency of ICD therapy in mutation carriers.

Keywords

dilated cardiomyopathy; genetics; mutation; single nucleotide polymorphism; *RBM20*

Introduction

Idiopathic dilated cardiomyopathy (DCM) is a common disease with an estimated prevalence of 36.5 per 100,000 individuals,¹ and is associated with substantial mortality.^{2–4} The pathophysiological mechanisms underlying DCM are heterogeneous, although there has been a longstanding appreciation of a heritable component to DCM.^{5, 6} Estimates suggest that up to one third of individuals with DCM have familial disease.^{7–11} Indeed, numerous mutations have been identified which are believed to underlie familial DCM.^{12–21} Among the most prevalent of these are mutations in lamin A/C and beta-myosin heavy chain, each accounting for up to 10% of cases of familial DCM in some series.^{22, 23}

In 2006, a susceptibility locus for DCM was mapped to chromosome 10q25 in two large families with DCM, fibrosis and sudden cardiac death.²⁴ Recently, Brauch et al. identified a missense mutation in exon 9 of the ribonucleic acid (RNA) binding motif protein 20 (*RBM20*) at the chromosome 10q25 locus that was responsible for the disease in one of the families.²⁵ Screening of the *RBM20* gene in an additional 278 additional probands with DCM of European descent identified three additional mutations. *RBM20* is highly expressed in cardiac tissues and regulates splicing by processing pre-messenger ribonucleic acid.²⁵ *RBM20* mutations in exon 9 were found in 3% of all the DCM cases tested and in over 13% of those with a history of sudden cardiac death (SCD). Out of the 44 subjects with *RBM20* mutations, 39 had clinically aggressive DCM and 9 had ventricular tachycardia (VT).²⁵

We therefore sought to: 1) determine the prevalence of mutations in *RBM20* in a large, multiracial cohort with DCM; 2) examine the clinical characteristics and outcomes of mutation carriers, including DCM-related outcomes such as atrial fibrillation (AF), ventricular arrhythmias, heart transplant, and mortality; and 3) determine if common genetic variation in *RBM20* alters clinical outcomes in patients with dilated or ischemic cardiomyopathy.

Methods

Study subjects

All subjects included in this analysis were enrolled in the Genetic Risk Assessment of Defibrillator Events (GRADE) Study.²⁶ Subjects with DCM recruited from the University of Pittsburgh Medical Center, Mid Ohio Cardiology, Emory University, Pittsburgh Veterans Affairs Medical Center, and Massachusetts General Hospital. Included subjects were at least 18 years of age and had significant left ventricular systolic dysfunction, defined as a left ventricular ejection fraction (EF) of 30% or less by transthoracic echocardiography, left ventriculography, or a multi-gated acquisition scan and increased left ventricular size as defined by a LVEDD>55 mm. Subjects were excluded if they were unable or unwilling to

provide written informed consent, had a life expectancy of less than 6 months from a non-cardiac disease, had received a cardiac transplant or left ventricular assist device, or had a history of ischemic cardiomyopathy (as defined by a documented history of myocardial infarction, percutaneous transluminal coronary angioplasty, coronary artery bypass graft or >50% diameter stenosis of any of the major coronary epicardial arteries).

Clinical characteristics, including information regarding implantable cardioverter-defibrillator (ICD) therapies, syncopal events, hospitalizations, heart failure episodes, New York Heart Association (NYHA) class, and medications were obtained in a standardized fashion at baseline and reassessed annually for each subject. All ICD therapies were obtained and reviewed by a central core laboratory at the University of Pittsburgh consisting of three clinical electrophysiologists. Appropriate ICD shocks for VT or ventricular fibrillation were used as a surrogate for SCD.

Prevalent AF was ascertained at enrollment using three methods: 1) Patient report at the time of a nurse administered enrollment interview, 2) review of the patient's medical history and records, and 3) for patients with an ICD at enrollment, a review of prior interrogations was performed. Data for incident AF is currently unavailable in the GRADE study.

All study procedures were reviewed annually by the local Institutional Review Boards at each site. Prior to any study procedures, written informed consent was obtained from each study subject.

RMB20 mutation screening

Genomic DNA was isolated from whole blood and oligonucleotide primers were designed for polymerase chain reaction (PCR) amplification of the coding region of RBM20 (NM_001134363.1). PCR was performed using standard techniques; amplicons were purified and was sequenced using the ABI PRISM dye terminator method (Applied Biosystems, Foster City, California, USA). Exons containing mutations in DCM cases were then screened in 500 healthy control subjects of European descent drawn from the local hospital catchment area and 100 individuals of African American decent from the Coriell repository.

Nonsynonymous and synonymous variants were classified as known polymorphisms if they were annotated in dbSNP (build 132),²⁷ as probable polymorphisms if they were identified with an allele frequency of 1% in ethnicity-matched control subjects, or as mutations if they were not identified in control individuals. dbSNP build 132 includes recent results from the 1000 genomes project pilots 1, 2, and 3, which is comprised of population based sequencing data.²⁸ Unfortunately, no data is available for RBM20 in recent exome variant server data release (<http://snp.gs.washington.edu/EVS>) that includes the exome sequencing data from more than 2000 individuals. The position of identified mutations is annotated according to the RBM20 protein sequence (NP_001127835.1).

Single nucleotide polymorphism genotyping

Two common *RBM20* coding SNPs with a minor allele frequency of >5% were genotyped (rs35141404 [c.90G>A] and rs942077 [c.3667G>C, p.Glu1223Gln]) using a TaqMan[®] genotyping assay.

Statistical analysis

Means and standard deviations or numbers and proportions were calculated and reported for subject characteristics. Continuous variables were compared between individuals with identified *RBM20* common variants and those without using two sample t-tests or the

Wilcoxon Rank Sum method as appropriate after assessing the normality of distributions for each characteristic. Categorical variables were assessed for independence between the two groups using the Fisher's exact test. In the population of patients with DCM, we examined the association between the presence or absence of *RBM20* mutations and ICD shock, death, and death or heart transplant separately using Cox proportional hazards regression. We examined associations between *RBM20* SNPs (rs35141404 and rs942077) and first appropriate ICD shock, death, and death or heart transplant separately using Cox proportional hazards regression in the entire GRADE cohort comprised of both DCM and ischemic cardiomyopathy. Associations between *RBM20* mutations or *RBM20* SNPs and first appropriate ICD shock, death, and death or heart transplant were also studied in the subset of patients of European ancestry to limit population stratification. For the analysis of ICD shock, observations were censored at death, transplant or loss to follow-up; for the analysis of death, observations were censored at heart transplant or loss to follow-up; and for the analysis of death or heart transplant, observations were censored at loss to follow-up. Variables in each model were tested for deviation from the proportional hazards assumption with a multiplicative interaction between each respective variable and time. We examined the association between SNPs and prevalent AF using logistic regression. We examined the association between SNPs and the baseline PR interval using linear regression, and excluded those with extreme PR intervals (< 80 msec or > 320 msec, $n=18$). All models were adjusted for age, sex, race, and ischemic or DCM. Linearity between age and each outcome was examined by comparing a model with an additional quadratic term for age to a reduced model with only a main effect for age, using a likelihood ratio test. The associations between SNP and outcomes were examined assuming an additive genetic model. Freedom from appropriate ICD therapies, death, and heart transplant were each estimated separately using the Kaplan-Meier method. Event rates were compared between genotypes using the log rank test. Results of mutational analysis were considered significant at the two-sided alpha threshold < 0.05 . For association studies of the common SNPs with appropriate ICD shocks, death, death or transplant and AF, results were considered significant at the two-sided alpha threshold < 0.05 . Analyses were performed using either SAS v9.1 (SAS Institute Inc., Cary, NC), R v2.11, or SPSS13 (SPSS Inc., Chicago, IL).²⁹

Identified *RBM20* mutations and variants were analyzed using SIFT (<http://sift.jcvi.org/>) and Polyphen (<http://genetics.bwh.harvard.edu/pph/>) to determine if they are likely to be deleterious.^{30,31}

Results

The total GRADE study consists of 1,465 individuals of which 283 individuals have DCM (Table 1). For individuals with DCM the mean age was 58 ± 13 years, 182 (64%) were men, and 204 (72%) were of European ancestry. Among the subjects with DCM, 30 experienced ICD shocks, 23 received subsequent heart transplants, and 32 died over a mean follow-up of 24.2 ± 17.1 months. Of the 1,182 subjects with ischemic cardiomyopathy, the mean age was 65 ± 10 years, 1,040 (88%) were men, and 1,028 (87%) were of European ancestry. Among the subjects with ischemic cardiomyopathy, 141 experienced ICD shocks, 45 received subsequent heart transplants, and 205 died over a mean follow-up of 28.1 ± 16.9 months.

Among the 283 subjects with DCM, we identified eight nonsynonymous variants in *RBM20* classified as mutations, occurring in exons 2, 4, 9, 11, and 14 (Table 2, Figure 1, Supplemental Figure 1 and Supplemental Figure 2). Five of the *RBM20* mutations were identified in patients of European ancestry, and three in patients of African American ancestry. No difference was observed in the proportion of variants in European versus African American descent ($p=0.45$). None of the identified *RBM20* mutations were present in control populations comprised of 500 subjects of European descent and 100 African

American subjects, nor were any annotated in dbSNP build 132. None of the identified mutations were discovered in pilot 1 of the 1000 genomes project (low coverage whole genome sequencing) which included the CEU sample comprised of 60 individuals and the YRI sample comprised of 59 individuals. Among the two trios in pilot 2, (one from the CEU, one YRI), none of the mutations were reported. *RBM20* was not selected for exon sequencing in pilot 3 of the 1000 genomes project (n=697 individuals).

Out of the eight mutations identified in the DCM subjects, seven were missense mutations and one was a nonsense mutation resulting in a premature stop codon. Most of the seven missense mutations altered conserved residues in *RBM20* (Supplemental Figure 1). One individual had a distinct mutation identified in exon nine, which has not been previously reported; the other mutations in exons 2, 4, 11 and 14 are also novel.²⁵

The mean follow-up among the eight patients with *RBM20* mutations was 27.4 ± 15.7 months. Appropriate ICD shocks occurred in one patient, one received a heart transplant, and none died. We did not observe an association between the presence of an *RBM20* mutation and ICD shocks (HR 1.15, 95% CI 0.16–5.48, $p=0.89$) or death or heart transplant (HR 0.51, 95% CI 0.07–3.70, $p=0.51$) (Figure 2A and Figure 2B). Among individuals of self-reported European ancestry, five of 188 DCM patients (2.7%) had *RBM20* mutations while three of the 76 DCM patients with African American ancestry (3.9%) had *RBM20* mutations. The difference in the proportion of mutations detected between races did not differ significantly ($p=0.69$) likely due to the small sample size. Three of eight DCM subjects (37.5%) with *RBM20* mutations had AF whereas 19 (7.4%) subjects without mutations had AF ($p=0.02$). Given the small number of individuals with mutations in each ancestral group, we did not attempt to examine the relations between clinical outcomes and the presence of mutations in each ancestral group.

Synonymous polymorphisms were common in patients with DCM (Supplemental Table 1), particularly in exon nine which contained three synonymous variants that were not identified in our control populations. Similarly, a number of novel nonsynonymous variants categorized as probable polymorphisms were identified.

We further examined the putative functional effects of the eight identified *RBM20* mutations using the Polyphen-2 and SIFT prediction algorithms.^{30,31} Polyphen-2 predicted that four of the eight nonsynonymous *RBM20* mutations were probably damaging (P638L, R703S, D888N and P1081R); two were predicted to be possibly damaging (L83I and E1206K); and one (S455L) was predicted to be benign. The G1031X *RBM20* mutation leads to a premature stop codon that we assume is deleterious, but was not processed by the Polyphen-2 algorithm since it does not estimate the deleterious nature of stop codons. Two of the eight identified nonsynonymous *RBM20* mutations were predicted to alter protein function (P1081R and E1206K) using SIFT, whereas the other six were predicted to be tolerated (Table 2).

Outcomes with common *RBM20* polymorphisms in the GRADE cohort

After the identification of *de novo* mutations in subjects with DCM, we sought to determine whether common genetic variants in *RBM20*, defined as SNPs with a minor allele frequency (MAF) >5%, were associated with adverse clinical outcomes among patients with DCM or ischemic cardiomyopathy. We therefore genotyped two common polymorphisms in *RBM20* in the entire GRADE cohort. The call rate for the *RBM20*rs35141404 polymorphism was 97.4% while the call rate for the *RBM20*rs942077 polymorphism genotyping was 97.6%. Allele frequencies are provided in Supplemental Table 1.

In multivariable models adjusted for age and sex, and stratified by dilated versus ischemic cardiomyopathy, we did not identify an association between either the *RBM20*rs35141404 A-allele (HR 1.15, 95% CI 0.92–1.45, $p=0.23$) or the *RBM20*rs942077 G-allele (c.3667G>C [p.E1223Q]) (HR 0.99, 95% CI 0.74–1.33, $p=0.98$) and ICD shocks (Figures 3A). Neither rs35141404 A-allele (HR 1.07, 95% CI 0.87 – 1.30, $p=0.54$) nor rs942077 G-allele (HR 0.97, 95% CI 0.76–1.25, $p=0.83$) were associated with death (Figure 3B, Figure 3D). The combined endpoint of heart transplant or death did not differ significantly between rs35141404 A-allele (HR 1.09, 95% CI 0.91–1.30, $p = 0.36$) or rs942077 G-allele (HR 0.96, 95% CI 0.77–1.20, $p = 0.71$). Among the subset of subjects of self-reported European ancestry, we did not identify an association between either the *RBM20*rs35141404 A-allele (HR 1.09, 95% CI 0.83–1.43, $p=0.53$) or the *RBM20*rs942077 G-allele (c.3667G>C [p.E1223Q]) (HR 0.85, 95% CI 0.59–1.24, $p=0.41$) and ICD shocks. Neither rs35141404 A-allele (HR 1.03, 95% CI 0.83 – 1.28, $p=0.79$) nor rs942077 G-allele (HR 0.88, 95% CI 0.66–1.17, $p=0.38$) were associated with death. The combined endpoint of heart transplant or death did not differ significantly between genotypes for rs35141404 A-allele (HR 1.07, 95% CI 0.88–1.30, $p = 0.48$) or rs942077 G-allele (HR 0.94, 95% CI 0.73–1.21, $p = 0.63$).

We examined whether common polymorphisms in *RBM20* were associated with AF and PR interval duration in the entire GRADE sample using multivariable logistic and multiple linear regression, respectively. The SNP rs35141404 A-allele was associated with AF at enrollment (OR 0.62, 95% CI 0.44–0.86, $p=0.006$), but not PR interval (β [se] 2.0 [1.9] msec, $p=0.28$). The SNP rs942077 G-allele was neither associated with AF (OR 1.12, 95% CI 0.76–1.62, $p=0.57$) nor PR interval (β [se] -1.9 [2.4] msec, $p=0.43$). Among those of self-reported European ancestry, rs35141404 A-allele was associated with AF at enrollment (OR 0.59, 95% CI 0.40–0.84, $p=0.006$), but not PR interval (β [se] 1.85 [2.11] msec, $p=0.38$). Among all GRADE subjects with DCM only, rs35141404 A-allele was associated with AF at enrollment (minor allele OR 0.58, $p=0.047$). The rs942077 G-allele was neither associated with AF (OR 1.13, 95% CI 0.73–1.69, $p=0.58$) nor PR interval (β [se] -2.2 [3.0] msec, $p=0.46$).

Discussion

In our large, multiethnic cohort of subjects with DCM, we identified mutations in coding regions of *RBM20* in about 3% of individuals.

The spliceosome is a complex of specialized RNA and SR protein subunits that removes introns from a transcribed pre-mRNA segment. SR proteins have 2 major domains: RS domain (rich in Arginine-Serine repeats) and RNA-recognition motif (RRM) that recognizes specific RNA sequences typically located within exons. *RBM20* is a member of SR protein family (Serine/Arginine-rich protein) that regulate and select alternative splice sites in eukaryotic pre-mRNA. *RBM20* is highly expressed in cardiac tissues (mean expression in the heart is > 8-fold higher than combined expression in 11 other tissues). *RBM20* is cardiac splicing regulator that was recently identified to play a role in the pathogenesis of DCM. Transcript splicing is regarded as one of the primary mechanisms by which phenotypic variation is generated.³² Alternative splicing is increasingly being recognized with evidence suggesting that up to 94% of human genes undergo alternative transcript splicing.^{33,34} Our report supports the role of *RBM20* as a proximal regulator in a pathway that regulates cardiac morphology and as a disease susceptibility locus for DCM. Perturbations of this pathway may result in cardiomyopathy, thereby conferring increased risks of heart failure and sudden cardiac death. We did not observe an increased risk of ventricular arrhythmias among DCM subjects with *RBM20* mutations as compared to those without such mutations. However the small number of events observed in our cohort limits our ability to draw meaningful conclusions about the influence of these variants on the incidence of death and/

or ventricular arrhythmias. The proximate mechanisms by which individual mutations in *RBM20* result in cardiomyopathy remain ambiguous, and may involve the interactions of numerous different alternatively spliced transcripts regulated by the gene. Whether specific DCM sub-phenotypes can be distinguished with more precise localization of mutations in exon 9 remains uncertain. Recently, DNA from 312 idiopathic DCM probands at the University of Miami was sequenced for nucleotide alterations in exons 6 through 9 of *RBM20*, and six *RBM20* rare variants in six unrelated probands (1.9%) were found. Four mutations, two of which were novel (R634W and R636C) and two previously identified (R634Q and R636H), were identified in a five amino acid hotspot in exon 6. Two other novel variants (V535I in exon 6 and R716Q in exon 9) were outside of this hotspot. Many subjects with these rare variants suffered severe heart failure resulting in early death or cardiac transplantation.³⁵

We found that AF was more common among *RBM20* mutation carriers and that the common polymorphism rs35141404 was associated with an increased risk of AF in the overall GRADE cohort as well as in the DCM subjects in the GRADE cohort. To adjust for population stratification as a confounding factor, we studied the cohort of European ancestry and found that *RBM20* mutation carriers and the common polymorphism rs35141404 were associated with an increased risk of AF. The role of *RBM20* in atrial signaling remains unexplored; however, our findings reiterate the close relation between electrical and mechanical function. While our findings that *RBM20* mutation carriers and that rs35141404 were both associated with an increased risk of AF, we would caution that both findings will require replication in independent cohorts with DCM.

Our study had several limitations. The study population was heterogeneous compared with other studies that restricted their study sample on the basis of race or heart failure etiology. Given the small number of identified *RBM20* mutations, we were unable to assess the associations between mutations and clinical outcomes in different ancestral groups. Our study had few Hispanics or Asians. We have limited clinical information on our control populations, while reportedly healthy, it is possible that some subjects may have occult cardiac dysfunction. Furthermore, we would note that our control populations did not undergo sequencing of the entire gene. It is possible that by fully sequencing *RBM20* we may have observed potentially damaging nonsynonymous polymorphisms in our control populations. Additionally, although a biologically plausible association might exist between the *RBM20* rs35141404 G allele and AF, it is possible that the association observed reflects another susceptibility locus in linkage disequilibrium with the *RBM20* rs35141404 G allele. Finally, we have not confirmed altered transcript splicing as a result of the individual mutations in our subjects. Further work will be needed to delineate the relation between *RBM20* and AF.

Conclusions

We have identified mutations in *RBM20* in approximately 3% of our sample of individuals with DCM. Mutations in *RBM20* did not adversely affect survival or ventricular arrhythmias in subjects with DCM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Topology and location of *RBM20* mutations in GRADE subjects with dilated cardiomyopathy.

Figure 2.

Outcomes in *RBM20* mutation carriers with DCM. A. Kaplan-Meier plots for freedom from appropriate ICD shocks in DCM subjects with or without a mutation in *RBM20* after 36 months of follow-up. B. Kaplan-Meier plots for survival in DCM subjects with or without a mutation in *RBM20* after 36 months of follow-up. None of the comparisons were statistically significant.

Figure 3. Relation between outcomes in the entire GRADE cohort and SNPs rs35141404 or rs942077. Kaplan-Meier plots for freedom from appropriate ICD shocks in the entire GRADE cohort after 60 months of follow-up for rs35141404 (A) or rs942077 (C). Kaplan-Meier plots for survival in the entire GRADE cohort after 60 months of follow-up in rs35141404 (B) or rs942077 (D). None of the comparisons were statistically significant.

Table 1

Characteristics of the GRADE study subjects.

	All subjects	DCM subjects
Number of subjects	1,465	283
Age at enrollment	64±11	58±13
Female (%)	17	36
Race		
White (%)	84	72
Black (%)	13	26
American Indian (%)	0.2	1
More than one race (%)	1.4	1
Asian (%)	0.6	0
NYHA class	2.2±0.7	2.1±0.65
I	189	45
II	827	168
III	407	66
IV	14	3
Coexisting conditions		
Hypertension (%)	64	53
Diabetes mellitus (%)	36	24
Hyperlipidemia (%)	68	36
Smoking (%)	54	34
Echocardiography		
Ejection fraction (%)	21±6	19±6
LVEDD (mm)	6.36±1.09	6.75±0.74
Electrocardiography		
Sinus rhythm (%)	91	92
Atrial fibrillation (%)	9	8
PR duration (msec)	172±44	171±81
QRS duration (msec)	137±37	140±35
QTc duration (msec)	471±54	480±50

Data reported as mean ± SD or %.

Table 2

RBM20 mutations in subjects with idiopathic dilated cardiomyopathy.

Sample number	Exon	Nucleotide position	Amino acid residue	Sex	Race	AF Status	Polyphen-2 predictions	SIFT predictions
3-DP213	2	c.247C>A	L83I	M	W	SR	Possibly damaging	Tolerated
2-DP012	4	c.1364C>T	S455L	F	W	SR	Benign	Tolerated
1-DR122	9	c.1913C>T	P638L	F	W	AF	Probably damaging	Tolerated
3-DP146	9	c.2109G>C	R703S	M	AA	AF	Probably damaging	Tolerated
3-DR087	11	c.2662G>A	D888N	F	W	AF	Probably damaging	Tolerated
3-DP171	11	c.3091G>T	G1031X	F	W	SR	-	-
1-DP180	11	c.3242C>G	P1081R	M	AA	SR	Probably damaging	Alter protein function
1-DP310	14	c.3616G>A	E1206K	M	AA	SR	Possibly damaging	Alter protein function

The *RBM20* nucleotide position is based on the NCBI reference sequence NM_001134363.1 and begins at the start codon. Amino acid positions are based on the NCBI reference sequence NP_001127835.1. M, Male; F, Female; W, White; AA, African American; AF, Atrial Fibrillation; SR, Sinus Rhythm; NA, Not Applicable.